

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

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ABSTRACT

Allergic contact dermatitis is a widespread disease with high clinical relevance affecting approximately 20% of the general population. Typically, contact allergens are low molecular weight electrophilic compounds which can activate the Keap1/Nrf2 pathway. We performed a proteomics study to reveal possible biomarkers for dendritic cell (DC) activation by contact allergens and to further elucidate the role of Keap1/Nrf2 signaling in this process. We used bone marrow derived dendritic cells (BMDCs) of wild-type (*nrf2*^{+/+}) and Nrf2 knockout (*nrf2*^{-/-}) mice and studied their response against the model contact sensitizers 2,4-dinitrochlorobenzene (DNCB), cinnamaldehyde (CA) and nickel(II) sulfate by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in combination with electrospray ionization tandem mass spectrometry (ESI-MS/MS). Sodium dodecyl sulfate (SDS, 100 μ M) served as irritant control. While treatment with nickel(II) sulfate and SDS had only little effects, CA and DNCB led to significant changes in protein expression. We found 18 and 30 protein spots up-regulated in wild-type cells treated with 50 and 100 μ M CA, respectively. For 5 and 10 μ M DNCB, 32 and 37 spots were up-regulated, respectively. Almost all of these proteins were not differentially expressed in *nrf2*^{-/-} BMDCs, indicating an Nrf2-dependent regulation. Among them proteins were detected which are involved in oxidative stress and heat shock responses, as well as in signal transduction or basic cellular pathways. The applied approach allowed us to differentiate between Nrf2-dependent and Nrf2-independent cellular biomarkers differentially regulated upon allergen-induced DC activation. The data presented might contribute to the further development of suitable *in vitro* testing methods for chemical-mediated sensitization.

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Abbreviations: 2D-PAGE, 2-dimensional polyacrylamide gel electrophoresis; ACD, allergic contact dermatitis; ALDH2, aldehyde dehydrogenase, mitochondrial; ALDOA, fructose-bisphosphate aldolase A; ARE, antioxidant response element; ATP, adenosine triphosphate; ATP5A1, ATP synthase subunit alpha, mitochondrial; ATP5B, ATP synthase subunit beta, mitochondrial; BMDC, bone marrow derived dendritic cell; CA, cinnamaldehyde; CAPG, macrophage-capping protein; CD, cluster of differentiation; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; DC, dendritic cell; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; DNCB, 2,4-dinitrochlorobenzene; ECVAM, European Centre for the Validation of Alternatives Methods; EEF1A1, elongation factor 1-alpha 1; ENO1, α -enolase; ER, endoplasmatic reticulum; ESI-MS/MS, electrospray ionization tandem mass spectrometry; EU, European Union; FTL1, ferritin light chain 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCLM, glutamate-cysteine ligase, modifier subunit; γ -GCS, γ -glutamylcysteine synthetase; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSTO1, glutathione S-transferase omega-1; HMOX1, heme oxygenase 1; HSPA9, stress-70 protein, mitochondrial; HSP90AA1, heat shock protein 90-alpha; IL18, interleukin 18; IL1 β , interleukin 1 beta; IFN γ , interferon- γ ; IMDM, Iscove's Modified Dulbecco's Medium; IPA, Ingenuity Pathway Analysis; IPG, Immobilized pH gradient; Keap1, kelch-like ECH-associated protein 1; LDHA, L-lactate dehydrogenase A chain; LLNA, local lymph node assay; MHC II, major histocompatibility complex II; MEST, mouse ear swelling test; NAD(P)H, Nicotinamide adenine dinucleotide phosphate; Ni²⁺, nickel ion; NiSO₄, nickel(II) sulfate; NQO1, NAD(P)H dehydrogenase, quinone 1; Nrf2, nuclear factor erythroid 2-related factor 2; *nrf2*^{+/+}, Nrf2 wild-type; *nrf2*^{-/-}, Nrf2 knockout; OECD, Organisation for Economic Co-operation and Development; P4HB, protein disulfide-isomerase; PBS, Phosphate-buffered saline; PCR, polymerase chain reaction; PKM, pyruvate kinase isozymes M1/M2; PRDX1, peroxiredoxin-1; PSMB7, proteasome subunit beta type-7; REACH, Regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals; SDS, sodium dodecyl sulfate; SOD2, superoxide dismutase [Mn], mitochondrial; TALDO1, transaldolase; TKT, transketolase; TNF- α , tumor necrosis factor alpha; TUBB2A, tubulin beta-2A; PGAM1, phosphoglycerate mutase 1; PGAM2, phosphoglycerate mutase 2; UCHL3, ubiquitin carboxyl-terminal hydrolase isozyme L3; TLR, toll-like receptor; VASP, vasodilator-stimulated phosphoprotein; VDACC1, voltage-dependent anion-selective channel protein 1.

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1. Introduction

Allergic contact dermatitis (ACD) represents a widespread hypersensitivity reaction in the skin, which may cause severe and long-lasting health problems. ACD also represents a major reason for occupational disability. Today >3000 allergens are known, among them preservatives, hair dyes or fragrances. The number of ACD patients is constantly growing in the Western hemisphere, reaching a prevalence of some 20% (Thyssen et al., 2007). The best strategy to contain ACD is the general avoidance of skin sensitizers. The testing of substances for their sensitizing potential is therefore of high importance for occupational and consumer safety.

Since 2013 the testing of cosmetic ingredients in living animals is prohibited in the EU (European Commission, 2009). This also includes the testing of possible sensitizing properties. At the same time, the reassessment of thousands of chemicals is being pursued in the EU in the context of REACH. Required testing under REACH is supposed to consume millions of animals in the years to come (Rovida and Hartung, 2009). Given these considerations, a validated *in vitro* and/or *in silico* testing strategy for sensitization is urgently needed. The complexity of the induction of ACD, however, renders it unlikely that a single assay will be sufficient to replace *in vivo* testing. Currently, the local lymph node assay (LLNA) is regarded as the *in vivo* gold standard for the testing of chemical-mediated sensitization. So, it is commonly assumed that a comprehensive and integrated testing strategy is needed to mirror the complete process of ACD development (Adler et al., 2011).

