Proteomic analysis of cellular changes after treatment of human monocytic cell lines and primary dendritic cell models with contact allergens

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Declaration of Independence

I hereby declare that I have independently prepared this thesis entitled ‘Proteomic analysis of cellular changes after treatment of human monocytic cell lines and primary dendritic cell models with contact allergens’ without the use of any other than the approved resources. All citations are marked as such.

The submitted work has not been accepted or found to be unsatisfactory in any previous doctoral proceedings.

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1 Summary

Allergic contact dermatitis (ACD) is one of the most common immunotoxicological disorders of the skin. ACD is provoked by exogenous contact allergens that are mistakenly identified as a danger by the immune system and orchestrated by a T cell-mediated adaptive immune response. However, before a symptomatic response occurs (elicitation), a clinically silent sensitization phase precedes. The sensitization phase is characterized by complex interactions of different cell types with dendritic cells (DC) playing a central role. DCs may take up complexes of chemically-modified self-proteins, process and present them on their surface for T cell recognition. Subsequently, they undergo a maturation process and migrate to lymph nodes to initiate the priming and proliferation of allergen-specific T cells. The DCs are thus of crucial importance for the development of contact allergy. Despite this central role, the exact cellular signaling pathways leading to allergen-induced maturation of dendritic cells are not yet fully understood. As the current knowledge is mainly based on transcriptome data, state of the art mass spectrometry-based proteomics are applied in this thesis to complement and extend existing data to enhance the understanding of ACD pathomechanisms.

In this work, it was shown for the first time that proteomic methods are suitable to distinguish activation of monocyte-derived dendritic cells (MoDC) triggered by the contact allergen $NiSO_4$ from the bacterial activator lipopolysaccharide (LPS). $NiSO_4$ caused not only a different MoDC phenotype but also the activation of divergent signaling pathways. Both treatments induced metabolic reprogramming towards aerobic glycolysis, which, however, manifested over a longer period in the case of $NiSO_4$ and eventually activated hypoxia-like signaling pathways such as hypoxia-inducible factor (HIF) 1α upregulation. $NiSO_4$ treatment further elicited a pronounced nuclear factor erythroid 2-related factor (Nrf) 2-dependent stress response in MoDCs. Most striking was the selective upregulation of cholesterol biosynthesis after treatment with $NiSO_4$ which was absent upon stimulation with LPS. Concomitantly, the cells exhibited a significant decrease in cellular cholesterol levels. Thus, this signaling pathway was identified as a promising approach in the search for predictive biomarker signatures for sensitization with contact allergens.

The second study was designed to verify the above-mentioned results in another cell model. Additional allergens were included to reveal similarities or differences in the activation of DCs by the allergens. THP-1 cells, which are standardly used for the prediction of contact allergens *in vitro*, were chosen as the cell model. The allergens $NiSO_4$, *p*-dinitrochlorobenzene (DNCB), *p*-benzoquinone (BQ), and *p*-nitrobenzyl bromide (NBB) induced comparable proteomic changes and differed mainly in magnitude of induced proteomic changes. DNCB and NBB were the most potent activators of the cells. In direct comparison with MoDCs, THP-1 cells showed a much more pronounced Nrf2-mediated stress response. Based on these results, the Nrf2-mediated stress response should also be

implemented into future *in vitro* DC test strategies for the prediction of contact allergens. Allergen-treated THP-1 cells developed a proinflammatory phenotype, which was supported by significant upregulation of the citrate cycle. Cholesterol biosynthesis was also significantly regulated in these cells - but in opposing direction to the findings from MoDCs.

Overall, the comparison of MoDCs and THP-1 cells revealed relevant differences in the regulation of many proteins and signaling pathways between the cell models, but still resulted in the activation of similar superordinate systemic endpoints. THP-1 cells are therefore limited in their representation of MoDCs as primary DCs model *in vitro*. The further development of already validated *in vitro* methods in THP-1 cells is should thus focus on proteins that are differentially regulated by contact allergens in both cell types.

2 Zusammenfassung

Die allergische Kontaktdermatitis (ACD) ist eine der häufigsten immunotoxikologischen Erkrankungen der Haut. Hierbei wird das körperfremde Kontaktallergen vom Immunsystem fälschlicherweise als Gefahr identifiziert und eine T zellvermittelte adaptive Immunreaktion initiiert. Bevor eine gezielte Immunantwort in der Auslösephase erfolgen kann, geht eine asymptomatische Sensibilisierung voran. Die Sensibilisierungsphase ist ein komplexes Zusammenspiel verschiedener Immunzellen. Eine zentrale Rolle spielen hierbei die dendritischen Zellen (DC). Diese können Komplexe aus körpereigenen Proteinen mit den Kontaktallergenen erkennen, aufnehmen, prozessieren und auf ihrer Oberfläche den T Zellen präsentieren. Hierbei durchlaufen sie einen Reifungsprozess und wandern in Lymphknoten, um dort die Bildung und Proliferation allergen-spezifischer T Zellen einzuleiten. Die DCs sind somit von elementarer Bedeutung für die Ausprägung einer Kontaktallergie. Trotz dieser zentralen Rolle, sind die exakten zellulären Signalwege, die zu der allergeninduzierten Reifung der dendritischen Zellen führen, noch nicht vollständig verstanden. Da der derzeitige Wissensstand hauptsächlich auf Transkriptomdaten beruht, werden in dieser Dissertation moderne massenspektrometrische Proteomanalysen durchgeführt, um die vorhandenen Daten zu ergänzen sowie zu erweitern und so die zugrundeliegenden Pathomechanismen von ACD besser zu verstehen.

Im Rahmen dieser Arbeit konnte erstmals gezeigt werden, dass proteomische Methoden geeignet sind, die Aktivierung von MoDCs durch das Kontaktallergen $NiSO_4$ von dem bakteriellen DC Aktivator Lipopolysaccharid (LPS) zu unterscheiden. $NiSO_4$ induzierte nicht nur einen anderen Phänotyp, sondern auch die Aktivierung divergenter Signalwege in den Zellen. Beide Behandlungen induzierten eine metabolische Reprogrammierung hin zur aeroben Glykolyse, welche sich jedoch im Fall von $NiSO_4$ über einen längeren Zeitraum manifestierte und schließlich Hypoxie-ähnliche Signalwege wie hypoxia-inducible factor (HIF) 1α aktivierte. Die $NiSO_4$-Behandlung löste weiterhin eine ausgeprägte nuclear factor erythroid 2-related factor (Nrf) 2-abhängige Stressantwort in MoDCs aus. Besonders prominent war zudem die selektive Hochregulation der Cholesterolbiosynthese nach Behandlung mit $NiSO_4$. Dies ging mit einer signifikanten Verminderung der zellulären Cholesterollevel einher. Dieser Signalweg stellt somit einen vielversprechenden Ansatz auf der Suche nach prädiktiven Biomarkersignaturen für die Sensibilisierung mit Kontaktallergenen dar.

In einer zweiten Studie sollten die Ergebnisse der ersten Studie in einem anderen Zellmodell verifiziert und weitere Allergene untersucht werden, um Gemeinsamkeiten oder Unterschiede in der Aktivierung von DCs durch die Allergene aufzuzeigen. Als Zellmodell wurden THP-1 Zellen gewählt, welche standardmäßig für die Vorhersage von Kontaktallergenen *in vitro* genutzt werden. Die Allergene $NiSO_4$, *p*-Dinitrochlorbenzol (DNCB), *p*-Benzoquinon (BQ) und *p*-Nitrobenzylbromid (NBB) induzierten vergleichbare proteomische Veränderungen und unterschieden sich hauptsächlich hinsichtlich ihrer

Potenz. DNCB und NBB waren die potentesten Aktivatoren der Zellen. Im direkten Vergleich zu den MoDCs zeigten THP-1 Zellen eine deutlich ausgeprägtere Nrf2-vermittelte Stressantwort. Basierend auf diesen Ergebnissen sollte die Nrf2-vermittelte Stressantwort künftig auch in *in vitro* Teststrategien für die Vorhersage von Kontaktallergenen in DCs implementiert werden. Allergen-behandelte THP-1 Zellen entwickelten einen proinflammatorischen Phänotyp, welcher durch signifikante Hochregulation des Citratzyklus gestützt wurde. Auch in diesen Zellen war die Cholesterolbiosynthese signifikant reguliert – jedoch gegensätzlich zu den Befunden in den MoDCs.

Insgesamt ergaben sich im Vergleich von MoDCs und THP-1 Zellen gravierende Unterschiede die Regulierung vieler Proteine und Signalwege betreffend, dennoch resultierte dies in der Aktivierung übergeordneter systemischer Endpunkte. THP-1 Zellen eignen sich folglich nur begrenzt als Modell für die Aktivierung von primären DCs *in vitro*. Die Weiterentwicklung von bereits validierten *in vitro* Methoden in THP-1 Zellen sollte sich somit auf Proteine stützen, die in beiden Zellentypen gleichsam durch Kontaktallergene exprimiert werden.

3 Abbreviations

A	
ACD	Allergic contact dermatitis
Akt	Protein kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
AOP	Adverse outcome pathway
APC	Antigen-presenting cell
ARE	Antioxidant response element
B	
BfR	German Federal Institute for Risk Assessment
BMDC	Bone marrow-derived dendritic cell
BQ	*p*-Benzoquinone
C	
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CLP	Classification, Labelling and Packaging
D	
DA	Defined approach
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNCB	*p*-Dinitrochlorobenzene
DPRA	Direct peptide reactivity assay
E	
ELISA	Enzyme-linked immunosorbent assay
G	
GARD	Genomic Allergen Rapid Detection
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H	
25HC	25-Hydroxycholesterol
hCLAT	Human cell line activation test
HIF	Hypoxia-inducible factor
HLA-DR	Human leukocyte antigen – DR isotype
I	
ICD	Irritant contact dermatitis
iDC	Immature dendritic cell
IKKε	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IL	Interleukin
INF	Interferon
IPA	Ingenuity Pathway Analysis
K	
KEAP	Kelch like-ECH-associated protein
$K_{o/w}$	Octanol-water partition coefficient
L	
L	Ligand
LC	Langerhans cell
LC-MS/MS	Liquid chromatography couple to tandem mass spectrometry
LFA	Lymphocyte function-associated antigen
LFQ	Label-free quantification
LLNA	Local lymph node assay
LPS	Lipopolysaccharide
LXR	Liver X receptor
M	

mDC	Mature dendritic cell
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cell
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
m/z	Mass to charge ratio
N	
NAM	New approach methodologies
NBB	*p*-Nitrobenzyl bromide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP	NACHT, LRR and PYD domains-containing protein
Nrf	Nuclear factor erythroid 2-related factor
O	
OECD	Organization for Economic Co-operation and Development
OXPHOS	Oxidative phosphorylation
P	
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PPD	*p*-Phenylenediamine
PRR	Pattern recognition receptor
R	
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
ROS	Reactive oxygen species
S	
SILAC	Stable isotope labelling by amino acids in cell culture
SREBP	Sterol response element binding protein
T	
TBK	TANK-binding kinase
TCA	Tricarboxylic acid
TCR	T cell receptor
T_c	$CD8^+$ cytotoxic T cell
TG	Test guideline
T_h	$CD4^+$ T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon beta

4 Introduction

4.1 Allergic contact dermatitis – epidemiology, diagnostic testing and treatment

Humans are continuously exposed to external influences of different sources such as UV radiation, pathogens, and xenobiotics. The skin is the largest human organ and its unique structure is an efficient protection shield against these external noxes. Yet, some chemicals possess the ability to transcend this ingenious barrier and subsequently cause an allergic reaction of the skin also known as ACD. ACD is a specific misdirected reaction of the immune system towards a compound dose that is typically well tolerated and does not cause immune reactions in the majority of the population. ACD can lead to severe clinical symptoms like inflammation, rashes, redness and skin lesions in affected individuals. Already centuries ago, in ancient Egypt, Rome, and Greece, people recognized a correlation between exposure to specific plants and skin disorders (Wright and Goldman, 1979; Smith, 2009). Since then, thousands of contact allergens were identified. Besides plant- and animal-derived products such as propolis and lanolin (Fransen et al., 2018; Nyman et al., 2021), cosmetics and commodities (jewelry, fragrances, preservatives) as well as pharmaceuticals were identified as sources of contact allergens. Of particular concern is a so-called occupational ACD that can be induced by the workplace environment and often affects hair dresser, health care personnel, constructors, and metal workers (Chu et al., 2020). There are also reports on occupational skin sensitization in the chemical and pharmaceutical industry (Goossens and Hulst, 2011; Bennett et al., 2016; Anderson et al., 2017). Importantly, not only active pharmaceutical components and their intermediates may act as contact allergens. Additives in pharmaceutical formulations such as fragrances and preservatives have also frequently been reported to induce ACD (Cheng and Zug, 2014; Liden et al., 2016; Deza and Gimenez-Arnau, 2017).

Patch testing is the gold standard for assessment of skin sensitization in patients. During the testing, the allergen is applied to the patient's skin in an occlusive chamber. After usually 48 h, the patch test is removed and inflammation and eczema of the skin are rated for the first time with later readings to follow (Dickel and Mahler, 2020). The frequency of contact allergy to a specific compound in the general public is dynamic and largely depends on exposure, which is also influenced by legislation, and awareness of the public and industry. Propolis, for example, was recently identified as emerging contact allergen due to its augmented use in cosmetics whereas numbers of methylisothiazolinone-sensitized patients currently decline following legal regulation after an European epidemic (Uter et al., 2020a; Uter et al., 2020b). The most frequent sensitizers (≥1% of patients react) are combined in sets recommended for testing in all patients, e.g. the European or German baseline series established by the European society on contact dermatitis and "Deutsche Kontaktallergiegruppe", respectively. The baseline series are regularly updated, with the help of epidemiological data collected from participating

clinics within the European Surveillance System on Contact Allergies in eleven European countries and the “Informationsverbund dermatologischer Kliniken” in Germany, Austria and Switzerland (Wilkinson et al., 2023). Till present, sensitization to nickel (Ni) is the most frequent cause of ACD (Uter et al., 2020b). Although release of Ni from consumer products intended for prolonged skin contact has been regulated by the EU nickel directive (European Union, 1994), 8 – 18 % of the European population remain sensitized to Ni (Ahlstrom et al., 2017; Alinaghi et al., 2019). Legal restriction is a powerful tool in protecting the general public and has led to decreasing numbers of affected individuals not only in the case of Ni but also for other allergens such as methylisothiazolinone (Ahlstrom et al., 2017; Uter et al., 2020a). However, other potent allergens like *p*-phenylene diamine (PPD) remain unregulated. The allergenic potential of PPD has been known for a long time and PPD was classified as a strong allergen (Warbrick et al., 1999). Basketter et al. (1994) investigated the sensitizing potential of PPD in a human maximization test, which resulted in 100% sensitization to PPD after induction with 10% PPD and challenge with 0.5% PPD respectively. In Europe, 0.8% of the general population are sensitized to PPD (Diepgen et al., 2016a). Yet, PPD is still marketed in hair dyes not only for professional application but also as over the counter product.

Exact numbers of sensitized patients are difficult to estimate since not every patient reports to the doctor and it is not always possible to correctly identify the causative allergen in order to reliably diagnose a patient with ACD. However, based on large patch test studies performed with the baseline series in the general public in Europe, it is assumed that 20% - 27% are sensitized to at least one contact allergen (Diepgen et al., 2016b; Alinaghi et al., 2019). Thus, ACD is to date the most prevalent form of immunotoxicity in Europe. In Germany, at least 142.000 patients were reported to be patch tested between 2007 - 2018 by the Information Network of Departments of Dermatology and approximately 15% of the patients were diagnosed with Ni allergy (Uter et al., 2020b).

Skin sensitization can occur at any stage of life. In principle, any person can develop ACD. However, there are conditions that facilitate skin sensitization. Exposure dose and time regimen to the offending allergen play an important role as high exposure levels as well as frequencies increase the probability to get sensitized. Another important factor is the condition of the skin. Injured skin is more susceptible to be sensitized. Nickel allergy, for example, is highly associated with the execution of body piercings (McDonagh et al., 1992; Nielsen and Menné, 1993). Furthermore, a reduced skin barrier function due to an existing lesion or irritation of the skin is prone for skin sensitization as the allergen can easily penetrate the protective skin barrier i.e. the stratum corneum (Proksch and Brasch, 2012). The skin barrier is also disrupted by exposure to chemicals such as solvents, detergents, and even water. Under experimental conditions, detergents like sodium lauryl sulfate for example were shown to cause skin irritation and thereby promoting sensitization with contact allergens (Kligman, 1966b;

Cumberbatch et al., 1993; Agner et al., 2002; Pedersen et al., 2004; De Rentiis et al., 2021). Co-exposure with other immune-stimulating agents such as LPS may facilitate skin sensitization (Kinbara et al., 2011; Strasser et al., 2017). The susceptibility of an individual to develop ACD can also be determined by genetic factors. Especially patients with atopic conditions are believed to be at higher risk to get sensitized due to impaired barrier function of the skin. However, despite the higher theoretical risk of atopic patients, Romita et al. (2019) did not find a higher rate of diagnosed ACD among atopic patients. Polymorphisms in genes relevant for the development of ACD were proposed in the past, however the scientific evidence is ambiguous in most cases (Schnuch et al., 2011; Choi et al., 2023). Petersen et al. (2019) demonstrated that mice with filaggrin deficiency were more responsive to contact allergens, in particular Ni, as control mice. Filaggrin is an integral protein of the skin and plays an important role during terminal differentiation and formation of the skin barrier. However, no general clinical evidence for a correlation between filaggrin mutations and elevated individual susceptibility to get sensitized was proven in humans (Lagrelius et al., 2020). Solely Ni contact allergic patients are known to display increased rates of gene mutations in the filaggrin gene (Thyssen et al., 2010).

Until today, sensitization to an allergen remains incurable and affected individuals have to avoid the culprit allergen and potential cross-reacting xenobiotics lifelong in order to prevent outbreak of an allergic reaction. Currently, the impact of immunosenescence on ACD in ageing patients is discussed. The prevalence of various allergens in different age groups was shown to differ. Nickel, for example, was shown to peak in the medium age range and decreasing with age (Balato et al., 2008; McSweeney et al., 2020), whereas allergy to the fragrance mix steadily increased with age (Buckley et al., 2003; Uter and Schnuch, 2004). The severity of ACD elicitation may also be reduced in elderly people (Kwangsukstith and Maibach, 1995). During the elicitation, acute symptoms can be alleviated with antihistaminics, glucocorticosteroids, calcineurin inhibitors, and immunosuppressive drugs like cyclosporine (Belsito et al., 2006; Li and Li, 2021). Novel antibody-based treatments are still under development (Bangsgaard et al., 2011; Chipalkatti et al., 2019). Especially dupilumab seems promising and was already shown to effectively alleviate ACD in sesquiterpene lactone-allergic patients.

4.2 Contact allergens

Only a minor amount of chemicals potentially acts as contact allergen as specific characteristics have to be fulfilled. First of all, the allergen has to penetrate the stratum corneum ergo the skin barrier which is facilitated by hydrophobicity and a low molecular weight. Potts and Guy (1992), for example, reported octanol-water partition coefficients (log $K_{o/w}$) in the range of -1 to 3 to correlate well with skin permeability. The $K_{o/w}$ is a measure for the lipophilicity of a chemical compound. The log$K_{o/w}$ of a

substance can range from -3 (very hydrophilic) to +10 (highly lipophilic) with $\log K_{o/w} = 1$ characterizing chemicals that are equally soluble in both phases. Most known contact allergens furthermore display a rather low molecular weight of smaller than 500 Da, however, also bigger molecules may act as skin sensitizer (Roberts et al., 2013; Fitzpatrick et al., 2017). Contact allergens are typically chemically reactive towards proteins. The binding of chemicals to skin self-proteins is referred to as haptenation. These altered self-proteins will be recognized by immune cells which will then induce the immunological response described in section 4.3. Contact allergens preferably bind to the nucleophilic side chains of lysine, cysteine, and histidine in proteins thereby acting as electrophiles (Divkovic et al., 2005; Aleksic et al., 2007). The most common reaction mechanisms for haptenation are Michael addition, nucleophilic substitution reactions and Schiff base formation (Roberts et al., 2007b). Metal allergens like Ni (Ni^{2+} ions) and cobalt (Co^{2+} ions), however, form non-covalent complexes with proteins (Zhang and Wilcox, 2002; Gamerdinger et al., 2003; Thierse et al., 2004). A high chemical reactivity towards proteins correlates with the potency of an allergen and is believed to entail numerous modification sites within the protein sequence by the allergen. This translates to a broad range of potential T cell epitopes. This diversity in T cell epitopes eventually results in a polyclonal and thus more potent T cell response (Martin et al., 2003; Moon et al., 2007; Esser et al., 2014). Indeed, research on haptenation sites was performed for various allergens applying mass spectrometric methods and multiple molecular targets were identified. Frequently modified proteins include human serum albumin, actin, and keratins, yet the altered target proteins were allergen-dependent (Guedes et al., 2016; Ndreu et al., 2020; Parkinson et al., 2020a; Parkinson et al., 2020b). A comprehensive overview on molecular haptenation sites can be found in section 6.1 of this thesis.

Some chemicals require initial activation to be able to act as an allergen. So-called prehaptens undergo abiotic activation induced by e.g. UV radiation or autoxidation (Hagvall et al., 2007; Sköld et al., 2008). Chemicals that depend on metabolic activation by skin-intrinsic enzymes such as cytochrome P450 or alcohol dehydrogenase are referred to as prohaptens (Smith et al., 2000; Bergström et al., 2007). To efficiently trigger an immunological response and induce sensitization, contact allergens need to possess inflammatory potential on the skin e.g. they are able to trigger a sterile inflammation (Martin, 2012). Contact allergens were thus shown to cause inflammation likewise to pathogens by triggering signaling via pattern recognition receptors (PRR) which induces the innate immune cascade (Martin et al., 2008; Schmidt et al., 2010; Esser et al., 2012a).

4.3 Pathomechanism of ACD

The pathogenesis of ACD is characterized by two successive phases: the sensitization and the elicitation phase (Figure 1). Clinical symptoms only emerge after sensitization during the elicitation phase. ACD is T cell-mediated and development of the allergic reaction as well as its symptoms usually manifest approximately one to several day(s) after re-exposure. Therefore, ACD is classified as type IV delayed hypersensitivity reaction. Sensitization to an allergen requires inflammation of the skin. The covalent binding of the allergen to a self-protein in the epidermis induces spatial conformation changes of the latter which drives inflammation via induction of the unfolded protein response (Gendrisch et al., 2022). The altered protein may furthermore be sampled by DCs and mistakenly recognized as non-self. These hapten-complexes act immunogenic alike to exogenous antigens. Besides this, the induction of reactive oxygen species (ROS) by contact allergens is a key driver of the induction of an immune response (Esser et al., 2012b). The production of ROS by the immune system is a defense strategy against microbial pathogens but ROS was also shown to damage the extracellular matrix by provoking for example high molecular hyaluronic acid breakdown (Monzon et al., 2010). The resulting low molecular hyaluronic acid acts pro-inflammatory and serves as endogenous activator of Toll-like receptors (TLR) 2 and 4 which ultimately drives inflammation of the skin (Termeer et al., 2002; Scheibner et al., 2006). Vennegaard et al. (2014) could show that induction of nickel allergy can also occur independent of TLR4 but in an interleukin (IL)-1- and myeloid differentiation primary response 88 (MyD88)-dependent manner in mice.

Upon haptenation, activated cells like keratinocytes subsequently secrete pro-inflammatory cytokines such as IL-1α, IL-1β, and IL-18 which support activation of DCs and other immune cells. DCs are professional antigen-presenting cells (APC) and reside in their immature phenotype (iDC) in the skin where they constantly sample their environment. Upon phagocytosis of an antigen and further stimulation by pro-inflammatory cytokines or other tissue damage signals, maturation of the DCs is initialized. DCs then process the antigen in their immunoproteasome and present fragments of the latter on their cell surface with the help of major histocompatibility complexes (MHC). Maturation of DCs is accompanied by major alterations of cellular metabolism, phenotype transformation, secretion of second messenger molecules, and migration to the local lymph nodes. The expression of adhesion molecules like cluster of differentiation (CD) 54 on the cells surface supports migration of the DCs through the lymphatics and costimulatory molecules such as CD40, CD80, and CD86 facilitate crosstalk to T cells. In the lymph node, mature DCs (mDC) present the allergen epitopes to naïve T cells thereby priming those with T cell receptor (TCR) specificity for the allergen-induced epitope. Priming of naïve T cells by maturated DCs is highly efficient. The main cell surface co-receptors involved in the initiation of the crosstalk are CD54, CD102, and CD58 on DCs and lymphocyte

function-associated antigen (LFA)-1 and CD2 on T cells. Once the antigen-loaded MHC of the APC has been specifically recognized via the TCR, a conformation change in LFA-1 is induced which enhances affinity to intercellular adhesion molecules like CD54 and CD102 on the cell surface of DCs. Binding of the TCR to the matching MHC along with binding of further costimulatory molecules like CD80 and CD86 expressed on the DC and sensing of relevant cytokines, eventually initiates cellular expansion of the naïve T cell and subsequent differentiation to T effector and memory cells. Another important costimulatory molecule expressed by DCs is CD40 which fosters T cell activation substantially via binding of its ligand, CD154. Precisely, differentiation and clonal expansion of naïve $CD4^+$ T helper (T_h) cells of the type T_h1 and T_h17 as well as generation of $CD8^+$ cytotoxic T cells (T_c) of the type T_c1 and T_c17 are induced. However, various contact allergens, including methylchloroisothiazolinone and methylisothiazolinone as well as metals like Ni and cobalt, were shown to induce Th2 cells or Th2-related cytokines as well (Minang et al., 2006; Quaranta et al., 2014b; Virgens et al., 2022). Differentiation of T cells to effector cells is accompanied by changes on cell surface which determine tissue homing of the cells. During this process, the lymph node homing molecules are downregulated and effector cells start migration towards the affected tissue. These skin-homing T cell clones migrate to the site of exposure in the skin where some cells become tissue-resident memory T cells (Freudenberg et al., 2009; Vocanson et al., 2009; Dyring-Andersen et al., 2013).

The elicitation phase is provoked upon exposure to the allergen in sensitized individuals. The exposure route can but does not have to be the same as during sensitization (Scheinman et al., 2021). Again, the above-mentioned innate immune response is triggered in antigen-presenting cells like DCs, Langerhans cells (LC), mast cells and also activated keratinocytes including presentation of the hapten-carrier-complex on their cell surface and secretion of pro-inflammatory cytokines. Skin-resident memory $CD4^+$ and $CD8^+$ T cells become reactivated and secretion of IL-17α and interferon (INF) γ induces T cell expansion, further activation of other cells like keratinocytes as well as recruitment of neutrophils and antigen-specific effector T cells (Vocanson et al., 2009; Weber et al., 2015; Schmidt et al., 2017; Murata and Hayashi, 2020; Funch et al., 2022). This antigen-specific immune response occurs without further need of T cell activation through e.g. costimulatory molecules or cytokines. Besides T cells, macrophages, mast cells, eosinophils, B cells, and natural killer cells are also involved in this inflammatory response which ultimately leads to redness, itchiness, burning, and other clinical symptoms of the affected skin (Honda et al., 2013).

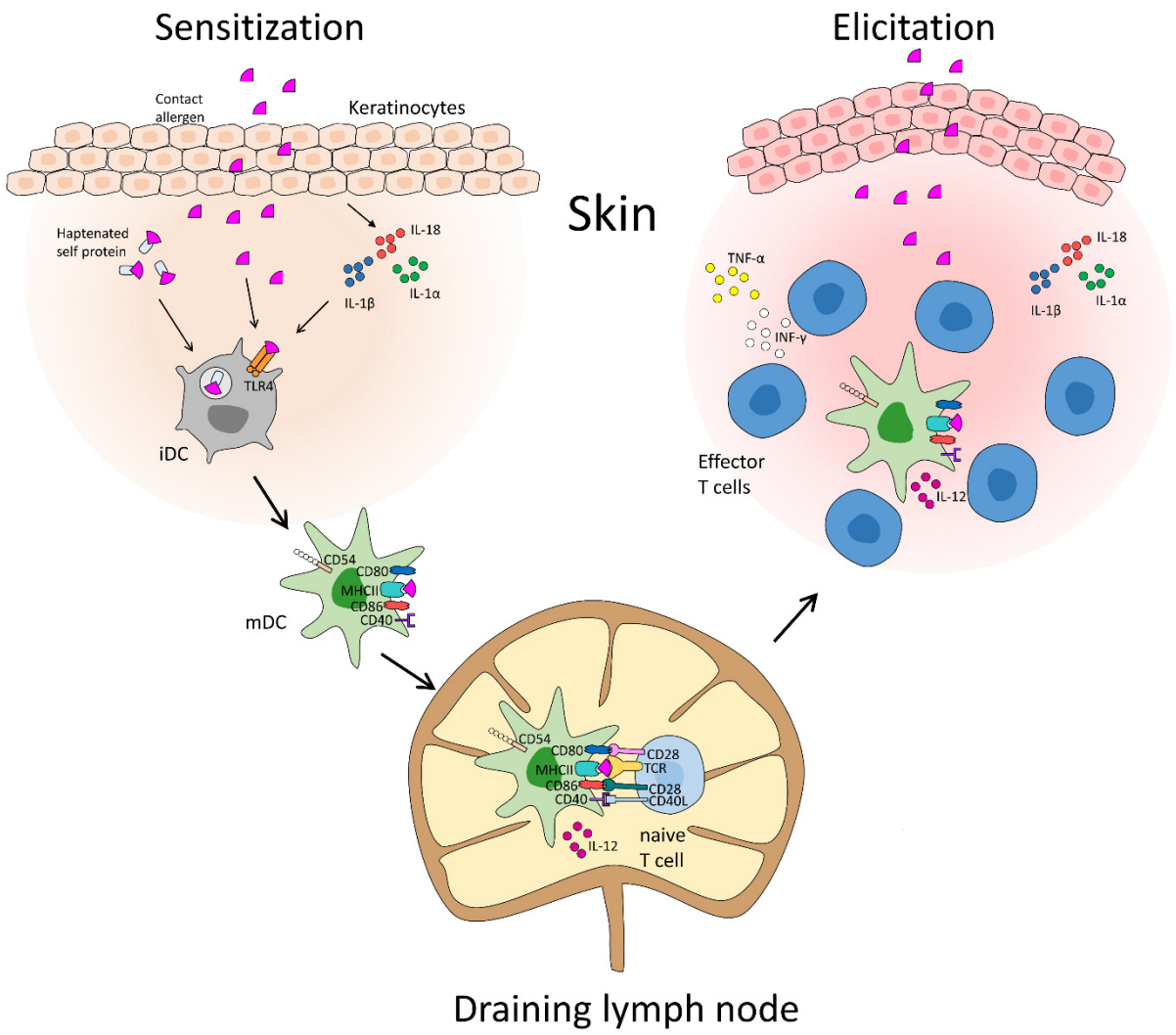

Figure 1 Schematic outline of ACD pathogenesis (modified after (Saito et al., 2016) and published in (Höper et al., 2017)). Briefly, the contact allergen penetrates the skin and binds to skin proteins which activates skin-resident cells like keratinocytes. iDC phagocytose the hapten-complex and subsequently undergo a maturation process. The mDCs migrate to afferent lymph nodes and prime naïve T cells. Antigen-specific T cells migrate back to the affected tissue and reside as memory cells. Upon second exposure during elicitation, memory T cells produce large numbers of effector T cells which than mediate an inflammatory reaction of the skin. CD: cluster of differentiation; iDC: immature dendritic cell; IL: interleukin; INF: interferon; L: ligand; mDC: mature dendritic cell; MHC: major histocompatibility complex; TCR: T cell receptor; TLR: Toll-like receptor; TNF: tumor necrosis factor.

