# Application of proteomics and metabolomics in molecular investigations of sensitization to contact allergens

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# 1. Introduction

## 1.1. Allergic contact dermatitis

Allergic contact dermatitis (ACD) represents a hypersensitivity reaction in the human skin leading to severe recurring medical conditions in patients. Cosmetics, consumables and commodities expose consumers to a growing number of contact allergens, most commonly nickel, fragrances and preservatives (Peiser et al. 2012). ACD also represents a major reason for occupational disability, *e.g.* among hairdressers, health care workers, construction workers and painters (Holness 2014). Consequently, the number of ACD patients increases constantly, currently revealing a prevalence of 15-20 % (Thyssen et al. 2007; Diepgen et al. 2016). Therefore, ACD probably qualifies as the most abundant immunotoxic disease among the general population (Kimber et al. 2002).

ACD represents a cell-mediated hypersensitivity reaction. Thus, ACD symptoms occur several days after exposure setting it apart from rapid antibody-mediated immune reactions, *e.g.* atopic dermatitis, and immediate skin reactions to chemical or physical stressors, *e.g.* irritant contact dermatitis (Tan et al. 2014). The development and mechanisms of ACD are rather complex as contact allergens represent a diverse group of substances and ACD involves the activation of the adaptive immune system. The main cell types involved in ACD are keratinocytes, dendritic cells (DCs) and various types of T cells (Martin 2015a).

At sites of exposure to specific contact allergens, the cellular mediated inflammation of the skin finally leads to repeatedly occurring, long-lasting contact eczema consisting of pruritic rashes, papules, blisters or vesicles. During further progression, contact eczema can ooze, drain or crust and become raw, scaled or thickened. Long-term exposure to contact allergens can result in chronic forms of ACD. In most cases, ACD represents a life-long condition without possibilities of curation. Therefore, therapies are restricted to symptomatic treatments, especially the administration of topical corticosteroids (Aslam et al. 2014). In the end, the best strategy to condemn ACD is the avoidance of contact allergens (Schwensen et al. 2016). Thus, regulatory authorities are under obligation to set up effective strategies for the identification and removal of ACD-triggering substances.

### 1.2. Contact allergens

Today, approx. 4,000 contact allergens are known (Martin 2015b). Despite their great diversity, contact allergens share physicochemical properties that enable them to elicit ACD (Kaplan et al. 2012). Firstly, most contact allergens are hydrophobic chemicals with a molecular weight lower than 500 Da. This enables them to penetrate the stratum corneum - the outer layer of the human skin – and to migrate further to deeper skin layers like the epidermis and the dermis. Skin lesions and co-exposure to irritants can affect the skin barrier function further and thus enhance penetration. Secondly, most contact allergens are electrophilic substances. Therefore, contact allergens may modify skin proteins and other cellular macromolecules at nucleophilic sites. Protein modification is essential for the development of ACD as contact allergens alone cannot serve as antigens for the adaptive immune system due to their small molecular size. The process of protein modification is referred to as haptenation.

Contact allergens classify as haptens if they directly bind to self-proteins. Other contact allergens need prior modifications to be able to react with self-proteins. Prehaptens undergo physiochemical modifications to become protein-reactive. Prohaptens need to be converted metabolically by enzymes before protein modification (Lepoittevin 2006). Protein modification during haptenation comprises covalent binding of chemicals following nucleophilic substitutions and metal ions binding to proteins by complexation reactions. Preferred sites of protein modifications include sulfhydryl and imidazole groups of the amino acids cysteine and histidine, respectively. Furthermore, side-chain amino groups of lysine and N-terminal amino groups are frequent targets of haptenation (Divkovic et al. 2005).

The exact target proteins and binding sites of contact allergens are still under investigation. Although it is known that certain contact allergens haptenate several abundant skin proteins (*e.g.* human serum albumin and keratin isoforms) the ultimate antigens leading to ACD are unknown (Höper et al. 2017).

### 1.3. Immunological mechanisms of ACD

The course of ACD comprises two phases. The first phase is referred to as sensitization and describes the immune mechanisms following the first exposure to a specific contact

allergen. The second phase called elicitation involves the memory response of the adaptive immune system upon further exposure to a contact allergen (Figure 1).

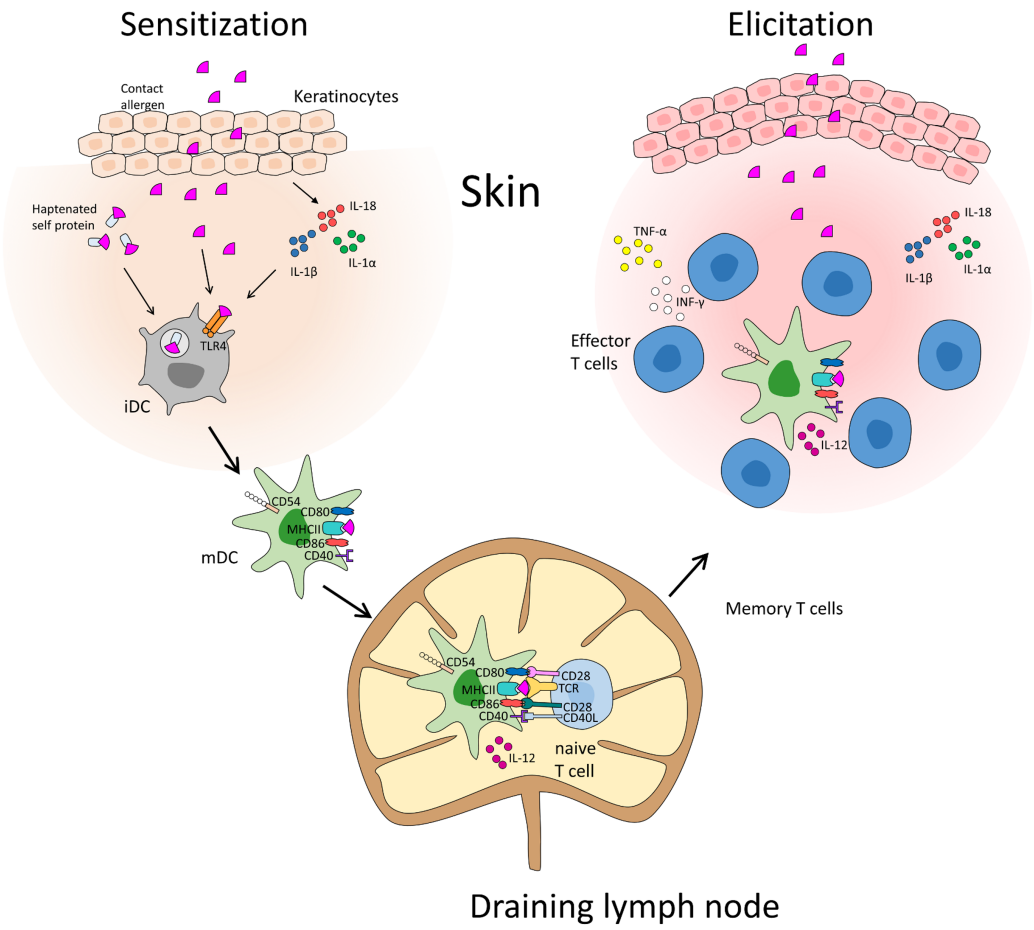


**Figure 1. Molecular and cellular mechanisms of ACD.** During the sensitization phase, keratinocytes activated by contact allergens secrete pro-inflammatory cytokines (*e.g.* IL-1α, IL-β, IL-18). Immature dendritic cells (iDCs) in the skin internalize haptenated proteins by phagocytosis. Binding of haptenated proteins to toll-like receptors (TLRs) activates iDCs and triggers the up-regulation of major histocompatibility complex class II (MHC) class II and co-stimulatory molecules like of CD40, CD80 and CD86 (phenotypic maturation). Mature dendritic cells (mDCs) migrate to the local lymph nodes and present processed haptenated proteins as antigens via MHC class II. Selection of antigen-specific T cells is mediated by the interaction of antigen-loaded MHC class II with antigen-specific T cell receptors (TCRs) and co-stimulatory molecules on mDCs and naïve T cells. Interaction with antigen-specific T cells elicits secretion of cytokines (*e.g.* IL-12) by mDCs (functional maturation) enabling T cell differentiation into effector T cells. Antigen-specific T cells migrate back to the site of exposure setting up the memory response. Re-exposure to the respective contact allergen during the elicitation phase leads to activation and proliferation of memory effector T cells secreting pro-inflammatory cytokines (e.g. TNFα, IFNγ). The resulting inflammatory response finally leads to visible contact eczema (Höper et al. 2017).

Skin sensitization is initiated by contact allergens penetrating the outmost layer of the epidermis, the *stratum corneum*. In the epidermis, keratinocytes become activated by contact allergen-induced, danger-associated molecular patterns (DAMPs), *e.g.* production of reactive oxygen species (ROS) or extra-cellular ATP. Activated keratinocytes then secrete pro-inflammatory cytokines like IL-1α, IL-1β or IL-18. Keratinocytes may also metabolize prohaptens into their protein-reactive metabolites. The pro-inflammatory milieu provokes the migration of immature dendritic cells (iDCs) to the site of exposure and supports their subsequent activation (Kaplan et al. 2012). iDCs steadily internalize and process proteins in their surroundings via phagocytosis. Upon activation by haptenated proteins, iDCs undergo phenotypic maturation resulting in the up-regulation of major histocompatibility complex (MHC) class II, cell adhesion molecules (*e.g.* CD54) and co-stimulatory molecules (*e.g.* CD80, CD86) on their surface. Mature DCs (mDCs) migrate to the local lymph nodes where they present fragments of processed haptenated proteins via MHC class II as possible antigens. Fully activated mDCs represent the most effective professional antigen-presenting cells (APCs) of the immune system (Banchereau and Steinman 1998). Presentation of haptenated protein fragments by mDCs facilitates the selection of naïve T lymphocytes with contact allergen- specific T cell receptors (TCRs) (Vocanson et al. 2009). The interaction of mDCs and T cells via MHC class II, TCR and co-stimulatory molecules elicits functional maturation of mDCs. Subsequent secretion of cytokines by mDCs (*e.g.* IL-12) induces T cell proliferation differentiation into effector T cells. Contact allergen-specific effector T cells then migrate to the site of exposure in the epidermis where they persist as memory effector T cells. The complete process of skin sensitization takes 10-15 days and is not connected to any symptoms of clinical relevance in most cases (Vocanson et al. 2009). Any further exposure to the respective contact allergen triggers the elicitation phase that also includes the activation of keratinocytes and DCs. The activation and proliferation of resting memory effector T cells in the skin leads to a pronounced inflammatory response. The memory response involves several effector T cell subsets including CD4+ $T_H1$, $T_H2$, $T_H9$, $T_H17$ and $T_H22$ T helper cells as well as CD8+ cytotoxic T cells. Within 24-72 hours, patients develop visible contact eczema (Vocanson et al. 2009; Cavani et al. 2012; Martin 2015a).

### 1.4. Dendritic cells

DCs belong to the innate immune system. However, their unique ability to select and activate naïve T cells makes them essential for the development of an adaptive immune response. The ontogeny of DCs in general is complex. Together with other types of mononuclear immune cells, they originate from myeloid progenitors. Consequently, DCs share phenotypical and functional characteristics with monocytes and macrophages, *e.g.* cell migration, phagocytosis, cytokine secretion and antigen presentation. Together, these cell types constitute the mononuclear phagocyte system (MPS) (Guilliams et al. 2014). Macrophages and subsets of DCs may originate from monocytes under inflammatory conditions, even though the exact lineage commitment is still under debate (Auffray et al. 2009; Chow et al. 2011). DCs divide into organ-specific subsets. Two major subsets of DCs involved in ACD reside in the skin. The so-called Langerhans cells and dermal DCs are located in the epidermis and dermis, respectively (Vocanson et al. 2009).