ACD represents a delayed-type hypersensitivity reaction which develops in two phases, called sensitization and elicitation. Typically, sensitizers are electrophilically reactive chemicals of low molecular weight (<500 Da) or metal ions. Upon skin contacts, keratinocytes encountering sensitizers secrete pro-inflammatory cytokines, such as IL1 β , IL18 or TNF- α , thereby attracting dendritic cells (DCs) to the site of exposure (Kaplan et al., 2012). Antigen presenting DCs in the skin are crucial during sensitization as they connect innate and adaptive immune responses. These specialized cells are enabled to internalize haptenated proteins and then, upon processing (i.e., limited digesting), present modified peptides as possible antigens loaded onto the major histocompatibility complex II (MHC II) at their cell surface. After migration to the local lymph nodes DCs help to select naïve T lymphocytes in a process called T cell priming that relies on the expression of further co-stimulatory molecules at the DC surface (Banchereau and Steinman, 1998). Primed T cells subsequently proliferate and migrate into the skin where they pass over into a standby position. Once the immune system has established memory T cells, any following exposure to the respective allergen will then lead to a visible inflammatory response causing the clinical aspect of contact eczema in the skin, which is referred to as the elicitation phase.

The Keap1/Nrf2 signaling pathway is one of the most important cellular cytoprotective pathways. It initiates cellular stress responses after exposure to a variety of disturbing agents, in particular oxidants and electrophiles (Kaspar et al., 2009). At steady-state conditions the transcription factor Nrf2 is bound to Keap1, an E3 ubiquitin ligase which is mediating rapid degradation of Nrf2. Under oxidative stress conditions, the oxidation of cysteine sulfhydryl groups induces a conformational change of Keap1 thereby leading to Nrf2 stabilization and its transfer into the nucleus (Kobayashi et al., 2006). The following binding to antioxidant response elements (ARE) triggers the expression of target genes. Nrf2 target genes are mainly involved in phase II metabolism and the antioxidant defense to counteract possible damage induced by oxidants and electrophiles. Additionally, Nrf2 also affects proteasomal protein degradation, cell growth and metabolism (Ma, 2013).

Sensitizing chemicals often induce the Keap1/Nrf2 pathway due to their electrophilic properties. However, the underlying mechanism between Nrf2 activation and sensitization remains unclear. Recent data suggest that Nrf2 in addition to its role in cellular stress response is involved in the regulation of the immune system. Nickel ions (Ni²⁺)

were reported to induce cytokine secretion of monocytic cells in an Nrf2-dependent manner (Lewis et al., 2006). Treatment of primary DCs or the monocytic human cell line THP-1 with several sensitizers induced the expression of typical Nrf2 target genes like NAD(P)H dehydrogenase, quinone 1 (NQO1) or heme oxygenase 1 (HMOX1) (Ade et al., 2009). Recently, Nrf2 was shown to affect the dermal barrier function as well. So, mice with enhanced Nrf2 activity exhibited epidermal thickening, hyperkeratosis and inflammation (Schäfer et al., 2012). Conversely, Nrf2 knockout mice showed an exacerbated skin inflammation in response to contact sensitizers that were known to be reactive against cysteine or lysine, thus pointing to an important role of Nrf2 in the suppression of skin sensitization (El Ali et al., 2013). Moreover, the knockout of Nrf2 leads to enhanced expression of co-stimulatory molecules on the surface of DCs, thus indicating an immune modulatory function of Nrf2 as well (Yeang et al., 2012). The Keap1/Nrf2 pathway has been proposed as marker toxicity pathway indicating cysteine-reactive sensitizers (Natsch, 2010). The KeratinoSens™ *in vitro* sensitization assay uses its properties in the assessment of contact allergenicity based on the quantification of Nrf2-dependent expression in keratinocytes via an ARE-luciferase reporter gene construct (Emter et al., 2010). Although this assay has been formally validated at the European Centre for the Validation of Alternatives Methods (ECVAM) and is available as an OECD test guideline (OECD TG 442D), it may suffer from false positive results in the case of substances that activate Nrf2 as chemical stressors like Tween80 (Emter et al., 2013).

A possible strategy to overcome current limitations in *in vitro* sensitization testing is to include additional biomarkers. Omics-based approaches, such as transcriptomics or proteomics, are well suited to uncover novel putative biomarkers. Several studies have already addressed transcriptomics alterations during activation of DCs (Johansson et al., 2011; Ott et al., 2010; Ryan et al., 2004). On the other hand, studying sensitizer-mediated DC activation at the proteomics level has not been done so far. To the best of our knowledge the present study is therefore the first to address proteomics alterations in DCs upon exposure to sensitizing agents. Since cell functions ultimately rely on functional proteins, proteomics approaches are expected to be particularly useful when it comes to the elucidation of molecular alterations underlying DC maturation in response to chemical sensitizers (Ferreira et al., 2010).

2. Materials and methods

2.1. Mice

Knockout *nrf2*^{-/-} mice were generated as described earlier (El Ali et al., 2013) and provided by the RIKEN BRC (Ibaraki, Japan). All mice investigated in the present study were generated from inbred C57BL/6J background *nrf2* heterozygous mice. Mice were backcrossed to C57BL/6J for at least 10 generations. Mice were housed in a pathogen-free facility and handled in accordance with the principles and procedures outlined in Council Directive 86/609/EEC. Genotyping was performed by PCR using genomic DNA that was isolated from tail snips as described (Itoh et al., 1997).

2.2. Cell culture, treatment and sample preparation

The method for generating murine BMDCs was carried out according to the literature (Lutz et al., 1999) with minor modifications. Briefly, cells derived from bone marrow collected from femur and tibia bones of 6 to 12 weeks old C57BL/6 *nrf2*^{+/+} and *nrf2*^{-/-} mice (Itoh et al., 1997) were seeded in bacteriological petri dishes in 10 ml Iscove's Modified Dulbecco's Medium (IMDM) containing 10% heat inactivated fetal calf serum, streptomycin and penicillin (IMDMc) (all from Gibco Invitrogen). Medium had been supplemented with 10% GM-CSF culture supernatant from the cell line J558 (kindly provided by Sebastian Amigorena, Institut Curie-France) and β -mercaptoethanol (50 μ M;

Sigma Aldrich, Lyon, France). At day 10, non-adherent cells and loosely adherent cells were collected by gentle pipetting and then centrifuged. The cells were then washed twice with IMDMc and incubated according to different protocols. The phenotype of BMDCs were characterized and analyzed by flow cytometry measuring the expression of cell surface markers CD11c^{high}, MHC II^{high} and CD86^{low}, CD80^{low}, CD103^{low}, CD207^{low}, Epcam^{low}. For treatment, cells were seeded at 1×10^6 cells/ml and treated for 8 h with cinnamaldehyde (CA; 50 μ M, 100 μ M; Sigma Aldrich, Lyon, France), 2,4-dinitrochlorobenzene (DNCB; 5 μ M, 10 μ M; Sigma Aldrich, Lyon, France), nickel(II) sulfate (NiSO₄; 250 μ M, 400 μ M; Sigma Aldrich, Lyon, France) and sodium dodecyl sulfate (SDS; 100 μ M; Sigma Aldrich, Lyon, France). Applied concentrations were derived in consideration of cytotoxicity data. We included the maximal tolerated dose and a second lower dose. Control cells were left untreated. In addition, vehicle controls were treated with DMSO only, using the same amount of DMSO as used for CA and DNCB treatments.