Due to similar symptoms and higher frequency in the general population, ACD can be mistaken for irritant contact dermatitis (ICD). ICD can be elicited after single exposure of the skin to xenobiotics that act damaging and inflammatory on the skin such as corrosive acids and bases or detergents. Sodium lauryl sulfate and sodium hydroxide, for example, cause an irritant contact dermatitis which

manifests with identical clinical symptoms as ACD (Nosbaum et al., 2009). In contrast to ACD, clinical symptoms during ICD occur shortly after exposure to the offending xenobiotic and the immunologic response is driven by innate immunity only and thus does not involve antigen-specific T cells (Nosbaum et al., 2009). Coutant et al. (1999) showed that irritants failed to induce activation marker expression like CD40, CD86, CD54, and HLA-DR as well as autologous T cell activation in human MoDCs. The non-induction of CD54 and CD86 by irritants is also used to discriminate skin sensitizers from non-sensitizers *in vitro* (Ashikaga et al., 2006b). On gene level, even more biomarker were found to discriminate irritants and skin sensitizer (Johansson et al., 2011b; Johansson et al., 2019).

4.4 Dendritic cell subsets in the skin

DCs are professional antigen-presenting cells of the innate immune system. Their biological function is the continuous sampling of potentially harmful material of exogenous or endogenous origin. DCs are considered as crucial link between innate and adaptive immunity due to their ability to migrate to afferent lymph nodes to induce antigen-specific T cell expansion. DCs are considered most efficient inducers of antigen-specific immunity amongst APCs since only professional APC can prime naïve T cells. In the bone marrow, DCs evolve from hematopoietic $CD34^+$ stem cells. In contrast to other cell types like T cells, B cells or monocytes, DCs are not characterized by a specific lineage marker like for example CD3 for T cells or CD14 and CD16 for monocytes. Over the years, a growing number of various DC subsets has been described depending on the tissue and species. Plasmacytoid and conventional DCs are the main DC cell types in humans and mice. Plasmacytoid DCs (pDC) are involved in antiviral defense and wound healing by efficient and rapid production of interferons, but are not found in steady-state skin (Gregorio et al., 2010).

In the human skin, various DC subsets with different functionalities during ACD have been described. The human epidermis is typically populated by LCs whereas DCs are mainly found in the dermis. In the dermis, three main DC subsets are known: $CD141^+$ DCs, $CD1c^+$ DCs, and $CD14^+$ DCs (Figure 2). The first two DC subsets are classified as conventional DCs (cDC). cDCs are specialized in T cell priming and are divided in cDC1 and cDC2 cells. cDC1 cells mainly express MHC I molecules in order to activate $CD8^+$ T cells after recognition of infected or transformed host cells (intracellular pathogens). Furthermore, cDC1 cells are superior to cDC2s in presenting antigens derived from damaged or dead cells (Ahrens et al., 2012; Zhang et al., 2012). Their phenotype is characterized by high levels of CD141. cDC2s on the other hand, display a high expression of CD1a and CD1c and present antigens from the extracellular space via MHC II to $CD4^+$ T cells (Scheib et al., 2022). An inflammatory response of the immune system may additionally induce differentiation of inflammatory $CD14^+$ DCs from monocyte progenitors (MoDC) that are uniquely expressed *in situ* under these conditions (Segura and Amigorena, 2013). $CD14^+$ DCs contribute to the induction of a humoral immune response as they are

efficient activators of T memory cells as well as antibody-secreting B cells (Caux et al., 1997; Matthews et al., 2012). $CD14^+$ DCs possess a low efficiency in priming naïve T cells (McGovern et al., 2014). As $CD14^+$ DCs are differentiated from monocytes, they share phenotypic and transcriptional characteristics with their progenitors as well as with macrophages (McGovern et al., 2014). In the skin, dermal DCs share some characteristics like typical dendrite morphology, migration to lymph nodes and antigen-presentation function with LCs located in the epidermis. For a long time, LCs were believed to be a special DC subset but are now classified as macrophages (Ginhoux et al., 2006; Hoeffel et al., 2012; Miller et al., 2012; Mass et al., 2016). LCs and dermal DCs are both efficient antigen-presenting cells. Both cell types eventually migrate to draining lymph nodes, presenting the loaded antigen and potentially activating T cells. During ACD to the allergen 2,4-dinitro-1-fluorobenzene in mice, numbers of LCs and $CD11b^+$ DCs were increased followed by migration of these cells to the draining lymph nodes (Tamoutounour et al., 2013). In the lymph node, allergen-specific $CD4^+$ and $CD8^+$ T cells are induced (Tamoutounour et al., 2013). $CD11b^+$ DCs in mice are the functional homologues to human $CD1c^+$ DCs. Concomitantly, monocytes migrate from the blood into the affected tissue and differentiate to $CD14^+$ DCs. These $CD14^+$ DCs displayed inferior migratory and T cell stimulatory functionality compared to $CD11b^+$ DCs (Tamoutounour et al., 2013). A superior role of LCs during the induction of ACD was suggested, however dermal DCs were shown to effectively induce sensitization in LC-knockout mice (Streilein, 1989).

In the tissue, DCs occur in two differentiation states that differ in function and phenotype: immature and mature DCs. This dichotomy enables DCs to optimally react to immunological triggers and induce a customized immune response. Immature DCs are usually characterized by high phagocytotic activity towards antigens. If maturation of immature DCs is induced, phagocytosis is notably diminished in favor of antigen presentation and priming of naïve T cells (Kiama et al., 2001; Kim and Kim, 2019). Upon maturation, DCs express costimulatory molecules on their cell surface that are essential for crosstalk with T cells. CD40 for example binds to CD154 on T cells and interaction of these binding partners triggers upregulation of further costimulatory molecules like CD80 and CD86 in DCs (Caux et al., 1994). CD80 and CD86 were found to play essential roles in T cell activation and proliferation by binding to CD28 on the latter (Sansom, 2000). Another well-known marker of mature DCs is CD83 (Prechtel and Steinkasserer, 2007). Migration of the activated DCs is initiated after down-regulation of tissue-homing cell surface marker and simultaneous upregulation of CCR7 which mediates chemotactic migration towards the lymphatic tissue (Ohl et al., 2004). These phenotypic alterations are supported by a so-called metabolic reprogramming in DCs.

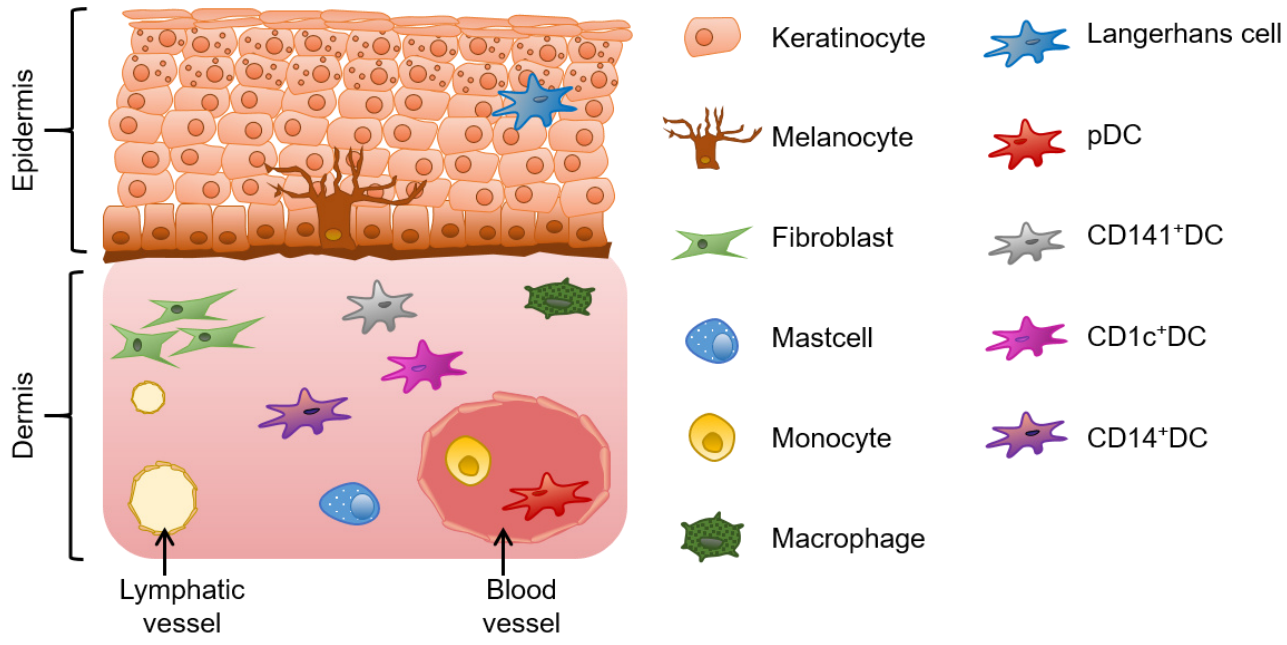

Figure 2 Distribution of dendritic cells and selected other cells in the human skin. At steady state, LCs are commonly found in the epidermis whereas DCs are found in the dermis. Dermal DCs can be divided in two major subsets: $CD1c^+$ and $CD141^+$ DCs. During inflammation, $CD14^+$ DCs are differentiated from blood and migrate into the skin. DC: dendritic cell; pDC: plasmacytoid dendritic cell

4.4.1 Dendritic cell populations, activation and maturation

Efficient activation of the innate immune response is fundamental for skin sensitization. In the presence of pathogens, DCs are activated by recognition of pathogen associated molecular patterns (PAMP) like LPS of bacterial cell membranes via their PRRs. One important group of PPRs are TLRs that are located on the plasma membrane. The exact cellular signaling induced by contact allergens in DCs is still not fully understood. Skin sensitizer differ in their reactivity towards skin proteins (Aleksic et al., 2007; Roberts et al., 2007a) and thus the exact mode of action of DCs activation depends on the culprit. TLR2 and TLR4 are of major importance during the induction of skin sensitization in a murine contact hypersensitivity model (Martin et al., 2008) and sensitization to the metal allergen Ni was demonstrated to be facilitated via direct interaction of the allergen with human TLR4 (Schmidt et al., 2010). Contact allergens that do not directly bind to PPRs were shown to activate the latter through damage associated molecular patterns (DAMPs). In the context of skin sensitization, DAMPs were shown to be ROS-induced endogenous degradation products of the extra cellular matrix like biglycans and hyaluronic acid fragments that are consecutively sensed as danger signal by PPRs namely TLR2 and TLR4 (Soltés et al., 2006; Martin et al., 2008; Babelova et al., 2009; Esser et al., 2012b). Due to their chemical properties, many contact allergens lead to oxidative stress which correlates with the

formation of ROS (Matsue et al., 2003; Mizuashi et al., 2005). The redox imbalance is counteracted by induction of an antioxidant defense mediated by Nrf2. Normally, cytoplasmic Nrf2 is constantly ubiquitinated by Kelch like-ECH-associated protein (KEAP) 1 and subsequently degraded in the proteasome. Under stress conditions, thiol groups of pivotal cysteine side chains in the KEAP1 are oxidized which impairs its function. As a consequence, Nrf2 is not anymore ubiquitinated and degraded but instead translocates to the nucleus where it acts as transcription factor for the antioxidant response element (ARE). Subsequently, transcription of genes coding for cytoprotective proteins like heme oxygenase, glutathione transferases and NAD(P)H quinone oxidoreductase are induced. Induction of Nrf2-linked pathways was widely described for different contact allergens in different cell types including keratinocytes as well as DCs and respective surrogate cell lines (Ade et al., 2009; Emter et al., 2010; El Ali et al., 2013; Mussotter et al., 2016). Furthermore, degradation products of the extracellular matrix can activate the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome which is involved in host defense and regulation of the immune response (Sebastião et al., 2020). This includes the proteolytic activation of caspase 1 which will then cleave the pro-inflammatory cytokines IL-1β and IL-18 into their active forms (Ghayur et al., 1997; Martinon et al., 2002). The crucial pro-inflammatory role of IL-1 during mediation of skin sensitization recently led to the development of an IL-1-based luciferase reporter assay for the prediction of contact allergens (Terui et al., 2021). The provoked inflammation in human monocyte-derived macrophages upon exposure to the skin sensitizer cobalt in alloys from implants was shown to be mainly dependent on NLRP3 inflammasome and only to a minor degree dependent on TLR4 signaling (Samelko et al., 2016). The cells secreted significantly augmented IL-1β amounts but failed to produce the anti-inflammatory cytokine IL-10 which is secreted after TLR4 stimulation (Gurung et al., 2015).

As aforementioned, the allergen itself or secondary molecules generated during inflammation caused by the allergen can bind to the TLRs. Binding of ligands to TLRs initiates receptor signaling via MyD88 and Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon beta (TRIF) (Martin et al., 2011). Down-stream of TLR stimulation, cellular signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) or p38 mitogen-activated protein kinase (MAPK) are triggered which leads to the maturation of the DCs and upregulation of pro-inflammatory genes (Kaplan et al., 2012). The metal skin sensitizer Ni, for example, activates NFκB and p38 MAPK pathways (Ade et al., 2007). Other allergens including the, in terms of potency classification, more potent skin sensitizer *p*-dinitrochlorobenzene, only activate p38 MAPK signaling (Mizuashi et al., 2005; Ade et al., 2007). p38 phosphorylation is also induced in THP-1 cells as response to haptenation with a series of different dinitrophenyl analogues and was much more pronounced than activation of Nrf2 and NFκB signaling (Megherbi et al., 2009). Importantly, non-allergic chemicals do not activate MAPK or NFκB and can thus be distinguished from contact allergens (Aiba et al., 2003; Mizuashi et al., 2005).

IL-8 is another central messenger secreted by activated DCs. IL-8 was shown to be regulated MAPK-dependent (Nukada et al., 2008) and was furthermore proven to be a reliable biomarker for the differentiation of skin sensitizer and non-sensitizer *in vitro* (Takahashi et al., 2011; OECD, 2022b). IL-8 induces chemotaxis of neutrophils towards the side of inflammation. Neutrophils have been shown to play a pivotal role during sensitization and elicitation of ACD (Weber et al., 2015).

4.4.2 Metabolic reprogramming of dendritic cells

The activation of the DCs triggers transcriptional and phenotypic alterations that pose an extremely high energetic challenge. To cope with the high energy demand, murine bone marrow-derived DCs (BMDC) undergo a so-called glycolytic burst and dramatically upregulate aerobic glycolysis thereby neglecting oxidative phosphorylation (OXPHOS) (Krawczyk et al., 2010). This metabolic shift is initiated already minutes after exposure to the activating agent and inhibition of glycolysis suppresses DC activation (Krawczyk et al., 2010; Everts et al., 2014). During metabolic reprogramming, two distinct phases can be distinguished. The early stage is fueled by extracellular glucose or depletion of cellular glycogen storage (Thwe et al., 2017). Orchestrated by TANK-binding kinase–inhibitor of nuclear factor kappa-B kinase subunit epsilon-protein kinase B (TBK1-IKKε-Akt) signaling, hexokinase 2 is activated and translocates to the mitochondrial membrane to enable a high glycolytic flux (Everts et al., 2014; Perrin-Cocon et al., 2018). Glucose serves as important building block for the lipid metabolism. Phenotypic alterations during maturation are driven by elevated fatty acid synthesis and subsequent expansion of Golgi apparatus and endoplasmic reticulum. Acetyl-CoA is also required for the biosynthesis of sterols like cholesterol and pro-inflammatory signaling molecules. After several hours, mammalian target of rapamycin (mTOR) can induce a long-term dependency of activated DCs to glycolysis and inhibition of mTOR can lead to a more tolerogenic phenotype in MoDCs which display extenuated levels of CD86 (Ferreira et al., 2015). The interplay of mTOR and 5' adenosine monophosphate-activated protein kinase (AMPK) was shown to be central for the regulation of glycolysis in DCs (Adamik et al., 2022). AMPK was active in both immature and mature DCs, but metabolic reprogramming is accompanied by transient mTOR activity (Adamik et al., 2022). mTOR dictates HIF1α signaling and thereby regulates inducible nitric oxide synthase (Everts et al., 2012). In DCs, nitric oxides affect maturation, migration and antigen presentation (as reviewed by Thwe and Amiel, 2018). In DC subsets that do not express inducible nitric oxide synthase, high glycolysis activity is maintained through HIF1α and type I interferons (Pantel et al., 2014).

Overall, this metabolic shift towards glycolysis is comparable to the Warburg effect in cancer cells (Pearce and Everts, 2015). In humans, different metabolic reprogramming regimes were elucidated for the distinct DC subsets (Sim et al., 2016; Basit et al., 2018). $CD1c^+$ myeloid DCs and MoDCs displayed a metabolic reprograming that was comparable to murine BMDCs (Basit et al., 2018).

4.5 Cell models to study DC biology in the context of allergic contact dermatitis

The frequency of DCs in various tissues is generally very low. For example, DCs represent less than 1% of cells in the human blood and 1-3% in skin and mucosa (Toebak et al., 2009; Orsini et al., 2012). This is why DC isolation is laborious and does not result in high yields. To overcome the shortage in DC availability, DCs are frequently generated from stem cells, monocytes and mouse bone marrow progenitors or are replaced by surrogate cell lines for experimental studies.

The differentiation of iDCs from $CD34^+$ stem cells or mouse bone marrow-derived DC progenitors results in little variability along with high yields (Hardonnière et al., 2022). However, generation of DCs from mouse bone marrow is obviously not an animal-free method and should with respect to the 3R principle be discussed critically. Furthermore, mouse DCs were in the past shown to be limited for assessing skin sensitization in human as they, for example, fail to correctly predict the metal allergen Ni due to missing histidines in the binding pockets of TLR4 (Schmidt et al., 2010).

MoDC are differentiated from monocytes typically isolated from human blood. Crucial for MoDC generation is supplementation of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. The transcriptome of obtained MoDCs resembles most closely inflammatory DCs (Segura et al., 2013) and their immature phenotype can, likewise to DCs, undergo maturation after exposure to external triggers (Sallusto and Lanzavecchia, 1994). Aiba et al. (1997) showed responsiveness of immature MoDCs towards the contact allergens Ni(II)chloride and *p*-dinitrochlorobenzene (DNCB) which manifested with elevated cell surface expressions of CD54, CD86, and human leukocyte antigen-DR isotype (HLA-DR) as well as secretion of significant levels of IL-1β. MoDCs are also able to express significant levels of CD40 and CD83 and show secretion of IL-6, tumor necrosis factor (TNF)α and IL-8 (Coutant et al., 1999; De Smedt et al., 2001; Aiba et al., 2003). On the downside, the quality of MoDCs is strongly affected by donor variation which sometimes causes significant CD86 expression already on immature MoDCs (Hardonnière et al., 2022).

As the differentiation of DCs from primary material is tedious and does not yield high cell numbers, surrogate cell lines have been established. However, most of the cell lines are monocytic leukemia cell lines. A frequently used cell model for studying DC response to contact allergens are THP-1 cells. THP-1 cells are monocytes that were isolated from the peripheral blood of a patient suffering from monocytic leukemia (Tsuchiya et al., 1980). Immature THP-1 cells treated for 24 h with contact allergens were shown to express activation markers of mature DCs like CD40, CD54, CD83, and CD86 which was concomitant with elevated secretion of IL-8 and TNFα (Miyazawa et al., 2007). Upregulation of CD54 and CD86 were proven to relatively reliably differentiate contact allergens and non-allergens *in vitro*. This assay is called human cell line activation test (hCLAT) and was adopted

as official OECD (Organization for Economic Co-operation and Development) test guideline for *in vitro* prediction of skin sensitizers (Ashikaga et al., 2006b; OECD, 2018).

Similar to THP-1 cells, U937 cells are employed for *in vitro* assessment of skin sensitizers based on the upregulation of the cell surface antigen CD86 (Natsch et al., 2013; OECD, 2018). Myeloid U937 cells are known to express monocytic markers and properties and originate from a histiocytic lymphoma (Sundström and Nilsson, 1976). U937 cells express significantly increased numbers of CD86 molecules and secrete second messenger molecules like IL-8 after treatment with contact allergens. Furthermore, the majority of contact allergens promotes gene expression of IL-1β and IL-8 in U937 cells (Python et al., 2007).

Another DC model are MUTZ-3 cells which were originally isolated from the blood of a patient who was diagnosed with acute myelomonocytic leukemia (Hu et al., 1996). Antigen-presenting properties via MHC molecules as well as induction of clonal expansion of T cells, expression of maturation markers of DCs like CD40, CD54, CD80, CD83, and CD86 and transcriptional similarity to MoDCs were proven for MUTZ-3 cells (Azam et al., 2006; Larsson et al., 2006; Santegoets et al., 2008; Johansson et al., 2011a).

4.6 New approach methodologies and regulatory framework

Chemicals that are produced over one ton/year and enter the European market have to be tested for their skin sensitizing potential beforehand to enable proper risk assessment under the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) legislation (European Commission, 2006; 2008). Also, medical products as well as pharmaceuticals (active substance and excipients) have to be tested for their skin sensitizing potential before testing in humans and marketing in the EU (EMA, 2015). The gold standard for assessment and classification of contact allergens is to date the local lymph node assay (LLNA) (OECD, 2010) and each year approximately 40,000 animals are used for toxicological assessment of skin sensitizers in the European Union and Norway (European Commission, 2020; 2021). However, because the large number of chemicals cannot be efficiently tested in animals (economic reasons) and data have limited transferability to humans (scientific reasons), new approach methodologies (NAM) are required for next generation risk assessment. Furthermore, since animal welfare and reduction of animal testings have become a major debate in the public as well as the scientific community, the 3R-principle was established and efforts to reduce animal testings were made. Therefore, the European Union has banned animal testings for cosmetics and the sale of animal-tested cosmetics since 2013 (EC, 2009). This ban fostered the development of several validated alternative testing methods, making skin sensitization assessment one of the most advanced NAM fields among regulatory settings.

According to the OECD's adverse outcome pathway (AOP) for skin sensitization (OECD, 2014), alternative testing strategies have been adopted and validated for the replacement of animal tests (Figure 3). The haptenation of an allergen to a self-protein in the skin was identified as key event 1 and can be assessed *in chemico* using, for example, the direct peptide reactivity assay (DPRA). Briefly, the covalent binding of a chemical to artificial peptides containing either cysteine or lysine is quantified using a liquid chromatography couple to ultraviolet detection readout. The depletion of the peptides is calculated and serves as a measure for the probability of general haptenation of self-proteins by the chemical tested (Gerberick et al., 2004). Emter et al. (2010) developed the KeratinoSens™ assay, a luciferase reporter gene assay, for the detection of keratinocyte activation in the context of skin sensitization. The assay measures the activation of genes regulated by the antioxidant response element in HaCaT keratinocytes thereby covering key event 2 of the AOP. The hCLAT on the other hand addresses the DC activation (key event 3) *in vitro*. Monocytic THP-1 cells are used as a DC surrogate and the upregulation of the cell surface markers CD54 and CD86 is measured by flow cytometry following 24 h exposure to the allergen (Ashikaga et al., 2006b). Key event 4, activation and proliferation of antigen-specific T cells, as well as the organism response can currently not be assessed with the help of validated alternative test protocols. However, NAMs addressing key event 4 are currently under investigation (Richter et al., 2013; Aparicio-Soto et al., 2020; Curato et al., 2022; Riedel et al., 2022).

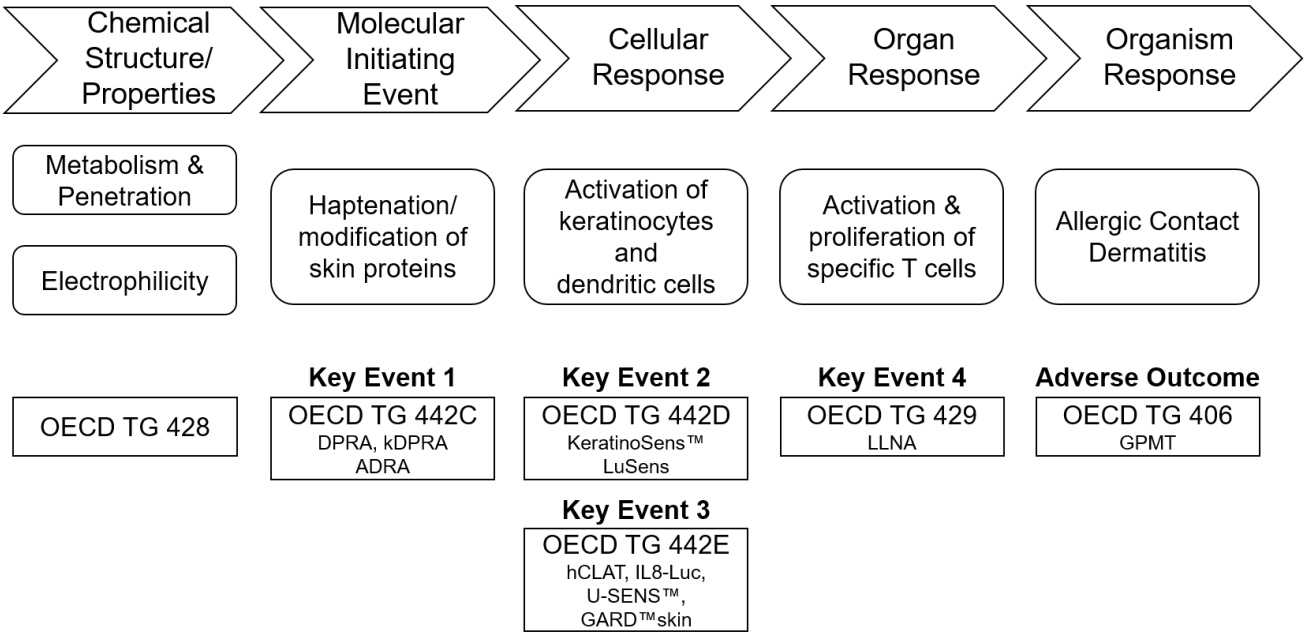

Figure 3 Schematic diagram of the adverse outcome pathway for skin sensitization including currently validated OECD test guidelines for the assessment of the molecular initiating event, key events and the adverse outcome. TG: test guideline, DPRA: direct peptide reactivity assay, kDPRA: kinetic direct peptide reactivity assay, ADRA:

amino acid derivative reactivity assay, hCLAT: human cell line activation assay, IL8-Luc: interleukin-8 reporter gene assay, LLNA: local lymph node assay, GPMT: guinea pig maximization test

Skin sensitization is a highly complex biological process involving multiple players on the cellular level as well as diverse sets of messenger molecules. Since the validated alternative methods are targeting individual key events, they are not used as stand-alone methods to assess skin sensitizer. This also implies that only limited mechanistic information can be derived from these testing methods. To overcome these limitations and to enhance accuracy and sensitivity, defined approaches (DA) have been developed and validated by the OECD (OECD, 2021). One DA follows the 2 out of 3 approach and combines the DPRA, hCLAT and KeratinoSens™ assay. This testing procedure was shown to outperform the LLNA in comparison to human reference data for the assessed chemical set (OECD, 2021).

In addition to the already mentioned limitations, the correct prediction of pre- and prohaptens was a major concern in the past. The 2 out of 3 workflow was shown to be well suited for this purpose. Prehaptens were best predicted with the DRPA and the hCLAT preformed best in predicting prohaptens, respectively (Patlewicz et al., 2016). Further technical limitations when performing *in vitro* assays are the applicability domains e.g. the physicochemical properties of the investigated chemicals. Lipophilic and highly cytotoxic chemicals as well as surfactants, metals or mixtures were shown to give ambiguous or false results in cell-based testing strategies such as the hCLAT (Sakaguchi et al., 2010; Bergal et al., 2020). Hence, limitations defined in the individual testing guidelines are also limiting established DAs or integrated testing strategies. Besides hazard identification as such, the potency classification of allergens according to the CLP (Classification, Labelling and Packaging) legislation is often necessary. The DA using 2 out of 3 was shown to be not yet suitable for potency estimation (OECD, 2021). However, computer-based regression models seem promising for potency classification but currently lack regulatory acceptance (Irizar et al., 2022; Natsch and Gerberick, 2022).

4.6.1 Categorization of contact allergens

Ideally, the potency of skin sensitizers should be derived from human data by determining substance-specific no-observed effect levels (NOEL). This was done in the past with the help of the human maximization test and repeated insult patch test (Kligman, 1966a; Politano and Api, 2008). As potency determinations cannot be performed in humans due to ethical reasons, LLNA data are used as surrogate. Based on LLNA data, skin sensitizing chemicals can be classified in four distinct groups: weak, moderate, strong and extreme. Grouping is based on the so called EC3 value which determines the dose of a test chemical that induces a 3-fold gain in lymph node weight due to allergen-specific T

cell proliferation in the affected lymph node. For extreme sensitizers, the EC3 threshold is typically reached for concentrations below 25 µg/cm^2 (Api et al., 2017).

The Global Harmonized System and CLP legislation require potency estimation for correct labeling of skin sensitizing chemicals. All chemicals that induce skin sensitization at concentrations below 500 µg/cm² are classified in category 1A as strong skin sensitizer. This value corresponds to a LLNA EC3 value of ≤ 2%. Category 1B sensitizers are moderate and weak sensitizers that may trigger skin sensitization at doses above 500 µg/cm² (UN, 2021). Based on NAMs, potency estimation is not yet fully resolved. Irizar et al. (2022) therefore proposed a list of reference allergens and their potencies that can be used to assess the quality of NAM-based potency classification and accelerate research in that field.

4.6.2 The human cell line activation assay (hCLAT)

In the context of skin sensitization, the activation of DCs can be determined using cell line-based assays. This includes the hCLAT which employs the cell surface markers CD54 and CD86 on THP-1 cells as measure for cellular activation and thus skin sensitizing capabilities of a compound (OECD, 2022b). CD86 is expressed in antigen-presenting cells and is an important costimulatory modulator of T cell response. Binding to its receptor CD28 induces a T cell response whereas binding to the receptor CTLA-4 has opposing effects (Coyle and Gutierrez-Ramos, 2001). CD54 is an intercellular adhesion molecule that is predominantly expressed on endothelial and immune cells. On antigen-presenting cells, CD54 serves as costimulatory molecule during CD28-independent activation of T cells (Gaglia et al., 2000). Taken together, evaluating the upregulation of these markers provides a good measure for THP-1 activation. Yet, this does neither provide functional information on actual T cell activation nor are these markers specifically induced by skin sensitizing compounds. Overall, the accuracy of the hCLAT was estimated to be approximately 84 % in comparison to the LLNA (Ashikaga et al., 2010). The sensitivity, the number of true positive chemicals in the test, was determined to be 88 % and the number of false positive chemicals was 30 % (Ashikaga et al., 2010). This indicates that the hCLAT overpredicts chemicals, which is good with regard to consumer protection. However, it also underlines that the use of general activation markers leads to false positive results, like, for example, in the case of 1-bromobutane which is classified as non-sensitizer by the LLNA but induces both markers, CD54 and CD86, and is thus wrongly classified as skin sensitizer by the hCLAT (Ashikaga et al., 2010).