Antigen-specific T cell selection requires DC activation including substantial phenotypical and functional alterations (Chapter 1.3., Figure 1). A central event during the activation of DCs and other cells of the MPS is the recognition of pathogen-associated molecular patterns (PAMPs) that bind to pattern-recognition receptors (PRRs) (Iwasaki and Medzhitov 2015). Toll-like receptors (TLRs) represent the most widely studied group of PRRs in DCs. Stimulation of TLRs is crucial for the functional maturation and specialization of DCs. The activation of PRRs in DCs triggers specific signaling cascades which cumulate in the activation of effector pathways via transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factors (IRF) or mechanistic target of rapamycin (mTOR). Activation of these pathways is essential for complete functional maturation of DCs, including the production of cytokines, chemokines and lipid mediators, like prostaglandins and leukotrienes (Dalod et al. 2014; Kelly and O'Neill 2015; Weichhart et al. 2015).

An essential event following TLR-dependent activation of DCs and other cells of the MPS represent the metabolic alterations referred to as metabolic reprogramming (O'Neill and Pearce 2016). Here, the induction of glycolysis to fuel lipid synthesis via intermediates of the tricarboxylic (TCA) cycle represents a common mechanism (Arts et al. 2016). Activation of DCs coincides with a metabolic switch from fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) to glycolysis and fatty acid synthesis (Figure 2). This metabolic switch resembles the so-called Warburg effect in rapidly growing cancer cells (Pearce and Everts 2015).

Metabolic reprogramming represents the prerequisite for phenotypic and functional DC maturation, stimulation of naïve T cells. Thus, the activation of DCs is essential for the induction of an adaptive immune response (Krawczyk et al. 2010; Everts et al. 2014). Metabolic reprogramming is mediated by the NO-induced inactivation of respiratory chain complexes and is further enhanced by a shortage in reducing equivalents – *i.e.* reduced nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide ($FADH_2$) – as increased fatty acid synthesis deprives the TCA cycle of citrate (Kelly and O'Neill 2015). As ATP production by OXPHOS diminishes, the shift to glycolysis during metabolic reprogramming ensures ATP supply independently of the oxygen concentrations in the surrounding tissues (aerobic glycolysis). During DC activation, fatty acids are needed for the synthesis of inflammatory lipid mediators. Even more importantly, fatty acids facilitate growth of the endoplasmic reticulum (ER) and the Golgi apparatus as a prerequisite for elevated protein synthesis to produce co- stimulatory molecules and especially cytokines and chemokines. During metabolic reprogramming induction of the unfolded protein response (UPR) helps to relieve ER stress induced by increased protein translation rates (Pearce and Everts 2015).

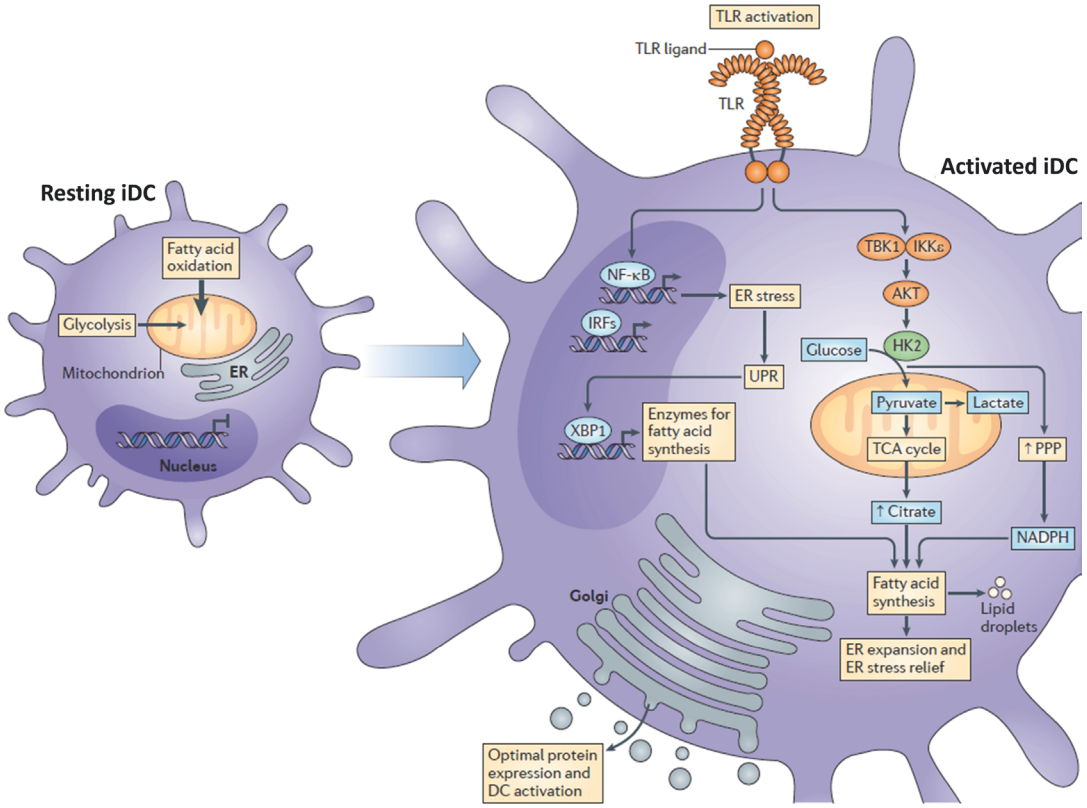


**Figure 2 TLR-dependent metabolic reprogramming of DCs.** In resting immature dendritic cells (iDCs) fatty acid oxidation is engaged and glycolysis rate is relatively low. Following iDC activation via TLRs, expression of nuclear factor-KB (NF-KB)- and interferon-regulatory factor (IRF)-responsive genes leads to an increased production of proteins needed for phenotypic and functional DC maturation. Increased protein translation rates cause ER stress and subsequent activation of the unfolded protein response (UPR). UPR activates the transcription factor X-box-binding protein 1 (XBP1) that induces the expression of enzymes for fatty acid synthesis. Concomitant activation of glycolysis via Akt, TANK-binding kinase 1 (TBK1), inhibitor of NF-KB kinase subunit-E (IKKE) and hexokinase 2 (HK2) results in an increase of citrate production via the tricarboxylic acid (TCA) cycle. Citrate is needed for the synthesis of fatty acids and lipids that facilitate ER expansion to relieve ER stress. Increased activity of the pentose phosphate pathway (PPP) ensures production of NADPH for fatty acid synthesis in the absence of a fully functional TCA cycle (Pearce and Everts 2015, modified).

The detailed mechanisms of DC activation by contact allergens remain largely unknown. However, it is assumed that binding of contact allergens to cellular macromolecules, especially proteins, facilitates the emulation of pathogen-associated patterns PAMPs and DAMPs and therefore the activation of PRRs (Martin 2012). Indeed, for metallic

contact allergens such as nickel the direct activation of TLR4 on the DC surface by complexation was already described (Schmidt et al. 2010). Metabolic reprogramming in case of TLR-dependent activation of DCs by contact allergens should be expected. However, metabolic alterations of DCs have merely been experimentally addressed in the context of sensitization to contact allergens.

### 1.5. The Keap1/Nrf2 pathway

The Keap1/Nrf2 pathway plays a central role in the protection of cells against oxidative or electrophilic stress induced by a multitude of stressors including xenobiotics, heavy metals and ionizing radiation (Kaspar et al. 2009; Ma 2013). Beyond its role as a regulator of the cellular stress response the Keap1/Nrf2 pathway affects fundamental cellular processes like metabolism (Mitsuishi et al. 2012), cell growth (Malhotra et al. 2010) and protein degradation (Kwak et al. 2003). Furthermore, multiple evidence supports an immune regulatory function of the Keap1/Nrf2 pathway (Thimmulappa et al. 2006; Kobayashi et al. 2016; Eitas et al. 2017).

In the steady state, the transcription factor Nrf2 is constantly expressed in human cells. However, Nrf2 is bound to the E3 ubiquitin ligase Kelch-like ECH-associated protein 1 (Keap1) leading to ongoing ubiquitination and proteasomal degradation of Nrf2 (Figure 3). Upon oxidation of specific cysteine sulfhydryl groups, Keap1 undergoes conformational changes and subsequently releases Nrf2 (Kobayashi et al. 2006). Following transfer to the nucleus, Nrf2 binds to so-called antioxidant response elements (ARE) of the cellular DNA. This enhances the expression of cytoprotective target genes mainly involved in phase II metabolism and the oxidative stress response.

The Keap1/Nrf2 pathway is of special interest in the context of ACD (Peiser et al. 2012). Due to their electrophilic properties, contact allergens may activate the Keap1/Nrf2 pathway in keratinocytes, DCs and DC-like cells. For instance, contact allergens can induce the expression of canonical Nrf2 targets like NAD(P)H dehydrogenase, quinone 1 (NQO1) or heme oxygenase 1 (HMOX1) in primary DCs and the monocytic cell line THP-1 (Ade et al. 2009). An immune regulatory function of Nrf2 during the response to contact allergens was also indicated in several studies. Nrf2 may be involved in the modulation of cytokine secretion by keratinocytes (van der Veen et al. 2014) and THP-1 cells (Lewis et al. 2006) treated with contact allergens. Furthermore, Nrf2 influences the expression of co-stimulatory molecules on the DC surface (Yeang et al. 2012). An

important role of Nrf2 for the systemic suppression of skin sensitization is indicated by an aggravated skin inflammation Nrf2 knockout mice showed treated with contact allergens (van der Veen et al. 2013; El Ali et al. 2013). Despite the manifold evidence for a functional role of Nrf2 during skin sensitization by contact allergens, further examination needed to clarify the underlying mechanisms.

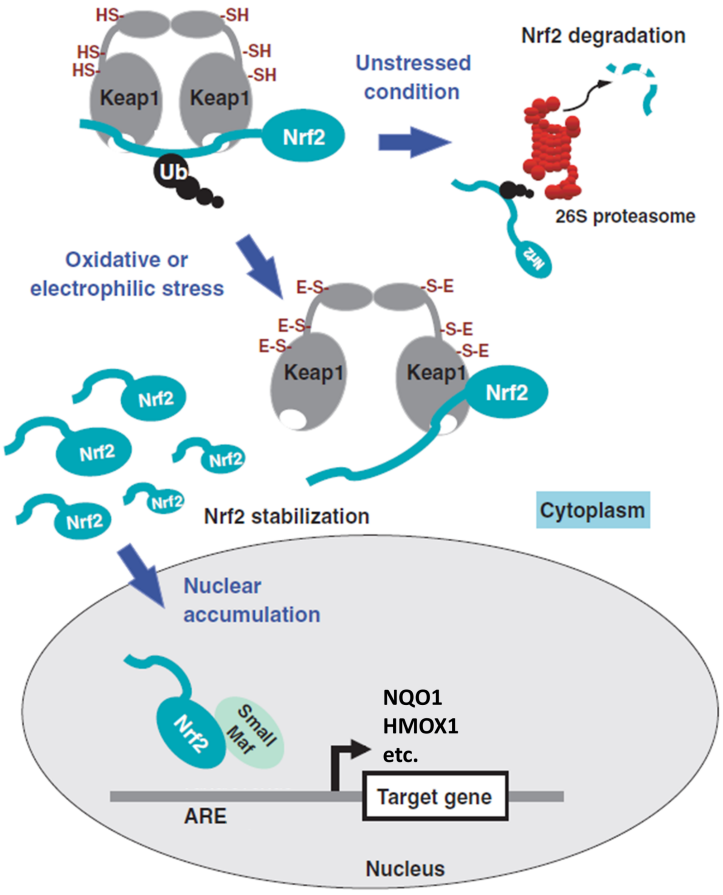


**Figure 3 The Keap1/Nrf2 pathway.** The transcription factor Nrf2 plays a central role in the response to oxidative and electrophilic stresses. Binding to Keap1 under unstressed conditions mediates constant degradation of Nrf2 via the ubiquitin-proteasome pathway. Oxidative or electrophilic stressors induce conformational changes of Keap1. Unbound Nrf2 translocates into the nucleus. Heterodimerization with small Maf proteins and binding to antioxidant response elements (ARE) induces the expression of cytoprotective target genes, *e.g.* NQO1, HMOX1 (Taguchi et al. 2011, modified).