Cells were harvested by centrifugation (200 $\times$ g, 5 min) and washed with PBS three times. Pellets were resuspended in 2D-PAGE lysis buffer [7 M urea (SERVA Electrophoresis GmbH, Heidelberg, Germany), 2 M thiourea (Sigma Aldrich, Munich, Germany), 4% CHAPS (Sigma Aldrich, Munich, Germany), 2% IPG buffer pH 3–10 (GE Healthcare Lifesciences, Freiburg, Germany), 2% DeStreak™ Reagent (GE Healthcare Lifesciences, Freiburg, Germany), 0.7% spermine tetrahydrochloride (Sigma Aldrich, Munich, Germany), 1% Protease Inhibitor Cocktail Set III (Merck Millipore, Darmstadt, Germany)] and incubated for 30 min at room temperature while rotating. Cell lysates were centrifuged (100,000 $\times$ g) for 1 h and supernatants retained for further analysis.

2.3. 2D-PAGE

Samples contained protein lysates from 2×10^6 cells each. Isoelectric focusing was performed on IPG strips (24 cm) with a non-linear pH gradient (3–10) using an Ettan IPGPhor3 Isoelectric Focusing System (GE Healthcare Lifesciences, Freiburg, Germany). Active rehydration and focusing was performed in 6 steps: 15 h at 30 V, 1.5 h at 200 V, 1 h at 500 V, 13.5 h gradient 500–1000 V, 3 h gradient 1000–8000 V and 6 h at 8000 V. Afterwards, proteins were reduced with 1% dithiothreitol (SERVA Electrophoresis GmbH, Heidelberg, Germany) and alkylated with 4% iodoacetamide (SERVA Electrophoresis GmbH, Heidelberg, Germany). Second dimension polyacrylamide gels were casted manually with 12.5% acrylamide/*N,N'*-methylene bisacrylamide (29:1) solution (SERVA Electrophoresis GmbH, Heidelberg, Germany). Electrophoresis was performed in 2 steps (30 min at 3 W per gel, 6 h at 15 W per gel) with an Ettan DALT twelve System second-dimension gel electrophoresis system (GE Healthcare Lifesciences, Freiburg, Germany). Gels were stained with ruthenium II-bathophenanthroline disulfonate chelate (Rabilloud et al., 2000) and scanned with a Typhoon FLA 9500 imager (GE Healthcare Lifesciences, Freiburg, Germany) using an LPG Channel 1 filter (excitation: 473 nm, detection: 610 nm). Gel images were analyzed with Delta2D image analysis software (Decodon GmbH, Greifswald, Germany). Single spots' fluorescence intensities were normalized to total fluorescence intensities of the respective gels. All samples were analyzed in three independently exposed replicates. Differentially expressed proteins (>1.5 times up- or down-regulated, unpaired two-sided Student's *t*-test $p < 0.05$) were selected for mass spectrometry identification.

2.4. Mass spectrometry and data analysis

Gel spots of interest were excised using spotXpress HT Spot Picking Robot (Proteome Factory, Berlin, Germany). In gel digestion was performed with trypsin (1.2 ng/ μ l, Promega, Mannheim, Germany). Peptides were extracted from the gel matrix and purified using C18 ZipTips (Merck Millipore, Darmstadt, Germany). Mass spectra were obtained by electrospray ionization tandem mass spectrometry (ESI-MS/

MS) using an Orbitrap velos system (Thermo Scientific, Braunschweig, Germany) as described previously (Zanker et al., 2015). Analyses were performed using the MASCOT Daemon and Swissprot database. Search parameters included one partial cleavage site, carbamidomethyl residues (Cys) as fixed modification and oxidation (Met) or deamidation (Gln, Asn) as variable modifications. Peptide tolerance was set to ± 20 ppm. MS/MS tolerance was set ± 0.6 Da. A significant MASCOT score was considered sufficient for reliable protein identification. In some cases, more than one protein could be identified from a single protein spot, which might be caused by persistent protein-protein interactions or similar sizes and isoelectric points. In such cases multiple identified proteins from a single spot were listed (Table S3).

2.5. Pathway analysis

Pathway analyses of regulated proteins were generated through the use of QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). Data sets of regulated proteins responding to single treatments were subjected to core analyses against the IPA knowledge base (build version 346717M, content version 21901358). Confidence level was set to “experimentally observed”. Subsequently comparison analysis was performed for protein enrichment in canonical pathways and diseases and biofunctions.

3. Results

To analyze alterations of the cellular proteome upon treatment with chemical sensitizers of different potency, *nrf2*^{+/+} BMDCs were exposed to two doses each of DNCB (strong sensitizer), and CA or NiSO₄ (moderate sensitizers) (Gerberick et al., 2001; Kimber et al., 2003). SDS was included to assess cellular responses to an irritant. Based on the observation that Nrf2-dependent protein induction is well detectable in DCs a few hours after sensitizer treatment (Migdal et al., 2013), we exposed BMDCs for 8 h prior to analysis. Proteomic alterations were compared by 2D-PAGE analysis. Protein spots with a fold change of >1.5 and $p < 0.05$ (Student's *t*-test) were considered statistically significantly regulated.

3.1. Spot patterns

In total, across all treatment groups 90 protein spots were found up- and 17 spots down-regulated in *nrf2*^{+/+} cells. Representative gel images are depicted in Fig. 1 and Fig. S1 and S2. Fig. 2 depicts Venn diagrams comparing numbers of regulated protein spots caused by different treatments.

The overall responses observed in the proteomics analyses nicely correlated with the potency of the sensitizer applied. So, the strongest upregulation was observed upon treatment with the strong sensitizer DNCB. The cysteine/lysine-reactive moderate sensitizer CA also provoked protein induction but to a lesser extent compared to DNCB. The metal sensitizer NiSO₄ and the irritant SDS induced only little changes in the spot patterns. Conversely, the majority of protein spots regulated in *nrf2*^{+/+} BMDCs appeared unaffected in *nrf2*^{-/-} cells (Table S1).

In detail, we found 32 spots up-regulated and 5 spots down-regulated after treatment of *nrf2*^{+/+} BMDCs with 5 μ M DNCB. After treatment with 10 μ M DNCB, 37 spots were up-regulated and 4 spots were down-regulated. Among both treatment groups there was a share of 17 protein spots being up-regulated (Fig. 2, Fig. S3).