4.7 The complex search for ACD-specific biomarkers

The identification of disease-specific indicators, so-called biomarkers, is valuable in medicine. With the help of a biomarker, correct diagnosis of the disease as well as monitoring of the progression are

more reliable. Additionally, biomarkers are excellent starting points for the development of treatment strategies. The sensitization to an allergen occurs spontaneously during life and cannot clinically be prevented nor can the medical disorder ACD be cured. A broad range of different studies including *in vitro* approaches as well as clinical studies in patients were conducted in the past to uncover ACD-specific biomarkers. However, most studies were limited in the cohort sizes as well as number of allergens analyzed within the study which is why till today, no global biomarker for ACD has been revealed and clinically validated. Most biomarker candidates that were proposed so far were deduced from transcriptomic studies (Dhingra et al., 2014; Quaranta et al., 2014a; Fortino et al., 2020). Quaranta et al. (2014a) were able to distinguish between psoriasis and eczematous skin biopsies based on differentially expressed genes. Gene transcription of skin suffering from eczema caused by Ni-induced ACD was furthermore shown to differ significantly from other eczematous lesions (Quaranta et al., 2014a). In another study, a set of approximately 20 differentially regulated genes was identified to distinguished irritant from allergic contact dermatitis in human patch test biopsies treated with allergens or irritants. For ACD, the genes *ADAM8* and *CD47* were associated with the highest potential to correctly classify ACD (Fortino et al., 2020). Lefevre et al. (2021) also proposed a gene signature of 50 genes which helps to discriminate between ACD and ICD in clinical biopsies. There was no overlap between this signature with the genes proposed by Fortino et al. (2020). This can be explained by the different time points investigated by the two groups (72 h vs. 48 h) and underlines how delicate biomarker identification is. Dhingra et al. (2014) conducted a study in patients that were exposed to the most frequent contact allergen classes in North America. In total, 149 genes related to immune regulation, inflammation, chemokines, and cell death were commonly regulated in allergen-treated patch test biopsies. Despite these shared genes, a large number of genes appeared to be regulated in an allergen-dependent manner (Dhingra et al., 2014). Apart from transcriptional changes, patch tests were also tested for cytokine expression levels. Especially IL-16 levels were found to be helpful to distinguish ACD and ICD (Brans et al., 2021). Furthermore, microRNA signatures were found to be distinct between ACD patients and control individuals (Werner et al., 2021; Zhang et al., 2023).

However, since the collection of skin biopsies is an invasive method, researchers have proposed altered plasma serum levels of cytokines as potential biomarker signatures for ACD (Bordignon et al., 2015; Zinkevičienė et al., 2015). The isolation of immune cells from patch tested patients' blood was frequently performed to assess the response of specific cellular subsets in terms of cytokine secretion, cell proliferation and gene transcription (Stejskal et al., 1990; Rustemeyer et al., 2004; Bordignon et al., 2008). Another non-destructive method is the analysis of tape strips from patch tests as performed by Tam et al. (2021).

Over the past years, *in vitro* testing has become more popular and the search for potential biomarkers for ACD has been extended to this research field as well. The transferability of biomarkers from *in vitro* testing to the clinical application is questionable as *in vitro* systems lack the biological complexity present *in vivo*. Yet, these biomarkers are still beneficial for improvement of existing *in vitro* test strategies and for mechanistic understanding of ACD.

Employing machine learning and microarray data of MUTZ-3 cells treated with skin sensitizers, irritants or control chemicals, Johansson et al. (2011a) were able to propose a gene signature of 200 differentially expressed genes that enable the prediction of skin sensitizers *in vitro*. The signature was further processed using Ingenuity Pathway Analysis (IPA) and the TOP3 pathways with the highest number of mapped genes were Nrf2-mediated oxidative stress response, xenobiotic metabolism signaling and protein ubiquitination pathway. This assay was published and validated as GARD (Genomic Allergen Rapid Detection) assay and became recently an official OECD *in vitro* testing method (Johansson et al., 2013; Johansson et al., 2019; OECD, 2022b).

Proteomics have so far been underemployed for the search of ACD specific biomarkers. Mussotter et al. (2016) analyzed the response of Nrf2-wildtype and knockout mice-derived BMDCs to a set of skin sensitizers and were able to suggest glycolytic enzymes supporting metabolic reprogramming in DCs as well as Nrf2-dependent proteins as potential biomarkers for ACD. Human $CD14^+$ monocytes were shown to respond to Ni^{2+}-treatment with significant changes in protein levels that were linked to apoptosis (Jakob et al., 2017). Using proteomics as well as metabolomics, metabolic reprogramming was shown to also be induced in THP-1 cells that were exposed to contact allergens. Furthermore, altered protein levels of fatty acid synthase and phospholipids were detected by Mussotter et al. (2018).

Taken together, many biomarker candidates were proposed for ACD as well as skin sensitization, but, due to the diversity of applied study designs, readouts, detection methods, sample material, and contact allergens screened, it is hardly possible to identify a mutual biomarker. Based on the diversity of candidates, it seems most promising to establish a signature of predictive molecules rather than relying on individual markers.

4.8 Proteomics data can complement transcriptome data in toxicological testing

To overcome the need of integrated testing strategies for proper risk assessment of potential skin sensitizers, current research focusses on developing more complex non-animal models that possess the ability to reflect the biological mechanisms involved during skin sensitization. The advancing biological complexity and reproducibility of skin models has led to the successful implementation of human skin models into testing strategies. The SENS-IS assay, for example, employs a reconstituted

human epidermis model to assess a gene signature which allows to discriminate between sensitizers, irritants and non-sensitizers (Cottrez et al., 2015). The application of so-called omics-technologies has furthermore enabled scientists to generate large data sets mirroring high biological complexity. Although the term ‘omics’ comprises a handful of disciplines, most studies have to date focused on transcriptomic signatures in the context of skin sensitization (Hooyberghs et al., 2008; Johansson et al., 2011a; Lambrechts et al., 2011). Global proteome changes remain largely underinvestigated in the context of ACD (cf. section 6.1). Transcriptomics for a long time outperformed other omics-techniques as even the lowest yield of RNA can be amplified, but advancing mass spectrometry technologies nowadays allow proteome analysis on the single cell level, rendering proteomics a tantamount technique well suited to complement transcriptome data. Proteomics can be applied to qualitatively and quantitatively investigate biomolecules of an organism in response to e.g. environmental exposures. The proteome is especially suitable as proteins reflect the true phenotype of the organisms at a specific time and gene induction does not necessarily correlate with protein levels due to post-transcriptional regulation.

4.8.1 Principles of untargeted proteomics

Most state-of-the-art proteomics workflows comprise the following sample preparation steps: lysis of the cells/biological material, enzymatic degradation of the proteins, desalting and enrichment of peptides, liquid chromatography for separation of the peptides, mass spectrometric analysis of samples, and data evaluation.

After separation of the peptides via liquid chromatography, peptides are often ionized in an electrospray ionization source. The generated ions are then transferred into the mass spectrometer where they are fragmented and finally detected based on their mass to charge (*m/z*) ratio. Fragmentation is typically performed twice. The precursor ion is usually used for quantification whereas its fragment ions are used for unambiguous identification of the peptide. Large data bases and complex bioinformatics algorithms are then employed to match the *m/z* ratios to the peptides they originated from and finally, peptides are mapped to proteins. Generally, one can distinguish between two different experimental setups: targeted and untargeted proteomics. As indicated by the name already, targeted proteomics aims at identifying and quantifying already known proteins in a sample. For this purpose, the specific proteins are often enriched or an inclusion list can be used during data acquisition. Targeted runs are typically rather short. Untargeted shotgun experiments on the other hand focus on elucidation of as many proteins as possible in a sample. Under optimal conditions, several thousand proteins can be identified within one sample, allowing to retrieve information on global protein changes in either specific cells, in a tissue, or any other matrix. Untargeted runs consume a lot of time on the liquid chromatography coupled to tandem mass spectrometry (LC-

MS/MS), as the complex peptide mixtures have to be separated by long chromatography gradients to reduce the complexity of the sample and avoid overload of the MS-detector. For the work presented in this thesis, exclusively untargeted proteomic workflows were used.

To be able to compare different treatment conditions or exposures amongst each other, protein quantification is mandatory. For untargeted proteomics, so called label-free or labeled quantification can be performed. Label-free quantification (LFQ) is in terms of consumables cheap but requires more measuring time on the MS as samples cannot be multiplexed. LFQ is based on precursor ion identification on MS1 level (before fragmentation) and the intensity of this peak. Alternatively, spectral counting can be performed. Briefly, the number of all spectra accounting to a specific protein is counted and then correlated with relative amount of protein in the sample. Prerequisite for LFQ is a stable and reproducible analytical method as the retention time is a central parameter for correct matching of ion intensities to peptides over all samples. Thus, LFQ is very sensitive to experimental and analytical errors, resulting in lower precision and reproducibility especially for low abundant proteins (Collier et al., 2010). As an alternative to LFQ, mass labels can be introduced to the samples to simplify quantification. These labels can either be incorporated via chemical reactions during sample preparation (e.g. TMT-labeling or iTRAQ) or in the case of stable isotope labelling by amino acids in cell culture (SILAC) metabolically during cultivation of cells. For SILAC, only cells that dived in culture can be used. The cell culture media is supplemented with isotope-labeled amino acids and treated cells and controls are cultured in media supplemented with different isotopically labeled amino acids. These amino acids are metabolically incorporated into all proteins during culture. Since the light and heavy isotope labeled amino acids are chemically identical, they are incorporated with equal kinetics. After harvest und lysis of the cells, same protein amounts of control and treatment cells are unified and processed together. During data evaluation, the groups can be distinguished based on the specific mass shift introduced by the labeled amino acid. SILAC is the more accurate strategy for quantitative proteomics due to its high reproducibility and precision (Li et al., 2012). The sample handling and data analysis are comparatively robust since treatment and control groups are combined directly after harvest of the cells. Since labeled amino acids are costly and only a limited number of suitable amino acids is commonly used, SILAC is constrained to duplex or triplex experiments. Compared to competing chemical labels which allow higher tiered multiplexing, sample preparation is less tedious. Chemical labeling requires a sophisticated workflow ensuring that labeling agents work properly and that same protein amounts are successfully tagged. During the work for this thesis, LFQ and SILAC were applied and compared.

5 Objective

Skin sensitization is a toxicological endpoint that has to be assessed for chemicals marketed in the European Union. As contact allergens are widely distributed in all kinds of consumer products, the prevalence of ACD is high amongst the general population. To keep sensitization levels as low as possible and to prevent epidemics like in the case of Ni and methylisothiazolinone in the past (Thyssen et al., 2007; Uter et al., 2020a), the molecular mechanisms of skin sensitization and ACD need to be better understood. For this, correct prediction and classification of skin sensitizers are critical for the risk assessment of regulatory bodies. Driven by the European ban of animal testing for cosmetic ingredients and the progressive implementation of the 3R principle, *in vitro* testing strategies are gaining importance for regulatory testings. To improve existing *in vitro* testing strategies, elucidation of the underlying mode of action and identification of predictive biomarker signatures for skin sensitization are essential.

The overarching goal of this thesis was the application of state-of-the-art proteomics techniques to identify commonly regulated proteins in immune cells induced by different contact allergens and deduce biomarker candidates or signatures for the prediction of skin sensitizing chemicals.

A systematic literature search had to be performed to identify data gaps and relevant proteomic targets for this thesis. The results of the literature search were to be published in a review paper. Proteome-based studies have to date only rarely been performed to study skin sensitization. Particularly the complex activation and maturation of DCs exhibits a great potential for the investigation of proteomic changes throughout the curse of skin sensitization. Thus, it was chosen to analyze cellular changes in human DC models after treatment with contact allergens.

As a first model, human MoDCs should be used to investigate proteomic changes following cellular activation via TLR4 with LPS, a classical DC activator, and $NiSO_4$, also a TLR4 activator and the most prevalent contact allergen in the European public. The molecular binding mechanisms of LPS and Ni to TLR4 are distinct. By analyzing differentially regulated proteins in both treatments compared to control (non-treated) cells, allergy-specific responses were to be identified and differentiated from bacterial activation of the MoDCs.

For this aim, the generation of immature MoDCs from blood donations had to be optimized followed by experiments to determine the treatment conditions like chemical treatment dose and exposure time to avoid toxic effects that could overshadow substance-specific effects. The successful generation of immature MoDCs had to be routinely tested based on characteristic cell surface marker expression with the help of flow cytometry. After maturation induced by the various treatment conditions, the cells

were to be characterized in detail by analyzing phagocytic activity, potential to activate T cells, cell surface marker expression, and cytokine secretion. Following proteomic analysis, proteins and pathways that were selectively induced by $NiSO_4$ had to be verified by complementary methods including immunoblotting, quantitative real-time polymerase chain reaction or by selective inhibition of specific pathways.

The German Federal Institute for Risk Assessment (BfR), as regulatory body, is highly interested in robust *in vitro* test strategies for risk assessment. Thus, the findings from MoDCs experiments were to be compared to results from THP-1 cells. Compared to MoDCs, THP-1 cells are easier to handle, more accessible as well as scalable and are already validated in a OECD test guideline for skin sensitization (OECD, 2022b). THP-1 cells had to be treated with $NiSO_4$ as well. Additionally, other strong contact allergens should be included to confirm potential overarching allergy-specific findings. Thus DNCB, BQ, and NBB were chosen as they have proven relevance for humans (Przybilla et al., 1983; Pambor and Wiesner, 1990; Lisi and Hansel, 1998). Furthermore, two different strategies for the quantification of the proteins (SILAC versus LFQ) were to be systematically tested with respect to their applicability to various cell models used for *in vitro* research on skin sensitization.

Overall, this work promotes the elucidation of allergen-specific activation of DCs in the context of skin sensitization by applying state of the art proteomics techniques. The findings contribute to the mechanistic understanding of skin sensitization and may ultimately serve as improvement of existing *in vitro* test methods for risk assessment and thus enhancing workmen and costumer protection.

6 Results

6.1 Paper 1

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

Tessa Höper, Franz Mußotter, Andrea Haase, Andreas Luch, and Tewes Tralau

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6.2 Paper 2

The Contact Allergen $NiSO_4$ Triggers a Distinct Molecular Response in Primary Human Dendritic Cells Compared to Bacterial LPS

Tessa Höper, Katherina Siewert, Verónica I. Dumit, Martin von Bergen, Kristin Schubert, and Andrea Haase

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The Contact Allergen $NiSO_4$ Triggers a Distinct Molecular Response in Primary Human Dendritic Cells Compared to Bacterial LPS

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Dendritic cells (DC) play a central role in the pathogenesis of allergic contact dermatitis (ACD), the most prevalent form of immunotoxicity in humans. However, knowledge on allergy-induced DC maturation is still limited and proteomic studies, allowing to unravel molecular effects of allergens, remain scarce. Therefore, we conducted a global proteomic analysis of human monocyte-derived dendritic cells (MoDC) treated with $NiSO_4$, the most prominent cause of ACD and compared proteomic alterations induced by $NiSO_4$ to the bacterial trigger lipopolysaccharide (LPS). Both substances possess a similar toll-like receptor (TLR) 4 binding capacity, allowing to identify allergy-specific effects compared to bacterial activation. MoDCs treated for 24 h with 2.5 μg/ml LPS displayed a robust immunological response, characterized by upregulation of DC activation markers, secretion of pro-inflammatory cytokines and stimulation of T cell proliferation. Similar immunological reactions were observed after treatment with 400 μM $NiSO_4$ but less pronounced. Both substances triggered TLR4 and triggering receptor expressed on myeloid cells (TREM) 1 signaling. However, $NiSO_4$ also activated hypoxic and apoptotic pathways, which might have overshadowed initial signaling. Moreover, our proteomic data support the importance of nuclear factor erythroid 2-related factor 2 (Nrf2) as a key player in sensitization since many Nrf2 targets genes were strongly upregulated on protein and gene level selectively after treatment with $NiSO_4$. Strikingly, $NiSO_4$ stimulation induced cellular cholesterol depletion which was counteracted by the induction of genes and proteins relevant for cholesterol biosynthesis. Our proteomic study allowed for the first time to better characterize some of the fundamental differences between $NiSO_4$ and LPS-triggered activation of MoDCs, providing an essential contribution to the molecular understanding of contact allergy.

Keywords: monocyte-derived dendritic cells, allergic contact dermatitis, proteomics, nickel, LPS

INTRODUCTION

Dendritic cells (DC) play a crucial role during the immune response given their linking function between innate and adaptive immunity. Immature DCs are phagocytic and sample antigens from surrounding tissues. The presence of additional stimuli, for instance activation via pattern recognition receptors such as toll-like receptors (TLR), triggers maturation of DCs. Upon maturation, the cells will partially lose their phagocytic characteristics and start migrating to draining lymph nodes while they undergo metabolic and phenotypic alterations (1–3) facilitating the presentation of the captured antigen to prime naïve T cells. These alterations include the secretion of different cytokines [e g., interleukin (IL) 1β, IL-6, IL-12 or tumor necrosis factor (TNF) α and the upregulation of antigen-presenting molecules and other DC surface proteins such as adhesion molecules, chemokine receptors, and co-stimulatory proteins. Particularly well described is the upregulation of the cluster of differentiation (CD) 86, CD54, CD40 and CD83 (4). A metabolic shift toward glycolysis supplies sufficient energy for the maturation of activated DCs (5).

DCs do not only play an essential role during host defense but also during chemical-induced immune responses like allergic contact dermatitis (ACD). It is assumed, that about 20% of the general population of Europe and North America are sensitized to at least one contact allergen (6). ACD is a prototypic T cell-mediated delayed-type hypersensitivity immune response that may be elicited after skin contact with organic chemicals or metal ions from cosmetics, jewelery or other commodities. Pathogenesis of ACD is sub-divided in two phases. Upon initial exposure to a contact allergen, the sensitization phase is initiated. Significant clinical symptoms will only emerge after re-exposure to the contact allergen during the so-called elicitation phase (7). The importance of DCs during the sensitization phase is well-understood and also reflected in the adverse outcome pathway on skin sensitization (8). To date, the maturation of DCs in the context of contact allergy has been insufficiently investigated. For instance, there is an ongoing debate, whether or not there are allergy-specific alterations of DCs, which may be used as allergy-specific biomarkers. Proteomics studies can help to elucidate molecular alterations induced by allergens. Proteins directly affect the phenotype of an organism and possess an enormous functional repertoire. They may, for instance, act as transporters, messenger molecules or as enzymatic catalyst. Furthermore, the number of genes is outnumbered by the number of proteins, e.g., due to manifold post-translational modifications. Thus, studying the proteome and metabolism of DCs after exposure to contact allergens is mandatory to get insights in the biological state of the cells (9).

Therefore, the aim of this study was to compare DC maturation induced by the metal allergen $NiSO_4$ to the bacterial trigger lipopolysaccharide (LPS). DC maturation by the latter has been investigated extensively (10–13). Nickel has been selected for several reasons. Firstly, nickel is the most common cause of ACD in Europe. Even though numbers of affected individuals are decreasing as the EU nickel directive restricts nickel release from products intended for prolonged skin contact, still 8–18% of the European population are sensitized to nickel (14). Secondly, it was shown that both Ni^{2+} and LPS act via TLR4 although with different molecular binding mechanisms. LPS binds to a specific pocket on myeloid differentiation factor 2 (MD-2) which forms a heterodimer with TLR4 (15), inducing dimerization and internalization of TLR4 (16, 17). In contrast, Ni^{2+} was shown to bind to three histidine residues on the ectodomain of human TLR4, which is distinct from the endotoxin binding site (18). TLR4 dimerization, which is mandatory for receptor activation, was found to occur independently of MD-2 in the presence of Co^{2+} and Ni^{2+} (19).

We were interested in elucidating whether these differences in binding to TLR4 might eventually induce different proteins and pathways. The comparison of an allergen with a bacterial stimulant allows the identification of allergy specific effects and possible biomarkers which may support current *in vitro* testing strategies for skin sensitization. Hence, we here present a global proteomic analysis of LPS and $NiSO_4$-treated primary human monocyte-derived DCs (MoDCs) using label-free quantification (LFQ). MoDCs were treated for 24 h with 400 μM $NiSO_4$ or 2.5 μg/ml LPS. Upon harvest, cells were prepared for proteomic analysis as well as for flow cytometry analysis of selected cell surface markers and cytokines. Proteomic data were validated using suitable methods like qPCR revealing differences in $NiSO_4$- and LPS-induced activation.

Abbreviations: ACD, allergic contact dermatitis; ACN, acetonitrile; ALDOA, aldolase A; APC, allophycocyanin; BV421, brilliant violet 421; CD, cluster of differentiation; CFSE, carboxyfluorescein succinimidyl ester; CYP51A1, lanosterol 14α-demethylase; DC, dendritic cell; EIF, eukaryotic initiation factor; FA, formic acid; FC, fold change; FITC, fluorescein isothiocyanate; GLUT, glucose transporter type; GM-CSF, granulocyte-macrophage colony-stimulating factor; GST, glutathione S-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIF, hypoxia-inducible factor; HK, hexokinase; HMGCS1, hydroxymethylglutaryl-CoA synthase; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HMOX, heme oxygenase; HPRT, hypoxanthineguanine phosphoribosyltransferase; HSA, human serum albumin; IL, interleukin; IPA, ingenuity pathway analysis; KEAP, kelch-like ECH-associated protein; LDHA, lactate dehydrogenase; LFQ, label-free quantification; LPS, lipopolysaccharide; MD-2, myeloid differentiation factor 2; MLR, mixed leukocyte reaction; MoDC, monocyte-derived dendritic cell; NEAA, non-essential amino acids; NFκB, nuclear factor κB; NQO, NAD(P)H dehydrogenase [quinone]; Nrf2, nuclear factor erythroid 2-related factor 2; OECD, Organization for Economic Co-operation and Development; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PE, phycoerythrin; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; PRDX, peroxiredoxin; q(RT)-PCR, quantitative real time polymerase chain reaction; SD, standard deviation; SLC, solute carrier; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; TLR, toll-like receptor; TNBS, trinitrobenzenesulfonic acid; TNF, tumor necrosis factor; TNP, trinitrophenyl-; TRAF, TNF receptor-associated factor; TREM, triggering receptor expressed on myeloid cells.

MATERIALS AND METHODS

Reagents

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS, P04-36500), RPMI 1640 (P04-17500), human serum (P-2701), HEPES (P05-01100), sodium pyruvate (P04-43100), penicillin-streptomycin (P06-07100) and non-essential amino acids (NEAA, P08-32100) were purchased from PAN.

MoDC Generation and Chemical Treatment

PBMCs were isolated by standard density gradient centrifugation with Ficoll Paque Plus (GE Healthcare, 17-1440-03) from human buffy coats. Buffy coats were obtained from German Red Cross (Berlin, Germany) according to the current version of the declaration of Helsinki with an approved ethic vote (Charité, Berlin Germany; EA4/071/13). Untouched $CD14^+$ and $CD14^+CD16^+$ monocytes were isolated by negative depletion using the human PAN monocyte isolation kit (Biolegend, 480060), following the manufacturer's instructions. Monocytes were cultured in commercial ready-to-use MoDC differentiation medium containing 400 IU/ml IL-4 and 500 IU/ml GM-CSF (Miltenyi Biotec, 130-094-812) at 37°C with 5% CO_2. Every two days, half of the culture medium was replaced with fresh medium. On day six, immature MoDCs were harvested, washed with PBS and resuspended in fresh medium at a density of 10^6 cells/ml. Cells were either treated with medium only as control, 2.5 μg/ml LPS (from Escherichia coli O111:B4, L3024-5MG) or 400 μM NiSO4 (31483, tested endotoxin-free using QCL-1000TM Endpoint Chromogenic LAL Assay (Lonza, 50-647U) according to the manufacturers protocol). For proteomic studies and flow cytometry, cells were harvested after 24 h of continuous chemical incubation; for quantitative RT-PCR, cells were incubated either for 24 h or over a 36 h period with sample collections at various time points.

Flow Cytometry

To characterize the phenotype of harvested MoDCs, cells were stained for 30 min at 4°C with BV421 anti-CD86 (FUN-1, 562433), FITC anti-CD40 (5C3, 555588), FITC anti–CD83 (HB15e, 556910), PE anti–CD80 (L307.4, 557227), APC anti-CD1a (HI149, 559775) (all BD Biosciences) and PE anti-CD14 (TÜK4, Miltenyi, 130-113-147). Viability was monitored using fixable near-IR dead cell stain (Thermo Fisher Scientific, L34976). For each antibody staining, a control isotype staining was included. Data were acquired using a FACSAria III flow cytometer (BD Biosciences) and analyzed with FlowJo software (V.10.6.1, FlowJo LLC, Ashland, OR, United States). An exemplary gating plot is depicted in **Supplementary Figure 1**. Graphical visualization was performed using GraphPad Prism 6 (GraphPad Inc., San Diego, CA, United States). On day 6 of MoDC differentiation, the immature state of MoDCs was confirmed by measuring downregulation of the monocytic marker CD14 and upregulation of the DC-marker CD1a (data not shown).

Phagocytosis of TNBS-Modified Human Serum Albumin

Human serum albumin (HSA, fraction V, Merck, 12668-10GM-M) was dissolved in PBS (10 mg/ml) and incubated at 37°C for 60 min with 5 mM trinitrobenzenesulfonic acid (TNBS, 92822-1ML) (mole ratio HSA:TNBS 1:300) following a modified protocol of Dietz et al. (20). Subsequently, free TNBS was removed with a 30 kDa cutoff spin filter and two consecutive washes with PBS. Purified trinitrophenyl (TNP)-modified HSA was resuspended in PBS, and protein concentration was determined using the Pierce BCA protein assay (Thermo Fisher Scientific, 23225). As a control, pure HSA dissolved in PBS was processed equally. MoDCs were treated for 3 h or 24 h with LPS or 400 μM $NiSO_4$. To monitor phagocytosis, cells were harvested, seeded at a density of 10^5 cells/well in a 96-well plate and incubated with HSA-TNP or pure HSA (200 μg/ml). After 3 h, cells were stained with fixable near-IR dead cell stain followed by an intracellular BV421 anti-TNP (A19-3, BD Biosciences, 562601) stain with inside stain kit (Miltenyi, 130-090-477).

Mixed Leukocyte Reaction (MLR)

Chemical-treated MoDCs were harvested after 24 h, washed with PBS and 6×10^4 cells/well were seeded in a 96-well U-bottom plate. Following the manufacturer's instructions, allogenic PAN T cells were negatively depleted from PBMCs with PAN T cell isolation kit (Miltenyi, 130-096-535). T cells were labeled with 4 nM CSFE (Thermo Fisher Scientific, C34554) to monitor cell proliferation and added to MoDCs at a concentration of 6×10^5 cells/well (ratio T cells: MoDCs = 10:1). As a negative control, T cells were cultured without MoDCs. As a positive control, a co-culture of T cells and medium-treated MoDCs, was stimulated with 1 μg/ml staphylococcal enterotoxin B superantigen (S4881-1MG). Cells were cultured in RPMI 1640 supplemented with 10% human serum, 2 mM GlutaMAX (Gibco, Thermo Fisher Scientific, 35050061), 10 mM HEPES, 1x NEAA, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 100 U/ml β-mercaptoethanol (Gibco, 21985023) at 37°C with 5 % CO_2. On day 4, proliferating T cells were identified by their reduced CFSE signal.

Inhibition of p38 and HIF1α

MoDCs were treated with 20 μM SB203580 (Biomol, AG-CR1-0030-M005) to inhibit p38 activity, or with 0.5 nM echinomycin (Biomol, BVT-0267-M001) to inhibit binding of hypoxia-inducible factor (HIF) 1α to hypoxia response elements for 30 min. Subsequently, LPS and $NiSO_4$ were added at final concentrations of 2.5 μg/ml and 400 μM, respectively. After 24 h, viability and CD86 expression were analyzed by flow cytometry.

Immunoblotting of HIF1α

Chemically treated MoDCs were collected after 24 h of treatment. Cells were washed with ice-cold PBS and cell lysis was performed with the lysis buffer described in the LC-MS/MS sample preparation section. Protein concentration was determined using the Pierce BCA protein assay. Subsequently, 20 μg protein were loaded on 10% SDS-PAGEs. Following the separation, proteins were transferred to nitrocellulose membranes (BioRad, 1620115). The immunoblots were blocked in 5% skimmed milk and incubated for 16 h at 4°C to allow binding of the primary anti-HIF1α antibody (H1alpha67, Novus Biologicals, NB100-105). Binding of the secondary antibody (horseradish peroxidase labeled, Dianova, 115-035-206) was performed for 1 h at room temperature. The blot was visualized using Pierce ECL West Pico Substrate (Thermo Fisher Scientific, 34078) using a Fusion FX6 gel documentation (Vilber, Eberhardzell, Germany). A HRP anti-β actin antibody (AC-15, Abcam, ab49900) was used as loading control. Blots were analyzed using the Image Lab 6.0.1 software

(BioRad). All values were background-corrected and the HIF1α-signal was normalized to the β-actin signal and medium control.

Quantification of Secreted Cytokines

MoDC culture supernatants were collected after chemical treatment for 24 h. Cells and debris were removed by centrifugation for 10 min at 300 g. Following the manufacturer's instructions, IL-1β, IL-6, IL-8, IL-10, IL12p70, IL-18 and TNFα were quantified with a customized bead-based LEGENDplex immunoassay (Biolegend) on a FACS Aria III. Data were analyzed using the provided LEGENDplex v8.0 software. Data were log2 transformed and visualized with GraphPad prism. Student's *t*-test was applied to calculate *p*-values relative to medium control.

Quantitative Real-Time PCR

MoDCs were collected after chemical treatment, washed with ice-cold PBS and resuspended in TRIzol reagent (Invitrogen, Thermo Fisher Scientific, 15596026). RNA was isolated from 10^6 cells following the TRIzol manufacturer's protocol. Quantification of isolated RNA was performed on a NanoDrop 1000 (VWR, Radnor, PA, United States). One microgram RNA was used for reverse transcription into cDNA using the High-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific, 4368813). Quantitative real-time PCR was performed on a TaqMan PCR 7500 fast system (Applied Biosystems) using Fast SYBR green master mix (Applied Biosystems, 4385618) and the following primers: HPRT DNA forward 5′-GTTCTGTGGCCATCTGCTTAG-3′, reverse 5′- GCCCAAAGGGAACTGATAGTC-3′; HIF1α DNA forward 5′-TTTTTGCTGAAGACACAGAAGC-3′, reverse 5′- GCTTGCGGAACTGCTTTCTA-3′; SLC2A1 DNA forward 5′- CCAGCAGCAAGAAGCTGAC-3′, reverse 5′- AGGATGCTCTCCCCATAGC-3′; HMGCR DNA forward 5′-TGTTTACTGGTAACAATAAGATCTGTG-3′, reverse 5′-GTTGACGTAAATTCTGGAACTGG-3′; SOD2 DNA forward 5′-TTGGCCAAGGGAGATGTTAC-3′, reverse 5′-AGTCACGTTTGATGGCTTCC-3′; NQO1 DNA forward 5′-GCACTGATCGTACTGGCTCA-3′, reverse 5′-GAACACTCGCTCAAACCAG-3′; TNFα DNA forward 5′-CTTCTGCCTGCTGCACTTTGGAG-3′, reverse 5′-GGCTACAGGCTTGTCACTCGG-3′; HK2 DNA forward 5′-GTTCCTGGCTCTGGATCTTG-3′, reverse 5′-GGCAATGTGGTCAAACAGC-3′; SREBF2 DNA forward 5′-CACCAAGCACGGAGAGGT-3′, reverse 5′-GGGGAGGAGAGGAAGGAGA-3′; PFKFB3 DNA forward 5′-AAAAGTGTTCAACGTCGGGG-3′, reverse 5'-CGAAAACCGCAATTTGTCCC-3'; SLC2A3 DNA forward 5'-GAGGACGTGGAGAAAACTTGC-3', reverse 5′-GCCAAATTGGAAAGAGCCGA-3′; SLC2A6 DNA forward 5′-ATCCCAGGCATCCTGGTTTG-3′, reverse 5′-GGTCGTTGAGGATCATGGCA-3′. The following genes were analyzed using QuantiTect Primer Assay (Qiagen): HMOX1 (#QT00092645); PRDX1 (#QT01005536); GSTO1 (#QT02394287); CAT1 (#QT00079674). cDNA was 1:10 fold diluted in water and 1 µl was used for qRT-PCR analysis. Gene expression was normalized to the housekeeping gene *HPRT*, and relative gene expression was determined using the ΔΔCT method (21). Significance relative to medium-control was calculated using Student's *t*-test in GraphPad Prism.