### 1.6. Regulatory testing of contact allergens

The lack of possibilities to cure ACD restricts therapies to symptomatic treatments and forces regulatory authorities to set up effective strategies for hazard identification and removal. The testing of substances for their sensitizing properties is therefore of high importance for occupational and consumer safety.

Strategies for the identification of contact allergens have relied on animal testing for decades. For a long time, the guinea pig represented the animal of choice for *in vivo* testing. Still, the Bühler test and the guinea pig maximization test (GPMT) are of high importance (OECD 1992). Nowadays, the murine local lymph node assay (LLNA) represents the gold standard for the identification and classification of contact allergens. The LLNA measures the proliferation of lymphocytes in the draining lymph nodes in mice (Gerberick et al. 2007; OECD 2010). Besides the identification of contact allergens animal testing enables their classification regarding their relative potency to elicit skin sensitization (Kimber et al. 2003).

However, the European Union (EU) prohibits *in vivo* testing of cosmetic ingredients since 2013 (European Commission 2009). Furthermore, the EU promotes the application of alternative test methods to meet the high demands in the context of Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (European Commission 2006). Both EU regulations include the testing for skin sensitization. Thus, the availability of robust and predictive alternative test methods for skin sensitization is growing in importance.

As the development of ACD represents a rather complex process, an integrated testing strategy of alternative methods is commonly regarded as a prerequisite to cover the disease sufficiently without using animals (Adler et al. 2011). To this end, the Organization for Economic Co-Operation and Development (OECD) formulated the adverse outcome pathway (AOP) for skin sensitization as a basis for the development of alternative test methods (OECD 2014). This AOP defines the activation of DCs as one of four key events during skin sensitization. Thus, the activation of DCs by contact allergens is regarded as a valuable target for the development of *in vitro* testing methods (Roggen 2014). For research on DC activation, human or murine primary DCs can be isolated from blood or spleen. Alternatively, DC precursors, *e.g.* monocytes or bone marrow-derived cells, can be differentiated into DC-like cells *in vitro*. However, low cell numbers, laborious cell culture conditions and/or donor variability of primary DC models

make cell lines an invaluable option (van Helden et al. 2008). *In vitro* assays monitoring DC activation by contact allergens commonly use myeloid leukemia-derived cell lines of monocytic character as surrogates for DCs. For instance, the *Myeloid U937 Skin Sensitization Test* (MUSST) measures the expression of CD86 by U937 cells after application of test substances (Natsch et al. 2013). Another example is the *Genomic Allergen Rapid Detection* (GARD) assay that quantifies a genomic signature of about 200 genes using MUTZ-3 cells as a DC model (Johansson et al. 2013). The *Human Gell Activation Test* (h-CLAT) represents the best-established alternative assay addressing the key event of DC activation (Ashikaga et al. 2006). This *in vitro* assay uses the monocytic THP-1 cell line as a surrogate for DCs and measures the expression of CD54 and CD86 after treatment with test substances. During their activation, THP-1 cells exhibit properties of activated monocytes, macrophages and DCs in response to immunogenic stimuli. Therefore, THP-1 cells serve as a well-established *in vitro* model for MPS cells in immunotoxicology. After validation by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM 2015), the h-CLAT was adopted as an OECD test guideline (OECD 2016). In an integrated testing battery, the h-CLAT can be combined with two other validated alternative assays for the detection of contact allergens (Urbisch et al. 2016). The Direct Peptide Activity Assay (DPRA) addresses the key event of protein haptenation and quantifies covalent binding of test substances to model peptides *in chemico* (Gerberick et al. 2004; OECD 2015a). The KeratinoSensM assay addresses the activation of keratinocytes *in vitro* by quantifying the expression of an ARE-luciferase reporter gene construct indicating Nrf2 activity (Emter et al. 2010; OECD 2015b).

Despite the ongoing progress, the development of alternative testing methods for contact allergens still faces certain challenges. Potency assessment and correct prediction of pre- and prohaptens still enforce improvement of existing testing strategies. A possible strategy to overcome current limitations of *in vitro* sensitization testing is to identify additional biomarkers for sensitization by contact allergens (Wong et al. 2015).

## 1.7. Omics technologies

Omics technologies have been identified as valuable tools to meet the challenges of modern toxicology (Hartung 2009). Omics-based approaches, such as transcriptomics, proteomics or metabolomics, are well suited to uncover novel biomarkers, biomarker signatures and toxicity pathways. Notably, the implementation of multi-omics approaches may support the development of alternative testing strategies (McBride 2017).

Transcriptomics was the first field of omics technologies to quantify cellular gene expression. The possibility to translate messenger RNA (mRNA) transcripts into stable complementary DNA (cDNA) that can be multiplied using techniques based on polymerase chain reaction (PCR) in combination with DNA sequencing makes transcriptomics sensitive and relatively convenient. Several studies analyzed transcriptome alterations during the activation of DCs by contact allergens (Ryan et al. 2004; Ott et al. 2010; Johansson et al. 2011). Furthermore, transcriptomics analysis in this context already entered the field of regulatory toxicology as the OECD Test Guideline Programme initiated formal validation of the GARD assay. However, the expression of mRNA transcripts not necessarily correlates with phenotypic gene expression on the proteome or metabolome level (Maier et al. 2009; Hoppe 2012).

Therefore, the use of proteomics or metabolomics techniques may provide a more precise description of a biological system's phenotypical state. However, these techniques are rather elaborate in terms of sample preparation and analytics. Both fields of omics rely on the usage of mass spectrometry (MS) for the identification and quantification of their respective target class of macromolecules.

In proteomics, the identification of proteins is usually connected to enzymatic digestion. In most cases, the resulting peptides are then separated by liquid chromatography (LC). Typically, mass-to-charge ratio (m/z) of peptides and peptide fragments are determined using matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) in combination with tandem mass spectrometry (MS/MS) techniques. Proteins are identified by comparison of the measurement results to databases providing predicted m/z ratios for the enzymatic digestion and MS/MS technique applied. Protein quantification can be achieved by methods based on sample separation in a gel matrix and/or isotope labeling of proteins before MS analysis. The most successful example for gel based methods is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Stable isotope labeling with amino acids in cell culture (SILAC) is a prominent example of the latter one. 2D-PAGE separates protein extracts stepwise by isoelectric point and molecular weight (Berth et al. 2007). After staining with protein-reactive dyes, the resulting protein spot patterns can be compared quantitatively using image analysis software before proteins from regulated spots are identified. SILAC relies on the incorporation of different isotope labelled amino acids during cell culture (Ong 2012). Cells incorporate amino acids into proteins independently of the specific isotopes used. Therefore, differently treated sample groups cultivated in different cell culture media can be unified before further analysis. During MS analysis, isotope labelling leads to peptide pairs of a specific mass shift between differently labelled sample groups. These peptide pairs can be relatively quantified by comparison of their peak intensities in the mass spectrum. Protein quantification then relies on integration of peptide ratios. Several studies applied quantitative proteomics successfully to analyze protein expression during DC activation (Pereira et al. 2005; Ferret-Bernard et al. 2008; Ceppi et al. 2009). However, only recently proteomics techniques were applied to investigate the DC activation by contact allergens (Mussotter et al. 2016; Zwicker et al. 2016). An exhaustive overview on the use of proteomics techniques in research on ACD is given in Chapter 3.1.

Metabolomics strives for the parallel analysis of a plethora of metabolites that may belong to several classes, *e.g.* lipids, amino acids or carbohydrates. Therefore, it is challenging to set up analytical strategies that meet the varying physicochemical properties of the target molecules. In metabolomics, LC, gas chromatography (GC) or flow injection analysis (FIA) coupled to MS/MS are mostly used for metabolite identification and quantification. Metabolomics approaches can be classified as targeted or untargeted. Untargeted metabolomics means to analyze the metabolome exhaustively using high resolution MS and elaborative data analysis. Targeted metabolomics examines a defined set of metabolites offering robust metabolite analysis less of an effort. Targeted metabolomics demonstrates its strength especially when promising target metabolite classes were identified before and in combination with additional (omics) techniques (Johnson et al. 2016).

Especially proteomics and metabolomics are regarded as invaluable in regulatory toxicology for the identification of biomarkers and toxicity pathways (Bouhifd et al. 2013; Johnson et al. 2016). Consequently, proteomics and metabolomics are progressively employed to study the mechanisms underlying the development of ACD (Zinkeviciene et

al. 2016; Zwicker et al. 2016; Jakob et al. 2017). Nevertheless, the use of proteomics and metabolomics approaches in research on DC activation by contact allergens is only emerging.

## 2. Objective

ACD represents a widespread hypersensitivity reaction of the human skin. Contact allergens are prevalent in all kinds of consumer products. Therefore, identification and containment of contact allergens is of high relevance for consumer protection. The activation of DCs during sensitization by contact allergens represents a key event during the sensitization to contact allergens. A deeper understanding of the mechanisms of DC activation by contact allergens may support the identification of novel biomarkers that advance the development of integrated alternative testing strategies.

The objective of the thesis on hand was to contribute to the understanding of sensitization to contact allergens. To this end, the responses of DC models to contact allergens were investigated on the molecular level. Different omics techniques were applied to identify differentially regulated biomolecules in DC models responding to contact allergens or irritant chemicals. The subordinated aims were the identification of significant pathways and valuable biomarker candidates for the activation of DC models by contact allergens *in vitro*.

In the first study, murine bone marrow derived DCs (BMDCs) treated with different contact allergens were analyzed using a gel-based proteomics approach. Data derived from wild-type and knockout mouse models was compared to further investigate the role of Nrf2 during this process.

The aim of the second study was to study the response of THP-1 cells as a DC surrogate after treatment with the strong contact allergen 2,4-dinitrochlorobenzene (DNCB). Based on findings of the first study, this study focused on the metabolic state of activated THP-1 cells using a multi-omics approach combining gel-free proteomics and targeted metabolomics.

# 3. Results

## 3.1. Application of proteomics in the elucidation of chemical-mediated allergic contact dermatitis

Summary:

The scope of this review is the application of proteomics in research on ACD. As an introduction, it gives an overview on the molecular and cellular mechanisms of ACD. Consecutively, toxicological concerns regarding animal and alternative testing of chemicals for skin sensitizing properties are summarized. After referring to general considerations on proteomics, the review outlines proteomics studies investigating key events of skin sensitization, including haptenation and the activation of keratinocytes and T cells. In the last part, the review summarizes applications of proteomics techniques to investigate the activation and maturation of DCs. Here, the review focusses on studies investigating DC models in the context of ACD.

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The author's contributions:

Preparation of manuscript and figures: 40 %

* Authors contributed equally.