All up-regulated protein spots in *nrf2*^{+/+} cells upon exposure to 5 μ M DNCB were not significantly regulated in *nrf2*^{-/-} BMDCs at the same treatment conditions (cf. Table 1 and Tables S1 and S2 for particular proteins identified). By contrast, from the 37 spots that were induced in *nrf2*^{+/+} cells at 10 μ M DNCB only 29 remained unaffected in *nrf2*^{-/-} cells (Table S1).

Upon treatment of *nrf2*^{+/+} cells with CA, we again observed a dose-dependent effect. Treatment with 50 μ M CA led to the induction of 18

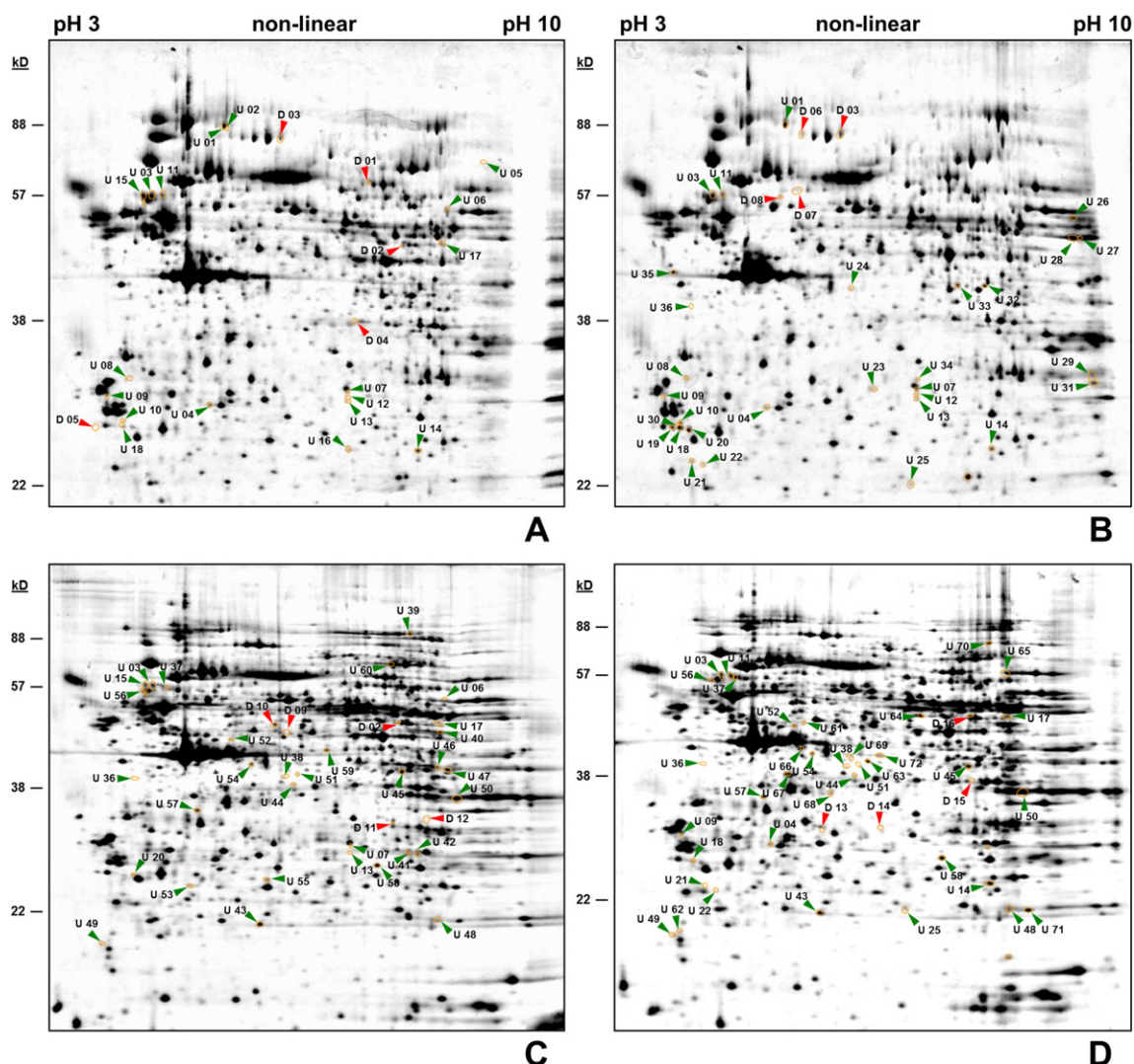


Fig. 1. Representative 2D-PAGE gels displaying up- and downregulated protein spots in *nrf2*^{+/+} BMDCs after treatment with low and high doses of CA and DNCB. (A): 50 μ M CA; (B): 100 μ M CA; (C): 5 μ M DNCB; (D): 10 μ M DNCB. Green and red arrows indicate up- and downregulated proteins, respectively ($n = 3$, regulation ≥ 1.5 or ≤ 0.67 , $p < 0.05$). For representative gels of all treatment groups see Fig. S1 and S2. Abbreviations: CA, cinnamaldehyde; DNCB, 2,4-dinitrochlorobenzene.

protein spots, while 100 μ M CA induced 30 spots. Among them 12 spots were similarly up-regulated through both treatments (Fig. 2). Conversely, 5 and 4 protein spots were down-regulated at 50 and 100 μ M CA, respectively, from which only one was shared between both treatment groups (Fig. S3).

Again, the majority was not induced in *nrf2*^{-/-} BMDCs, i.e. 16 of 18 spots detected in *nrf2*^{+/+} were not up-regulated in *nrf2*^{-/-} BMDCs after treatment with 50 μ M CA. For cells treated with 100 μ M CA, 20 spots appeared to be induced in an Nrf2-dependent manner (Table S1).

Upon treatment of *nrf2*^{+/+} cells with NiSO₄ 17 spots were found up-regulated at both low (250 μ M) and high dose (400 μ M) treatments. Twelve protein spots among them appeared at both concentrations (Fig. 2). Thus, we observed much weaker cellular responses against NiSO₄ when compared to the other two sensitizers applied. Furthermore, there was no indication for a dose-dependent effect in the case of NiSO₄, which was well observed for DNCB and CA (Fig. S3). Also, treatments with NiSO₄ did not lead to down-regulation of any protein spots. The majority of NiSO₄ regulated spots appeared to be dependent on Nrf2 as only one spot was induced at 250 μ M and none at 400 μ M in *nrf2*^{-/-} BMDCs (Table S1).