Cholesterol Quantification

To evaluate effects of chemical treatment on cellular cholesterol content, MoDCs were treated with LPS and $NiSO_4$ as stated above. As a positive control for the inhibition of the cholesterol biosynthesis, medium-treated MoDCs were treated with 40 µM lovastatin (M2147-25MG). After 24 h, cells were harvested and washed with PBS. Lipids were extracted following the Folch protocol (22). Briefly, cells were resuspended in 500 µL chloroform-methanol (2:1, 1024451000, 1060072500), and lipids were extracted for 20 min by shaking and occasional vortexing at 4°C. Extraction was repeated after addition of 125 µL water. The suspension was centrifuged for 10 min at 1,000 g and 300 µL of the organic phase were transferred to a new Eppendorf tube and vacuum-dried. Proteins were obtained from the interphase and their concentration was determined using the Pierce BCA protein assay. Cholesterol levels of the samples were quantified using the Amplex Red Cholesterol Assay Kit (Thermo Fisher Scientific, 10236962). Desiccated lipids were dissolved in assay buffer and analyzed following the manufacturer's protocol. Fluorescence was measured at 590 nm emission on a Synergy Neo2 plate reader (BioTek, Winooski, VT, United States) with excitation at 540 nm. Total cholesterol levels were standardized to the respective protein concentration and normalized to the untreated control. *P*-values were calculated using Student's *t*-test relative to the medium control.

LC-MS/MS

Cell Lysis

After chemical treatment, MoDCs were collected, washed twice with ice-cold 0.9% NaCl and lysed in 100 µL lysis buffer per 10^6 cells. The lysis buffer was composed of 150 mM NaCl (S7653-250G), 10 mM TRIS pH 7.2 (T1503-250G), 5 mM EDTA (E5134-250G), 0.1% SDS (436143-25G), 1% Triton X-100 (T8787-100ML), 1% sodium deoxycholate (30970-100G), 200 µM phenylmethylsulfonyl fluoride (P7626-1G), 1 mM sodium orthovanadate (S6508-10G) and cOmplete protease inhibitor cocktail (Roche, 1.167.498.001). Cells were vortexed, incubated on ice for 15 min and centrifuged. The protein concentration of the supernatant was determined using Pierce BCA protein assay.

Sample Preparation

An untargeted proteomics approach was applied using label-free quantification as described before (23). Briefly, 30 µg protein per sample was reduced with 0.1 µmol tris(2-carboxyethyl) phosphine (C4706-10G), followed by alkylation with 0.2 µmol iodoacetamide (Merck, 8.04744.0025). Protein solutions were acidified and acetonitrile (ACN, Carl Roth, AE70.2) was added to reach more than 50 % (v/v) organic content facilitating protein binding to SpeedBeads™ magnetic carboxylate modified particles (SP3 beads, GE Healthcare, 65152105050250). Proteins were loaded on 20 µg beads, followed by a first washing step with 70% (v/v) ethanol (Merck, 22.462.500) in water and a

second step with 100 % (v/v) ACN. Proteins were digested with trypsin (enzyme:protein ratio 1:50, Promega, V5117) in 100 mM tetraethylammonium bromide (T7408-100ML). Digestion was stopped by addition of 100 % (v/v) ACN to reach ≥ 95% (v/v) organic content, thus again allowing the peptides to bind to the beads. Peptides were again cleaned-up using 100% (v/v) ACN. Elution of peptides was carried out in two steps with 87% (v/v) ACN in ammonium formate (pH 10) (Agilent Technologies, AGG1946-85021), followed by 2% (v/v) dimethyl sulfoxide (D2650-5x-10ml), resulting in two fractions per sample. Samples were evaporated to dryness and reconstituted in 0.1% (v/v) formic acid (FA, Fluka, Thermo Fisher Scientific, 56302-50ML-F) before measurement.

LC-MS/MS

Samples were analyzed on a UPLC system (Ultimate 3000, Dionex, Thermo Fisher Scientific) coupled to a Q Exactive HF (Thermo Fisher Scientific) as described previously (23). Peptides were loaded on an Acclaim PepMap 100 C18 trap column (3 µm, nanoViper, 75 µm × 5 cm, Thermo Fisher Scientific, PN164535) at a flow rate of 5 µl/min using an eluent composed of 2% (v/v) ACN and 0.05% (v/v) trifluoroacetic acid (Biosolve, 202341A8) in water. Peptides were separated by a 150 min non-linear gradient from 0 to 80% ACN in 0.1% FA on a reversed-phase column (Acclaim PepMap 100 C18, 3 µm, nanoViper, 75 µm × 25 cm, Thermo Fisher Scientific, PN164569). A chip-based ESI source (Nanomate, Advion, Ithaca, NY, United States) was used for ionization at 1.7 kV and coupled to the Q Exactive HF. The MS1 scans were acquired at a resolution of 120K in a range of 350–1,550 *m/z*. AGC target was set to 3×10^6 with a maximal injection time of 100 ms. The top 10 most abundant peptides were isolated for MS2 acquisition with an isolation window of 1.4 *m/z*. Peptides were fragmented at normalized collision energy of 28, and the fragment ion spectra were acquired at a resolution of 15K using AGC target of 2×10^5 and maximal IT of 100 ms. All spectra were acquired using XCalibur (Version 3.0).

Analysis

MS raw data were processed with MaxQuant Version 1.6.3.10 (24). If not stated otherwise, default parameters were used. Peptides were identified using a database search against the *Homo sapiens* UniProtKB reference proteome (24-09-2019, 74349 reviewed and unreviewed entries). Carbamidomethylation of cysteine was chosen as fixed, whereas oxidation of methionine and acetylation of protein N-terminus were set as variable modifications. A minimum of two peptides with at least one unique peptide was required for protein identification applying FDR ≤ 0.01. Match between runs was activated. Proteins were quantified based on two unique peptides. Protein contaminants and reverse hits were excluded before further use. LFQ protein intensities were processed, and results were visualized in R-3.5.0. Accordingly, the data were log2-transformed, filtered for proteins that were quantified in a minimum of four replicates under at least one condition, followed by variance-stabilization. Imputation was performed using the DEP package (25) (fun = "MinProb," $q = 0.01$) for proteins not quantified in any of the replicates under the particular condition. Fold changes (FCs) and *p*-values were calculated using Student's *t*-test relative to the medium control. Proteins with a *p*-value ≤ 0.05 were considered as significantly changed.

Pathway Enrichment

Significantly enriched pathways were identified using ingenuity pathway analysis (IPA, Qiagen), considering significantly enriched proteins.

RESULTS

Phenotypical and Functional MoDC Characterization

The effects of $NiSO_4$ and LPS on the phenotype and function of primary human monocyte-derived DCs (MoDCs) were investigated first. MoDCs were generated from buffy coats of five individual donors, and treated with LPS (2.5 µg/mL) or $NiSO_4$ (400 µM) for 24 h. Cell viability and upregulation of the maturation markers CD86, CD83, CD40 and CD80 on their surface was measured (**Supplementary Figure 1**). Cell viability was assured to be above 75% for all samples (**Supplementary Figure 1A**), in agreement with the acceptance criteria of the human Cell Line Activation Test (26). MoDCs were used in further experiments, if maturation markers were upregulated after LPS- and $NiSO_4$-treatment (**Supplementary Figures 1B–E**). Overall, changes induced by LPS were more pronounced.

Furthermore, the gene expression of the allergy relevant cytokine TNFα was determined by qRT-PCR. The *TNFA* gene is directly induced after TLR4 signaling and activates nuclear factor κB (NFκB). To capture the dynamics of *TNFA* expression, we measured its gene expression over 36 h at various time points. The time course of gene induction was comparable for LPS and $NiSO_4$ peaking 2 h after treatment. However, LPS acted as a much more potent inducer (**Supplementary Figure 2**).

Moreover, selected inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, IL12p70, IL-18, and TNFα) were quantified in the cell culture supernatants using a bead-based immunoassay (**Supplementary Figure 3**). The production of IL-8 for example is used as a biomarker for DC activation (26). All cytokines were significantly enriched in the cell culture supernatant of both treatments compared to the unstimulated control. Only for IL-10, $NiSO_4$-induced release was not significantly altered compared to medium control. Overall, the secreted amounts of all analyzed cytokines were higher in LPS-treated compared to $NiSO_4$-treated cells.

The Phagocytotic Activity of LPS- and $NiSO_4$-Treated MoDCs Is Markedly Reduced

It is widely accepted that mature DCs are less phagocytotic compared to their immature progenitors. The degree of reduction of phagocytosis depends on the stimuli as well as on the DC maturation state (27–29). Thus, we aimed to functionally compare dendritic cells that were maturated with either LPS or $NiSO_4$ by monitoring uptake of trinitrophenyl-modified HSA. MoDCs maturated for 3 h with either substance showed no significant reduced phagocytotic activity compared

to untreated cells (**Supplementary Figure 1G**). However, prolonged chemical treatment of 24 h reduced phagocytosis of MoDCs (**Supplementary Figure 1F**). The degree of reduction in phagocytotic activity was comparable for LPS and $NiSO_4$.

LPS- and $NiSO_4$-Treated MoDCs Induce Proliferation of Allogenic T Cells

To test whether $NiSO_4$ affects the ability of MoDCs to activate T cells, we performed a mixed leukocyte reaction. Control, LPS- or $NiSO_4$-treated MoDCs were incubated for 4 days with allogenic T cells, and proliferation of the latter was determined by CFSE dilution. LPS- and $NiSO_4$-treated MoDCs induced stronger proliferation of T cells compared to control MoDCs with LPS being the more potent inducer (**Supplementary Figure 4A**). These results are in concordance with the upregulation of co-stimulatory molecules on the DC surface. As control, T cells were incubated in the absence of MoDCs, which resulted in hardly any proliferation compared to T cells cultured in the MLR (negative control, data not shown) while the superantigen staphylococcal enterotoxin B induced a strong T cell proliferation (positive control, data not shown).

Overall, the upregulation of activation markers, expression of inflammatory cytokines, reduced antigen-uptake and the ability to induce T cell proliferation prove the activation of MoDCs after treatment with $NiSO_4$ and LPS. Thus, even if less pronounced compared to LPS, immunological signaling induced by $NiSO_4$ was demonstrated.

Proteomic Analysis

To analyze the underlying cellular effects and to identify similarities and changes of LPS- and $NiSO_4$-activated DCs, we applied a global proteomics approach. A principal component analysis was employed to assess the similarity of the treatment groups. Biological replicates show little variance, indicative for the high quality of our proteomic dataset. $NiSO_4$-samples cluster between LPS- and control-samples (**Figure 1A**), pointing toward an intermediate cellular state of $NiSO_4$-treated cells. In total, more than 3,300 proteins were identified using a label-free quantification approach (**Supplementary Table 1**). Of these, the majority was identified in both treatment groups (**Figure 1B**). Analysis of significantly altered proteins relative to the control exhibited 493 significantly altered proteins by LPS, while 402 proteins were changed by $NiSO_4$-treatment and 144 proteins were altered by both treatments with $p \leq 0.05$ (**Figure 1B**). Furthermore, the z-score of each fold change was calculated and plotted in a heat map (**Figure 1C**), which allowed comparison across replicates and treatments. Biological replicates show minimal variance, which allowed the identification of clusters of significantly altered proteins compared to the control. Interestingly, we observed different clusters of upregulated proteins for $NiSO_4$-treated cells compared to LPS-treatments, indicating a distinct mode of action. Next, we visualized the data using volcano plots to identify statistically significantly regulated proteins based on their *p*-value compared to the magnitude of change. As shown in **Figure 1D**, LPS induced a more pronounced spread of the data. Overall, upregulation was favored over downregulation for both chemicals.

To differentiate the effects of LPS- or $NiSO_4$-treatment on cellular pathways, significantly altered proteins ($p \leq 0.05$) were used to identify enriched pathways using QIAGEN's Ingenuity Pathway Analysis (IPA). Notably, LPS-treatment induced more significantly enriched pathways than $NiSO_4$ (**Figure 1E**). Shared pathways between the two groups included Nrf2-mediated oxidative stress response, CD40 signaling, triggering receptor expressed on myeloid cells (TREM) 1 signaling and acute phase response signaling. Pathways that were uniquely induced after $NiSO_4$-treatment comprised the superpathway of cholesterol biosynthesis, the unfolded protein response and eukaryotic initiation factor (EIF) 2 signaling.

$NiSO_4$-Treatment Activates Nrf2 Target Genes in MoDCs

The nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated oxidative stress response was predicted to be highly activated in the ingenuity pathway analysis for $NiSO_4$-treated MoDCs. Furthermore, this pathway was among the few pathways that were induced much stronger by $NiSO_4$ compared to LPS (**Figure 1E**). Hence, we ought to investigate the differences between the two stimuli concerning the Nrf2 pathway in more detail. Gene expression of Nrf2-target genes was analyzed by quantitative RT-PCR (**Figure 2**). Indeed, 4 out of 6 genes, namely *GSTO1*, *GSTM1*, *NQO1*, and *PRDX1*, were induced only due to $NiSO_4$- but not after LPS-treatment. *HMOX1* gene expression was more pronounced after $NiSO_4$-treatment; however, the effect was not statistically significant. *SOD2* gene levels were exacerbated after both treatments, rendering *SOD2* the only Nrf2 target gene that was found to be significantly upregulated after LPS-treatment. On the protein level, heme oxygenase (HMOX) 1 was confirmed in the proteomic data set as significantly upregulated after $NiSO_4$-treatment. Superoxide dismutase (SOD) 2 showed strong upregulation on protein level after both treatments. However, only LPS induced changes were significant. From this data, it can be concluded that $NiSO_4$ causes a distinct oxidative stress level in MoDCs which may strongly contribute to the activation of the cells. Thus, induction of oxidative stress may be an additional mode of action besides TLR4 activation in $NiSO_4$-treated MoDCs.

$NiSO_4$ Leads to Cholesterol Depletion but Induces Cholesterol Biosynthesis Pathway

Pathway enrichment analysis by IPA revealed the superpathway of cholesterol biosynthesis as one of the strongly activated pathways after treatment of MoDCs with $NiSO_4$ (**Figure 1E**). Proteins matched to this pathway were significantly upregulated and comprised proteins like lanosterol 14α-demethylase (CYP51A1), hydroxymethylglutaryl-CoA synthase (HMGCS1) and 7-dehydrocholesterol reductase. We therefore quantified cellular cholesterol levels using the Amplex Red Cholesterol assay and confirmed a significant depletion of cholesterol in $NiSO_4$-treated MoDCs (**Figure 3A**). The strength of this effect

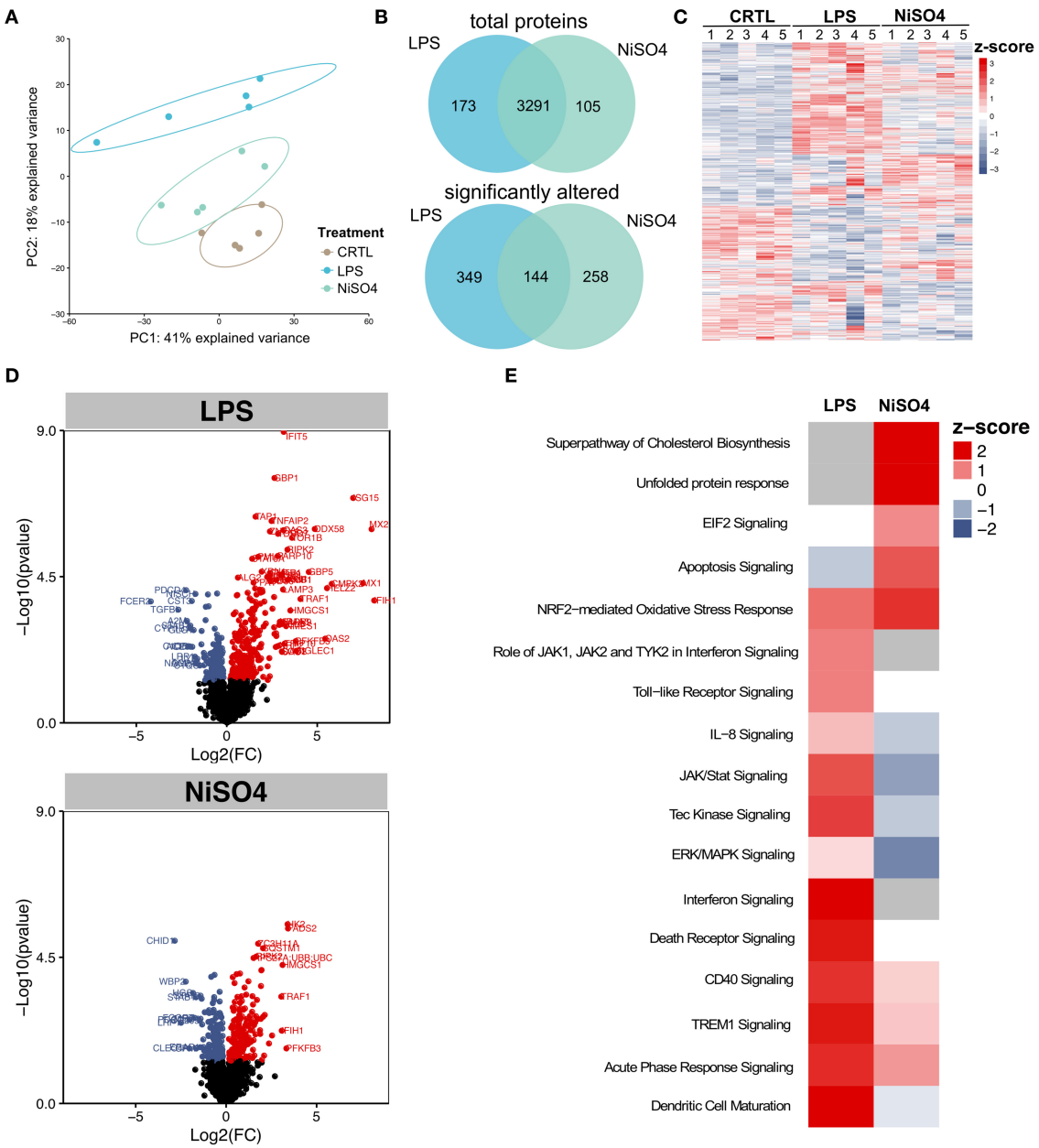

FIGURE 1 | Quantitative proteomic analysis of differentially regulated proteins in LPS- and $NiSO_4$-treated MoDCs. **(A)** Principal component analysis of the five biological replicates showing a distinct clustering of each group, **(B)** Venn-diagrams of total as well as significantly altered proteins in the LPS-group (blue) and $NiSO_4$-group (green), **(C)** heat map of significantly regulated proteins. Plotted are z-scores for all identified proteins, **(D)** volcano plots showing significantly regulated proteins for LPS- and $NiSO_4$-treated MoDCs compared to untreated MoDCs ($p \leq 0.05$). Mean values of the log2-fold change are displayed and plotted against –log10(*p*-value), red = upregulation, blue = downregulation, **(E)** significantly changed pathways in LPS and $NiSO_4$-treated MoDCs. Canonical IPA pathways with a positive z-score (red) are predicted to be activated; blue represents a negative z-score and inactivation of the pathway. Gray indicates unmatched pathways. IPA analysis was based on proteins that were significantly changed compared to control ($p \leq 0.05$). Within the IPA software, z-scores were calculated and only experimental data of human origin was allowed. The tissue specificity was set to immune cells. Significance was calculated using Student's *t*-test.

was comparable to that of cells treated with lovastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), which is the rate-limiting enzyme of the cholesterol biosynthesis. In contrast, LPS did not induce changes in cellular cholesterol levels. Based on these findings, we analyzed the gene expression of HMGCR and sterol regulatory element-binding protein (SREBB) 2, the regulating transcription factor of this pathway. Both genes were found to be constantly upregulated in $NiSO_4$-treated MoDCs but were not induced (or even slightly downregulated) after treatment with LPS (**Figures 3B,C**).

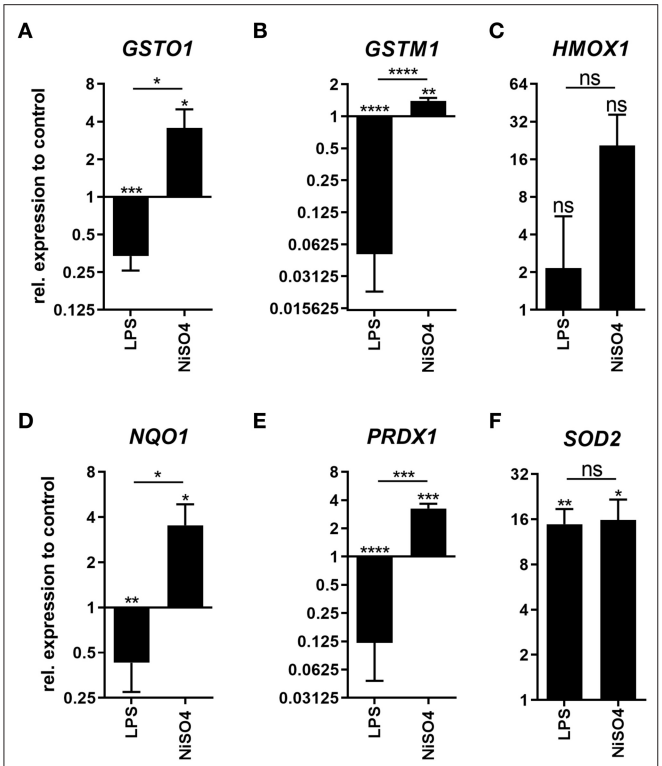

FIGURE 2 | Expression of Nrf2-target genes in LPS- and $NiSO_4$-treated MoDCs. Depicted is the gene expression of glutathione S-transferase omega (GSTO1, **A**), glutathione S-transferase mu (GSTM1, **B**), heme oxygenase (HMOX1, **C**), NAD(P)H dehydrogenase (NQO1, **D**), peroxiredoxin (PRDX1, **E**) and superoxide dismutase (SOD2, **F**) as measured by the qRT-PCR after treatment of MoDCs with medium only, 2.5 μg/ml LPS or 400 μM $NiSO_4$ for 24 h. Gene expression was normalized to the gene expression of the housekeeping gene HPRT and medium control. Thereof, mean and SD are shown ($n = 3$ donors). Applying the Student's *t*-test, significance was calculated to medium control (**p*-value ≤ 0.05; ***p*-value ≤ 0.01; ****p*-value ≤ 0.001; *****p*-value < 0.0001).

LPS and $NiSO_4$ Induce Metabolic Shifts That Lead to Hypoxic Conditions in Case of $NiSO_4$

Metabolic reprogramming is essential during DC maturation since the metabolic switch from oxidative phosphorylation to glycolysis ensures adequate energy supply to support cellular reconstructions. Thus, we aimed to investigate the effects of $NiSO_4$ on the metabolism of DCs. In the proteomic data set, proteins associated with anaerobic glycolysis were significantly upregulated after treatment with $NiSO_4$, including lactate dehydrogenase (LDHA) along with the glycolytic enzymes aldolase A (ALDOA), hexokinase (HK) 2 and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) 3. These findings were supported by elevated expressions of proteins from the glucose transporter (GLUT) family which facilitate the transport of sugars. Namely, solute carrier (SLC) 2A1, SLC2A3/2A14 and SLC2A6 expression were significantly increased after $NiSO_4$-treatment (**Figure 4A**).

In contrast, cells treated with LPS induced only upregulation of PFKFB3 and SLC2A6. SLC2A1 was not detected, and SLC2A3 was significantly downregulated in this group (**Figure 4A**). Thus, we aimed to investigate these differences in more detail using RT-PCR. Gene expression analysis suggests different kinetics for *HK2* gene expression. Both treatments resulted in an increasing *HK2* gene expression over the first 6 h of treatment. After 6 h a plateau was reached, which $NiSO_4$-treated MoDCs maintained over the investigated time. Yet, LPS-treated MoDCs appear to counteract this gene induction leading to subsequent downregulated expression of *HK2* (**Figure 4B**). Upregulation of PFKFB3 induced by LPS and $NiSO_4$ was verified by measuring gene expression after 24 h (**Figure 4C**). Protein expression of the GLUT family proteins was confirmed by measuring the gene expression of the respective genes 24 h after treatment (**Figures 4D,E**, **Supplementary Figure 5B**). Stimulation of MoDCs with LPS was shown to cause metabolic reprogramming that was mainly dependent on p38 mitogen-activated protein kinase signaling leading to HIF1α accumulation and elevated HK2 gene expression and activity (30). In our study, expression of the *HIF1A* gene was downregulated for both groups (**Supplementary Figure 5A**) but quantitative immunoblot analysis revealed increased cellular HIF1α protein levels after 24 h of treatment (**Supplementary Figure 5C**). Since we found HK2 upregulated on protein and gene level, we were interested in studying whether inhibition of p38 using the inhibitor SB203580 had similar effects in LPS- and $NiSO_4$-stimulated cells. Indeed, CD86 expression was decreased after inhibition of p38. However, the reduction was only significant in LPS-treated MoDCs (**Supplementary Figure 6A**). Additionally, we analyzed the effect of echinomycin-induced HIF1α inhibition on the MoDCs. Inhibition of HIF1α induced a significant reduction of the CD86 fluorescence signal in $NiSO_4$- treated cells (**Supplementary Figure 6B**). The pronounced dependency of $NiSO_4$-treated cells on HIF1α along with increased protein levels and gene expression of glycolytic enzymes and glucose transporters can be indicative for a prolonged stabilization of HIF1α and hypoxia-like conditions (31).

Figure 5 summarizes the main findings of this study in a comprehensive and visual way.

DISCUSSION

The present study aimed at elucidating the cellular mode of action of $NiSO_4$-stimulation in primary human MoDCs in comparison to LPS activation. Nickel is a well-known allergen, whereas LPS belongs to pathogen-associated molecular patterns of Gram-negative bacteria. Both agents are known to activate DCs by ligation to TLR4 (15, 18). As the molecular binding mechanism is not identical (19), we studied if this may lead to the differential regulation of allergy-specific proteins in $NiSO_4$-treated MoDCs. To investigate proteome alterations and underlying mechanisms, we applied different approaches including proteomics, quantitative RT-PCR and flow cytometry. MoDCs generated from five individual donors were treated with 2.5 μg/ml LPS or 400 μM $NiSO_4$ to induce maturation of DCs. We confirmed maturation of MoDCs by both stimuli along with reduced phagocytosis, secretion of immunostimulatory cytokines

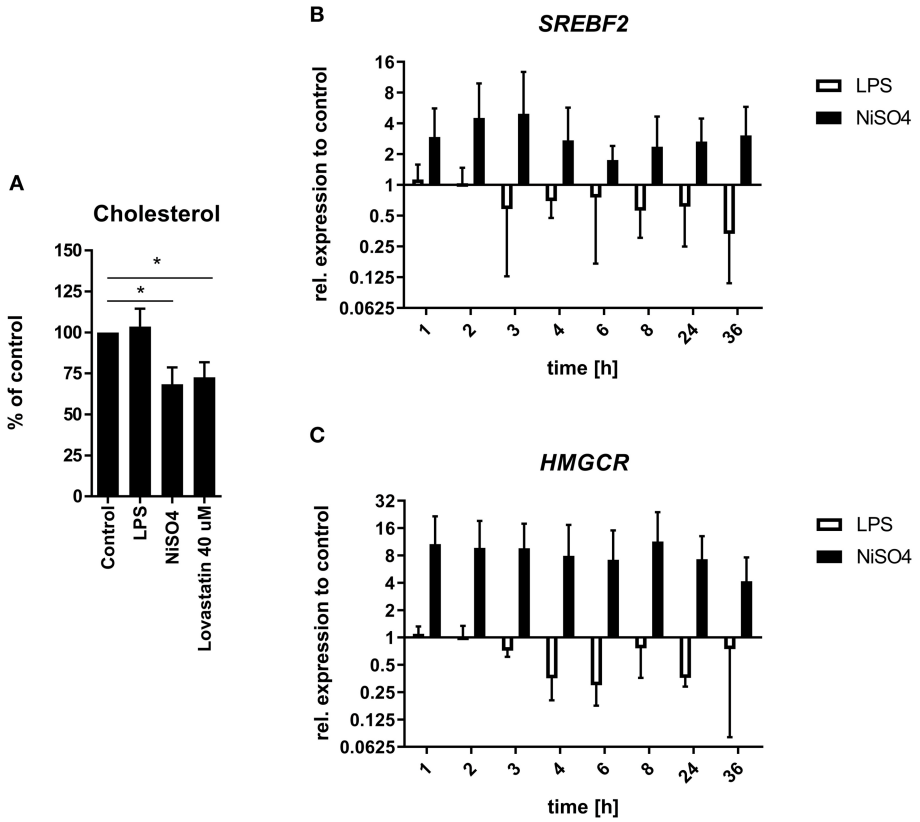

FIGURE 3 | Cellular cholesterol quantification and relative gene expression of relevant genes in LPS- and $NiSO_4$-treated MoDCs. **(A)** Cellular cholesterol levels were determined fluorometrically using the Amplex Red Cholesterol Assay kit after lipid extraction from MoDCs treated for 24 h. The fluorescence intensity was normalized to the protein concentration of the respective sample. Cholesterol/protein-ratios were then further normalized to the untreated control. Significance to medium control was calculated using Student's *t*-test and is indicated with $^*p \leq 0.05$. The relative gene expression of sterol regulatory element-binding protein 2 (SREBF2, **B**) as well as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR, **C**) were analyzed at selected time points after treatment of MoDCs. Gene expression was measured by qRT-PCR and normalized to the gene expression of HPRT (housekeeping gene) and medium control. Plotted are mean and SD of the normalized data ($n = 3$ donors). For each assay MoDCs were treated with medium only, 2.5 μg/ml LPS, 400 μM $NiSO_4$ or 40 μM lovastatin.

as well as induction of T cell proliferation. Overall, $NiSO_4$-treatment appears to elicit a phenotype comparable to LPS.