## 3.2. Proteomics analysis of dendritic cell activation by contact allergens reveals possible biomarkers regulated by Nrf2

Summary:

In this study, the response of BMDCs after treatment with contact allergens was analyzed by quantitative proteomics. BMDCs of wild-type (*nrf2*$^{+/+}$) and Nrf2 knockout (*nrf2*$^{-/-}$) mice were treated with two doses each of model contact allergens of different potencies, *i.e.* the strong contact allergen DNCB and the moderate contact allergens cinnamaldehyde (CA) and nickel(II) sulfate ($NiSO_4$). Treatment with sodium dodecyl sulfate (SDS) was included to discriminate against the response to an irritant chemical. Sample protein extracts were separated by 2D-PAGE. Differentially regulated protein spots between sample groups were identified by comparative image analysis. Extracted proteins from regulated protein spots were identified by electrospray ionization tandem mass spectrometry (ESI-MS/MS).

Concluding from regulated protein spot numbers, treatments with CA and DNCB induced significant perturbations on the proteome level that were dependent on potency and dosage of the contact allergen applied. In comparison, $NiSO_4$ and SDS provoked protein changes to a lesser extent and were missing dose-dependency. Significantly regulated protein spots in *nrf2*$^{+/+}$ samples were compared to respective protein spots in *nrf2*$^{-/-}$ samples. For all treatments applied, most of these protein spots were regulated in an Nrf2-dependent manner indicating Nrf2-dependent regulation of the respective proteins.

A set of 100 proteins from regulated spots in nfr2$^{+/+}$ could be identified by ESI-MS/MS. Identification of oxidative stress response proteins confirmed Nrf2-dependent induction of canonical Nrf2 targets after treatment with contact allergens but not with the irritant SDS. Additionally, three Nrf2 targets could be confirmed on the protein level for the first time. Furthermore, several putative Nrf2 targets could be identified.

Protein identification and subsequent pathway analysis confirmed the perturbation of basic cellular pathways involved in DC activation by contact allergens including unfolded protein response, cell signaling, protein translation and re-organization of the cytoskeleton. Remarkably, contact allergens induced several glycolytic proteins indicating an increased glucose consumption by DCs in response to contact allergens.

In summary, pathway analysis indicated Nrf2-dependent induction of stress response mechanisms and cell survival.

Integration of literature information led to the identification of promising biomarker candidates by concentrating the data set to proteins solely regulated after treatment with contact allergens that were reported before to be Nrf2-dependent involved in processes of DC functioning.

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The author's contributions:

Project planning: 40 %

Experimental work: 70 %

Evaluation and interpretation of experimental data: 80 %

Preparation of manuscript and figures: 80 %

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

Summary:

In this study, an integrated approach combining proteomics and metabolomics was employed to investigate the metabolic state of human THP-1 cells in response to the strong contact allergen DNCB. Cells were treated with three concentrations of DNCB for 4, 8, and 24 h, respectively. SDS served as irritant control. A targeted metabolomics approach allowed for the quantification of 188 endogenous metabolites including phospholipids, acylcarnitines, amino acids and hexoses. Metabolites were analyzed by LC-MS/MS and FIA-MS/MS. Untargeted quantitative proteomics analysis was based on SILAC. Proteins were identified and quantified using MALDI-TOF-MS/MS.

Multivariate analysis of metabolomics data proved separation of treatment groups from each other for all time-points. Hierarchical cluster analysis revealed clustering of different metabolite classes separating amino acids and short-chained acylcarnitines from phospholipids and long-chained acylcarnitines in a time- and treatment-dependent manner. Ranking of metabolites after Jonckheere-Terpstra testing indicated a shift from the regulation of mainly biogenic amines, acylcarnitines and amino acids after 4 h and 8 h to mainly phosphatidylcholines after 24 h. Analysis of the THP-1 proteome revealed regulation of proteins mainly after 24 h. Regulated proteins involved fatty acid synthase, a key enzyme of lipid synthesis.

Integration of significantly regulated metabolites and proteins helped to identify relevant pathways. In summary, consistent alterations in the metabolome and the proteome indicated metabolic reprogramming of THP-1 cells resulting in augmented lipid synthesis in response to treatment with DNCB but not SDS. XY plotting of significantly regulated metabolites supported the identification of biomarker candidates for the identification of contact allergens *in vitro*.

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

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[a] 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; CPTl, 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, endoplasmin; 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; Hl, sum of hexoses; h-CLAT, human cell line activation test; HEPES, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; HNRHl, heterogeneous nuclear ribonucleoprotein H; iNOS, inducible nitric oxide synthase KPYM, Pyruvate kinase isozymes Ml/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; PDIAl, 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.

**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-l cells in response to the strong contact allergen, 2,4-dinitrochlorobenzene (DNCB). Cells were treated with 5, l0 and 20 µM DNCB for 4, 8, and 24 h, respectively. Sodium dodecyl sulfate (SDS) at l00 µM served as irritant control. Using a targeted metabolomics approach, we quantified levels of l88 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-l cells by DNCB but not SDS. 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.

**Keywords:** Allergic Contact Dermatitis · Sensitization · THP-l cells · Metabolic reprogramming · Metabolomics · Proteomics

## 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., 20l2).

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., 20l4). 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., 20ll).

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, l998). The field of immunometabolism is currently gaining attention as knowledge is increasing on how metabolic states co-decide about the fate and activity of immune cells like monocytes, macrophages, and DCs (O'Neill et al., 20l6). Together, these cell types constitute the mononuclear phagocyte system (MPS) (Guilliams et al., 20l4). 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., 20l6). Accordingly, metabolic reprogramming could be

detected in primary monocytes (Lachmandas et al., 20l6), macrophages (Jha et al., 20l5) and DCs (Rehman et al., 20l3) 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., 20l0). Consistent with this, we recently provided evidence for metabolic reprogramming in DCs activated by selected electrophilically reactive contact allergens (Mussotter et al., 20l6).

Mechanisms of DC activation have proven a promising target for the development of *in vitro* testing methods (Roggen, 20l4). One prominent example is the human Cell Line Activation Test (h-CLAT) which uses the monocytic THP-l 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, 20l5; OECD, 20l6). 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., 20l5). 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., 20l7; Johansson et al., 20l3; Zwicker et al., 20l6).

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-l cells by the contact allergen 2,4-dinitrochlorobenzene (DNCB) compared to the response obtained upon treatment with sodium dodecyl sulfate (SDS). THP-l cells were exposed to three concentrations of the contact allergens DNCB (5, l0 and 20 µM) and one concentration of the irritant SDS (l00 µM) 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.

## Materials and Methods

### 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 RPMI1640 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 (1mM) 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 $1x10^5$ cells per ml in T75 flasks. Cells were incubated in a standard incubator at 37 °C, 5 % $CO_2$ and 95 % humidity. For SILAC experiments, RPMI media were supplemented with 10 % (v/v) dialyzed FBS obtained from Biochrom. Carbon-12 ($^{12}C$) and Carbon-13 ($^{13}C$) labeled L-lysine and L-arginine were obtained from Silantes (Munich, Germany) and added in the same concentrations as in standard RPMI medium composition.

### 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 ($1x10^5$ cells per well at $1x10^6$ per ml). Cells were incubated for 24 h with ascending concentrations of DNCB and SDS (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 %. SDS dissolved in phosphate-buffered saline (PBS; PAN-Biotec, Aidenbach, Germany). 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).

Assessment of cell activation

Expression of cell surface markers CD86 and CD54 was observed by flow cytometry. THP-l were treated as stated above for 24 h, then harvested by centrifugation (200 x 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). l0,000 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 (Vl0; FlowJo, LLC, Ashland, Oregon, USA).

Cell culture for metabolomics experiments

THP-l cells were seeded and directly treated in T25 flasks in standard cell culture medium at $lxl0^6$ cells per ml. Cells were harvested by centrifugation 4, 8 and 24 h after treatment (200 x 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 (l55 mM, pH 7.4). Supernatants were removed after centrifugation (200 x 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 (l2,000 x g, 30 min, 4 °C) and analyzed immediately using the Biocrates AbsoluteIDQ® pl80 Kit (Biocrates, Innsbruck, Austria).

Targeted metabolomics approach

In total, l88 metabolites were analyzed, including amino acids (AAs) and biogenic amines (BAs), acylcarnitines (ACs), the sum of hexoses (90 - 95 % glucose; Hl), 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., 20l2).

The AbsoluteIDQ® pl80 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 (l:l0, v/v). For flow injection analysis-tandem mass spectrometry (FIA-MS/MS) analysis, Biocrates' kit running solvent was used (l:50, v/v). Extracts were analyzed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) using an Agilent l200 Series HPLC (Agilent, Waldbronn, Germany) coupled to a QTRAP 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) controlled by the Analyst l.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-Cl8 column (3 x l00 mm, 3.5 µm; Agilent, Waldbronn, Germany). The FIA-MS/MS procedure was used for the quantification of ACs, PCaa's, PCae's, LPCs, SMs and Hl 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.

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 l23 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 l00 %.

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 (V2l; IBM Analytics, Ehningen, Germany) including Z-score normalization and Bonferroni correction (Potratz et al., 20l6). The data set was split by time points. Metabolites with a significant group difference ($p < 0.00l$) 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 (Vl4; MKS Data Analytics Solutions, Umeä, 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, l0 µM DNCB, 20 µM DNCB and the other three conditions (untreated controls, vehicle controls, l00 µM

SDS). 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 l23 analyzed metabolites was performed using the Bonferroni method for control of the family-wise error rate. *P*-values < 0.05 were considered significant.

Cell culture for proteomics experiments

THP-l cells were grown in SILAC media for 7 days before treatments yielding incorporation rates of > 95 %. Controls were grown in $^{l3}C$ medium, treatment groups in $^{l2}C$ medium. THP-l cells were seeded and directly treated in T25 flasks in standard cell culture medium at lxl0$^6$ cells per ml. Cells were harvested by centrifugation 4 h, 8 h and 24 h after treatment (200 x 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 l:l.

Proteomics analyses

Sample preparation followed the filter-aided sample preparation (FASP) protocol (Wisniewski et al., 2009). In short, cells were lysed in 4 % (w/v) SDS, l00 mM Tris/HCl pH 7.6, 0.l M dithiothreitol (DTT) at 95 °C for 5 min. DNA was sheared by sonication and lysates clarified by centrifugation (l6,000 x g; 5 min; RT). Lysates were mixed with UA buffer (8 M urea in 0.l 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 (l4,000 x g; l5 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 $NH_4HCO_3$). 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.l % trifluoroacetic acid (TFA; ratio l:25). Peptide yields were monitored using the Protein A280 method of a NanoDrop l000 Spectrophotometer v3.7 (Thermo Fisher Scientific, Wilmington, DE).

Peptides were purified using Cl8 ZipTip® pipette tips (Merck Millipore; Darmstadt, Germany). In short, ZipTips® were prepared by sequential washing with acetonitrile (ACN), 7:3 ACN/0.l % TFA and 0,l % TFA. Subsequently, peptides were loaded on ZipTips® and washed with 0.l % TFA. Peptides were eluted with 7:3 ACN/0.l % TFA.

Purified peptides were separated using a Dionex UltiMate 3000 RSLCnano system equipped with an Acclaim® PepMapl00 Cl8, 3 µm, l00 A, Separation Bed 75 µm i.d. x 2 cm trap column and an Acclaim® PepMap™ RSLC Cl8, 2 µm, l00 A, 75 µm i.d. x 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.l % 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-l0 % B for **1** min; l0-50 % B for l64 min; 50-95 % B for **1** min; 95 % B for 20 min; 95-4 % B for **1** min; 4 % B for 20 min. l5 s fractions were collected and mixed with matrix solution (90 % ACN; 4.5 % of a solution saturated with a-cyano-4-hydroxycinnamic acid; 0.l % 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 l:200 in calibrant matrix solution (80 % ACN; 4.5 % of a solution saturated with a-cyano-4-hydroxycinnamic acid; 0.l % 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 >l0 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:$^{l3}$C(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 l.0 %, significance threshold was set to $p < 0.05$. Mascot's ion score threshold for significant peptide identification was set to l5.0. At least two significant 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.