Treatment of cells with 100 μ M SDS provoked an up-regulation of 16 protein spots (Fig. 2). In terms of protein spot numbers, this was

comparable to the results obtained upon NiSO₄ treatment. One protein spot was found down-regulated after treatment with 100 μ M SDS (Fig. S3). None of the regulated spots appeared to be changed in *nrf2*^{-/-} BMDCs (Table S1).

Taken together, the strong sensitizer DNCB concentration dependently induced the highest numbers of protein spots, thereby indicating stronger cellular responses when compared to the moderate sensitizers CA and NiSO₄, or the irritant SDS.

3.2. Protein identification

We succeeded to identify a total of 100 proteins regulated by the sensitizers and the irritant applied in *nrf2*^{+/+} BMDCs (Table 1 and Table S2 and S3). Among all proteins identified, 76 were up-regulated, 13 down-regulated and 11 were either down- or up-regulated, depending on the kind of treatment. Expression data of these proteins across different treatment groups could be extracted from corresponding 2D gels and subsequently characterized (Table 1 and Table S2).

DNCB and CA strongly affected proteins associated with oxidative stress responses [e.g., HMOX1, glutamate-cysteine ligase, modifier subunit (GCLM)] and phase II metabolism [e.g., mitochondrial aldehyde dehydrogenase (ALDH2)]. Peroxiredoxin-1 (PRDX1) and mitochondrial

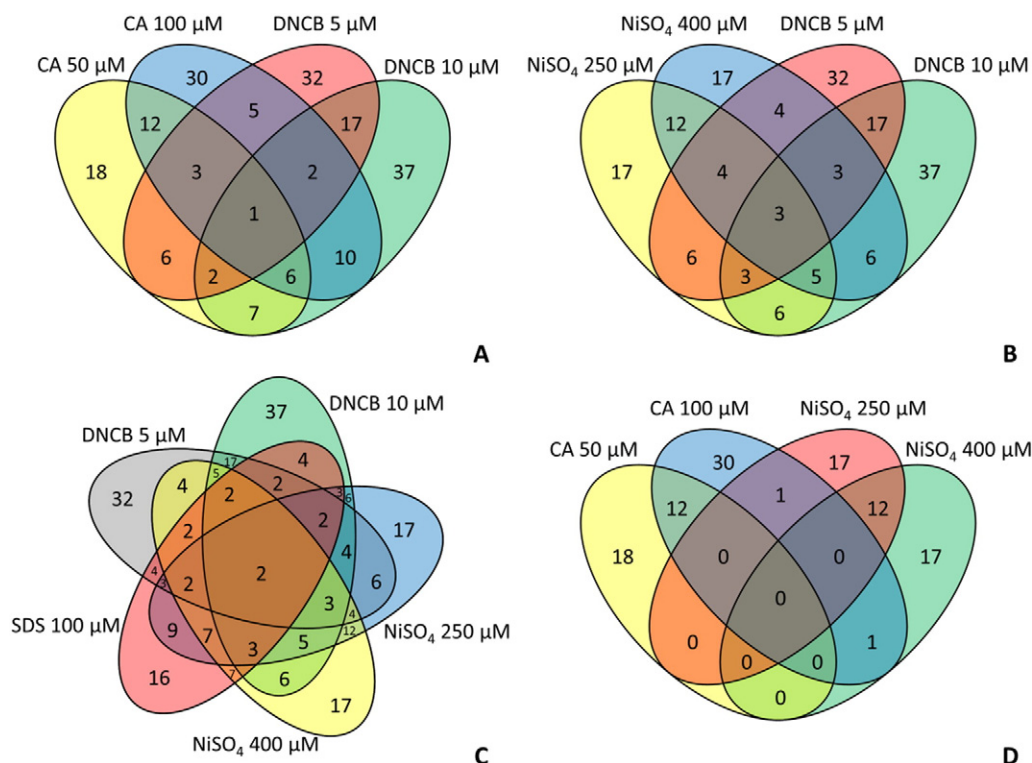


Fig. 2. Venn diagrams of overlapping upregulated protein spots after treatment of *nrf2*^{+/+} BMDCs with low and high doses of CA, DNCB, NiSO₄ and SDS. (A): CA ∩ DNCB; (B): NiSO₄ ∩ DNCB; (C): DNCB ∩ NiSO₄ ∩ SDS; (D): CA ∩ NiSO₄ (*n* = 3, upregulation ≥ 1.5, *p* < 0.05). Abbreviations: CA, cinnamaldehyde; DNCB, 2,4-dinitrochlorobenzene; NiSO₄, nickel sulfate; SDS, sodium dodecyl sulfate.

superoxide dismutase [Mn] (SOD2) were found induced after treatment of cells with DNCB and NiSO₄. By contrast, SDS did not regulate any stress response proteins.

Sensitizers also influenced the expression of proteins related to protein folding and degradation. So, CA, DNCB, and NiSO₄ altered the expression of 11, 7 and 2 chaperones and proteins related to protein degradation, respectively [e.g., heat shock protein 90- α (HSP90A1), mitochondrial stress-70 protein (HSPA9), protein disulfide-isomerase (P4HB), proteasome subunit beta type-7 (PSMB7), and ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCHL3)]. In addition, 14-3-3 protein isoforms were also found induced by CA and DNCB.

Moreover, many glycolytic enzymes were up-regulated, especially upon treatment of cells with DNCB or CA [e.g., pyruvate kinase isozymes M1/M2 (PKM), GAPDH, fructose-bisphosphate aldolase (ALDOA), α -enolase (ENO1), transaldolase (TALDO1), transketolase (TKT), and isoforms of phosphoglycerate mutase (PGAM1, PGAM2)]. By comparison, enzymes linked to glycolysis were induced far less through NiSO₄ and SDS treatments [e.g., GAPDH, L-lactate dehydrogenase A chain (LDHA)].

In addition, CA and DNCB but not NiSO₄ or SDS regulated a number of proteins involved in gene transcription and protein translation such as isoforms of ribosomal proteins and heterogeneous nuclear riboproteins (Table S2). Furthermore, cytoskeletal proteins (e.g., actin or tubulin isoforms) and regulators of the cytoskeleton [e.g., vasodilator-stimulated phosphoprotein (VASP), macrophage-capping protein (CAPG)] were found affected in cells treated with CA and DNCB. Conversely, NiSO₄ and SDS had only a minor impact on tubulin regulation and none on actin.