At the protein level, LPS-treated cells in our study elicited proteins and signaling as reported previously. Arya et al. (13) conducted a study to characterize proteomic changes induced in MoDCs after LPS treatment. The pathways of interferon signaling and IL-9 signaling and the proteins nuclear factor NF-kappa-B p105 subunit (NFKB1), nuclear factor NF-kappa-B p100 subunit (NFKB2), IL-1β, TNF receptor-associated factor (TRAF) 1, E3 ubiquitin-protein ligase TRIM4, CD54, fascin and signal transducer and activator of transcription 1 were also significantly altered by LPS in our study. $NiSO_4$-treatment likewise lead to significantly altered levels of NFKB2, fascin, CD54 and TRAF1. Hussaarts et al. (3) analyzed proteomic changes in MoDCs that were incubated with LPS for 32 h. The authors identified TRAF1, myristoylated alanine-rich C-kinase substrate, human leukocyte antigen B as well as fascin as TOP4 upregulated proteins. We could confirm these findings for LPS-treated MoDCs. However, after $NiSO_4$-treatment, only TRAF1 and fascin were significantly altered. The respective TOP4 downregulated proteins in the study by Hussaarts et al. (3) were cathepsins, ganglioside GM2 activator and macrophage mannose receptor 1, which again were also significantly downregulated after LPS-treatment in our proteomic data. Macrophage mannose receptor 1 was the sole protein that was significantly downregulated after $NiSO_4$-treatment, too. Despite these eight proteins, also other proteins were identified to be significantly regulated after LPS-treatment in both studies, such as SOD2, WARS and CD54 indicating that the maturation of MoDCs due to LPS is generally robust. In the context of allergy, Strasser et al. (32) published a proteomic study in MoDCs reacting to the prominent birch pollen allergen Bet v 1. The pure recombinant allergen induced only minor changes in MoDC. However, changes caused by birch pollen extract were comparable to LPS induced changes. The authors therefore suspect that Bet v 1 only displays its allergenic potential in combination with additional danger signals such as LPS. The data presented for the birch pollen extract and LPS show high similarities with the data set presented here. The allergenic pollen extract and $NiSO_4$ induce similar IPA pathways including Nrf2 mediated oxidative stress response, TREM1 signaling and

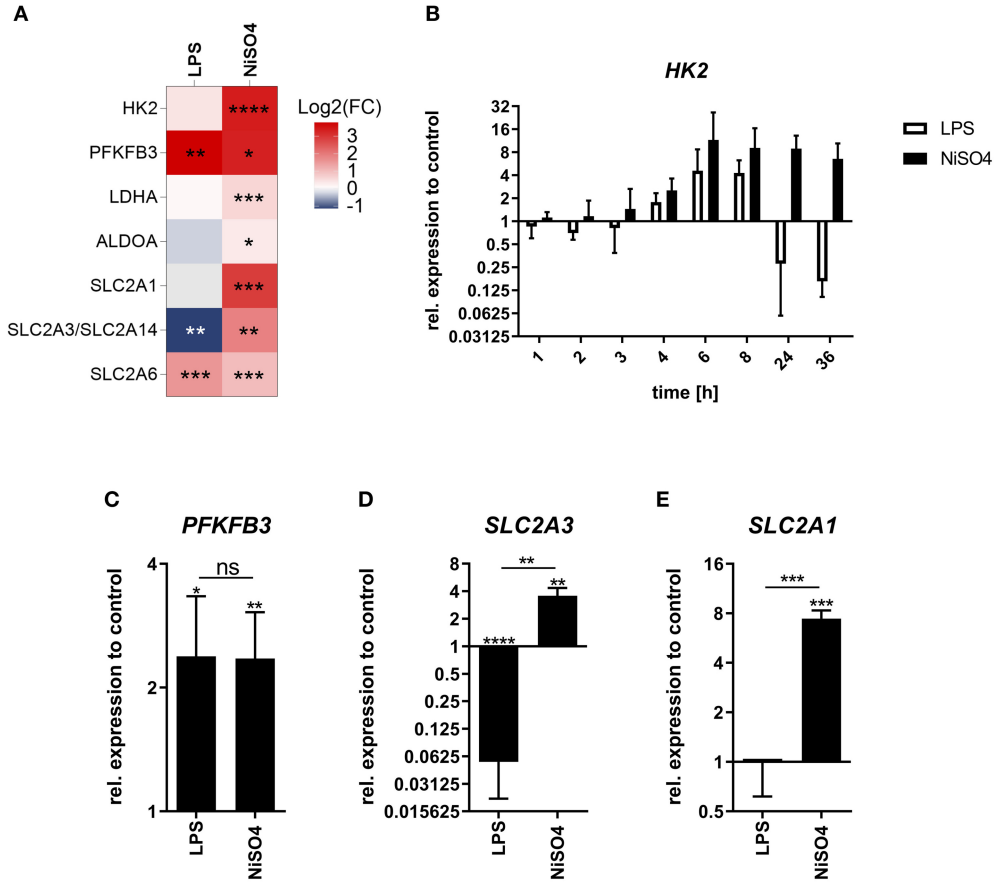

FIGURE 4 | Fold change of glycolytic proteins and their respective gene expression in LPS- and $NiSO_4$-treated MoDCs. **(A)** Heat map of differentially upregulated proteins in the proteomics data set linked to glycolysis and glucose transport. Red indicates upregulation of the respective protein and blue refers to downregulation (n = 5 donors). **(B–E)** Relative gene expression of hexokinase 2 (HK2, B), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3, C), solute carrier family 2 member 3 (SLC2A3, D) and solute carrier family 2 member 1 (SLC2A1, E). Gene expression was measured by qRT-PCR at indicated time points **(B)** or after 24 h of treatment for **(C–E)**. Gene expression was normalized to the gene expression of HPRT (housekeeping gene) and medium control. Mean and SD of the normalized data are shown (n = 3 donors). Significance to medium control was calculated using Student's t-test (ns: not significant; *p-value ≤ 0.05; **p-value ≤ 0.01; ***p-value ≤ 0.001; ****p-value < 0.0001). MoDCs were treated with medium only, 2.5 μg/ml LPS or 400 μM $NiSO_4$.

ERK/MAPK signaling. Furthermore, we were able to identify HMOX1, SOD2, sequestosome-1, TRAF1 and nicotinamide phosphoribosyltransferase as significantly changed due to $NiSO_4$. Proteomic changes induced by the investigated $NiSO_4$ are hence more comparable with the birch pollen extract than with the recombinant protein itself. It can be suspected that $NiSO_4$ does possess the ability to induce danger signaling that Bet v 1 seems to lack. These findings also point to TREM1 as one important signaling pathway in the induction of an allergy.

In our study, both stimuli, LPS and $NiSO_4$, induced protein and gene expression changes that are characteristic for TLR4 signaling, reflected by upregulation of NFκB2 on the protein level. NFκB signaling was identified as a crucial pathway in DC maturation in response to $NiSO_4$ as it is tightly connected to the expression of maturation markers like CD40, HLA-DR, CD86 and the secretion of pro-inflammatory cytokines like IL-8 and IL-6 (33). Furthermore, NFκB signaling is indicative for the activation of TLR4 by $NiSO_4$. The central role of NFκB in mediating the response to $NiSO_4$ in MoDCs was also reflected in the strong upregulation of proteins such as SAM and SH3 domain-containing protein (SASH) 1 and TRAF1 which are known to positively regulate NFκB activity (34, 35) and which were also significantly upregulated in our proteomic data set for $NiSO_4$-exposed MoDCs. Downstream of NFκB, we identified increased gene expression for TNFα and could also show that $NiSO_4$ triggered elevated secretion of this cytokine along with IL-8. Again, LPS induced more pronounced alterations, suggesting a more substantial capacity to activate TLR4 compared to $NiSO_4$.

One of the most distinct differences between LPS- and $NiSO_4$-treatment was the induction of the Nrf2-pathway after treatment with $NiSO_4$. Nrf2 is an important transcription factor for cytoprotective genes. Under normal conditions, Nrf2 is located in the cytoplasm where it is associated with a protein complex including the kelch-like ECH-associated protein (KEAP) 1. KEAP1 continually promotes ubiquitination and proteasomal degradation of Nrf2 (36). Electrophilic and oxidative stress

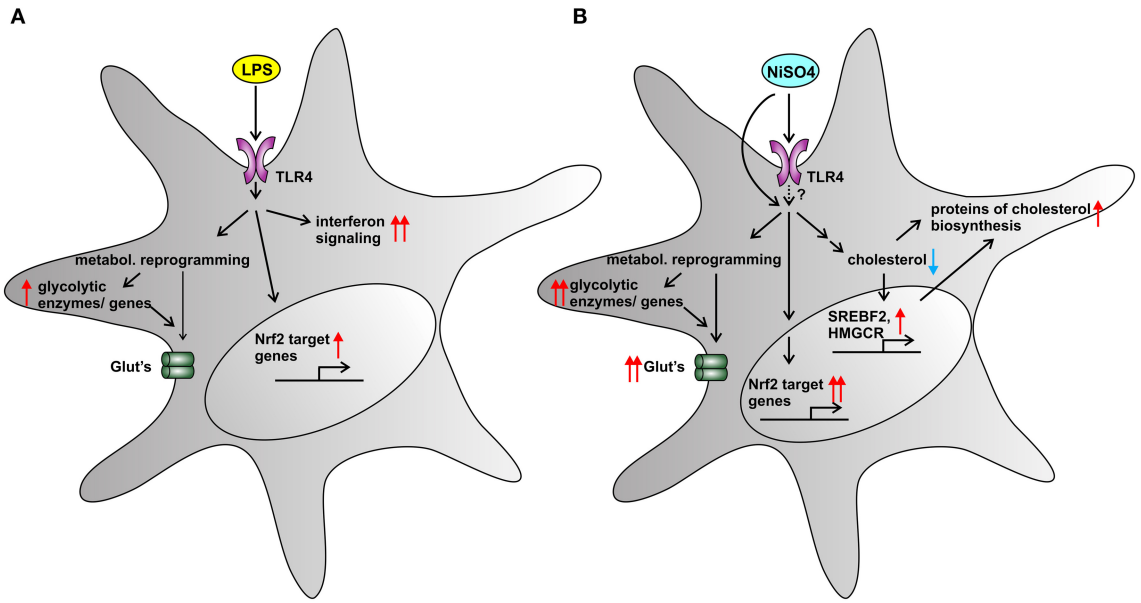

FIGURE 5 | Summarizing Figure. Cellular effects of two TLR4-activators on MoDCs: LPS **(A)** and $NiSO_4$ **(B)**. **(A)** LPS-treatment induced a metabolic shift toward glycolysis and a weak Nrf2-dependent stress response. IPA pathway analysis revealed a strong upregulation of interferon signaling. **(B)** $NiSO_4$-treated cells showed a distinct upregulation of glycolytic enzymes both on mRNA and protein level, suggesting metabolic reprogramming and hypoxia-like conditions. The metal allergen $NiSO_4$ also triggered a pronounced Nrf2-dependent stress response that culminated in upregulation of Nrf2-target genes. Most strikingly, $NiSO_4$ induced a cellular cholesterol depletion in MoDCs, which was reflected in the upregulation of genes and proteins involved into cholesterol biosynthesis. We speculate that haptenation to other cellular proteins than TLR4 also contributed to the observed changes. A blue arrow indicates downregulation and red arrows indicate upregulation. A pronounced change is indicated by two arrows.

induces a conformational change in KEAP1 which suppresses Nrf2 ubiquitination and enables translocation of Nrf2 to the nucleus. In the nucleus, Nrf2 binds to antioxidant-responsive elements and induces transcription of target genes (37). Nrf2-dependent stress response was identified as a major player in the immunological reaction to allergens (38). Specifically, the high relevance of Nrf2 during skin sensitization was reflected in the upregulation of target genes like HMOX1 and NAD(P)H dehydrogenase [quinone] (NQO) 1 at the protein level in CD34-derived dendritic cells and THP-1 cells (39). A prior proteomic study of our lab used Nrf2 knockout mice for the identification of potential biomarkers for skin sensitization among them HMOX1 and PRDX1 (40). Previously, activation of Nrf2 due to treatment with $NiCl_2$ was shown in monocytic THP-1 cells (41). Our findings also underline the importance of Nrf2 in response to $NiSO_4$ since the Nrf2 stress response signaling was identified as highly activated pathway using IPA analysis. In this context, we were able to identify significantly increased gene expression levels for the Nrf2 target genes peroxiredoxin (PRDX) 1, SOD2, NQO1, glutathione S-transferase (GST) M1 and GSTO1 as well as elevated protein levels of SOD2 and HMOX1. Besides $NiSO_4$, LPS is also able to induce oxidative stress in dendritic cells which is believed to support the maturation of the cells (42, 43). We identified SOD2 as strongly upregulated protein after treatment with LPS which was verified by quantitative RT-PCR. Proteomic upregulation of SOD2 after LPS-treatment was also reported by Hussaarts et al. (3). Thus, Nrf2-dependent proteins play a major role during cellular response to an allergen. However, biomarker candidates from this group of proteins have to be selected carefully since other substances like LPS that induce DC maturation also regulate Nrf2-dependent proteins.

The induction of TLR4 signaling pathways by binding of nickel undoubtedly contributed to the activation of the cells as proposed by Schmidt et al. (18). However, Vennegaard et al. (44) showed that sensitization to Ni^{2+} occurs independent of TLR4 in mice. MyD88-dependent and IL-1-related pathways mediated the immunological reaction to Ni^{2+} in their *in vivo* model. In our study with human MoDCs, we found significantly elevated secretion levels of IL-1β after treatment with $NiSO_4$, which may be indicative for an involvement of IL-1-related pathways during sensitization to $NiSO_4$. We also believe that the haptenation of nickel to other cellular proteins led to the induction of a distinct oxidative stress response which eventually resulted in cellular activation and the observed proteomic phenotype of the MoDCs. The haptenation mechanisms of metal allergens remain to date under-investigated. Yet, nickel was shown to bind to a broad range of cellular proteins, including many heat shock proteins and cytoskeletal proteins (45, 46). Binding of nickel to serum albumin was shown to bypass antigen-processing of antigen-presenting cells by a transfer of the Ni^{2+}-ion to peptide-major histocompatibility complexes inducing subsequent T cell activation (45). Due to the *modus operandi* in this study, further nickel-interacting proteins could not be detected.

IPA results revealed the superpathway of cholesterol biosynthesis as strongly induced due to $NiSO_4$- but not after LPS-treatment. $NiSO_4$-treated MoDCs exhibited significantly reduced cellular cholesterol levels which were accompanied by upregulation of genes and proteins involved in the cholesterol biosynthesis. Cellular cholesterol homeostasis is a delicate equilibrium. Under cholesterol deficiency, sterol response element-binding proteins (SREBP) become activated and subsequently activate genes of the mevalonate pathway like HMGCR to fuel cholesterol accumulation (47). Changes in cholesterol levels can lead to fatal effects with major impacts on DCs role in immunity. Disrupted cholesterol homeostasis may affect DC differentiation and maturation, antigen presentation, migration, as well as priming of T cells (48). During DC maturation cholesterol highly supports the synthesis of membranes needed for cellular expansion. Especially the formation of lipid rafts in the cell membrane is sensitive to changed cholesterol levels. Since TLR or major histocompatibility complexes are located in these rafts, receptor-induced signaling like NFκB- or IFN-signaling is directly affected upon perturbed cholesterol levels (48–51). Excessive cholesterol biosynthesis may also be a cellular mechanism to restore and maintain cell membranes. Cholesterol decreases membrane fluidity and therefore stabilizes the latter (52).

Cholesterol is also known to play a pivotal role in cellular stress response and during hypoxia. Under hypoxic conditions, sterol levels were depleted in the yeast *Saccharomyces pombe*, which was counteracted by activation of Sre1 (the analog of SREBPs in eukaryotes) until normal levels of sterol synthesis were again reached after a few hours (53). In our experiment, the gene expression of SREBP and HMGCR was continuously upregulated in $NiSO_4$-treated cells. Thus, potentially being a compensatory effect for the cellular cholesterol depletion induced by $NiSO_4$. A recently published study on the lipid composition in maturated human MoDCs, revealed that mature MoDCs are stiffer than their immature progenitors. These findings were explained by an altered lipid class composition in mature MoDCs. Strikingly the performed lipidomic analysis also disclosed reduced cholesterolester levels in mature MoDCs (54), which supports our findings of cellular cholesterol depletion. However, the role and fate of cholesterol during DC maturation is to date not fully understood.

We suspect that diminished cellular cholesterol levels can be explained by the formation and secretion of extracellular vesicles. Extracellular vesicles were proven to play a pivotal role in allergy and immunity in general (55). Exosomes of dendritic cells may transport allergens and thereby activate T cells (56). Another example for the relevance of DC exosomes is their use as vaccine in cancer immunotherapy (57). Taken together, the impact of $NiSO_4$ on the cellular fate of cholesterol is an exciting target for future research. Hence, we already conducted a subsequent proteomic experiment to unravel whether the observed changes in cholesterol biosynthesis are unique for $NiSO_4$. Appling a stable isotope labeling with amino acids in cell culture (SILAC), we analyzed the effects of organic allergens, including the strong allergens *p*-benzoquinone and 2,4-dinitrochlorobenzene, on the proteome of THP-1 cells. THP-1 cells are validated for usage within the human Cell Line Activation Test to assess skin sensitizers (26). We were able to confirm the upregulation of pathways connected to the cholesterol biosynthesis after treatment of THP-1 cells with all contact sensitizers tested (unpublished data). These data support the evidence presented in our study in MoDCs and underline the need for further research in this field which may eventually lead to a better understanding of cellular mechanisms in the context of ACD.

Due to strict search criteria for the IPA pathway analysis, no metabolic pathways were identified as significantly regulated. However, as already described in the literature, phenotypic alterations and cellular reconstruction during DC maturation are supported by a metabolic reprogramming, i.e. cellular metabolism is shifted from oxidative phosphorylation toward glycolysis which fuels the cells with sufficient energy. The metabolic shift toward glycolysis is triggered by strong and weak DC activators likewise and is essential for DC migration (58). However, long-term dependency on glycolysis was only induced by potent DC activators and is achieved by HIF1α stabilization (59, 60). As reported before, LPS triggered a metabolic shift toward glycolysis by increasing the gene expression of HK2 (30). Although protein levels of HK2 were not significantly changed due to LPS, HK2 was one of the proteins with the most pronounced differential expressions after $NiSO_4$-treatment. HK2 protein expression was significantly altered and 3.4-fold upregulated, respectively. Additionally, we identified another protein supporting the shift toward glycolysis: PFKFB3 was significantly upregulated after LPS- and $NiSO_4$-treatment. PFKFB3 is a kinase producing fructose 2,6-bisphosphate which is an allosteric activator of the phosphofructokinase, a bottleneck enzyme in the glycolysis. PFKFB3 was reported to increase glycolytic activity in cells in the context of the Warburg effect (61) as well as after LPS stimulation of macrophages (60). As the PFKFB3 gene promotor possesses a binding site for HIF1α (62), it is also induced under hypoxic and hypoxia-like conditions, e.g. triggered by Co^{2+} (63).

The findings, as mentioned above, can be matched to the proteomic data generated from MoDCs treated with $NiSO_4$. However, $NiCl_2$ was reported to induce distinct hypoxia-like conditions via induction of HIF1α (64, 65). Accordingly, increased gene expression for glycolytic enzymes like HK2, LDHA and SLC2A1 was reported (66). These proteins were also significantly changed after treatment of MoDCs with $NiSO_4$ in our study, and additionally, elevated protein levels of HIF1α were detected. MoDCs stimulated with $NiSO_4$ were more sensitive to HIF1α-inhibition than cells stimulated with LPS. $NiSO_4$ and cobalt are known to induce TLR4-dependent IL-8 production, which is defined as a key event for skin sensitization. However, both metals also elicit hypoxia. Asakawa et al. (67) showed that $NiCl_2$ binds to heat shock protein 90β in THP-1 cells and thus increases the activity of HIF1α. Elevated HIF1α activity eventually resulted in elevated IL-8 expression. TREM1 protein expression and IL-8 secretion were both previously reported to be increased under hypoxic conditions (68). We could show that $NiSO_4$-treated MoDCs also induced TREM1 signaling as well as IL-8 secretion. Additionally, Viemann et al. (65) described a central role of HIF1α in endothelia cells in response to

nickel. Besides pro-inflammatory signaling by NFκB, HIF1α was identified as a central inducer of genes that act on cell survival and metabolism. Hypoxia and HIF1α were found to be elementary drivers during DC activation and thereby enhancing the immunological response of the latter. Precisely, hypoxia was shown to enhance pro-inflammatory signaling, cellular glucose metabolism as well as cell surface maker expression in mouse-derived DCs. This eventually resulted in an amplified ability to stimulate T cell proliferation (59). We could confirm the metabolic shift in $NiSO_4$-treated cells along with the upregulation of important cell surface makers and the ability to stimulate lymphocyte proliferation. Hence, the induction of a pronounced hypoxia by $NiSO_4$ contributed strongly to the immunological response of the MoDCs. However, more data is needed to allow distinct discrimination between TLR4-dependent and hypoxia-induced proteins in $NiSO_4$-treated MoDCs. Furthermore, the pronounced Nrf2-mediated stress response may also favorably contribute to HIF1α-dependency in $NiSO_4$-treated MoDCs since Nrf2 directly regulates HIF1α gene expression (69).

CONCLUSION

In the present work, we extensively studied the activation of MoDCs by mass spectrometry-based proteomics. We aimed to elucidate the cellular mode of action of $NiSO_4$-induced MoDC maturation concerning nickel allergy by comparing MoDC activation induced by $NiSO_4$ to the well-studied bacterial LPS. Overall, the results for LPS-treated MoDCs are in concordance with literature data, confirming that our technical approach detects DC activation as such. $NiSO_4$-treated cells upregulated relevant activation markers, such as CD83 and CD86, and pathways that point toward signaling in immunology, like CD40 and TREM1 signaling. However, the immunological response triggered by $NiSO_4$ is partly overshadowed by oxidative stress that is potentially caused by the metallic character of Ni^{2+}. This implicates a subordinate role of TLR4-activation and -signaling in $NiSO_4$-activated MoDCs for the chosen experimental settings. $NiSO_4$-induced oxidative stress is likely the responsible event upstream of the upregulation of Nrf2-target genes, as detected at mRNA as well as at protein level. The metabolic shift observed for $NiSO_4$-treated MoDCs resembles that of LPS-treated MoDCs. Nevertheless, prolongation of the upregulation of detected glycolytic enzymes, in connection with elevated HIF1α levels, suggest a hypoxia-like cellular state, which is remarkably different from LPS-induced effects. The link between cellular cholesterol depletion and activation of DCs triggered by $NiSO_4$ remains to be elucidated by future studies.

Taken together, our study suggests immunostimulatory and cytotoxic effects of $NiSO_4$ leading to DC activation, and possibly culminating in sensitization to nickel *in vivo*. Our results illuminate the phenotype of activated DCs during sensitization to an allergen and thereby enhance our understanding of nickel-induced molecular pathways.

DATA AVAILABILITY STATEMENT

The dataset presented in this study can be found online in the PRIDE repository with the dataset identifier PXD022599 (70). A full list of all proteins including the protein names and accession numbers can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Charité, Berlin Germany; EA4/071/13. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TH and AH conceived and designed the experiments. TH performed the experimental work. Data analysis was done by TH, VD, and KSc. Figures were created by TH and KSc. KSi and TH conceived, conducted, and analyzed the mixed leukocyte reaction. MB provided conceptual input. The manuscript was written by TH, KSc, and AH with contributions from all co-authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.644700/full#supplementary-material

REFERENCES

1. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. *Nat Immunol*. (2014) 15:323–32. doi: 10.1038/ni.2833
2. Everts B, Pearce EJ. Metabolic control of dendritic cell activation and function: recent advances and clinical implications. *Front Immunol*. (2014) 5:203. doi: 10.3389/fimmu.2014.00203
3. Hussaarts L, Kaisar MMM, Guler AT, Dalebout H, Everts B, Deelder AM, et al. Human dendritic cells with Th2-polarizing capacity: analysis using label-free quantitative proteomics. *Int Arch Allergy Immunol*. (2017) 174:170–82. doi: 10.1159/000479512
4. Dudek AM, Martin S, Garg AD, Agostinis P. Immature, semi-mature, and fully mature dendritic cells: toward a DC-cancer cells interface that augments anticancer immunity. *Front Immunol*. (2013) 4:438. doi: 10.3389/fimmu.2013.00438

5. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, et al. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood*. (2010) 115:4742–9. doi: 10.1182/blood-2009-10-249540
6. Alinaghi F, Bennike NH, Egeberg A, Thyssen JP, Johansen JD. Prevalence of contact allergy in the general population: a systematic review and meta-analysis. *Contact Dermatitis*. (2019) 80:77–85. doi: 10.1111/cod.13119
7. Kimber I, Basketter DA, Gerberick GF, Dearman RJ. Allergic contact dermatitis. *Int Immunopharmacol*. (2002) 2:201–11. doi: 10.1016/S1567-5769(01)00173-4
8. OECD. *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins*. Paris (2014).
9. Hoper T, Mussotter F, Haase A, Luch A, Tralau T. Application of proteomics in the elucidation of chemical-mediated allergic contact dermatitis. *Toxicol Res*. (2017) 6:595–610. doi: 10.1039/C7TX00058H
10. Angenieux C, Fricker D, Strub J-M, Luche S, Bausinger H, Cazenave J-P, et al. Gene induction during differentiation of human monocytes into dendritic cells: an integrated study at the RNA and protein levels. *Funct Integr Genomics*. (2001) 1:323–9. doi: 10.1007/s101420100037
11. Watarai H, Hinohara A, Nagafune J, Nakayama T, Taniguchi M, Yamaguchi V. Plasma membrane-focused proteomics: dramatic changes in surface expression during the maturation of human dendritic cells. *Proteomics*. (2005) 5:4001–11. doi: 10.1002/pmic.200401258
12. Buschow SI, Lasonder E, van Deutekom HWM, Oud MM, Beltrame L, Huynen MA, et al. Dominant processes during human dendritic cell maturation revealed by integration of proteome and transcriptome at the pathway level. *J Proteome Res*. (2010) 9:1727–37. doi: 10.1021/pr9008546
13. Arya S, Wiatrek-Moumoulidis D, Synowsky SA, Shirran SL, Botting CH, Powis SJ, et al. Quantitative proteomic changes in LPS-activated monocyte-derived dendritic cells: a SWATH-MS study. *Sci Rep*. (2019) 9:4343. doi: 10.1038/s41598-019-40773-6
14. Ahlstrom MG, Thyssen JP, Menne T, Johansen JD. Prevalence of nickel allergy in Europe following the EU Nickel Directive - a review. *Contact Dermatitis*. (2017) 77:193–200. doi: 10.1111/cod.12846
15. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*. (2009) 458:1191–5. doi: 10.1038/nature07830
16. Kagan JC, Medzhitov R. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell*. (2006) 125:943–55. doi: 10.1016/j.cell.2006.03.047
17. Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol*. (2008) 9:361–8. doi: 10.1038/ni1569
18. Schmidt M, Raghavan B, Muller V, Vogl T, Fejer G, Tchaptchet S, et al. Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat Immunol*. (2010) 11:814–9. doi: 10.1038/ni.1919
19. Raghavan B, Martin SF, Esser PR, Goebeler M, Schmidt M. Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2. *EMBO Rep*. (2012) 13:1109–15. doi: 10.1038/embor.2012.155
20. Dietz L, Esser PR, Schmucker SS, Goette I, Richter A, Schnolzer M, et al. Tracking human contact allergens: from mass spectrometric identification of peptide-bound reactive small chemicals to chemical-specific naive human T-cell priming. *Toxicol Sci*. (2010) 117:336–47. doi: 10.1093/toxsci/kfq209
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*. (2001) 25:402–8. doi: 10.1006/meth.2001.1262
22. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. (1957) 226:497–509. doi: 10.1016/S0021-9258(18)64849-5
23. Wang Z, Karkossa I, Großkopf H, Rolle-Kampczyk U, Hackermüller J, von Bergen M, et al. Comparison of quantitation methods in proteomics to define relevant toxicological information on AhR activation of HepG2 cells by BaP. *Toxicology*. (2020) 448:152652. doi: 10.1016/j.tox.2020.152652
24. Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics*. (2014) 13:2513–26. doi: 10.1074/mcp.M113.031591
25. Zhang X, Smits AH, van Tilburg GB, Ovaa H, Huber W, Vermeulen M. Proteome-wide identification of ubiquitin interactions using UbIA-MS. *Nat Protoc*. (2018) 13:530–50. doi: 10.1038/nprot.2017.147
26. OECD. *Test No. 442E: in vitro Skin Sensitisation*. Paris (2018).
27. Gil-Torregrosa BC, Lennon-Duménil AM, Kessler B, Guermonprez P, Ploegh HL, Fruci D, et al. Control of cross-presentation during dendritic cell maturation. *Eur J Immunol*. (2004) 34:398–407. doi: 10.1002/eji.200324508
28. Alloatti A, Kotsias F, Magalhaes JG, Amigorena S. Dendritic cell maturation and cross-presentation: timing matters! *Immunol Rev*. (2016) 272:97–108. doi: 10.1111/imr.12432
29. Kim MK, Kim J. Properties of immature and mature dendritic cells: phenotype, morphology, phagocytosis, and migration. *RSC Adv*. (2019) 9:11230–8. doi: 10.1039/C9RA00818G
30. Perrin-Cocon L, Aublin-Gex A, Diaz O, Ramiere C, Peri F, Andre P, et al. Toll-like receptor 4-induced glycolytic burst in human monocyte-derived dendritic cells results from p38-dependent stabilization of HIF-1alpha and increased hexokinase II expression. *J Immunol*. (2018) 201:1510–21. doi: 10.4049/jimmunol.1701522
31. Lee P, Chandel NS, Simon MC. Cellular adaptation to hypoxia through hypoxia inducible factors and beyond. *Nat Rev Mol Cell Biol*. (2020) 21:268–83. doi: 10.1038/s41580-020-0227-y
32. Strasser L, Dang HH, Schwarz H, Asam C, Ferreira F, Horejs-Hoeck J, et al. Unbiased quantitative proteomics reveals a crucial role of the allergen context for the activation of human dendritic cells. *Sci Rep*. (2017) 7:16638. doi: 10.1038/s41598-017-16726-2
33. Ade N, Antonios D, Kerdine-Romer S, Boisleve F, Rousset F, Pallardy M. NF-kappaB plays a major role in the maturation of human dendritic cells induced by NiSO(4) but not by DNCB. *Toxicol Sci*. (2007) 99:488–501. doi: 10.1093/toxsci/kfm178
34. Lavorgna A, De Filippi R, Formisano S, Leonardi A. TNF receptor-associated factor 1 is a positive regulator of the NF-kappaB alternative pathway. *Mol Immunol*. (2009) 46:3278–82. doi: 10.1016/j.molimm.2009.07.029
35. Dauphinee SM, Clayton A, Hussainkhel A, Yang C, Park YJ, Fuller ME, et al. SASH1 is a scaffold molecule in endothelial TLR4 signaling. *J Immunol*. (2013) 191:892–901. doi: 10.4049/jimmunol.1200583
36. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev*. (1999) 13:76–86. doi: 10.1101/gad.13.1.76
37. Ma Q. Role of Nrf2 in oxidative stress and toxicity. *Ann Rev Pharmacol Toxicol*. (2013) 53:401–26. doi: 10.1146/annurev-pharmtox-011112-140320
38. Helou DG, Martin SF, Pallardy M, Chollet-Martin S, Kerdine-Römer S. Nrf2 involvement in chemical-induced skin innate immunity. *Front Immunol*. (2019) 10:1004. doi: 10.3389/fimmu.2019.01004
39. Ade N, Leon F, Pallardy M, Peiffer JL, Kerdine-Romer S, Tissier MH, et al. HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: Role of the Keap1/Nrf2 pathway. *Toxicol Sci*. (2009) 107:451–60. doi: 10.1093/toxsci/kfn243
40. Mussotter F, Tomm JM, El Ali Z, Pallardy M, Kerdine-Romer S, Gotz M, et al. Proteomics analysis of dendritic cell activation by contact allergens reveals possible biomarkers regulated by Nrf2. *Toxicol Appl Pharmacol*. (2016) 313:170–9. doi: 10.1016/j.taap.2016.11.001
41. Lewis JB, Messer RL, McCloud VV, Lockwood PE, Hsu SD, Wataha JC. Ni(II) activates the Nrf2 signaling pathway in human monocytic cells. *Biomaterials*. (2006) 27:5348–56. doi: 10.1016/j.biomaterials.2006.06.007
42. Matsue H, Edelbaum D, Shalhevet D, Mizumoto N, Yang C, Mummert ME, et al. Generation and function of reactive oxygen species in dendritic cells during antigen presentation. *J Immunol*. (2003) 171:3010–8. doi: 10.4049/jimmunol.171.6.3010
43. Yamada H, Arai T, Endo N, Yamashita K, Fukuda K, Sasada M, et al. LPS-induced ROS generation and changes in glutathione level and their relation to the maturation of human monocyte-derived dendritic cells. *Life Sci*. (2006) 78:926–33. doi: 10.1016/j.lfs.2005.05.106
44. Vennegaard MT, Dyring-Andersen B, Skov L, Nielsen MM, Schmidt JD, Bzorek M, et al. Epicutaneous exposure to nickel induces nickel allergy in mice via a MyD88-dependent and interleukin-1-dependent pathway. *Contact Dermatitis*. (2014) 71:224–32. doi: 10.1111/cod.12270