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 > l.3 (standard deviation (SD) of < 0.l). 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 30 of the 36 measured samples hierarchical clustering was performed using Pearson correlations as similarity measure and the average linkage method to calculate the distance between clusters.

## Results

Dose selection for omics studies and monitoring of THP-l cell activation

To select appropriate doses of DNCB and SDS, the cytotoxicity was assessed using the MTT assay in regular and in SILAC RPMI medium (Figure Sl). For DNCB two clearly non-toxic doses, i.e. 5 µM (cell viability comparable to controls), l0 µM (90 % cell viability compared to controls), and one slightly cytotoxic dose, i.e. 20 µM (75 % cell viability compared to controls) have been selected. For SDS one concentration, i.e. l00 µM, was chosen, which was not cytotoxic (90% cell viability compared to controls).

THP-l cell activation was monitored by flow cytometry. Treatment with l0 µM and 20 µM DNCB resulted in significant up-regulation of the surface activation markers CD86 and CD54, whereas untreated cells, vehicle controls, 5 µM DNCB or SDS treatment did not modulate surface marker expression (Figure S2).

Metabolomics analysis

Metabolic changes during THP-l 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 :: 0.00l). This resulted in 42, 3l and 74 metabolites after 4 h, 8 h and 24 h, respectively. Subsequent PCA analysis revealed a sound separation of samples treated with 20 µM DNCB at all time points, whereas controls without treatment and vehicle controls clustered together. Samples treated with l0 µM DNCB were completely separated after 4 h. Despite a partial overlap with controls, 5 µM DNCB and SDS samples, the l0 µM DNCB sample group still separated reasonably well after 8 h and 24 h. Cells treated with 5 µM DNCB mainly clustered with the control groups. SDS samples largely separated from controls and DNCB samples after 4 h and 8 h (Figure l).

Hierarchical cluster analysis revealed grouping of different metabolite classes in prominent clusters (Figure 2). In general, amino acids and short-chain acylcarnitines (cluster A) 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 and revealing lowest levels after 24 h at DNCB 20 µM (clusters 3, 5 and 7). In SDS treated samples, these metabolite classes seemed to be induced after 24 h. These samples were assigned to clusters 2 and 6 for SDS treatment due to slight differences in phospholipid levels. 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 5 and 6). These metabolites remained largely unchanged in SDS treated samples (cluster 2-4). Untreated samples, vehicle controls and 5 µM DNCB did not induce frequent significant alterations of metabolite levels although some exceptions were detected. For instance, a decrease of phosphatidylcholines and lysophosphatidylcholines led to clustering of samples mainly belonging to these treatment groups and samples treated with SDS for 8 h (cluster l). Indifferencies 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 (Figure S3).

In addition, we used the Jonckheere-Terpstra test to identify significantly regulated metabolites. Time point-separated rankings were compiled sorting metabolites from lowest to highest *p*-values (Table 1 and Table Sl). While after 4 h and 8 h mainly biogenic amines, acylcarnitines and amino acids belonged to the top l0 metabolites, phosphatidylcholines dominated after 24 h.

Figure 3 contains exemplary metabolite profiles that will be discussed in detail below. A compilation of all l23 metabolites that were statistically analyzed is given in the supplementary section (Figure S4).

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

Proteomics analysis

THP-l cells were treated in the same manner as done for the metabolomics experiments (cf. above). Proteins with at least l.3-fold up- or down-regulation and a standard deviation below 0.l were regarded as significantly regulated. However, only treatment with 20 µM 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 (PDIAl), heterogeneous nuclear ribonucleoprotein H (HNRHl) and endoplasmin (ENPL). Pyruvate kinase isozymes Ml/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 (Figure S5). Selected protein profiles are depicted in Figure 5. Complete protein expression data are given in the supplementary section.

**Discussion**

Using THP-l cells as a model, we could detect characteristic changes in the metabolome after treatment with the strong contact allergen DNCB compared to treatment with the irritant SDS and to controls. Metabolic reprogramming has already been reported for the activation of MPS cells in general (O'Neill and Pearce, 20l6), but has yet not been investigated in the context of ACD. Our study provides evidence for metabolic reprogramming of THP-l cells upon activation by the contact allergen DNCB but not after treatment with the irritant SDS. Based on the changes in the metabolome we could largely separate the l0 and 20 µm DNCB treatments from SDS and control treatments using PCA.

We could identify characteristic profiles of regulated metabolites for DNCB treatments that clearly differed from SDS treated samples. For example, the biogenic amines spermine and taurine as well as the long-chain phosphatidylcholines PCae_C42:l and PCae_C44:4 responded concentration- and/or time-dependently to DNCB treatment (Figure 3 and Figure S4). XY plotting of these metabolites confirmed their capability to separate these samples (Figure 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 l0 µM DNCB. However, induction of the surface markers CD86 and CD54 confirmed THP-activation at both DNCB doses. Additionally, phospholipid levels increased at the medium DNCB dose, indicating the capability of THP-l cells to perform the necessary metabolic reactions. Significant changes on the proteome level only occurred after treatment with 20 µM DNCB. Possibly, THP-l cells in

the steady state provide sufficient metabolic enzymes to elicit the less pronounced changes on the metabolome level after treatment with l0 µM DNCB. Notably, weak responses of THP-l cells on the proteome level after treatment with contact allergens was reported before (Zwicker et al., 20l6). Sub-cellular fractionation of THP-l 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-l cells by treatment with DNCB had an impact on the overall intracellular hexose (Hl) levels. Especially incubation with 20 µM DNCB led to a significant drop in Hl 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 and SDS treated 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., 20l6). 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 Ml macrophages (Lachmandas et al., 20l6; O'Neill and Pearce, 20l6). 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, 20l5). 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 (CPTl) 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 CPTl represents a rate-limiting step in FAO (Houten and Wanders, 20l0). Therefore, a decrease of short-chain acylcarnitines indicates diminished FAO and provides further evidence for a diminished OXPHOS after THP-l activation.

In addition, we provide evidence that activation of THP-l 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., 20l4). 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., 20l0). BMDCs and macrophages exhibited an up-regulation of FAS and other proteins involved in lipid metabolism during differentiation (Becker et al., 20l2). 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., 20ll). Other recent studies also reported a dominance of phosphatidylcholines in MPS cell models (Ecker et al., 20l0; Martins et al., 20l6; Santinha et al., 20l2). 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 a-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. Spermidine was also induced to some extent after treatment with SDS. 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 PDIAl 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, 20l5). UPR can also influence lipid synthesis and antigen presentation (Grootjans et al., 20l6). PDI isoforms have also been found induced by the contact allergens DNCB and CA during BMDC activation in our previous study (Mussotter et al., 20l6).

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, KPYM - the key enzyme of glycolysis - was down-regulated. This is consistent with findings in LPS-activated THP-l 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., 20l2, 20ll).

**Conclusion**

We identified several characteristic metabolic changes that indicate metabolic reprogramming in THP-l 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 provides 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-l 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:l 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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## Figures

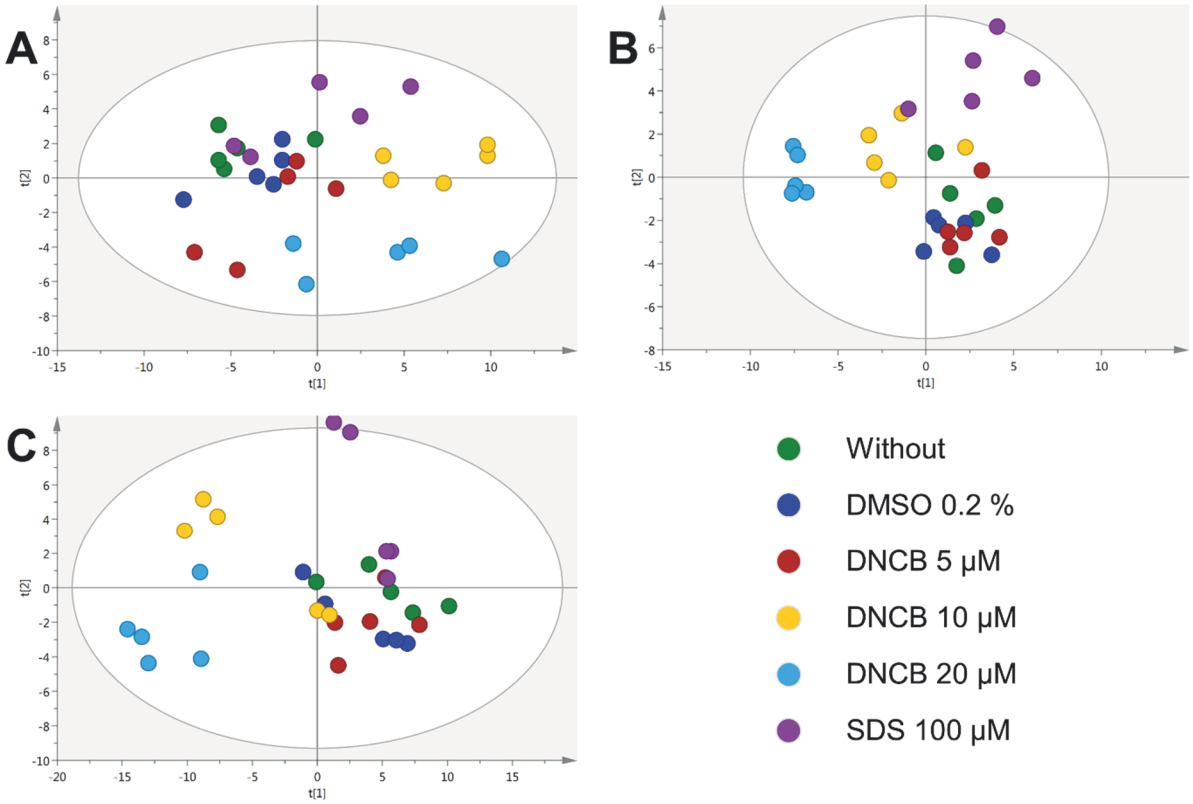


**Figure 1.** Principal component analysis (PCA) of metabolomics data. Metabolites that were significantly affected by one of the treatments, i.e. those with a *p*-value :: 0.00l according to a multivariate analysis of variance (MANOVA) using IBM SPSS Statistics (V2l), were subjected to PCA performed with SIMCA-P+ (Vl4). PCA was performed for each time point individually with 4h 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; SDS, sodium dodecyl sulfate; WO, without treatment.