3.3. Pathway analysis

Regulated proteins were further analyzed with respect to the canonical pathways and predicted downstream effects using Ingenuity Pathway Analysis software (IPA, Ingenuity® Systems). Comparative analyses of pathway affiliations suggested a major impact of sensitizing

agents on cell signaling, metabolism and oxidative stress responses in *nrf2*^{+/+} BMDCs (Fig. 3A). Pathways related to cell-cell interaction and phagocytosis were also affected. Compared to the experiments with wild-type cells most of the relevant pathways seemed to remain unaffected in *nrf2*^{-/-} BMDCs. Furthermore, comparison of possible downstream effects in *nrf2*^{+/+} BMDCs with IPA predicted, among others, counter-regulation of cell death, inflammation and oxidative stress (Fig. 3B). Concomitantly, the strengthening of cell survival, cellular immune response, cell migration and virus infection were suggested by the software algorithm.

3.4. Biomarker candidates for sensitization

Of particular interest are proteins that appeared regulated after treatment with different sensitizers since these might represent promising biomarker candidates for contact allergen identification *in vitro*. Indeed, we observed a considerable overlap in up-regulated proteins between *nrf2*^{+/+} cells either treated with CA or DNCB (Table 1). Only three of these proteins were also affected in BMDCs treated with the irritant SDS [i.e., ATP synthase subunit alpha (ATP5A1), LDHA, CAPG]. P4HB, ATP synthase subunit beta (ATP5B) and elongation factor 1- α 1 (EEF1A1) were up-regulated in BMDCs by CA and DNCB, irrespective of the concentration applied (Table 1). Furthermore, there was a significant overlap between proteins that have been found up-regulated in BMDCs upon treatment with either NiSO₄ or DNCB (Table 1). From the three protein spots equally affected by DNCB and NiSO₄ irrespective of the concentration applied (Fig. 2B), ATP5A1, LDHA, GAPDH, tubulin beta-2A (TUBB2A) chain and PRDX1 could be isolated and subsequently characterized (Table S1). While the former four proteins were also found up-regulated in cells exposed to SDS, PRDX1 was affected by these two sensitizers only. Only three proteins were conjointly affected in wild-type BMDCs treated with CA and SDS (i.e., ATP5A1, LDHA, CAPG). Finally, only one protein spot for each of the NiSO₄ doses applied was also found in BMDCs after treatment with

Table 1

Regulated proteins extracted from protein spots emerging in 2D gels (selection).

Protein	Gene	<i>nrf2</i> ^{+/+}							<i>nrf2</i> ^{-/-}						
		CA	CA	DNCB	DNCB	NiSO ₄	NiSO ₄	SDS	CA	CA	DNCB	DNCB	NiSO ₄	NiSO ₄	SDS
		50 μM	100 μM	5 μM	10 μM	250 μM	400 μM	100 μM	50 μM	100 μM	5 μM	10 μM	250 μM	400 μM	100 μM
Actin, cytoplasmic 1	ACTB	0.31	2.03	1.68	2.06				—	3.96	—	4.39			
Aldehyde dehydrogenase, mitochondrial	ALDH2	1.58		1.60	1.81				—		—	0.20			
α-Enolase	ENO1		5.40	0.48						5.43	—				
ATP synthase subunit α, mitochondrial	ATP5A1		2.15	3.74	5.30	3.90	2.66	3.66		—	—	—	—	—	—
ATP synthase subunit β, mitochondrial	ATP5B	2.78	1.94	2.05	2.03				—	5.43	—	1.69			
Ferritin light chain 1	FTL1			1.77	1.79						—	—			
Fructose-bisphosphate aldolase A	ALDOA		4.93		3.71					—		—			
Glutamate-cysteine ligase, regulatory subunit	GCLM	2.05	1.79		1.61				—	—		—			
Glutathione S-transferase o-1	GSTO1			3.76							—				
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH			2.34	3.17	1.91	2.04	1.69			—	—	—	—	—
Heat shock protein90-α	HSP90AA1	13.23	16.05						4.61	4.93					
Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	0.46		1.59					—		—				
Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1	4.61	10.67	1.59					—	—	—				
Heme oxygenase 1	HMOX1	3.35	4.89	3.69					—	—	—				
L-Lactate dehydrogenase A chain	LDHA	1.58		1.60	1.81	1.67	1.61	1.64	—		—	0.20	—	—	—
Macrophage-capping protein	CAPG		4.75	2.16		1.82		1.84		4.24	—		1.57		—
Myosin-9	MYH9			2.00	1.80						—	—			
Peroxiredoxin-1	PRDX1			2.02	2.40	1.95	2.27				—	0.57	—	—	
Phosphoglycerate mutase 1	PGAM1			1.88	2.02						—	—			
Proteasome subunit β type-7	PSMB7	2.49	1.62		1.59				—	—		1.86			
Protein disulfide-isomerase	P4HB	2.78	1.94	2.05	2.03				—	—	—	1.69			
Pyruvate kinase isozymes M1/M2	PKM	1.89	5.40	1.85					—	5.43	—				
Superoxide dismutase [Mn], mitochondrial	SOD2				1.54		1.58					—		—	
Stress-70 protein, mitochondrial	HSPA9	3.95							2.26						
Transaldolase	TALDO1			2.58	2.30						—	—			
Transketolase	TKT				2.27							—			
Triosephosphate isomerase	TPI1	0.46							—						
Tubulin β-2A chain	TUBB2A			3.74	5.30	3.90	2.66	3.66			—	—	—	—	—
Ubiquitin carboxyl-terminal hydrolase isozyme L3	UCHL3	1.56	11.24		1.77				—	6.90		2.22			
Vasodilator-stimulated phosphoprotein	VASP	1.58		1.60	1.81				—		—	0.20			
Voltage-dependent anion-selective channel protein 1	VDAC1		2.44							—					

Values represent x-fold regulation in *nrf2*^{+/+} BMDCs upon exposure to the respective compound in comparison with vehicle control ($n = 3$, regulation ≥ 1.5 or ≤ 0.67 , $p < 0.05$). “—” indicates no significant regulation or spot intensity below the detection limit. Abbreviations: CA, cinnamaldehyde; DNCB, 2,4-dinitrochlorobenzene; NiSO₄, nickel sulfate; SDS, sodium dodecyl sulfate.