45. Thierse HJ, Moulon C, Allespach Y, Zimmermann B, Doetze A, Kuppig S, et al. Metal-protein complex-mediated transport and delivery of Ni2+ to TCR/MHC contact sites in nickel-specific human T cell activation. *J Immunol*. (2004) 172:1926–34. doi: 10.4049/jimmunol.172.3.1926
46. Heiss K, Junkes C, Guerreiro N, Swamy M, Camacho-Carvajal MM, Schamel WWA, et al. Subproteomic analysis of metal-interacting proteins in human B cells. *Proteomics*. (2005) 5:3614–22. doi: 10.1002/pmic.200401215
47. Luo J, Yang H, Song B-L. Mechanisms and regulation of cholesterol homeostasis. *Nat Rev Mol Cell Biol*. (2020) 21:225–45. doi: 10.1038/s41580-019-0190-7
48. Wang SH, Yuan SG, Peng DQ, Zhao SP. HDL and ApoA-I inhibit antigen presentation-mediated T cell activation by disrupting lipid rafts in antigen presenting cells. *Atherosclerosis*. (2012) 225:105–14. doi: 10.1016/j.atherosclerosis.2012.07.029
49. Hiltbold EM, Poloso NJ, Roche PA. MHC class II-peptide complexes and APC lipid rafts accumulate at the immunological synapse. *J Immunol*. (2003) 170:1329–38. doi: 10.4049/jimmunol.170.3.1329
50. Cheng AM, Handa P, Tateya S, Schwartz J, Tang C, Mitra P, et al. Apolipoprotein A-I attenuates palmitate-mediated NF-kappaB activation by reducing Toll-like receptor-4 recruitment into lipid rafts. *PLoS ONE*. (2012) 7:e33917. doi: 10.1371/journal.pone.0033917
51. York AG, Williams KJ, Argus JP, Zhou QD, Brar G, Vergnes L, et al. Limiting cholesterol biosynthetic flux spontaneously engages type I IFN signaling. *Cell*. (2015) 163:1716–29. doi: 10.1016/j.cell.2015.11.045
52. Simons K, Ikonen E. How cells handle cholesterol. *Science*. (2000) 290:1721–6. doi: 10.1126/science.290.5497.1721
53. Hughes AL, Todd BL, Espenshade PJ. SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast. *Cell*. (2005) 120:831–42. doi: 10.1016/j.cell.2005.01.012
54. Lühr JJ, Alex N, Amon L, Kräter M, Kubánková M, Sezgin E, et al. Maturation of monocyte-derived DCs leads to increased cellular stiffness, higher membrane fluidity, and changed lipid composition. *Front Immunol*. (2020) 11:590121. doi: 10.3389/fimmu.2020.590121
55. Nazimek K, Bryniarski K, Askenase PW. Functions of exosomes and microbial extracellular vesicles in allergy and contact and delayed-type hypersensitivity. *Int Arch Allergy Immunol*. (2016) 171:1–26. doi: 10.1159/000449249
56. Vallhov H, Gutzeit C, Hultenby K, Valenta R, Gronlund H, Scheynius A. Dendritic cell-derived exosomes carry the major cat allergen Fel d 1 and induce an allergic immune response. *Allergy*. (2015) 70:1651–5. doi: 10.1111/all.12701
57. Pitt JM, Andre F, Amigorena S, Soria JC, Eggermont A, Kroemer G, et al. Dendritic cell-derived exosomes for cancer therapy. *J Clin Invest*. (2016) 126:1224–32. doi: 10.1172/JCI81137
58. Guak H, Al Habyan S, Ma EH, Aldossary H, Al-Masri M, Won SY, et al. Glycolytic metabolism is essential for CCR7 oligomerization and dendritic cell migration. *Nat Commun*. (2018) 9:2463. doi: 10.1038/s41467-018-04804-6
59. Jantsch J, Chakravortty D, Turza N, Prechtel AT, Buchholz B, Gerlach RG, et al. Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. *J Immunol*. (2008) 180:4697–705. doi: 10.4049/jimmunol.180.7.4697
60. Ruiz-García A, Monsalve E, Novellasdemunt L, Navarro-Sabaté A, Manzano A, Rivero S, et al. Cooperation of adenosine with macrophage Toll-4 receptor agonists leads to increased glycolytic flux through the enhanced expression of PFKFB3 gene. *J Biol Chem*. (2011) 286:19247–58. doi: 10.1074/jbc.M110.190298
61. Shi L, Pan H, Liu Z, Xie J, Han W. Roles of PFKFB3 in cancer. *Sig Transduct Target Ther*. (2017) 2:17044. doi: 10.1038/sigtrans.2017.44
62. Obach M, Navarro-Sabate A, Caro J, Kong X, Duran J, Gomez M, et al. 6-Phosphofructo-2-kinase. (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. *J Biol Chem*. (2004) 279:53562–70. doi: 10.1074/jbc.M406096200
63. Minchenko A, Leshchinsky I, Opentanova I, Sang N, Srinivas V, Armstead V, et al. Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. *J Biol Chem*. (2002) 277:6183–7. doi: 10.1074/jbc.M110978200
64. Salnikow K, Blagosklonny MV, Ryan H, Johnson R, Costa M. Carcinogenic nickel induces genes involved with hypoxic stress. *Cancer Res*. (2000) 60:38–41. Available online at: https://cancerres.aacrjournals.org/content/60/1/38.full-text.pdf
65. Viemann D, Schmidt M, Tenbrock K, Schmid S, Muller V, Klimmek K, et al. The contact allergen nickel triggers a unique inflammatory and proangiogenic gene expression pattern via activation of NF-kappaB and hypoxia-inducible factor-1alpha. *J Immunol*. (2007) 178:3198–207. doi: 10.4049/jimmunol.178.5.3198
66. Salnikow K, Davidson T, Costa M. The role of hypoxia-inducible signaling pathway in nickel carcinogenesis. *Environ Health Perspect*. (2002) 110(Suppl. 5):831–4. doi: 10.1289/ehp.02110s5831
67. Asakawa S, Onodera R, Kasai K, Kishimoto Y, Sato T, Segawa R, et al. Nickel ions bind to HSP90β and enhance HIF-1α-mediated IL-8 expression. *Toxicology*. (2018) 395:45–53. doi: 10.1016/j.tox.2018.01.006
68. Mishra KP, Jain S, Ganju L, Singh SB. Hypoxic stress induced TREM-1 and inflammatory chemokines in human peripheral blood mononuclear cells. *Indian J Clin Biochem*. (2014) 29:133–8. doi: 10.1007/s12291-013-0345-9
69. Lacher SE, Levings DC, Freeman S, Slattery M. Identification of a functional antioxidant response element at the HIF1A locus. *Redox Biol*. (2018) 19:401–11. doi: 10.1016/j.redox.2018.08.014
70. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res*. (2019) 47:D442–D450. doi: 10.1093/nar/gky1106

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6.3 Paper 3

A comparative proteomics analysis of four contact allergens in THP-1 cells shows distinct alterations in key metabolic pathways

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Data analysis (60%)

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A comparative proteomics analysis of four contact allergens in THP-1 cells shows distinct alterations in key metabolic pathways

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ABSTRACT

Allergic contact dermatitis (ACD) is the predominant form of immunotoxicity in humans. The sensitizing potential of chemicals can be assessed *in vitro*. However, a better mechanistic understanding could improve the current OECD-validated test battery. The aim of this study was to get insights into toxicity mechanisms of four contact allergens, *p*-benzoquinone (BQ), 2,4-dinitrochlorobenzene (DNCB), *p*-nitrobenzyl bromide (NBB) and $NiSO_4$, by analyzing differential proteome alterations in THP-1 cells using two common proteomics workflows, stable isotope labeling by amino acids in cell culture (SILAC) and label-free quantification (LFQ). Here, SILAC was found to deliver more robust results. Overall, the four allergens induced similar responses in THP-1 cells, which underwent profound metabolic reprogramming, including a striking upregulation of the TCA cycle accompanied by pronounced induction of the Nrf2 oxidative stress response pathway. The magnitude of induction varied between the allergens with DNCB and NBB being most potent. A considerable overlap between transcriptome-based signatures of the GARD assay and the proteins identified in our study was found. When comparing the results of this study to a previous proteomics study in human primary monocyte-derived dendritic cells, we found a rather low share in regulated proteins. However, on pathway level, the overlap was high, indicating that affected pathways rather than single proteins are more eligible to investigate proteomic changes induced by contact allergens. Overall, this study confirms the potential of proteomics to obtain a profound mechanistic understanding, which may help improving existing *in vitro* assays for skin sensitization.

1. Introduction

Developing alternative testing strategies for chemical safety testing has been an overarching topic in regulatory toxicology for decades. The strict ban on animal testing for cosmetic ingredients in the European Union (EC, 2009) has led to great advances in this field. For simple toxicological endpoints, such as skin irritation or skin corrosion, several test guidelines accepted by the Organization for Economic Co-operation and Development (OECD) have been available for a long time. The more complex endpoints like skin sensitization remained challenging as multiple key events had to be identified and covered by test systems. The Adverse Outcome Pathway (AOP) that the OECD published for skin

Abbreviations: ACLY, ATP-citrate synthase; ACN, acetonitrile; AOP, adverse outcome pathway; BIN2, bridging integrator 2; BQ, p-benzoquinone; COX, cytochrome *c* oxidase; DC, dendritic cell; DMSO, dimethyl sulfoxide; DNAAJA, DnaJ homolog subfamily A member; DNAJC, DnaJ homolog subfamily C member; DNCB, 2,4-dinitrochlorobenzene; EPHX, epoxide hydrolase; FC, fold change; GARD, Genomic Allergen Rapid Detection; GCLM, glutamate-cysteine ligase; GSR, glutathione reductase; h-CLAT, human Cell Line Activation Test; HMGCS, hydroxymethylglutaryl-CoA synthase; IDH, isocitrate dehydrogenase; KE, key event; LFQ, label-free quantification; MGST, microsomal glutathione S-transferase; NBB, p-nitrobenzyl bromide; NDUF, NADH dehydrogenase; Ni, NiSO4; NQO, NAD(*P*)H dehydrogenase (quinone); OXPHOS, oxidative phosphorylation; PCA, principal component analysis; PPP, pentose phosphate pathway; SOD, superoxide dismutase; SUCNR, succinate receptor 1; TOMM40, mitochondrial import receptor subunit TOM40 homolog; TXNRD, thioredoxin reductase; WO, with out.

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sensitization in 2012 (OECD, 2014) paved the way for several alternative test methods, each addressing key events (KEs) of the AOP, ultimately leading to the Defined Approach released by OECD in 2021 (OECD, 2021). The skin sensitization AOP starts from the haptenation of allergens with proteins in the skin as the molecular initiating event, which can be assessed *in chemico* using the direct peptide reactivity assay (Gerberick et al., 2004; OECD, 2022a). The next KEs are the cellular activation of keratinocytes and dendritic cells (DC), which can be evaluated *in vitro* (OECD, 2022b, 2022c). The KeratinoSens™ is validated for estimating keratinocyte activation based on the gene expression of the antioxidant response element as well as Nrf2 (Emter et al., 2010; OECD, 2022b), and activation of DCs can be determined based on the expression of selected cell surface proteins. The human cell line activation test (hCLAT) for example utilizes upregulation of CD54 and CD86 on THP-1 cells as a measure for DC activation after exposure to sensitizing chemicals (Ashikaga et al., 2006; OECD, 2022c). Activated DCs then migrate to the lymph nodes, where they present the hapten to naïve T cells, inducing their activation and proliferation. T cell proliferation in the lymph nodes was recognized in the AOP as the organ response. However, to date no validated alternative test method for the assessment of T cell activation exists.

Despite these substantial achievements, the currently available alternative test methods for skin sensitization have several limitations. For example, pre- and prohaptens are difficult to predict since some test systems lack the metabolic competency to convert the chemicals into their reactive metabolite. For instance, *in chemico* methods like the direct peptide reactivity assay entirely lack metabolic activity and also cell-based test systems like the hCLAT were shown to have limitations regarding metabolic activation of substances (Gerberick et al., 2004; Ashikaga et al., 2010; Fabian et al., 2013; Oesch et al., 2014). Also, most assays are not designed for the testing of lipophilic compounds (Gerberick et al., 2007; Ashikaga et al., 2010; Takenouchi et al., 2013). Furthermore, the accurate potency classification of sensitizers is not yet fully resolved (Nukada et al., 2013; Jaworska et al., 2015; Reisinger et al., 2015). Thus, further work is needed. In particular a more detailed mechanistic understanding can be helpful, as the current methods provide only very limited insights into the underlying modes of action. For this purpose, omics approaches are especially well suited as they deliver comprehensive mechanistic insights. To date, mostly transcriptomics was used to characterize cellular changes induced by contact allergens in patient's biopsies as well as *in vitro* (Johansson et al., 2011; Dhingra et al., 2014; Lefevre et al., 2021), which also led to the development of the GARD (Genomic Allergen Rapid Detection) assay. The GARD assay comprises a signature of 200 transcripts that allows to discriminate between contact allergens and non-allergens (Johansson et al., 2013). The identity of the discriminating genes is disclosed but further information (*e.g.* direction of regulation) has not been published. Recently, the GARD assay underwent official validation and became an OECD test guideline for skin sensitization (OECD, 2022c). In contrast to transcriptomics, the potential of proteomics has not been fully exploited, yet. Proteomics is typically applied to identify cellular haptenation sites (Parkinson et al., 2014; Guedes et al., 2016; Parkinson et al., 2018; Parkinson et al., 2020), while proteomic studies that aim to unravel cellular changes in allergen-treated DCs remain scarce (as reviewed by Höper et al., 2017). DCs play a major role during skin sensitization as they act as link between adaptive and innate immune system and thereby commence the cell-mediated allergic immune response. Exposure of DCs to contact allergens induces maturation accompanied by reconstruction of the cell organelles and membranes. Upregulation of protein expression serves the elevated energy demand necessary for maturation as well as the crosstalk with other immune cells, particularly T cells. Hence, our aim was to conduct a comprehensive proteomic study to unravel regulated proteins. For this purpose, we selected THP-1 cells, since this is the utilized cell model in the OECD-validated hCLAT assay. THP-1 cells were treated with the four contact allergens, *p*-benzoquinone (BQ), 2,4-dinitrochlorobenzene (DNCB), *p*-nitrobenzyl bromide (NBB) and $NiSO_4$ (Ni) to assess whether the different allergens induce similar proteins and pathways in the cells, which could potentially be used as a predictive protein-based signature for skin sensitization. As we expected that potent allergens most likely induce more alterations compared to moderate or weak allergens, we intentionally selected DNCB, BQ and NBB, which have been classified as extreme sensitizers in the LLNA (Gerberick et al., 2005). The potency of nickel salts is ranked as weak to moderate, yet nickel is the most prevalent allergen in the EU (Oosterhaven et al., 2019). In addition, nickel appeared relevant to us as, in contrast to other allergens, it can directly interact with and bind to the TLR4 and, therefore, does not rely on indirect receptor activation (Martin et al., 2011). Furthermore, we applied and compared two common proteomic quantification approaches, SILAC (stable isotope labeling by amino acids in cell culture) and LFQ (label-free quantification). SILAC is a very common approach for cell cultures and is considered a very robust quantification technique (Li et al., 2012). LFQ was included as it is frequently used due to its wide range of applications. Moreover, we used this approach in our previous proteomics study in primary human dendritic cells (Höper et al., 2021). As debates in the scientific community are still ongoing whether THP-1 cells are a good model to properly mimic responses of DCs, we also compared the proteomic data derived from this study to the ones obtained in primary human DCs earlier (Höper et al., 2021).

2. Material and methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS, P04–36500), RPMI 1640 (P04–17500), HEPES (P05–01100), sodium pyruvate (P04–43100), L-glutamine (P04–80100) and penicillin-streptomycin (P06–07100) were purchased from PAN.

2.1. Cell culture

THP-1 cells were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were grown in RPMI 1640 media supplemented with 10% (*v*/v) fetal bovine serum (Merck, S0615), 10 mM HEPES, 2 mM L-glutamine (2 mM), sodium pyruvate (1 mM) and penicillin/streptomycin (100 U/ml) at 37 °C in an incubator with 5% CO_2. Every 3 to 4 days, cells were passaged and seeded at 1×10^5 cells per ml in T75 flasks.

2.2. Cell culture for SILAC experiments

For SILAC experiments, cells were grown in SILAC RPMI media (Thermo Fisher Scientific, A33823) supplemented with 10% (*v*/v) dialyzed fetal bovine serum (Thermo Fisher Scientific, 26400044) and isotope labeled amino acids. Medium control cells were grown in medium containing $^{13}C_6$-L-lysine (Silantes, 211204102) and $^{13}C_6$-L-arginine (Silantes, 201204102). Chemical-treated cells as well as vehicle controls were cultured in medium containing the light amino acids $^{12}C_6$-L-lysine (Silantes, 211003902) and $^{12}C_6$-L-arginine (Silantes, 201004102), respectively. Complete incorporation of the amino acids after 7 days was verified beforehand and cells were treated as mentioned below.

2.3. Chemical treatment

Cells were seeded in 6-well plates with a density of 1×10^6 cells per well and immediately treated for 24 h with 400 μM Ni (31483), 20 μM BQ (B10358), 10 μM DNCB (237329) or 2 μM NBB (N13054). The metal allergen Ni was dissolved in cell culture medium, whereas the other organic allergens were dissolved in dimethyl sulfoxide (DMSO, Merck, D2438). The stock solutions were diluted in medium and the final DMSO concentration in the cell suspension was <0.2% (*v*/v). DMSO

concentrations were in accordance with maximum levels allowed in OECD TG 442 E (OECD, 2022c). Medium-only treated cells were included as control and DMSO-treated cells were used as vehicle controls.

2.4. Viability assessment

Cell viability was assessed by flow cytometry. Cells were harvested, centrifuged (5 min, 300 xg, RT) and washed with PBS. Staining for flow cytometry was performed for 30 min at 4 °C using fixable near-IR dead cell stain (Thermo Fisher Scientific, L34976). Data were acquired using a FACSAria III flow cytometer (BD Biosciences) and analyzed with FlowJo software (V.10.7.1, FlowJo LLC, Ashland, OR, United States).

2.5. Proteome analysis

2.5.1. Cell harvest and lysis

After 24 h of treatment, cells were harvested by centrifugation (5 min, 300 xg, RT). Cell pellets were washed twice with ice-cold PBS, and the washed cells were lysed in 100 µl lysis buffer per 10^6 cells. The lysis buffer was composed of 150 mM NaCl (S7653-250G), 10 mM TRIS pH 7.2 (T1503-250G), 5 mM EDTA (E5134-250G), 0.1% (*v*/v) SDS (436143-25G), 1% (v/v) Triton X-100 (T8787-100ML), 1% (v/v) sodium deoxycholate (30970-100G), 200 µM phenylmethylsulfonyl fluoride (P7626-1G), 1 mM sodium orthovanadate (S6508-10G) and cOmplete protease inhibitor cocktail (Roche, 1.167.498.001). Cells were thoroughly vortexed, incubated on ice for 15 min and sonicated to shear DNA. Subsequently, the samples were centrifuged (10 min, 16,000 g, 4 °C), and the supernatant was collected. The protein concentration of the supernatant was determined using Pierce BCA protein assay (Thermo Fisher Scientific, 23225). For SILAC experiments, equal protein numbers of treated and control cells were combined in a fresh tube.

2.5.2. Sample preparation

For each sample condition, 30 µg protein were diluted in 100 mM triethylammonium bicarbonate buffer (T7408). Proteins were reduced using 200 mM tris(2-carboxyethyl)phosphine (Serva, 36970.01) and subsequently alkylated with 375 mM iodoacetamide (Serva, 26710.02). For tryptic digestion of the proteins, the lysis buffer was removed using SpeedBeads™ magnetic carboxylate modified particles (SP3 beads, GE Healthcare, 65152105050250). Organic concentration of the samples was adjusted to >50% (*v*/v) acetonitrile (ACN, 1000292500) to enable binding of the proteins to the beads. The beads were washed twice with 70% (*v*/v) ethanol (1117272500) and once with ACN. Proteins were digested using trypsin (1:50 ratio, Promega, V5117) in 50 mM ammonium bicarbonate buffer. After tryptic digestion, the aqueous supernatant containing the peptides was collected and transferred to a fresh tube. The beads were washed with 50 mM ammonium bicarbonate and the supernatant was collected into the same tube as before. Peptides were then prepared for LC-MS analysis using solid phase extraction cartridges (Waters, 186000383), vacuum-dried and reconstituted in 0.1% (*v*/v) formic acid (00940) prior to measurement.

2.5.3. LC-MS/MS

An UPLC system (Ultimate 3000, Dionex, Thermo Fisher Scientific) coupled to a Q Exactive HF (Thermo Fisher Scientific) was used to analyze the samples as described previously for LFQ (Wang et al., 2020). Peptides were injected to an Acclaim PepMap 100 C18 trap column (3 µm, nanoViper, 75 µm × 5 cm, Thermo Fisher Scientific, PN164535) at a flow rate of 5 µl/min using a loading eluent composed of 2% (v/v) ACN and 0.05% (v/v) trifluoroacetic acid (Biosolve, 202341A8) in water. Peptides were subsequently separated by a 150 min non-linear gradient from 0 to 80% (v/v) ACN in 0.1% (v/v) formic acid on a reversed-phase column (Acclaim PepMap 100 C18, 3 µm, nanoViper, 75 µm × 25 cm, Thermo Fisher Scientific, PN164569). Ionization was performed with a chip-based ESI source (Nanomate, Advion, Ithaca, NY, United States). The MS1 scans were acquired at a resolution of 120 K in a range of 350–1550 *m/z*. AGC target was set to 3×10^6 with a maximal injection time of 10 ms. MS2 data acquisition was based on a Top 10 approach with an isolation window of 1.4 *m/z*. Peptides were fragmented at normalized collision energy of 28, and the fragment ion spectra were acquired at a resolution of 15 K using AGC target of 2×10^5 and maximal IT of 100 ms. Dynamic exclusion was set to 20 s. All spectra were acquired using XCalibur (Version 4.2).

2.5.4. Data analysis

MaxQuant Version 1.6.2.10 (Cox et al., 2014) was used to process the raw MS data using the default parameters if not indicated otherwise. For peptide identification, a database search against the *Homo sapiens* UniProtKB reference proteome (07-10-2021) was performed. Carbamidomethylation of cysteine was set as fixed, whereas oxidation of methionine and acetylation of protein N-terminus were set as variable modifications. Protein identification was performed applying a false discovery rate ≤ 0.01 to proteins, with a minimum of two peptides and at least one unique peptide. The protein quantification was performed on the basis of two unique peptides. Match between runs was activated. Protein contaminants, identification only by site and reverse hits were excluded before further use. SILAC and LFQ protein intensities were processed, and results were visualized in R-3.5.0 using the following packages: plyr (Wickham, 2011), reshape2 (Wickham, 2007), xlsx (Adrian and Cole, 2018), DEP (Zhang et al., 2018), mixOmics (Rohart et al., 2017), pheatmap (Kolde, 2019), ggsci (Nan, 2018), circlize (Gu, 2014), calibrate (Jan, 2019), ggplot2 (Hadley, 2016), dendsort (Sakai, 2015), readxl (Hadley and Jennifer, 2019), qpcR (Andrej-Nikolai, 2018), splitstackshape (Ananda, 2019), tidyr (Hadley and Lionel, 2019), and Tmisc (Stephen, 2019) Accordingly, the data were Log2-transformed, filtered for proteins that were quantified in a minimum of three replicates under at least one condition, followed by variance-stabilization. Fold changes (FCs) were calculated relative to medium control. For SILAC, p-values were calculated using Student's *t*-test based on the Log2(FC) of the replicates tested against 0. For LFQ samples, p-values were calculated relative to medium control replicates. P-values were adjusted according to Benjamini and Hochberg. Proteins with an adjusted p-value ≤0.05 were considered regulated.

2.5.5. Pathway enrichment

Enriched pathways were identified using Ingenuity Pathway Analysis (IPA) (Qiagen, 2023; Krämer et al., 2013), considering regulated proteins (adjusted p-value ≤0.05). For this purpose, the database was filtered for human data, and the tissue specificity was set to immune cells. Obtained z-scores reflect the regulation direction and Benjamini and Hochberg adjusted p-values indicate significance of enrichment. Pathways were considered significantly enriched with adjusted p-value ≤0.05.

3. Results

3.1. SILAC quantification outperforms LFQ with respect to the number of regulated proteins

The aim of this study was to obtain novel insights into proteomic changes during cellular activation of contact allergen-treated THP-1 cells exposed to 20 µM BQ, 10 µM DNCB, 2 µM NBB or 400 µM Ni for 24 h. Cell viability was ensured to be at least 75% (Supplementary Fig. 1), in concordance with the CV75 value of the h-CLAT assay (OECD, 2022c). First, we compared two untargeted proteomics approaches, SILAC and LFQ, regarding their potential to identify regulated proteins.

Overall, 1997 and 2200 proteins were reliably quantified using SILAC and LFQ, respectively, of which 1904 proteins were identified by both methods (Fig. 1A). A principal component analysis (PCA) of SILAC-quantified proteins revealed that proteomic changes induced by the contact allergens DNCB and NBB led to a clear separation from vehicle

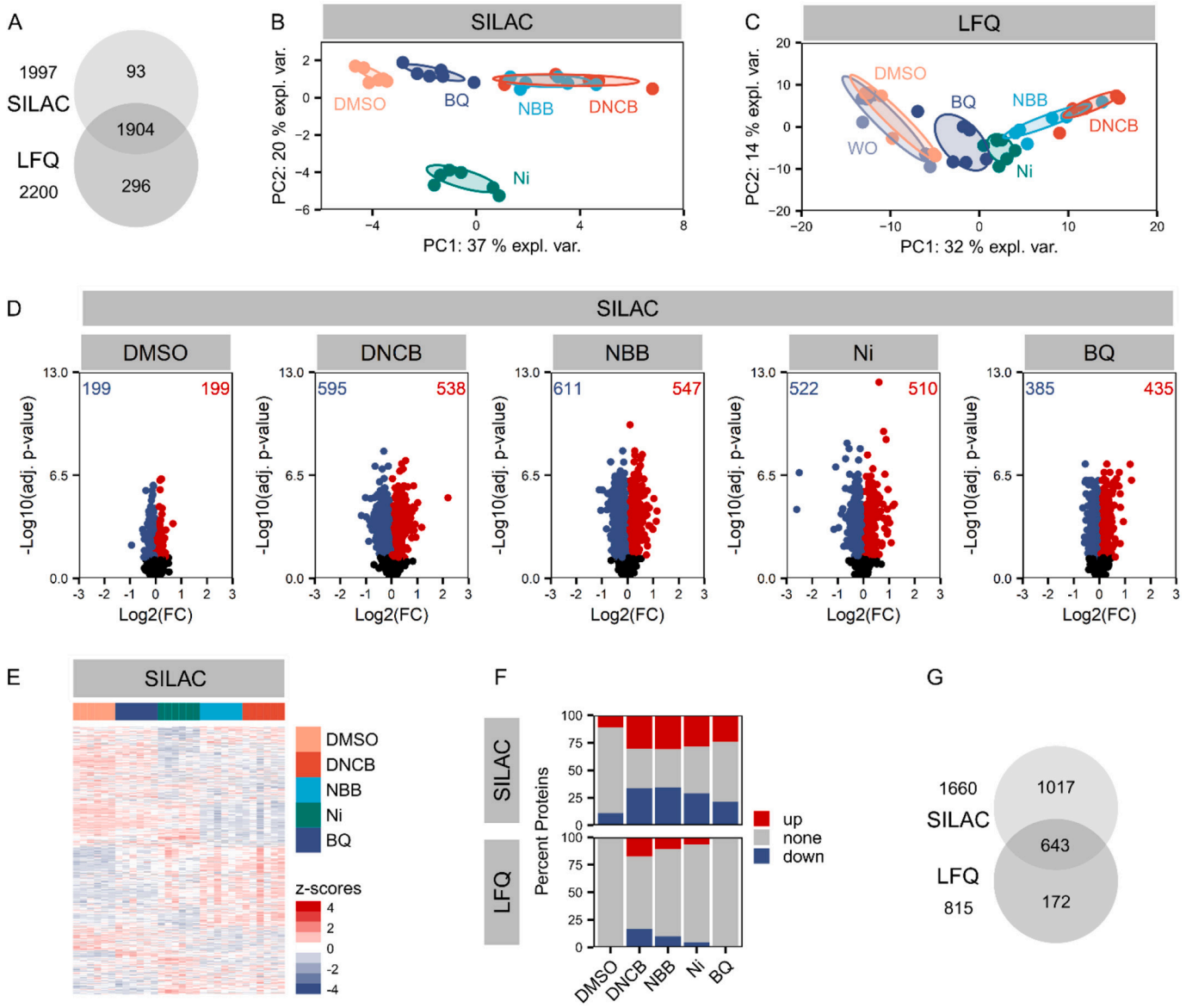

Fig. 1. Quantitative proteomic analysis in THP-1 cells treated with skin sensitizers. THP-1 cells were treated with the allergens BQ, DNCB, NBB or Ni. DMSO (solvent control) and medium only (WO) samples were used as controls. The number of quantified proteins is displayed in (A). PCA revealed general differences between treated cells and controls for SILAC (B) and LFQ (C) data. Volcano plots showing Log2(FCs) and –Log10(adjusted p-values) of allergen-treated THP-1 cells indicated changes induced by the allergens tested here. Numbers of regulated proteins (adjusted p-value $\leq$0.05; up: Log2(FC) > 0, red; down: Log2(FC) < 0, blue) are provided in the corners (D). Clustering of regulated proteins was determined using z-scored replicate data (E). Percentages of regulated proteins were compared for SILAC and LFQ data (F). Regulated proteins shared between SILAC and LFQ data were determined (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controls (DMSO). Separation of Ni-treated cells from controls and other treatments was distinct (Fig. 1B). LFQ also resulted in a separation of the allergens DNCB, NBB and Ni from controls. BQ-treated cells clustered in between. Overall, separation of the different treatments was not as clear compared to SILAC quantification (Fig. 1C). Next, regulated proteins (adjusted p-value $\leq$0.05) were determined, and comparing the distribution of Log2(FCs) and -Log10(adjusted p-values) for SILAC (Fig. 1D) and LFQ data (Supplementary Fig. 2A), it was noted that Log2(FC) ranges were smaller in SILAC data than in LFQ data. Notably, a high reproducibility and treatment-specific cluster formation of regulated proteins was observable with both methods (SILAC: Fig. 1E, LFQ: Supplementary Fig. 2B). Again, DNCB- and NBB-induced clusters differed clearly from control cells, whereas BQ-induced proteins predominantly clustered with control cells. Ni-treatment induced clusters that differed from controls as well as the other treatments. Interestingly, when comparing the numbers of regulated proteins, we found that SILAC resulted in a higher percentage for each treatment (Fig. 1F) and also a higher number of regulated proteins in at least one treatment than LFQ (Fig. 1G). Although 643 proteins were regulated in both SILAC and LFQ data, the majority of regulated proteins (1017) were identified exclusively in SILAC data (Fig. 1G).