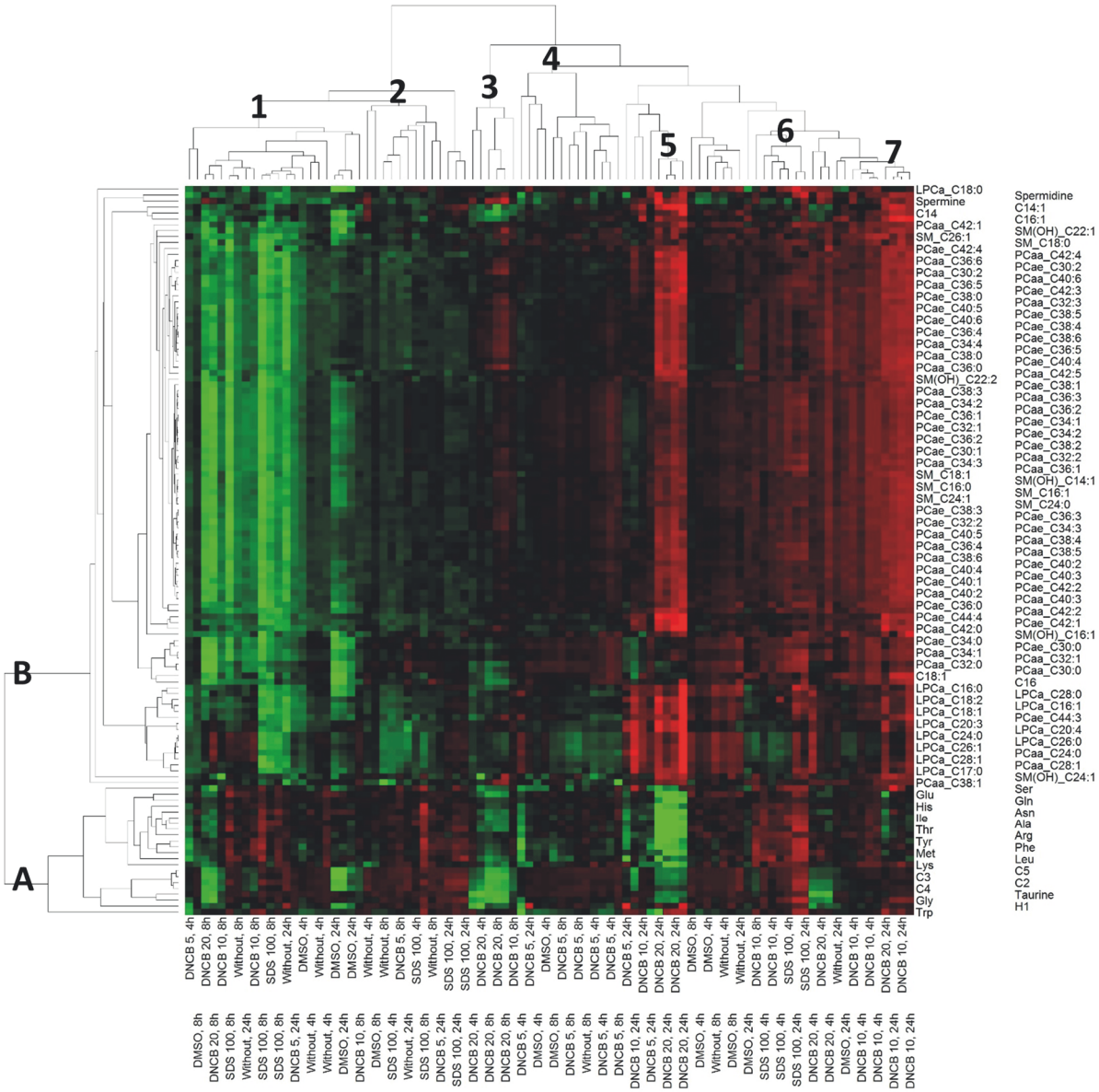


**Figure 2.** Hierarchical cluster analysis of metabolite levels. Relative metabolite levels were log2-transformed. Hierarchical clustering was performed using Pearson correlation as similarity measure and average linkage as a distance measure. Abbreviations: Ala, alanine; Arg, arginine; Asn, asparagine; DMSO, dimethyl sulfoxide; C_x:y, acylcarnitine; DNCB, 2,4-dinitrochlorobenzene; Gln, glutamine; Glu, glutamate; Gly, glycine; Hl, sum of hexoses (90 - 95 % glucose); His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; LPC_Cx:y, lysophosphatidylcholine; Met, methionine; PCaa_Cx:y, Phe, phenylalanine; phosphatidylcholine diacyl; PCae_Cx:y, phosphatidylcholine acyl-alkyl; Pro, proline; SDS, sodium dodecyl sulfate; Ser, serine; SM_Cx:y, sphingomyelin; SM(OH)_Cx:y,

hydroxysphingomyelin; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; WO, without treatment; x, number of carbon atoms in the lipid chain; y, number of double bonds. Green: down-regulated; red: up-regulated.

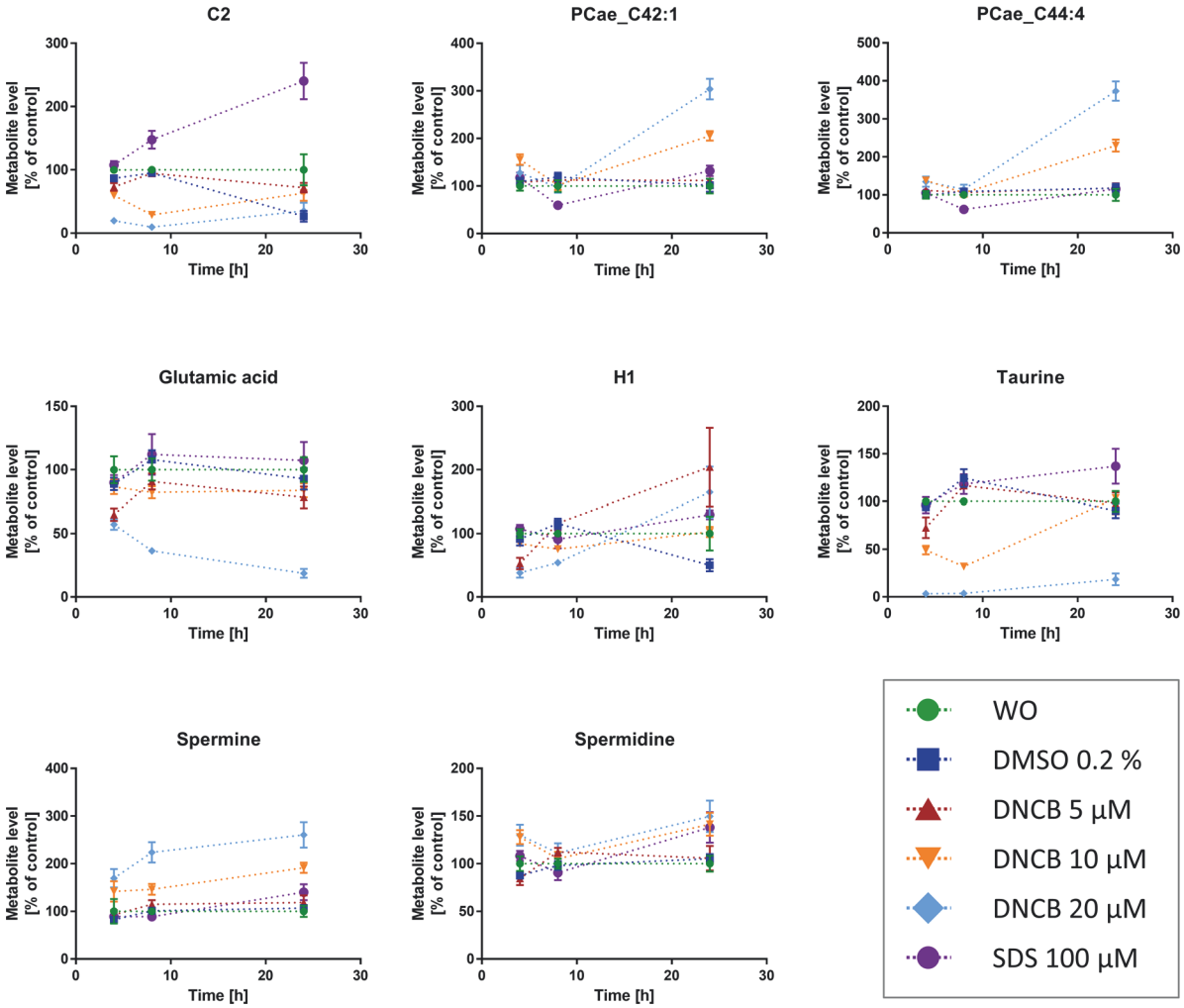


**Figure 3.** Selected metabolite profiles. Values represent percentage of untreated controls, which were set as l00 %. Error bars represent standard errors of the mean (SEM) (n=5). Abbreviations: DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; Hl, sum of hexoses (90 - 95 % glucose); PCae_Cx:y, phosphatidylcholine acyl-alkyl; SDS, sodium dodecyl sulfate; WO = without treatment; x, number of carbon atoms in the lipid chain; y, number of double bonds.

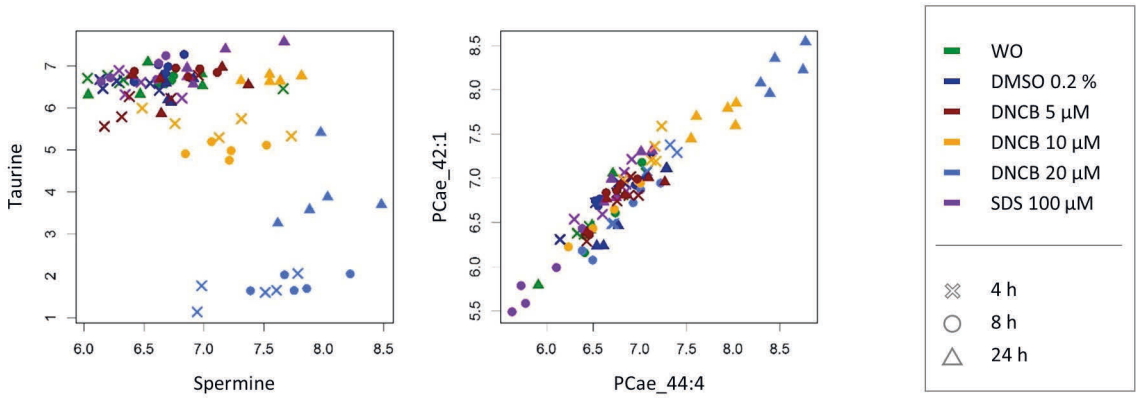


**Figure 4.** XY plots of significantly regulated metabolites. Plots of all time points were generated using the R programming language. Values represent log2-transformed metabolite levels. Abbreviations: DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; Hl, sum of hexoses (90 - 95 % glucose); PCae_Cx:y, phosphatidylcholine acyl-alkyl; SDS, sodium dodecyl sulfate; WO = without treatment, x, number of carbon atoms in the lipid chain; y, number of double bonds.

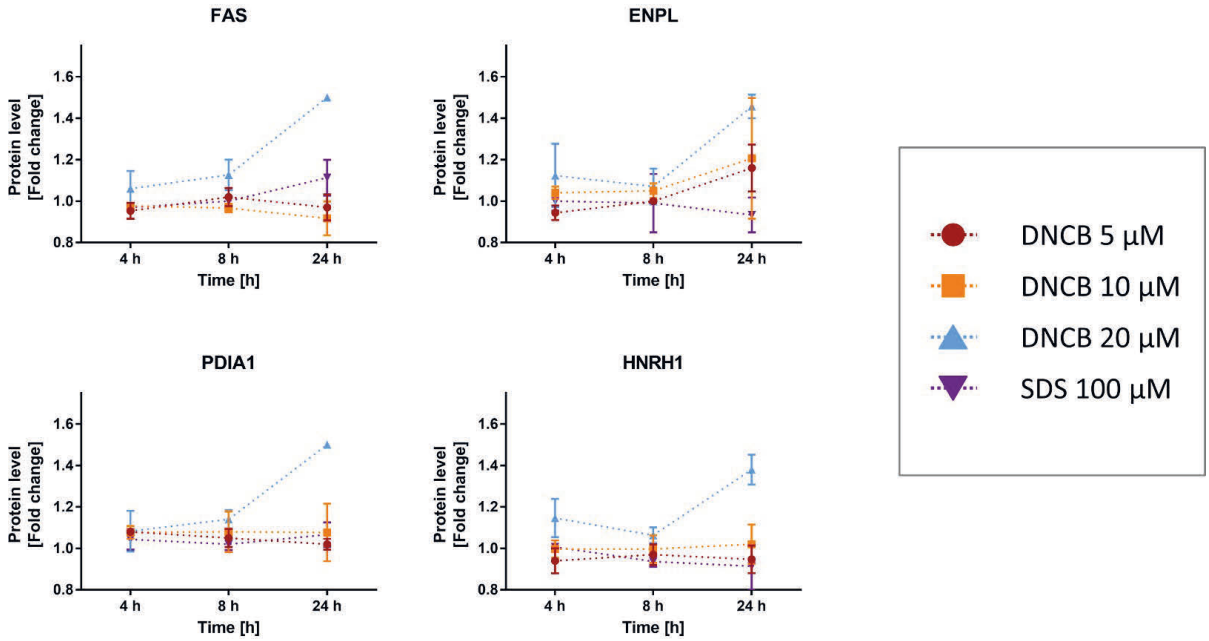


**Figure 5.** Expression profiles of selected regulated proteins. Values represent fold changes compared to controls. Error bars represent standard deviations (SD). Proteins with a regulation > l.3-fold (SD < 0.l) were regarded as significantly regulated. Abbreviations: DNCB, 2,4-dinitrochlorobenzene; ENPL, endoplasmin; FAS, fatty acid synthase; HNRHl, heterogeneous nuclear ribonucleoprotein H; PDIAl, protein disulfide isomerase; SDS, sodium dodecyl sulfate.