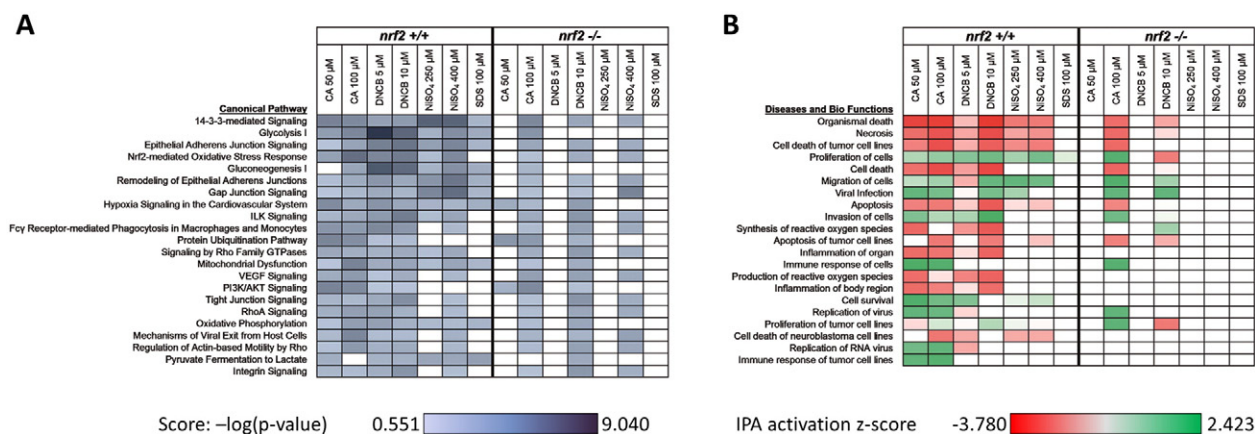


Fig. 3. Comparative Ingenuity Pathway Analysis (IPA) of the most significant canonical pathways, diseases and biofunctions. (A): Comparative analysis of the most significant canonical pathways. Data sets for core analysis comprised all significantly regulated proteins from each treatment. Pathways are sorted according to score. Color intensity represents $-\log(p\text{-value})$ indicating significance of canonical pathway. (B): Comparative analysis of diseases and biofunctions. Data sets for core analysis comprised all significantly regulated proteins from each treatment. Pathways are sorted according to score. Colors indicate predicted pathway activation (green) or inhibition (red). Color intensity represents activation z-score. Abbreviations: CA, cinnamaldehyde; DNCB, 2,4-dinitrochlorobenzene; NiSO_4 , nickel sulfate; SDS, sodium dodecyl sulfate.

100 μM CA (Fig. 2D). Nevertheless, analyses at the single protein level revealed that at least CAPG, LDHA and ATP5A1 were affected by both sensitizers, yet in dependence on the concentration applied (Table 1). An extensive literature search was performed to extract such identified proteins from those previously described as being Nrf2-dependent, and involved in DC functioning or regulated during sensitization. The results are shown in Fig. 4.

4. Discussion

Proteomic changes detected in this study clearly point to DC activation by sensitizer treatments. For instance, proteins related to cell signaling, cellular transport and gene expression were up-regulated. Induction of these processes is essential for DC activation as it facilitates phenotypic alterations needed for DC function. Consistently, 14-3-3 proteins (e.g., Ferret-Bernard et al., 2012), cytoskeletal components like actin isoforms (e.g., Ferret-Bernard et al., 2008) and proteins of the gene expression machinery like ribosomal proteins (Ceppi et al., 2009) have already been found up-regulated during DC maturation before.

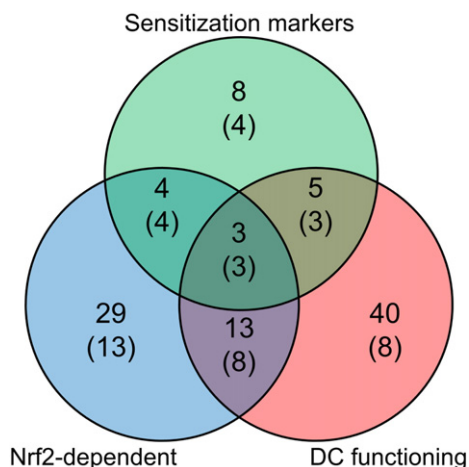


Fig. 4. Venn diagram depicting overlap of proteins identified in our study with known Nrf2 dependency, involvement in dendritic cell (DC) functioning and sensitization according to published literature. Numbers in brackets represent proteins that correspond to genes which are controlled via antioxidant response elements (ARE). Information was extracted from the literature under guidance of the keywords Nrf2 networks, DC biology and sensitization.

Furthermore, sensitizers induced manifold metabolic enzymes involved in carbohydrate, lipid and amino acid metabolism. Thus, it appears that metabolic reprogramming is linked to DC activation by sensitizing agents. Sufficient availability and consumption of glucose after toll-like receptor (TLR) activation in DCs is important for subsequent expression of co-stimulatory molecules, cytokine secretion and T cell stimulation. For proper functioning DCs perform a metabolic shift from oxidative phosphorylation towards aerobic glycolysis as a main pathway for ATP production (O'Neill, 2014). Increased metabolic activity is a prerequisite for DCs to maintain sufficient viability (Krawczyk et al., 2010; Park et al., 2006).

Here we could confirm the up-regulation of several glycolytic enzymes after treatment of BMDCs with different sensitizers which can be attributed to metabolic activation of DCs. This facilitates the phenotypic alterations during DC maturation by re-directing citrate to lipid synthesis. Increased lipid synthesis is needed for prostaglandin synthesis and the expansion of the endoplasmic reticulum (ER) and Golgi apparatus. Growth of these compartments enables enhanced protein synthesis (Everts et al., 2014). Long-term energy supply by glycolysis in DCs resembles the so-called Warburg effect that helps cancer cells to guarantee permanent and rapid cell growth (Krawczyk et al., 2010). A key enzyme during Warburg transition is the glycolytic enzyme PKM (Grüning et al., 2010). PKM was up-regulated by both sensitizers CA and DNCB. Interestingly, metabolic programming and enhanced cell proliferation of cancer cells was described recently to be controlled by Nrf2 (Mitsuishi et al., 2012). Additionally, Nrf2 was described as a regulator of energy metabolism that facilitates elevated energy utilization (Vomhof-Dekrey and Picklo, 2012). Stimulation of lung epithelial cells with toluene or benzene induced Nrf2-dependent oxidative stress response accompanied with induction of the central carbohydrate metabolism (Murugesan et al., 2013).