Overall, the allergens clearly induced effects, which were detectable using SILAC and LFQ. Since SILAC yielded more regulated proteins (Fig. 1G) and showed a clearer separation of treatments (Fig. 1B), we decided to focus on SILAC data for subsequent analyses. Nevertheless, corresponding LFQ results are available in the supplementary material.

3.2. Contact allergen-exposed THP-1 cells show pronounced metabolic reprogramming

Next, an IPA pathway analysis was employed for regulated proteins (adjusted p-value ≤0.05) to gain functional insights into the effects of contact allergens on the pathway level. The majority of the significantly (adjusted p-value ≤0.05) enriched IPA pathways (74) was shared for SILAC and LFQ but again SILAC outperformed LFQ as more pathways were found significantly enriched (Fig. 2A). Among the TOP 30 significantly enriched pathways identified using SILAC, most pathways were regulated uniformly by the four allergens tested (Fig. 2B). The magnitude of induction varied with DNCB and NBB being the most potent inducers of cellular response mechanisms. Interestingly, five pathways were directly related to cellular metabolism: Oxidative phosphorylation (OXPHOS), gluconeogenesis, TCA cycle and pentose phosphate pathway were upregulated, pointing towards an increased cellular energy demand. β-oxidation of fatty acids was significantly upregulated after treatment with DNCB, NBB and Ni but was downregulated in BQ-treated cells (Fig. 2B). Of these pathways, OXPHOS was strongly upregulated after contact allergen exposure of the THP-1 cells (Fig. 2B). OXPHOS serves to supply the cell with ATP produced by a series of five protein complexes in the inner mitochondrial membrane. Several isoforms and subunits of the enzymes belonging to the electron transport chain were found to be upregulated, including NADH dehydrogenase (NDUF, complex I), cytochrome *c* oxidase (COX, complex IV) and ATP synthase (ATP, complex V) (Fig. 2C). Furthermore, the TCA cycle was highly affected (Fig. 2B). The conversion of isocitrate to α-ketoglutarate is the rate-limiting step of the TCA cycle and is catalyzed by the enzyme isocitrate dehydrogenase (IDH). Here, we identified two subunits of IDH (IDH3A and B) to be upregulated after treatment with DNCB and NBB.

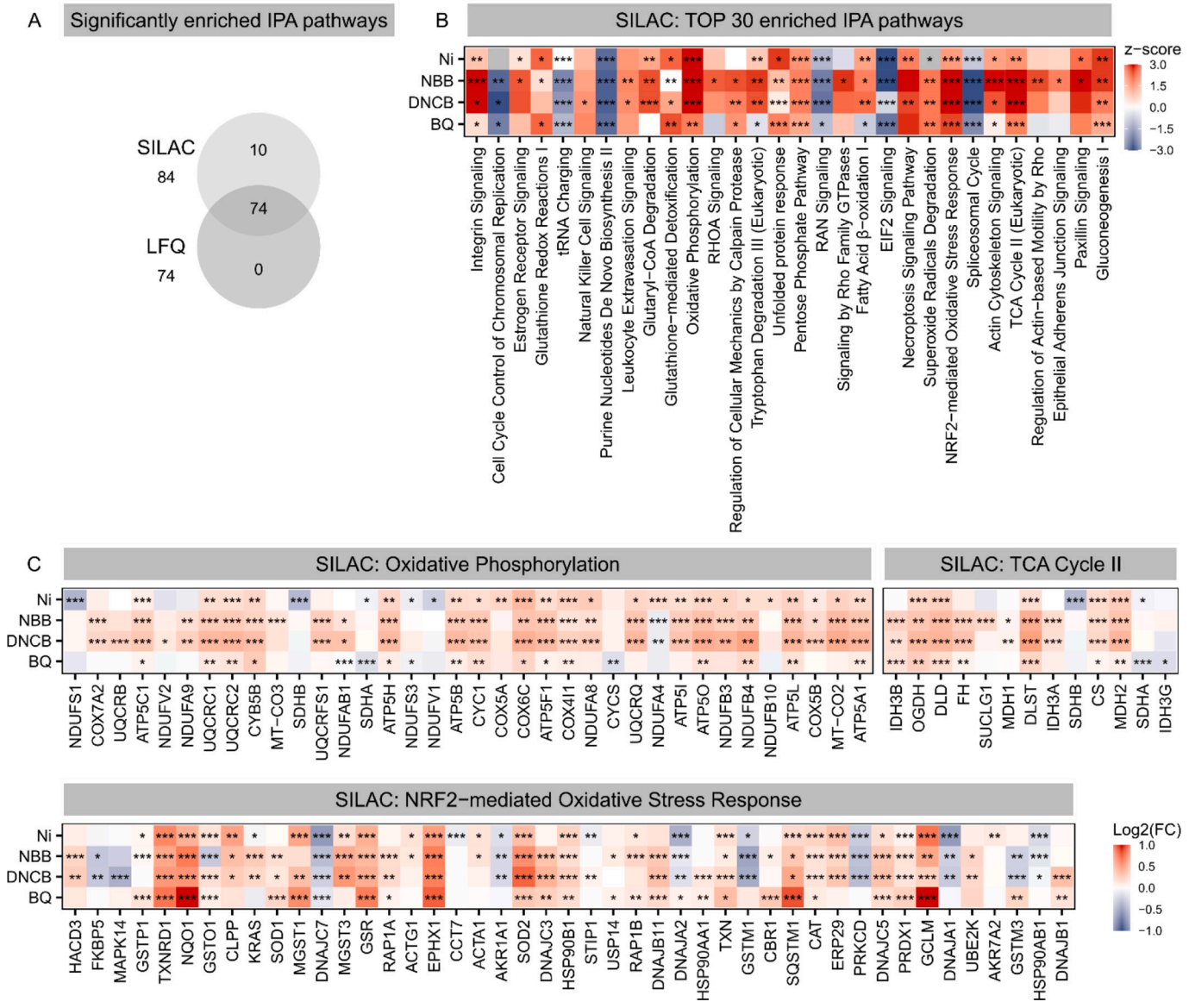

Fig. 2. Significantly enriched IPA pathways and regulated proteins assigned to selected pathways. Regulated proteins (adjusted p-value ≤0.05) were subjected to the enrichment of canonical pathways using IPA, resulting in adjusted p-values describing the significance of the enrichment and z-scores reflecting the direction of the regulation (activation (red): z-score > 0, inhibition (blue): z-score < 0). Overlaps of significantly enriched pathways (adjusted p-value ≤0.05) were compared for SILAC and LFQ data (A). TOP 30 pathways were extracted based on the adjusted p-values across all treatments for SILAC data (B). SILAC-based regulated proteins were depicted for oxidative phosphorylation, TCA cycle II and Nrf2-mediated oxidative stress response (C). Significances are indicated by asterisks: * adjusted p-value ≤0.05; ** adjusted p-value ≤0.01; *** adjusted p-value ≤0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Treatment with BQ selectively led to the upregulation of IDH3B and the metal allergen Ni did not induce IDH at all. Succinate dehydrogenase was the only protein of the TCA cycle that was found to be down-regulated after treatment with BQ and Ni or remained unchanged for DNCB and NBB (Fig. 2C). This points towards a high succinate withdrawal from the cycle.

Moreover, the exposure to the contact allergens induced pronounced cellular stress, which was reflected in the induction of Nrf2-mediated oxidative stress response, superoxide radicals degradation, glutathione-mediated detoxification, glutathione redox reactions and unfolded protein response (Fig. 2B). With 44 assigned regulated proteins, Nrf2-mediated oxidative stress response seemed to be very relevant. Among the highly upregulated proteins, we found proteins like NAD(*P*)H dehydrogenase (quinone) 1 (NQO1), epoxide hydrolase 1

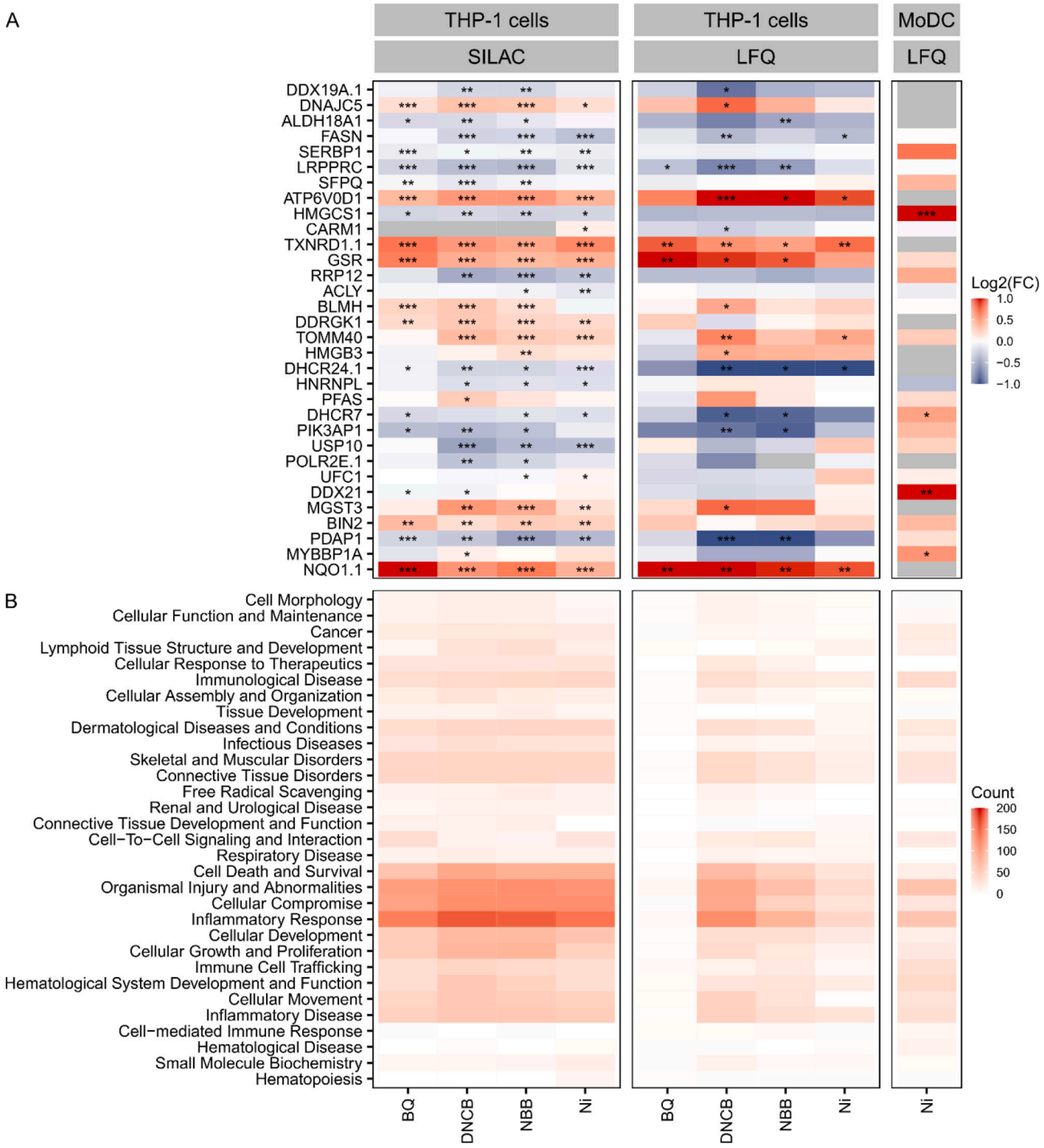

Fig. 3. Comparison of results from THP-1 cells and MoDCs. The gene signature employed by Johansson et al., 2011 to distinguish allergens from non-allergens was matched to regulated proteins in the allergen-treated THP-1 cells and the previously described MoDCs, focusing on the candidates being regulated in SILAC data. Shown are Log2(FCs) and significances for matching proteins (* adjusted p-value ≤0.05; ** adjusted p-value ≤0.01; *** adjusted p-value ≤0.001, for MoDCs, raw p-values were used) (A). According to the previously described procedure (Johansson et al. (2011)), triggered IPA diseases and biological functions were determined and filtered for those with at least 15 matching regulated proteins (adjusted p-value ≤0.05 for THP-1 cells, raw p-values ≤0.05 for MoDCs) in at least one treatment (B).

(EPHX1), thioredoxin reductase 1 (TXNRD1), superoxide dismutase 2 (SOD2), glutamate-cysteine ligase (GCLM) and glutathione reductase (GSR). The co-chaperones DnaJ homolog subfamily A member 1 (DNAJA1) and DnaJ homolog subfamily C member 7 (DNAJC7) were downregulated due to allergen treatment (Fig. 2C).

Furthermore, allergen-treatment induced downregulation of pathways involved in cell cycle maintenance and mRNA translation. Reconstruction of the actin cytoskeleton was also represented in the TOP 30 IPA pathways (Fig. 2B). Analogous figures for LFQ data are provided in the supplement (Supplementary Figs. 3 and 4).

3.3. Proteomics data reflect GARD gene signature and underline central role of Nrf2-mediated stress response as well as cholesterol biosynthesis

To assess the suitability of proteomics for detection of skin sensitizing chemicals in THP-1 cells, we compared our data to complementary data from a transcriptomic study, which defined the so-called GARD signature, a set of 200 genes that was found to be applicable to distinguish allergens from irritants and non-allergens in MUTZ-3 cells (Johansson et al., 2011). In total, 43 proteins relating to the signature genes were found in the THP-1 cells investigated here (SILAC and LFQ). For SILAC data, 38 proteins were part of the signature, of which 32 were regulated by at least one allergen. With 20 regulated proteins out of 41 assigned proteins, LFQ was again outperformed by SILAC and we thus decided to focus on the SILAC candidates as before (Fig. 3A).

Matched proteins were mostly unidirectionally regulated across the four allergens investigated here. Since directionality of transcriptional regulation was not published together with the GARD signature, comparison of regulation directionality between gene and protein level was impossible. Interestingly, the signature included proteins related to cholesterol biosynthesis, including hydroxymethylglutaryl-CoA synthase (HMGCS1) and ATP-citrate synthase (ACLY). Furthermore, proteins of the antioxidant response were successfully matched to the gene signature, *i.e.* the induced proteins NQO1, TXNRD1, microsomal glutathione S-transferase 3 (MGST3) and GSR (Fig. 3A).

We then compared the data retrieved from the THP-1 cells here to data from MoDCs. MoDCs are a frequently used *in vitro* model for DCs and are derived from human blood cells. For the comparison of THP-1 cells and MoDCs, we used our previously published proteomics data, where the effects of Ni were investigated using LFQ (Höper et al., 2021). Notably, the share of regulated proteins was rather low between THP-1 cells and MoDCs (Supplementary Fig. 5), which was also reflected by the low overlap in regulated proteins among the GARD signature genes (Fig. 3A). In total, 65 proteins of the MoDC data set were assigned to the GARD signature. However, only 14 of them were regulated after treatment with Ni. In most cases, these proteins were regulated opposite for THP-1 cells compared to MoDCs. Proteins that were found to be regulated unidirectional included GSR, bridging integrator 2 (BIN2) and mitochondrial import receptor subunit TOM40 homolog (TOMM40). These proteins are involved in oxidative stress response, cell motility and mitochondrial shuttling processes, respectively. In summary, the picture that emerges is that proteomic studies reflect the GARD signature to a limited extent. Also, the comparison of two different cell models on protein level does not yield congruent results, which was independent of the quantification method used.

3.4. Similar diseases and functions were affected in THP-1 cells compared to MoDCs

The low overlap on single protein level between THP-1 cells and MoDCs (Supplementary Fig. 5) prompted us to investigate whether this would still induce a comparable effect on enrichment level. For doing so, IPA was used to analyze diseases and functions based on regulated proteins in THP-1 cells compared to MoDC data (Fig. 3B). These provide information on superordinate toxicological functions, diseases and biological processes that are regulated and thereby provide more causal and holistic information on the organism under investigation than the analysis of canonical pathways alone. As done by Johansson et al. (2011) to determine the dominating functions of the GARD prediction signature, only diseases and functions with at least 15 matched regulated proteins were considered for this comparison. Among those, diseases and functions related to inflammation and especially inflammatory responses ranked highest (Fig. 3B). Other terms related to inflammation and cell damage, like cellular compromise and organismal injury abnormalities, were also affected after treatment with the four contact allergens investigated (Fig. 3B). This inflammatory status of the cells was accompanied by biological functions involved in the cellular adaption to these conditions like cellular development, cellular function and maintenance, cell-to-cell signaling and interaction as well as cell morphology (Fig. 3B). As expected, some of the identified diseases and functions were linked to contact allergy, such as dermatological disease and conditions and cell-mediated immune response. The comparison of these results with those previously described in MoDCs treated with Ni (Höper et al., 2021) revealed similar diseases and functions to be affected (Fig. 3B). Yet, the cellular response of the MoDCs to Ni-exposure seems to be less augmented compared to THP-1 cells (Fig. 3B).

Overall, the induced diseases and functions well reflect the cellular stress induced by allergen-treatment and point towards inflammatory signaling, which was less evident in the canonical pathway analysis. Notably, we observed the induction of the very same diseases and functions in two proteomic sets with low overlap in regulated proteins, suggesting the pathway/enrichment level to be better suited for dataset comparison than the single protein level.

4. Discussion

4.1. SILAC outperforms LFQ in terms of regulated proteins and enriched pathways

The determination of the skin-sensitizing potential of chemicals is essential for proper chemical safety assessment. Hence, the research on cellular mechanisms during the induction of skin sensitization as well as the development of animal-free test guidelines have gained much attention in the field of toxicology over the last decades. In general, omics techniques were already applied in research on alternative methods for skin sensitization. Transcriptomics, for example, were successfully deployed for the discrimination of contact allergens from non-sensitizers in DC models (Hooyberghs et al., 2008; Johansson et al., 2011; Lambrechts et al., 2011). Although the proteome maps the phenotype of the biological organism more accurately, only few studies applying proteomics to investigate cellular mechanisms in the context of contact allergy were published to date (Höper et al., 2017; Koppes et al., 2017). Our group conducted a preceding study in human MoDCs to reveal differences in the cellular proteome after treatment with the metal contact allergen nickel *versus* the bacterial endotoxin LPS and found significant differences between the two treatments (Höper et al., 2021). Ni-treatment induced metabolic reprogramming, a pronounced Nrf2-mediated stress response, hypoxia as well as cholesterol depletion in MoDCs, whereas LPS-treated MoDCs displayed interferon signaling additionally to metabolic reprogramming and Nrf2 activation (Höper et al., 2021). Now, we aimed at screening multiple allergens to broaden the proteomic understanding of contact allergy. For this purpose, we selected THP-1 cells as surrogate model for DCs, as cell lines are better suited for screenings of broad sets of chemicals. Nevertheless, critical discussions on suitable cell models are inevitable in toxicology. Even though THP-1 cells are the selected cell model in the validated h-CLAT assay, they have been questioned due to their monocytic character and leukemia background.

For quantification of the proteomic data, we performed SILAC and LFQ, both of which are common approaches. LFQ can be applied to any kind of experimental setup and moreover was used for our preceding MoDC study. SILAC was selected as it is well-known for its high

reproducibility and precision (Li et al., 2012). Overall, both quantification strategies delivered comparable results in terms of affected pathways. Yet, more regulated proteins and pathways were identified based on SILAC data. Both techniques have their justification, but for cell lines SILAC is preferable, as it is easier to handle, requires substantial less measuring time at the mass spectrometer, minimizes measuring bias due to multiplexing and moreover, led to more detailed mechanistic insights due to the higher number of proteins found regulated. Thus, we recommend to use SILAC quantification when working with cell lines. Alternatively, chemical labeling like tandem mass tags (TMT) can be applied, which allows multiplexing of up to 16 samples and can be applied to any kind of sample as LFQ (Stepath et al., 2020; Wang et al., 2020).

4.2. Contact allergen-exposed THP-1 cells undergo pronounced metabolic shifts

Exposure of THP-1 cells to electrophils, such as contact allergens, results in a distinct cellular stress response that is largely mediated by Nrf2. Earlier *in vivo* studies with contact allergens have already suggested a substantial role of Nrf2 during skin sensitization (El Ali et al., 2013). Enzymes like NQO1, SOD2, glutathione s-transferases, heme oxygenase or catalase have been reported to be induced in allergen-treated THP-1 cells and DCs in the past (Lewis et al., 2006; Ade et al., 2009; Mussotter et al., 2016; Höper et al., 2021). Nrf2-mediated stress response can thus be considered a hallmark in DCs during skin sensitization. The central role of Nrf2 was also confirmed in keratinocytes, which led to the development of the KeratinoSens™ assay that has become an official OECD test guideline (OECD, 2018). Due to the power of proteomics, we are the first to report >40 regulated proteins upon contact allergen treatment linked to Nrf2-mediated stress response (Fig. 2C). We therefore recommend to use an additional Nrf2-related readout directly in DCs or DC surrogates.

In an antecedent multi-omics study, we already found evidence for metabolic reprogramming in THP-1 cells after treatment with the extreme skin sensitizer DNCB. The metabolome of DNCB-exposed cells was highly affected, pointing towards upregulation of glycolysis, TCA cycle and lipid synthesis (Mussotter et al., 2018). The current study confirms and expands these findings. Several metabolic pathways like TCA cycle, pentose phosphate pathway and oxidative phosphorylation were induced after treatment with all allergens investigated. In DCs, metabolic reprogramming was found to be crucial to support cellular activation following exposure to danger signals (Krawczyk et al., 2010). This metabolic adaption is typically characterized by upregulation of glycolysis and pentose phosphate pathway (PPP) with simultaneous downregulation of OXPHOS. DC activation *via* TLR massively increases aerobic glycolysis to a similar level as observed in cancer cells that display the typical Warburg effect (Kelly and O'Neill, 2015). The rapid metabolic switch supports maturation of the cells to enable migration to the draining lymph nodes and thereby ultimately enables immunological signaling as well as the induction of a systemic immune response. Induction of glycolytic proteins in contact allergen-exposed DCs was already reported in mouse bone marrow-derived DCs as well as in human MoDCs (Mussotter et al., 2016; Höper et al., 2021). Compared to the here investigated THP-1 cells, metabolic reprogramming in MoDCs was far less pronounced on proteome level (Höper et al., 2021). Elucidation of the underlying cellular mechanisms is complex and cannot be resolved using proteomics only. We speculate that the more pronounced metabolic activation of the THP-1 cells can partly be attributed to their cancer background (Warburg effect). Furthermore, the potency of a stimuli determines the degree of metabolic reprogramming both in DCs and monocytes (Lachmandas et al., 2016; Guak et al., 2018). Thus differences in metabolic shift can also be induced by different stimulatory potency of the contact allergens in different cells types.

Downstream of glycolysis, glycolytic products are fed into the TCA cycle, which was an activated metabolic pathway in the here investigated THP-1 cells. It has been shown that LPS-activated DCs rapidly upregulate glycolysis to increase the production of citrate *via* the TCA cycle (Everts et al., 2014). Citrate, which is transported to the cytosol to serve as a precursor for fatty acid and cholesterol biosynthesis, is required for maturation, involving restructuring of the plasma membrane. Our study confirms that most enzymes of the TCA cycle are upregulated after treatment of THP-1 cells with contact allergens (Fig. 2C). This is further supported by the upregulation of the PPP, which we also observed. Upregulation of PPP was also found in THP-1 cells treated with the sensitizer 2-hydroxyethylmethacrylate (Samuelsen et al., 2019). Importantly, the PPP supplies the cells with the reducing equivalent NADPH that is needed for fatty acid and cholesterol synthesis.

Interestingly, in the last ten years, it has become clear that the TCA cycle is not only important to deliver precursors for several synthesis routes but also is able to control various biological processes, including regulating cellular immunity. Since biological diseases and functions linked to inflammation were found to be upregulated in allergen-treated THP-1 cells, we suggest that the pronounced induction of the enzymes in the TCA cycle strongly supports a proinflammatory phenotype of the cells by providing respective metabolites. Citrate, for instance, has been shown to play an important role in key inflammatory pathways being relevant for macrophages as well as for DCs (Williams and O'Neill, 2018). Citrate is essential for the production of proinflammatory signaling molecules, including ROS and prostaglandin E2 (Infantino et al., 2014). Prostaglandin E2 is mandatory for the synthesis of pro-IL-1β after exposure of macrophages to LPS (Zasłona et al., 2017), and IL-1β was shown to play a major role during mediation of ACD (Yeung et al., 2021). Moreover, high levels of citrate and acetyl-CoA were shown to increase the expression of glycolytic enzymes in tumor cells (Lee et al., 2014).

Of all TCA cycle enzymes detected in this study, the only enzyme downregulated was succinate dehydrogenase, pointing towards a high succinate demand in the activated cells. Succinate is known to act as a proinflammatory signal in immune cells (Rubic et al., 2008; Tannahill et al., 2013). Furthermore, succinate was shown to enhance immunological signaling in DCs (Rubic et al., 2008) and to stabilize HIF-1α in the cytosol, thereby inducing genes like IL-1β important for glycolysis, inflammation and inflammasome activation (Tannahill et al., 2013; Li et al., 2016). Another potential fate of succinate could be the succinylation of lysine residues. Lysine succinylation can directly affect cellular metabolism by increasing enzyme activity of enzymes involved in glycolysis and TCA cycle (Park et al., 2013). Mitochondrial succinate can leak from dysfunctional mitochondria, and especially macrophages were shown to secrete succinate under inflammatory conditions (Littlewood-Evans et al., 2016). Extracellular succinate binds to its receptor SUCNR1 (succinate receptor 1) expressed on the plasma membrane of immune cells including DCs (Rubic et al., 2008; Littlewood-Evans et al., 2016), which is considered an immunological danger signal. Furthermore, succinate stimulates cell migration in a chemokine-like manner and enhances antigen-presentation to T cells as reported by Rubic et al. (2008). Indeed, *Suncr1*-deficient mice did not show elevated T cell activation after re-exposure of pre-sensitized mice to the contact allergen oxazolone (Rubić-Schneider et al., 2017).

To further elucidate the fate of TCA cycle metabolites in contact allergen-treated THP-1 cells, the proteomic data presented here should be complemented by a metabolomics experiment. In addition, mitochondrial respiration and glycolysis can for example be tracked using the seahorse analyzer. A metabolic flux analysis using isotope-labeled glucose or glutamine could reveal whether the disrupted TCA cycle that was described for LPS-treated macrophages and DCs (Everts et al., 2014; Galván-Peña and O'Neill, 2014) is also featured in THP-1 cells. Furthermore, inhibition of selected enzymes of the TCA cycle and analysis of downstream effects can be employed to support the proteomics data.

4.3. Affected pathways rather than single proteins are suitable for the comparative investigation of skin sensitization in THP-1 cells and MoDCs

One of our aims was to elucidate whether THP-1 cells are an appropriate surrogate to study proteomic changes during skin sensitization in DCs. Thus, we compared the effects uncovered here with effects observed in our previous study with MoDCs (Höper et al., 2021). We found that the overlap of regulated proteins was low between the two cell models (Supplementary Fig. 5). Based on the small overlap between the two cell systems on protein level, THP-1 cells should be considered a limited model for DCs. However, on enrichment level (pathways, diseases and functions) we obtained comparable results.

Exposure of DCs with contact allergens alters a broad range of proteins that orchestrate the complex immune response induced in the cells, eventually enabling presentation of the antigen to T cells. For T cells, antigen-specificity is well known (Chaplin, 2010) and specific T cell receptor repertoires were described for metal- as well as for chemical-induced allergy (Curato et al., 2022; Riedel et al., 2022). However, DCs are able to recognize, process and present a large range of antigens and thereby induce the antigen-specific immune response. Depending on the DC population, route of antigen uptake and the antigen itself, activation and maturation of DCs may vary and is not considered antigen-specific (Kamphorst et al., 2010; Alloatti et al., 2016). Due to this lack in specificity, it seems unlikely that specific protein biomarkers for skin sensitization can be identified in DCs. Thus, the use of predictive signatures seems more promising. Johansson et al. (2011) suggested a gene signature for the prediction of skin sensitizing chemicals *in vitro*. It is commonly known that the overlap between gene regulation and altered protein levels is not necessarily high. Yet, the integrative application of transcriptomic and proteomic data may support the identification of biomarkers, as it helps to uncover transcriptionally active mRNAs during the complex course of skin sensitization. We compared the GARD assay mRNA signature to regulated proteins in THP-1 cells after treatment with contact allergens, revealing 32 matching regulated proteins (Fig. 3A, SILAC). Among those, we found proteins linked to oxidative stress response like NQO1 and GSR, further underlining Nrf2-regulated pathways as hallmark during sensitization to exogenous chemicals. Also, proteins related to the cholesterol biosynthesis like HMGCS1 and CYP51A1 were regulated on mRNA level in MUTZ-3 cells as well as on protein level in THP-1 cells and MoDCs. Yet, directionality of protein regulation was contrary. These findings underline the evidence that cholesterol biosynthesis plays a pivotal role in allergen-treated DCs. However, due to equivocal regulation of proteins, this pathway has to be investigated further to assess its suitability as potential hallmark for skin sensitization. Notably, Lindberg et al. (2020) conducted a proteomics study in MUTZ-3 cells combined with the GARD assay to assess the skin sensitizing potential of glyphosate and its formulations. Here, 3 proteins were successfully matched to the predictive GARD signature and these proteins were also involved in cholesterol biosynthesis. Overall, we believe that the establishment of a proteomic signature of specific proteins and triggered cellular pathways might be more conducive than the search for individual biomarkers. Yet, a pathway-signature has to be chosen carefully to securely predict contact allergens based on proteomic data.

To be able to assess the suitability of a proteomic signature for the prediction of contact sensitizers in more depth, a larger set of contact sensitizers of different potency as well as irritants and non-allergenic chemicals should be analyzed to withdraw profound information for both cell systems, THP-1 cells and MoDCs, as the classification of allergens, irritants and non-allergenic chemicals is critical for proper risk assessment. The inclusion of irritants and non-allergens would thus increase the predictive power of the signature, as unspecific pathways can be identified and removed from the set of signature pathways. Furthermore, data from untargeted proteomic analyses could be used to develop a targeted proteomic method for routine testing of identified biomarkers and biomarker signatures. Moreover, THP-1 cells should be discussed more critically in the future when used as a tool for predicting contact allergens based on proteomic studies. Further proteomics studies should be conducted to investigate if THP-1 cells, differentiated into a DC-like phenotype before contact allergen treatment, resemble DCs more closely. Furthermore, a comparative proteomic study comparing THP-1 and MUTZ-3 cells could provide information on the suitability of these cell lines for the proteomic investigation of skin sensitization.