## Tables

**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 | 3.35E-08 | C2 | 3.28E-06 | Ala | 2.86E-05 |
| 2 | Taurine | 5.50E-07 | Spermine | 5.02E-06 | PCae_C44:4 | 9.40E-05 |
| 3 | C4 | 5.25E-05 | Glu | 7.0lE-03 | His | 9.40E-05 |
| 4 | C3 | 4.67E-03 | Taurine | 3.45E-02 | Ile | l.64E-04 |
| 5 | Hl | 5.76E-03 | Gly | 9.6lE-02 | Thr | l.64E-04 |
| 6 | PCaa_C38:0 | 7.06E-03 | C5 | 9.6lE-02 | PCaa_C42:2 | 3.63E-04 |
| 7 | Gly | 7.06E-03 | Asn | 3.52E-0l | PCae_C42:l | 6.0lE-04 |
| 8 | Spermine | l.05E-02 | Gln | 4.96E-0l | PCaa_C42:4 | 7.68E-04 |
| 9 | PCae_C38:6 | l.28E-02 | C3 | 4.96E-0l | Asn | 9.77E-04 |
| l0 | PCaa_C36:0 | l.55E-02 | C4 | 4.96E-0l | PCaa_C28:l | 9.77E-04 |

The Bonferroni method was used for multiple testing correction. *P*-values < 0.05 were considered significant. For complete lists, refer to Table Sl. Abbreviations: Ala, alanine; Asn, asparagine; C_x:y, acylcarnitine; Gln, glutamine; Glu, glutamate; Gly, glycine; Hl, sum of hexoses (90 - 95 % glucose); His, histidine; Ile, isoleucine; LPC_Cx:y, lysophosphatidylcholine; PCaa_Cx:y, phosphatidylcholine diacyl; PCae_Cx:y, phosphatidylcholine acyl-alkyl; Ser, serine; Thr, threonine; x, number of carbon atoms in the lipid chain; y, number of double bonds.

# 4. Discussion

In the first experimental study (Chapter 3.2.), alterations in the proteome of *nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ BMDCs after treatment with contact allergens were analyzed. This study was the first that quantitatively analyzed the response of DCs to several contact allergens in comparison to an irritant substance using proteomics. Proteins found regulated in this study confirmed results of proteomics studies investigating DC maturation before (Ferreira et al. 2008; Ferret-Bernard et al. 2008; Ceppi et al. 2009; Ferret-Bernard et al. 2012).

The proteomic alterations induced by contact allergens in *nrf2*$^{+/+}$ could be interpreted in the context of DC activation by contact allergens as many regulated proteins could be mapped to essential processes during phenotypical and functional DC maturation. In *nrf2*$^{+/+}$ BMDCs contact allergens induced manifold metabolic enzymes. Especially the up-regulation of glycolytic enzymes pointed to DC activation in response to treatment with contact allergens as the induction of glycolysis is the essential step during metabolic reprogramming of activated DCs (Park et al. 2006; Krawczyk et al. 2010). The up-regulation of proteins involved in gene transcription and protein translation supported the assumption that BMDCs increased protein translation rates in response to the treatment with contact allergens. Furthermore, contact allergens induced the expression of several chaperones and proteasomal constituents, which is an essential part of the UPR. Partly, these proteins may have encountered contact allergen induced misfolding of cellular proteins. However, UPR can also be induced during DC activation as increased protein translation rates may cause endoplasmic stress and misfolding of proteins (Pearce and Everts 2015). Additionally, chaperones and the proteasome are also functionally involved in the processing and presentation of antigens (Yang et al. 2008; Wang et al. 2008; Tsan and Gao 2009). The *nrf2*$^{+/+}$ data set also contained many proteins that were described as Nrf2-dependent in the literature before and that were mainly associated with phase II metabolism and oxidative stress responses. The up-regulation of Nrf2 targets confirmed the activation of the Keap1/Nrf2 during the DC response to treatment with contact allergens (Ade et al. 2009). Comparison of *nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ data indicated Nrf2-dependent regulation of proteins. For heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), myosin-9 (MYH9) and triosephosphate isomerase (TPI1), an Nrf2-dependent regulation predicted on the transcript level could be confirmed on the protein level for the first time. Furthermore, proteins that have not been reported in the context

of Nrf2 before could be identified as putative Nrf2 targets, *e.g.*, heat shock protein 90-alpha (HSP90AA1) and 14-3-3 epsilon. In summary, this proteomics study supported the hypothesis that Nrf2 is functionally involved during the response of DCs to contact allergens as regulated proteins contribute to essential processes of DC maturation. Especially the potentially Nrf2-dependent induction of glycolytic enzymes indicating metabolic reprogramming should be regarded here as it was reported before that Nrf2 controls metabolic reprogramming and cell growth of cancer cells (Mitsuishi et al. 2012). Furthermore, an induction of Nrf2-dependent oxidative stress response and carbohydrate metabolism by xenobiotics was reported before (Murugesan et al. 2013).

In the second study (Chapter 3.3.), the response of THP-1 cells to DNCB or SDS was analyzed in a multi-omics approach. The aim of this study was to support the findings on metabolic reprogramming in BMDCs treated with contact allergens (Chapter 3.2.). The hypothesis that THP-1 cells would undergo metabolic reprogramming in response to DNCB treatment was supported by several molecular alterations on the metabolome and proteome level. Glycolysis seemed to be induced in response to DNCB treatment. Intracellular hexose levels were reduced after 4 h and 8 h after treatment with DNCB but not SDS. Furthermore, the results indicated an induction of lipid synthesis in THP-1 cells treated with DNCB. Phospholipid levels increased over time and fatty acid synthase (FAS) was up-regulated after DNCB treatment. FAS represents the key enzyme of fatty acid synthesis and therefore an essential enzyme for the generation of lipids. DNCB-treated THP-1 cells also revealed decreasing amino acid levels. This strengthens the assumption that in activated THP-1 cells the TCA cycle is mainly used to produce citrate that fuels fatty acids and lipid synthesis. Thus, amino acid synthesis may have been impaired as the TCA cycle may not have provided the necessary intermediates produced in the following enzymatic reactions. Impairment of the TCA cycle can be overcome by conversion of glutamate to a-ketoglutarate (O'Neill 2015) which was indicated by concentration-dependent reduction of glutamate in THP-1 cells treated with DNCB. In addition, the decline in amino acids may have been enforced by the boosted synthesis of biogenic amines as DNCB treatment led to increased levels of spermidine and spermine. These biogenic amines may have supported the induction of protein translation (Igarashi and Kashiwagi 2015) which again could have contributed to reduced levels of free amino acids. Several regulated proteins spoke for an enhanced protein synthesis during activation of THP-1 cells by DNCB. These included the translation-related proteins 60S acidic ribosomal protein P2 (RLA2) and heterogeneous nuclear

ribonucleoprotein H (HRNH1) and the chaperones endoplasmin (ENPL) and protein disulfide-isomerase (PDIA1), which are both involved in the UPR.

Both studies presented in this thesis helped to generated information that may contribute to the advancement of alternative testing strategies to detect contact allergens in the future. In Chapter 3.2., several possibly Nrf2-controlled proteins involved in DC biology were identified as promising biomarker candidates for chemical sensitization. Seven ARE-containing Nrf2 targets were induced by contact allergens and could be proposed as novel biomarker candidates, *i.e.* mitochondrial stress-70 protein (HSPA9), voltage-dependent anion-selective channel protein 1 (VDAC1), glutathione S-transferase omega-1 (GSTO1), ferritin light chain 1 (FTL1), peroxiredoxin-1 (PRDX1), mitochondrial superoxide dismutase (SOD2) and transketolase (TKT). Among these, FTL1, PRDX1 and SOD2 stand out as promising biomarker candidates as they were regulated Nrf2-dependently have been reported to be regulated during DC differentiation and maturation before (Gadgil et al. 2003; Pereira et al. 2005; Ferreira et al. 2010). In Chapter 3.3., regulated metabolites for DNCB treatments clearly differed from SDS treated samples. Especially the biogenic amines spermine and taurine as well as the long-chain phosphatidylcholines PCae C42:1 and PCae C44:4. may be regarded as valuable biomarker candidates as their regulation was concentration- and/or time-dependent after treatment with DNCB and they facilitated separation of differentially treated samples and controls. On the protein level, FAS represents a promising biomarker candidate as the FAS transcript is also included in the biomarker signature of the GARD assay (Johansson et al. 2011).

## 5. Conclusion and outlook

The studies presented in the scope of this thesis affirm that proteomics and metabolomics are invaluable when investigating the sensitization to contact allergens. The analysis of regulated proteins and metabolites in DC models treated with contact allergens allowed the identification of crucial cellular pathways involved in mechanisms DC activation. Therefore, the thesis on hand contributes to the understanding of sensitization to contact allergens. Furthermore, valuable biomarker candidates could be proposed that may serve as initial points for the advancement of *in vitro* testing strategies for the identification of contact allergens in the future.

Future experiments should include additional contact allergens and irritants to verify the performance of the biomarker candidates proposed including the classification of contact allergens according to potency. Based on the results obtained in this thesis, two strategies could be followed to design follow-up studies.

Firstly, omics analysis could be improved on several levels. Regarding proteomics analysis, a gel-free approach using an ESI-MS/MS setup will increase proteome coverage and reduce measuring time. Furthermore, coverage of functionally relevant membrane proteins could be increased by enrichment of cellular membranes. Especially the plasma membrane via subcellular fractioning will be a promising target fraction. With respect to the metabolic changes during DC activation, enrichment of mitochondria be another promising approach. Regarding metabolomics analysis, analysis of a larger number of metabolites will result in a better coverage of metabolic pathways of interest. In combination with multivariate statistics, an increase in the coverage of the proteome and/or metabolome could facilitate the implementation of biomarker signatures for the identification and classification of contact allergens *in vitro*.

Secondly, single biomarker candidates on the proteome and metabolome level could be validated using targeted biochemical assays. Protein biomarker candidates could be verified using specific antibodies. Depending on the subcellular location, biochemical properties and abundance of the respective proteins, immunoblotting, enzyme-linked assays or flow cytometry could be applied. In the context of metabolic reprogramming, assays measuring glycolysis rate, cellular respiration rate or (phospho-)lipid synthesis could serve as promising read-outs to be assayed.

Furthermore, the hypothesis that Nrf2 may be involved in the control of metabolic reprogramming in DC models responding to contact allergens should be addressed in future experiments. To this end, experiments should include additional contact allergens, non-sensitizing Nrf2-activators and inhibitors of glycolysis.