Several chaperones and proteasomal components were induced by the sensitizers CA, DNCB or NiSO_4 but not the irritant SDS. In most cases protein expression seemed to be Nrf2-dependent. These proteins can be linked to protein quality control and may be needed in increased amounts due to induction of oxidative stress by sensitizers, resulting in oxidative modifications, misfolding and aggregation of proteins in the cell. Chaperones can assist refolding of denatured proteins. In cases where renaturation is not possible proteins become degraded by the proteasome to protect cells. Consistently, expression levels of proteasomal proteins have already been described to be dependent on the Nrf2/Keap1 pathway (Kwak et al., 2003a, 2003b). However, chaperones and proteasomal components are also involved in antigen processing and presentation by antigen presenting cells (Tsan and Gao, 2009;

Yang et al., 2008). Such proteins were reported to be up-regulated during DC maturation (Ferreira et al., 2008; Ferret-Bernard et al., 2012). In addition, up-regulation of chaperones may also reflect increased protein translation rates during DC maturation. This is supported by the fact that several proteins involved in gene transcription and protein translation were also up-regulated in our study.

Our previous study highlighted that Nrf2 plays a major role in the control of both phases of ACD: sensitization and elicitation (et al. et al., 2013). We showed that Nrf2 crucially impacts the irritation potency of contact allergens, suggesting a control of the inflammatory process that may play the role of danger signal during the sensitization phase. Using the mouse ear swelling test (MEST) and LLNA as readouts, in *nrf2*^{-/-} mice, we showed an exacerbation of the inflammatory response in the presence of contact allergens. Nrf2-deficiency resulted in an enhancement of ear swelling by amplification of skin irritation and increased lymphocyte proliferation. Possibly, Nrf2-deficiency and the concomitant higher ROS production in *nrf2*^{-/-} mice could also contribute to a higher basal activation level of innate cells such as DCs, augmenting the capacity of these cells to elicit an adaptive immune response. However, DC activation was not monitored in this study, albeit preliminary data showed that *nrf2*^{-/-} BMDCs, compared to *nrf2*^{+/+} BMDCs, had elevated levels of the co-stimulatory surface molecules CD86 and CD80. Our proteomics analysis focused on the response of BMDCs to contact allergens. Therefore, the interplay of DCs, keratinocytes and T cells was missing and the complex immunoregulatory network of sensitization could not be observed. However, an ACD-enhancing effect of Nrf2-deficiency is not necessarily contradicting a functional role of Nrf2 during DC activation. DC activation in Nrf2-deficient mice may still lead to a proper sensitization as Nrf2 may not be regarded as the only factor enabling DC activation and T cell priming is still possible. It may then lead to an enhanced inflammatory response during elicitation caused by a loss of electrophilic stress response leading to exacerbated irritation and more frequent antigen formation. Indeed, a supporting function of Nrf2 during DC activation is indicated by the correlation of CD86 expression and Nrf2 activity in human CD34⁺-derived dendritic cells and the THP-1 myeloid cell line after treatment with several contact allergens (Ade et al., 2009).

The set of 100 proteins identified in our study included 29 proteins described as Nrf2-dependent and that mainly associate with phase II metabolism and oxidative stress responses. However, only for 13 of them ARE sequences were identified in the promoter regions (e.g., Thimmulappa et al., 2002; Kwak et al., 2003b; Lee et al., 2003).

From the set of putative Nrf2 targets without identified ARE sequences we could confirm an Nrf2-dependent regulation for heterogeneous nuclear ribonucleoprotein A1, myosin-9, triosephosphate isomerase on the protein level for the first time. In addition, we found several proteins induced in *nrf2*^{+/+} but not in *nrf2*^{-/-} BMDCs which have not been described in the literature as Nrf2 targets before. Our data indicate a possible Nrf2-dependent regulation for 62 such proteins. They may be regarded as putative new Nrf2 targets. However, it should be noted that analysis in *nrf2*^{-/-} cells was hampered by reduced overall protein levels. Lower protein levels interfere with the detection of protein spots, especially of low abundant proteins. Thus, a few of the missing spots in *nrf2*^{-/-} gels may derive from lower proteins levels.

Some of these potential new Nrf2 targets were found to be regulated in *nrf2*^{-/-} BMDCs as well, but regulation was reduced compared to *nrf2*^{+/+} (e.g., HSP90AA1, 14-3-3 epsilon). 48 of these proteins were either not regulated or the corresponding spots were completely missing in *nrf2*^{-/-} BMDCs (see supplementary Table S1 for examples).

The data set contained several regulated proteins that were described as contact allergen responsive before. ARE-regulated proteins known to be influenced by sensitizers were GCLM, HMOX1, ALDH2 and TALDO1 which were found induced by CA and DNCB but not by NiSO₄ or SDS (e.g., Thimmulappa et al., 2002; Kitteringham et al., 2010). It seems reasonable to regard possible Nrf2-controlled proteins involved in DC biology as promising biomarker candidates for

sensitization. We found 7 ARE-containing Nrf2 targets solely induced by sensitizers and not reported in sensitization studies before including HSPA9, voltage-dependent anion-selective channel protein 1 (VDAC1), glutathione S-transferase omega-1 (GSTO1), ferritin light chain 1 (FTL1), PRDX1, SOD2 and TKT. Especially FTL1, PRDX1 and SOD2 seem to be interesting as their regulation was Nrf2-dependent in this study and they have already been reported to be regulated during DC differentiation and maturation before (e.g., Gadgil et al., 2003; Pereira et al., 2005; Ferreira et al., 2010).

Notably, as glycolysis rate increases quickly and induction lasts for several hours metabolic activation can be an attractive endpoint for *in vitro* testing strategies in DC models or other immune cell types.

5. Conclusion

In this study proteomic changes in BMDCs derived from *nrf2*^{+/+} and *nrf2*^{-/-} mice after treatment with three different sensitizers and an irritant were analyzed. In total 100 proteins were identified, which were regulated in *nrf2*^{+/+} BMDCs by at least one of the treatments. Among them we identified many known Nrf2-dependent proteins to be up-regulated after treatment with sensitizers, in particular stress response proteins such as GCLM or HMOX1. In addition, we found evidence for metabolic activation, the induction of signaling cascades and alterations in cell cytoskeleton. Mostly these responses could not be detected anymore in *nrf2*^{-/-} BMDCs.

We could identify several promising biomarker candidates for sensitizers. Our study gives further evidence to the hypothesis that DC activation by sensitizers is linked to metabolic reprogramming in the cells. In particular glycolysis seems to be induced by sensitizers as we found many glycolytic enzymes up-regulated after CA and DNCB treatments. A key enzyme is PKM, which we found induced by both sensitizers. Other promising biomarker candidates are proteins, which have already been reported to be regulated during DC differentiation and maturation and which we could confirm to be induced after sensitizer treatment. In particular, FTL1, PRDX1 and SOD2 could be interesting.

Thus, our study clearly suggests a role for Nrf2 in maintaining DC function and also points to a functional involvement in DC maturation in response to contact allergens.

Conflict of interest

The authors declare no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.taap.2016.11.001.

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