## 6. Summary

ACD represents a widespread adaptive immune reaction in the human skin elicited by substances contained in consumable products. Mediating the selection and activation of contact allergen-specific T cells during sensitization, DCs play a central role during the development of ACD. Reliable identification and classification of contact allergens is of high importance to curtail ACD. Analyses of the sensitization to contact allergens on the molecular level may contribute to the advancement of alternative testing methods. In the thesis on hand, proteomics and metabolomics analyses were conducted to investigate the sensitization to contact allergens on the molecular level using DC models.

In the first study, gel-based proteomics was applied to identify differentially regulated proteins in *nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ BMDCs after treatment with the contact allergens DNCB, CA, $NiSO_4$ and the irritant SDS. Alterations on the proteome level were identified using 2D-PAGE and ESI-MS/MS. Regulated proteins in *nrf2*$^{+/+}$ BMDCs could be mapped to essential processes of BMDC activation including unfolded protein response, cell signaling, protein expression and re-organization of the cytoskeleton. A central finding was that contact allergens induced enzymes of the carbohydrate metabolism indicating metabolic reprogramming in BMDCs. Comparative analysis of *nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ data confirmed Nrf2 targets on the protein level for the first time and identified further putative Nrf2 targets. On the pathway level, differential regulation of oxidative stress response proteins confirmed the induction of the Keap1/Nrf2 pathway by contact allergens. In summary, this study confirmed a central role of Nrf2 during the response of BMDCs treated with contact allergens and led to the identification of promising biomarker candidates for the identification of contact allergens *in vitro*.

In the second study, the response of human THP-1 cells to the contact allergen DNCB and the irritant SDS was investigated using a multi-omics strategy. For the targeted analysis of metabolites LC-MS/MS and FIA-MS/MS was employed. Untargeted quantitative proteomics analysis was based on SILAC and MALDI-TOF-MS/MS. Consistent findings on the metabolome and proteome level indicated metabolic reprogramming in THP-1 cells treated with DNCB. An induction of lipid synthesis was confirmed by the up-regulation of phospholipids and fatty acid synthase, a key enzyme of lipid synthesis. Additionally, proteins involved in protein synthesis and UPR were induced and an interruption of the TCA cycle could be concluded. Biogenic amines and long-chained phospholipids could be proposed as promising biomarker candidates for

the activation of THP-1 cells by contact allergens. In summary, this study supported the hypothesis that contact allergens may induce metabolic reprogramming in THP-1 cells. Furthermore, the results confirmed that the analysis of metabolic endpoints may represent a promising approach for the identification of contact allergens *in vitro*.

## 7. Zusammenfassung

Allergische Kontaktdermatitis (ACD) stellt eine weitverbreitete, adaptive Immunreaktion der menschlichen Haut dar, welche durch Substanzen in verbrauchernahen Produkten ausgelöst werden kann. Dendritische Zellen (DC) spielen eine entscheidende Rolle während der Entstehung von ACD, indem sie die Selektion und Aktivierung kontaktallergen-spezifischer T-Zellen während der Sensibilisierung vermitteln. Die zuverlässige Identifizierung und Klassifizierung von Kontaktallergenen spielt eine wichtige Rolle bei der Eindämmung von ACD. Analysen der Sensibilisierung gegen Kontaktallergene auf molekularer Ebene können zur Verbesserung alternativer Testmethoden beitragen. In der vorliegenden Arbeit wurden proteomische und metabolomische Analysen an verschiedenen Modellen für DC durchgeführt, um die Sensibilisierung gegen Kontaktallergene auf der molekularen Ebene zu untersuchen.

In der ersten Studie der Arbeit wurden murine, aus dem Knochenmark gewonnene dendritische Zellen (Bone Marrowed Derived Dendritic Cells, BMDC) mit den Kontaktallergenen 2,4-Dinitrochlorbenzol (DNCB), Zimtaldehyd (CA) und Nickel(II)-sulfat ($NiSO_4$) sowie dem Irritanz Natriumdodecylsulfat (SDS) behandelt. Veränderungen auf Proteom-Ebene wurden mittels 2-dimensionaler Polyacrylamidgelelektrophorese (2D-PAGE) und Elektrosprayionisation-gekoppelter Tandem-Massenspektrometrie (ESI-MS/MS) identifiziert. Regulierte Proteine in wildtypischen BMDC (*nrf2*$^{+/+}$) konnten essentiellen Prozessen der Aktivierung von BMDC zugeordnet werden, einschließlich der Antwort auf ungefaltete Proteine, Signaltransduktion, Expression von Proteinen und Reorganisation des Cytoskeletts. Ein zentrales Ergebnis war, dass Kontaktallergene Enzyme des Stoffwechsels von Kohlenhydraten induzierten, was eine metabolische Reprogrammierung von BMDC anzeigte. Die vergleichende Analyse von Daten aus *nrf2*$^{+/+}$ und BMDC mit Nrf2-Knockout (*nrf2*$^{-/-}$) bestätigte vorhergesagte Nrf2-Zielmoleküle zum ersten Mal auf der Proteinebene und konnte weitere Kandidaten für Nrf2-Zielmoleküle identifizieren. Auf der Signalweg-Ebene bestätigte die differentielle Regulierung von Proteinen der oxidativen Stressantwort die Aktivierung des Keap1/Nrf2-Signalweges durch Kontaktallergene. Zusammengefasst bestätigte diese Studie eine zentrale Rolle für Nrf2 während der Antwort von BMDC auf die Behandlung mit Kontaktallergenen und führte zur Identifizierung von vielversprechenden Biomarker-Kandidaten für die Identifizierung von Kontaktallergenen *in vitro*.

In der zweiten Studie wurde die zelluläre Antwort humaner THP-1 Zellen nach Behandlung mit dem Kontaktallergen DNCB oder dem Irritanz SDS mit Hilfe von Metabolomics und Proteomics analysiert. Für eine gezielte Metabolit-Analyse wurden LC-MS/MS und FIA-MS/MS angewendet. Die ungezielte Protein-Analyse basierte auf SILAC und MALDI-TOF-MS/MS. Konsistente Ergebnisse auf Metabolom- und Proteom-Ebene zeigte eine metabolische Reprogrammierung der mit DNCB behandelten THP-1 Zellen an. Eine Aktivierung der Lipid-Synthese wurde durch die Hochregulierung von Phospholipiden und der Fettsäure-Synthase, einem zentralen Enzym der Lipid-Synthese, bestätigt. Zusätzlich wurden Proteine induziert, die an Protein-Synthese und der Antwort auf ungefaltete Proteine beteiligt sind. Außerdem konnte auf eine Beeinträchtigung des Citratzyklus geschlossen werden. Langkettige Phospholipide sowie die biogenen Amine Taurin und Spermin konnten als vielversprechende Biomarker-Kandidaten für die Aktivierung von THP-1 Zellen durch Kontaktallergene vorgeschlagen werden. Zusammengefasst unterstützt diese Studie die Hypothese, dass Kontaktallergene metabolische Reprogrammierung von THP-1 Zellen auslösen können. Außerdem bestätigten die Ergebnisse, dass Analysen metabolischer Endpunkte ein vielversprechender Ansatz für die Identifizierung von Kontaktallergenen *in vitro* sein können.

## 8. Abbreviations

| | |
|---|---|
| 2D-PAGE | Two-dimensional polyacrylamide gel electrophoresis |
| ACD | Allergic contact dermatitis |
| AOP | Adverse outcome pathway |
| APC | Antigen-presenting cell |
| ARE | Antioxidant response element |
| ATP | Adenosine triphosphate |
| BMDC | Bone marrow-derived dendritic cell |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| Da | Dalton |
| DAMP | Danger-associated molecular pattern |
| DC | Dendritic cell |
| DNA | Deoxyribonucleic acid |
| DNCB | 2,4-dinitrochlorobenzene |
| DOI | Digital Object Identifier |
| DPRA | Direct peptide reactivity assay |
| ENPL | Endoplasmin |
| ER | Endoplasmic reticulum |
| ESI | Electrospray ionization |
| ESI-MS/MS | Electrospray ionization tandem mass spectrometry |
| EU | European Union |
| EURL ECVAM | European Union Reference Laboratory for alternatives to animal testing |

| | |
|---|---|
| $FADH_2$ | Flavin adenine dinucleotide |
| FAO | Fatty acid oxidation |
| FAS | Fatty acid synthase |
| FIA | Flow injection analysis |
| FTL1 | Ferritin light chain 1 |
| GARD | Genomic Allergen Rapid Detection |
| GPMT | Guinea pig maximization test |
| GSTO1 | Glutathione S-transferase omega-1 |
| h-CLAT | Human Cell Line Activation Test |
| HK2 | Hexokinase 2 |
| HMOX1 | Heme oxygenase 1 |
| HNRNPA1 | Heterogeneous nuclear ribonucleoprotein A1 |
| HRNH1 | Heterogeneous nuclear ribonucleoprotein H |
| iDC | Immature dendritic cell |
| IFNγ | Interferon gamma |
| IKKE | Inhibitor of NF-κB kinase subunit-E |
| IL | Interleukin |
| IRF | Interferon regulatory factors |
| Keap1 | Kelch-like ECH-associated protein 1 |
| LC | Liquid chromatography |
| LC-MS/MS | Liquid chromatography-tandem mass spectrometry |
| LLNA | Local lymph node assay |
| m/z | Mass-to-charge ratio |
| MALDI | Matrix-assisted laser desorption/ionization |
| mDC | Mature dendritic cell |

| | |
|---|---|
| MHC | Major histocompatibility complex |
| MPS | Mononuclear phagocyte system |
| mRNA | Messenger RNA |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| mTOR | Mechanistic target of rapamycin |
| MUSST | Myeloid U937 Skin Sensitization Test |
| MYH9 | Myosin-9 |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| Nrf2 | Nuclear factor (erythroid-derived 2)-like 2 |
| *nrf2*$^{+/+}$ | Nrf2 wild-type |
| *nrf2*$^{-/-}$ | Nrf2 knockout |
| $NiSO_4$ | Nickel(II) sulfate |
| NQO1 | NAD(P)H dehydrogenase, quinone 1 |
| OECD | Organization for Economic Co-Operation and Development |
| OXPHOS | Oxidative phosphorylation |
| PAMP | Pathogen-associated molecular pattern |
| PCR | Polymerase chain reaction |
| PDIA1 | Protein disulfide-isomerase |
| PPP | Pentose phosphate pathway |
| PRDX1 | Peroxiredoxin-1 |
| PRR | Pattern recognition receptor |

| | |
|---|---|
| REACH | Registration, Evaluation, Authorisation and Restriction of Chemicals (Regulation (EC) No 1907/2006) |
| RLA2 | translation-related proteins 60S acidic ribosomal protein P2 |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| SDS | Sodium dodecyl sulfate |
| SILAC | Stable isotope labeling with amino acids in cell culture |
| SOD2 | Mitochondrial superoxide dismutase |
| TBK1 | TANK-binding kinase 1 |
| TCA | Tricarboxylic acid |
| TCR | T cell receptor |
| TKT | Transketolase |
| TLR | Toll-like receptor |
| TNFα | Tumor necrosis factor alpha |
| TOF | Time-of-flight |
| TPI1 | Triosephosphate isomerase |
| UPR | Unfolded protein response |
| VDAC1 | Voltage-dependent anion-selective channel protein 1 |
| XBP1 | X-box-binding protein 1 |

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## 10. List of publications

Mussotter, F., Potratz, S., Budczies, J., Luch, A. Haase, A., 201x. A multi-omics analysis reveals metabolic reprogramming in THP-1 cells upon treatment with the contact allergen DNCB. Submitted.

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