5. Conclusion

Overall, our study underlines the power of proteomics to unravel toxicity mechanisms. When comparing the two different proteomics approaches applied here, SILAC and LFQ, certainly both have their justification. However, when working with cell lines, SILAC should be preferred as it delivers more robust data and yields a higher number of regulated proteins.

The four allergens BQ, DNCB, NBB and Ni overall showed comparable responses in THP-1 cells, which underwent profound metabolic reprogramming that was accompanied by pronounced induction of proteins of the Nrf2 oxidative stress response pathway. The TCA cycle seems to play a central role and might be connected to the proinflammatory response. Compared to Ni-treated MoDCs, similar IPA diseases and functions as well as pathways were triggered in THP-1 cells but the overlap on the protein level was rather low. When aiming to improve existing *in vitro* assays by proteomic signatures, one should therefore focus on proteins that are regulated by allergens in both, THP-1 cells and primary MoDCs. This includes proteins of the Nrf2 pathway along with proteins from glycolytic and lipid metabolism. For the elucidation of cellular modes of action, primary cells seem to be superior, as THP-1 cells were reacting unidimensional and showed a much less complex network of induced IPA pathways compared to MoDCs.

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CRediT authorship contribution statement

T. Höper: Conceptualization, Investigation, Methodology, Project administration, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **I. Karkossa**: Methodology, Data curation, Formal analysis, Software, Visualization, Writing – original draft, Writing – review & editing. **V. I. Dumit**: Conceptualization, Supervision, Writing – review & editing. **M. von Bergen**: Conceptualization, Resources, Writing – review & editing. **K. Schubert**: Conceptualization, Writing – review & editing. **A. Haase**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The dataset presented in this study can be found online in the PRIDE repository (Perez-Riverol et al., 2019) with the dataset identifier PXD031017. A full list of all proteins, including the protein names and accessions as well as Log2(FCs) and adjusted p-values can be found in the article supplementary material.

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Appendix A. Supplementary data

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References

Ade, N., Leon, F., Pallardy, M., Peiffer, J.L., Kerdine-Romer, S., Tissier, M.H., Bonnet, P. A., Fabre, I., Ourlin, J.C., 2009. HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: role of the Keap1/Nrf2 pathway. Toxicol. Sci. 107, 451–460.

Adrian, A.D., Cole, A., 2018. xlsx: Read, Write, Format Excel 2007 and Excel 97/2000/XP/2003 Files. R Package Version 0.6.1. https://CRAN.R-project.org/package=xlsx.

Alloatti, A., Kotsias, F., Magalhaes, J.G., Amigorena, S., 2016. Dendritic cell maturation and cross-presentation: timing matters! Immunol. Rev. 272, 97–108.

Ananda, M., 2019. splitstackshape: Stack and Reshape Datasets After Splitting Concatenated Values. R Package Version 1.4.8. https://CRAN.R-project.org/package=splitstackshape.

Andrej-Nikolai, S., 2018. qpcR: Modelling and Analysis of Real-Time PCR Data. R Package Version 1.4–1. https://CRAN.R-project.org/package=qpcR.

Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama, K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., Toyoda, H., 2006. Development of an in vitro skin sensitization test using human cell lines: the human cell line activation test (h-CLAT). I. Optimization of the h-CLAT protocol. Toxicol. in Vitro 20.

Ashikaga, T., Sakaguchi, H., Sono, S., Kosaka, N., Ishikawa, M., Nukada, Y., Miyazawa, M., Ito, Y., Nishiyama, N., Itagaki, H., 2010. A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). Altern. Lab. Anim 38, 275–284.

Chaplin, D.D., 2010. Overview of the immune response. J. Allergy Clin. Immunol. 125, S3–23.

Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N., Mann, M., 2014. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol. Cell. Proteomics 13, 2513–2526.

Curato, C., Aparicio-Soto, M., Riedel, F., Wehl, I., Basaran, A., Abbas, A., Thierse, H.J., Luch, A., Siewert, K., 2022. Frequencies and TCR repertoires of human 2,4,6-Trinitrobenzenesulfonic acid-specific T cells. Front. Toxicol. 4, 827109.

Dhingra, N., Shemer, A., Correa da Rosa, J., Rozenblit, M., Fuentes-Duculan, J., Gittler, J. K., Finney, R., Czarnowicki, T., Zheng, X., Xu, H., Estrada, Y.D., Cardinale, I., Suárez-Fariñas, M., Krueger, J.G., Guttman-Yassky, E., 2014. Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response. J. Allergy Clin. Immunol. 134, 362–372.

EC, 2009. Regulation (EC) no 1223/2009 of the European Parliament and of the council of 30 November 2009 on cosmetic products. Off. J. Eur. Union L342, 59–209.

El Ali, Z., Gerbeix, C., Hemon, P., Esser, P.R., Martin, S.F., Pallardy, M., Kerdine-Römer, S., 2013. Allergic skin inflammation induced by chemical sensitizers is controlled by the transcription factor Nrf2. Toxicol. Sci. 134, 39–48.

Emter, R., Ellis, G., Natsch, A., 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. Toxicol. Appl. Pharmacol. 245, 281–290.

Everts, B., Amiel, E., Huang, S.C., Smith, A.M., Chang, C.H., Lam, W.Y., Redmann, V., Freitas, T.C., Blagih, J., van der Windt, G.J., Artyomov, M.N., Jones, R.G., Pearce, E. L., Pearce, E.J., 2014. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nat. Immunol. 15, 323–332.

Fabian, E., Vogel, D., Blatz, V., Ramirez, T., Kolle, S., Eltze, T., van Ravenzwaay, B., Oesch, F., Landsiedel, R., 2013. Xenobiotic metabolizing enzyme activities in cells used for testing skin sensitization in vitro. Arch. Toxicol. 87, 1683–1696.

Galván-Peña, S., O'Neill, L.A.J., 2014. Metabolic reprograming in macrophage polarization. Front. Immunol. 5.

Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W., Lepoittevin, J.P., 2004. Development of a peptide reactivity assay for screening contact allergens. Toxicol. Sci. 81, 332–343.

Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y., Basketter, D.A., 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis 16, 157–202.

Gerberick, G.F., Vassallo, J.D., Foertsch, L.M., Price, B.B., Chaney, J.G., Lepoittevin, J.P., 2007. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol. Sci. 97, 417–427.

Gu, Z., 2014. Circlize implements and enhances circular visualization in R. Bioinformatics 30, 2811–2812.

Guak, H., Al Habyan, S., Ma, E.H., Aldossary, H., Al-Masri, M., Won, S.Y., Ying, T., Fixman, E.D., Jones, R.G., McCaffrey, L.M., Krawczyk, C.M., 2018. Glycolytic metabolism is essential for CCR7 oligomerization and dendritic cell migration. Nat. Commun. 9, 2463.

Guedes, S., Neves, B., Vitorino, R., Domingues, R., Cruz, M.T., Domingues, P., 2016. Contact dermatitis: in pursuit of sensitizer's molecular targets through proteomics. Arch. Toxicol. 1–15.

Hadley, W., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Hadley, W., Jennifer, B., 2019. readxl: Read Excel Files. R Package Version 1.3.1. https://CRAN.R-project.org/package=readxl.

Hadley, W., Lionel, H., 2019. tidyr: Tidy Messy Data. R Package Version 1.0.0. https://CRAN.R-project.org/package=tidyr.

Hooyberghs, J., Schoeters, E., Lambrechts, N., Nelissen, I., Witters, H., Schoeters, G., Van Den Heuvel, R., 2008. A cell-based in vitro alternative to identify skin sensitizers by gene expression. Toxicol. Appl. Pharmacol. 231, 103–111.

Höper, T., Mussotter, F., Haase, A., Luch, A., Tralau, T., 2017. Application of proteomics in the elucidation of chemical-mediated allergic contact dermatitis. Toxicol. Res. 6, 595–610.

Höper, T., Siewert, K., Dumit, V.I., von Bergen, M., Schubert, K., Haase, A., 2021. The contact allergen NiSO4 triggers a distinct molecular response in primary human dendritic cells compared to bacterial LPS. Front. Immunol. 12.

Infantino, V., Iacobazzi, V., Menga, A., Avantaggiati, M.L., Palmieri, F., 2014. A key role of the mitochondrial citrate carrier (SLC25A1) in TNFα- and IFNγ-triggered inflammation. Biochim. Biophys. Acta (BBA) - Gene Regul. Mech. 1839, 1217–1225.

Jan, G., 2019. calibrate: Calibration of Scatterplot and Biplot Axes. R Package Version 1.7.5. https://CRAN.R-project.org/package=calibrate.

Jaworska, J.S., Natsch, A., Ryan, C., Strickland, J., Ashikaga, T., Miyazawa, M., 2015. Bayesian integrated testing strategy (ITS) for skin sensitization potency assessment: a decision support system for quantitative weight of evidence and adaptive testing strategy. Arch. Toxicol. 89, 2355–2383.

Johansson, H., Lindstedt, M., Albrekt, A.-S., Borrebaeck, C.A., 2011. A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. BMC Genomics 12, 399.

Johansson, H., Albrekt, A.S., Borrebaeck, C.A., Lindstedt, M., 2013. The GARD assay for assessment of chemical skin sensitizers. Toxicol. in Vitro 27, 1163–1169.

Kamphorst, A.O., Guermonprez, P., Dudziak, D., Nussenzweig, M.C., 2010. Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes. J. Immunol. 185, 3426.

Kelly, B., O'Neill, L.A., 2015. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell Res. 25, 771–784.

Kolde, R., 2019. pheatmap: Pretty Heatmaps [Online]. Available: https://CRAN.R-project.org/package=pheatmap [Accessed 26.04.2021].

Koppes, S.A., Engebretsen, K.A., Agner, T., Angelova-Fischer, I., Berents, T., Brandner, J., Brans, R., Clausen, M.L., Hummler, E., Jakasa, I., Jurakic-Toncic, R., John, S.M., Khnykin, D., Molin, S., Holm, J.O., Suomela, S., Thierse, H.J., Kezic, S., Martin, S.F., Thyssen, J.P., 2017. Current knowledge on biomarkers for contact sensitization and allergic contact dermatitis. Contact Dermatitis 77, 1–16.

Krämer, A., Green, J., Pollard Jr., J., Tugendreich, S., 2013. Causal analysis approaches in ingenuity pathway analysis. Bioinformatics 30, 523–530.

Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J. R., Jung, E., Thompson, C.B., Jones, R.G., Pearce, E.J., 2010. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood 115, 4742–4749.

Lachmandas, E., Boutens, L., Ratter, J.M., Hijmans, A., Hooiveld, G.J., Joosten, L.A.B., Rodenburg, R.J., Fransen, J.A.M., Houtkooper, R.H., van Crevel, R., Netea, M.G., Stienstra, R., 2016. Microbial stimulation of different toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. Nat. Microbiol. 2, 16246.

Lambrechts, N., Nelissen, I., Van Tendeloo, V., Witters, H., Van Den Heuvel, R., Hooyberghs, J., Schoeters, G., 2011. Functionality and specificity of gene markers for skin sensitization in dendritic cells. Toxicol. Lett. 203, 106–110.

Lee, J.V., Carrer, A., Shah, S., Snyder, N.W., Wei, S., Venneti, S., Worth, A.J., Yuan, Z.F., Lim, H.W., Liu, S., Jackson, E., Aiello, N.M., Haas, N.B., Rebbeck, T.R., Judkins, A., Won, K.J., Chodosh, L.A., Garcia, B.A., Stanger, B.Z., Feldman, M.D., Blair, I.A., Wellen, K.E., 2014. Akt-dependent metabolic reprogramming regulates tumor cell histone acetylation. Cell Metab. 20, 306–319.

Lefevre, M.-A., Nosbaum, A., Rozieres, A., Lenief, V., Mosnier, A., Cortial, A., Prieux, M., De Bernard, S., Nourikyan, J., Jouve, P.-E., Buffat, L., Hacard, F., Ferrier-Lebouedec, M.-C., Pralong, P., Dzviga, C., Herman, A., Baeck, M., Nicolas, J.-F., Vocanson, M., 2021. Unique molecular signatures typify skin inflammation induced by chemical allergens and irritants. Allergy 76, 3697–3712.

Lewis, J.B., Messer, R.L., McCloud, V.V., Lockwood, P.E., Hsu, S.D., Wataha, J.C., 2006. Ni(II) activates the Nrf2 signaling pathway in human monocytic cells. Biomaterials 27, 5348–5356.

Li, Z., Adams, R.M., Chourey, K., Hurst, G.B., Hettich, R.L., Pan, C., 2012. Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. J. Proteome Res. 11, 1582–1590.

Li, Y., Zheng, J.Y., Liu, J.Q., Yang, J., Liu, Y., Wang, C., Ma, X.N., Liu, B.L., Xin, G.Z., Liu, L.F., 2016. Succinate/NLRP3 inflammasome induces synovial fibroblast activation: therapeutical effects of clematichinenoside AR on arthritis. Front. Immunol. 7, 532.

Lindberg, T., de Ávila, R.I., Zeller, K.S., Levander, F., Eriksson, D., Chawade, A., Lindstedt, M., 2020. An integrated transcriptomic- and proteomic-based approach to evaluate the human skin sensitization potential of glyphosate and its commercial agrochemical formulations. J. Proteome 217, 103647.

Littlewood-Evans, A., Sarret, S., Apfel, V., Loesle, P., Dawson, J., Zhang, J., Muller, A., Tigani, B., Kneuer, R., Patel, S., Valeaux, S., Gommermann, N., Rubic-Schneider, T., Junt, T., Carballido, J.M., 2016. GPR91 senses extracellular succinate released from

inflammatory macrophages and exacerbates rheumatoid arthritis. J. Exp. Med. 213, 1655–1662.
Martin, S.F., Esser, P.R., Weber, F.C., Jakob, T., Freudenberg, M.A., Schmidt, M., Goebeler, M., 2011. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. Allergy 66, 1152–1163.
Mussotter, F., Tomm, J.M., El Ali, Z., Pallardy, M., Kerdine-Romer, S., Gotz, M., von Bergen, M., Haase, A., Luch, A., 2016. Proteomics analysis of dendritic cell activation by contact allergens reveals possible biomarkers regulated by Nrf2. Toxicol. Appl. Pharmacol. 313, 170–179.
Mussotter, F., Potratz, S., Budczies, J., Luch, A., Haase, A., 2018. A multi-omics analysis reveals metabolic reprogramming in THP-1 cells upon treatment with the contact allergen DNCB. Toxicol. Appl. Pharmacol. 340, 21–29.
Nan, X., 2018. ggsci: Scientific Journal and Sci-Fi Themed Color Palettes for 'ggplot2'. R Package Version 2.9. https://CRAN.R-project.org/package=ggsci.
Nukada, Y., Miyazawa, M., Kazutoshi, S., Sakaguchi, H., Nishiyama, N., 2013. Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Toxicol. in Vitro 27, 609–618.
OECD, 2014. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins.
OECD, 2018. Test No. 442D: In Vitro Skin Sensitisation.
OECD, 2021. Guideline No. 497: Defined Approaches on Skin Sensitisation.
OECD, 2022a. Test No. 442C: In Chemico Skin Sensitisation.
OECD, 2022b. Test No. 442D: In Vitro Skin Sensitisation.
OECD, 2022c. Test No. 442E: In Vitro Skin Sensitisation.
Oesch, F., Fabian, E., Guth, K., Landsiedel, R., 2014. Xenobiotic-metabolizing enzymes in the skin of rat, mouse, pig, guinea pig, man, and in human skin models. Arch. Toxicol. 88, 2135–2190.
Oosterhaven, J.A.F., Uter, W., Aberer, W., Armario-Hita, J.C., Ballmer-Weber, B.K., Bauer, A., Czarnecka-Operacz, M., Elsner, P., García-Gavín, J., Giménez-Arnau, A. M., John, S.M., Kręcisz, B., Mahler, V., Rustemeyer, T., Sadowska-Przytocka, A., Sánchez-Pérez, J., Simon, D., Valiukevičienė, S., Weisshaar, E., Schuttelaar, M.L.A., 2019. European surveillance system on contact allergies (ESSCA): contact allergies in relation to body sites in patients with allergic contact dermatitis. Contact Dermatitis 80, 263–272.
Park, J., Chen, Y., Tishkoff, Daniel X., Peng, C., Tan, M., Dai, L., Xie, Z., Zhang, Y., Zwaans, Bernadette M.M., Skinner, Mary E., Lombard, David B., Zhao, Y., 2013. SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. Mol. Cell 50, 919–930.
Parkinson, E., Boyd, P., Aleksic, M., Cubberley, R., O'Connor, D., Skipp, P., 2014. Stable isotope labeling method for the investigation of protein haptenation by electrophilic skin sensitizers. Toxicol. Sci. 142, 239–249.
Parkinson, E., Aleksic, M., Cubberley, R., Kaur-Atwal, G., Vissers, J.P.C., Skipp, P., 2018. Determination of protein haptenation by chemical sensitizers within the complexity of the human skin proteome. Toxicol. Sci. 162, 429–438.
Parkinson, E., Aleksic, M., Kukic, P., Bailey, A., Cubberley, R., Skipp, P., 2020. Proteomic analysis of the cellular response to a potent sensitiser unveils the dynamics of haptenation in living cells. Toxicology 445, 152603.
Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D. J., Inuganti, A., Griss, J., Mayer, G., Eisenacher, M., Perez, E., Uszkoreit, J., Pfeuffer, J., Sachsenberg, T., Yilmaz, S., Tiwary, S., Cox, J., Audain, E., Walzer, M., Jarnuczak, A.F., Ternent, T., Brazma, A., Vizcaino, J.A., 2019. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res. 47, D442–D450.
Qiagen. Ingenuity Pathway Analysis [Online]. Available: https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/.
Reisinger, K., Hoffmann, S., Alépée, N., Ashikaga, T., Barroso, J., Elcombe, C., Gellatly, N., Galbiati, V., Gibbs, S., Groux, H., Hibatallah, J., Keller, D., Kern, P., Klaric, M., Kolle, S., Kuehnl, J., Lambrechts, N., Lindstedt, M., Millet, M., Martinozzi-Teissier, S., Natsch, A., Petersohn, D., Pike, I., Sakaguchi, H., Schepky, A., Tailhardat, M., Templier, M., van Vliet, E., Maxwell, G., 2015. Systematic evaluation of non-animal test methods for skin sensitisation safety assessment. Toxicol. in Vitro 29, 259–270.
Riedel, F., Aparicio-Soto, M., Curato, C., Münch, L., Abbas, A., Thierse, H.J., Peitsch, W. K., Luch, A., Siewert, K., 2022. Unique and common TCR repertoire features of Ni(2+) -, Co(2+) -, and Pd(2+) -specific human CD154 +CD4+ T cells. Allergy 78, 270–282.
Rohart, F., Gautier, B., Singh, A., Le Cao, K.A., 2017. mixOmics: an R package for 'omics feature selection and multiple data integration. PLoS Comput. Biol. 13, e1005752.
Rubic, T., Lametschwandtner, G., Jost, S., Hinteregger, S., Kund, J., Carballido-Perrig, N., Schwärzler, C., Junt, T., Voshol, H., Meingassner, J.G., Mao, X., Werner, G., Rot, A., Carballido, J.M., 2008. Triggering the succinate receptor GPR91 on dendritic cells enhances immunity. Nat. Immunol. 9, 1261–1269.
Rubić-Schneider, T., Carballido-Perrig, N., Regairaz, C., Raad, L., Jost, S., Rauld, C., Christen, B., Wieczorek, G., Kreutzer, R., Dawson, J., Lametschwandner, G., Littlewood-Evans, A., Carballido, J.M., 2017. GPR91 deficiency exacerbates allergic contact dermatitis while reducing arthritic disease in mice. Allergy 72, 444–452.
Sakai, R., 2015. dendsort: Modular Leaf Ordering Methods for Dendrogram Nodes. R Package Version 0.3. 3.
Samuelsen, J.T., Michelsen, V.B., Bruun, J.A., Dahl, J.E., Jensen, E., Ortengren, U., 2019. The dental monomer HEMA causes proteome changes in human THP-1 monocytes. J. Biomed. Mater. Res. A 107, 851–859.
Stepath, M., Zülch, B., Maghnouj, A., Schork, K., Turewicz, M., Eisenacher, M., Hahn, S., Sitek, B., Bracht, T., 2020. Systematic comparison of label-free, SILAC, and TMT techniques to study early adaption toward inhibition of EGFR signaling in the colorectal cancer cell line DiFi. J. Proteome Res. 19, 926–937.
Stephen, T., 2019. Tmisc: Turner Miscellaneous. R Package Version 0.1.22. https://CRAN.R-project.org/package=Tmisc.
Takenouchi, O., Miyazawa, M., Saito, K., Ashikaga, T., Sakaguchi, H., 2013. Predictive performance of the human cell line activation test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients. J. Toxicol. Sci. 38, 599–609.
Tannahill, G.M., Curtis, A.M., Adamik, J., Palsson-McDermott, E.M., McGettrick, A.F., Goel, G., Frezza, C., Bernard, N.J., Kelly, B., Foley, N.H., Zheng, L., Gardet, A., Tong, Z., Jany, S.S., Corr, S.C., Haneklaus, M., Caffrey, B.E., Pierce, K., Walmsley, S., Beasley, F.C., Cummins, E., Nizet, V., Whyte, M., Taylor, C.T., Lin, H., Masters, S.L., Gottlieb, E., Kelly, V.P., Clish, C., Auron, P.E., Xavier, R.J., O'Neill, L.A.J., 2013. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature 496, 238–242.
Wang, Z., Karkossa, I., Großkopf, H., Rolle-Kampczyk, U., Hackermüller, J., von Bergen, M., Schubert, K., 2020. Comparison of quantitation methods in proteomics to define relevant toxicological information on AhR activation of HepG2 cells by BaP. Toxicology 152652.
Wickham, H., 2007. Reshaping data with thereshapePackage. J. Stat. Softw. 21.
Wickham, H., 2011. The split-apply-combine strategy for data analysis. J. Stat. Softw. 40.
Williams, N.C., O'Neill, L.A.J., 2018. A role for the Krebs cycle intermediate citrate in metabolic reprogramming in innate immunity and inflammation. Front. Immunol. 9, 141.
Yeung, K., Mraz, V., Geisler, C., Skov, L., Bonefeld, C.M., 2021. The role of interleukin-1β in the immune response to contact allergens. Contact Dermatitis 85, 387–397.
Zasłona, Z., Pålsson-McDermott, E.M., Menon, D., Haneklaus, M., Flis, E., Prendeville, H., Corcoran, S.E., Peters-Golden, M., O'Neill, L.A.J., 2017. The induction of pro-IL-1β by lipopolysaccharide requires endogenous prostaglandin E(2) production. J. Immunol. 198, 3558–3564.
Zhang, X., Smits, A.H., van Tilburg, G.B., Ovaa, H., Huber, W., Vermeulen, M., 2018. Proteome-wide identification of ubiquitin interactions using UbIA-MS. Nat. Protoc. 13, 530–550.

7 Discussion

Skin sensitization poses a major challenge for economy and public health as well as individual well-being in Europe since approximately 20 - 27% in the general public are skin-sensitized (Diepgen et al., 2016b; Alinaghi et al., 2019). The occurrence of several contact allergen epidemics in the recent past (Thyssen et al., 2007) further emphasized how important correct prediction of chemical allergens is to prevent future outbreaks. This thesis provides novel insights into the cellular changes induced by skin sensitizers through the generation of comprehensive proteomic data sets in a human primary dendritic cell model (MoDCs) as well as a human cell line (THP-1) upon exposure to human relevant contact allergens ($NiSO_4$, DNCB, BQ, NBB) (Höper et al., 2017; Höper et al., 2021; Höper et al., 2023). Furthermore, the suitability of proteomic techniques for the investigation of cellular modes of action during skin sensitization and future applications are outlined.

7.1 Proteomics – not only a matter of generation of extensive data sets

Proteomics have been proven to be a useful tool to get deeper insights into the still largely elusive cellular mechanisms during skin sensitization (Mussotter et al., 2016; Strasser et al., 2017; Mussotter et al., 2018; Höper et al., 2021; Höper et al., 2023). In contrast to gene signatures, the proteome reflects the actual phenotype of an organism at a given time. MS-based techniques offer the advantage of highly sensitive qualitative detection coupled with quantification of proteins. In contrast to immunoassays such as enzyme-linked immunosorbent assays (ELISA), a large number of proteins can be detected at the same time, which increases sample throughput. Compared to ELISAs, the amount of material required and the cost per sample are lower for LC-MS/MS measurements, yet the initial costs for the mass spectrometer are higher. However, LC-MS/MS methods are generally equally or more sensitive and selective than ELISA-based methods. In particular, selectivity can be critical with ELISA, as it is highly dependent on the quality of the antibody and minor variances of the antigen (such as isoforms or post-translational modifications) often cannot be distinguished (Fuchs et al., 2011; Denburg et al., 2016; Dingess et al., 2021). Limits of detection of MS and ELISA were shown to be comparable for food allergens (Holzhauser et al., 2020) as well as for the detection of various clinical diagnosis markers (Fortin et al., 2009; Faupel-Badger et al., 2010; Dingess et al., 2021). Nguyen et al. (2019) showed that high-resolution targeted proteomics can be applied to detect attomoles of low abundant proteins like transcription factors and challenging membrane proteins at low cost and with little sample preparation effort. However, untargeted proteomic workflows are not designed to primarily detect low abundant proteins but rather serve the analysis of global protein changes. For the proteomic studies presented here, data-dependent acquisition was used. This term refers to a specific measuring mode at the mass spectrometer. Generally, data acquisition is subdivided in different cycles. Every cycle, all ions that reach the first quadrupole, are detected. The

mass spectrometer then choses the so-called ‘Top N’ ions for further fragmentation in the collision cell and detection of the fragment ions in the analyzer. We used a Top 10 approach meaning that the 10 most intense ions were automatically selected for further fragmentation. Subsequently, these *m/z* ratios are transferred to a temporary exclusion list and the mass spectrometer proceeds with fragmentation of the next Top 10 ion package. Fragmentation of serial Top 10 ion packages is continued until the cycle time has elapsed. Then, a new list of incoming ions is generated in the first quadrupole and gradually fragmented like during the first cycle. Hence, low abundant proteins are underrepresented in the generated data if the cycle time is shorter than the theoretical time needed for fragmentation of all incoming ions. Untargeted proteomics, as applied within this thesis, are also limited in terms of data analysis and interpretation. The complex mass spectrometry data require automated data analysis using curated data bases. This biases the data towards proteins that have already been identified in the past and are therefore listed in the data base. Identification of novel proteins does usually not occur within these workflows. Additionally, data interpretation with the help of software tools like Qiagen’s Ingenuity Pathway Analysis provides helpful guidance for data interpretation, yet they are restricted towards pathways, diseases, and adverse effects that have previously been described for a specific species, organ or cell type. Consequently, it obliges on the researcher in charge to scientifically evaluate and manually rework the data if necessary. Despite known limitations, the sensitive detection, price effectiveness and sample handling time underline the great potential of proteomics-based analytical methods for the sensitive detection of biomarkers.

Yet, apart from the GARD assay, omics-techniques are till today not commonly applied for regulatory purposes although transcriptomics, metabolomics, and proteomics likewise generate extensive data at low costs for a specific endpoint and the underlying mechanisms. The main reason for the poor acceptance can also be attributed to low standardization in the field of omics. Since omics are a fast-evolving field, data acquisition and processing parameters are subject to frequent changes and optimizations. Especially data processing is crucial for omics as one deals with a huge quantity of data which is highly affected by the bioinformatics applied and the curated data bases behind it. Furthermore, data reporting and storage have not been harmonized, yet. In Höper et al. (2023) we conducted a study to test the robustness and reproducibility of proteomic data by comparing two different quantitation methods: LFQ and SILAC. Aside from the introduction of stable isotope-labeled amino acids for SILAC quantification, samples were treated identically and also the MS method as well as data analysis was identical. We found that, a greater number of proteins was identified with LFQ quantification (2200 proteins versus 1997 proteins for SILAC), yet more significantly altered proteins were identified using SILAC (1660 proteins versus 815 proteins for LFQ). SILAC delivered more robust data in our experimental setup. This study illustrates well that modifications to a

proteomics workflow such as a changed quantitation strategy, may lead to differences in the number of regulated proteins, which of course also impacts downstream interpretation of data.

To overcome these challenges and in order to define best practices, several working groups have been established in the field. Already in 2002, the Human Protein Organization has founded the Proteomics Standard Initiative with a particular focus on data standardization (Orchard et al., 2003; Deutsch et al., 2017). Also, the OECD has established a framework program for omics-technologies in chemical testings. Based on the work of Harrill et al. (2021), the OECD has now published a guidance document and reporting framework for metabolomics and transcriptomics data in regulatory toxicology (OECD, 2022a). The framework is designed to facilitate assessment of data quality by providing information on study design and execution within the data report. However, data interpretation was not yet been included. It remains unclear why proteomics have been excluded from this framework program. However, it can be suspected that this is due to the nature of proteomics as it is difficult to scale the method up. Sample material is a limiting factor and cannot be multiplied as for example DNA. Furthermore, sample collection in patients is invasive. Mostly, biopsies of the tissue under investigation have to be taken in order to analyze proteomic changes. Metabolomic analyses in contrast are often done using body liquids e.g. blood serum which is usually much easier to obtain. The Human Metabolome Database for 2022 lists ~1700 endogenous human metabolites which have different levels of confidence including predicted as well as already experimentally quantified metabolites (Wishart et al., 2022). In contrast to this, ~21.000 protein-coding genes are annotated in the human reference proteome in the UniProt database whereas approximately 82.000 unique proteins have been described (UniProt Consortium, 2017). This illustrates the immense quantity of proteins in the human body which renders proteomics data complex for interpretation and biomarker identification.

7.2 Advantages and limitations of DC models

Besides technical considerations, the choice of the biological model under investigation is crucial. Due to the low occurrence of DCs in the skin and the blood, studies on human DC biology often rely on surrogate models such as cell lines or MoDCs. For the experimental work of this thesis, MoDCs and THP-1 cells were used to elucidate proteomic changes in these cells after treatment with selected contact allergens. The study using MoDCs as surrogates for dermal DCs has revealed $NiSO_4$-specific and thus potentially skin sensitization-specific protein changes in the cells (Höper et al., 2021). Bacterial and chemical activation were clearly distinct. Some of these findings could be verified in allergen-treated THP-1 cells (Höper et al., 2023). However, merely 50% of regulated proteins in MoDCs were also regulated in THP-1 cells. This raises the question of suitability of the two cell models used to investigate cellular mode of actions of skin sensitizing agents (Höper et al., 2023).

In the human body, different DC subsets exist (cf. section 4.4) challenging the researchers studying DCs in different contexts. Since the frequency of DCs is rather low in tissues and blood (Merad and Manz, 2009; Orsini et al., 2012), several DC models were established in order to cultivate and expand DCs *in vitro*. Manifold protocols serving this purpose exist and the cells can for example be obtained from bone marrow, from blood, and via differentiation of monocytes or pluripotent stem cells (Luo and Dalod, 2020). MoDCs are differentiated from isolated blood monocytes treated with a cytokine cocktail *in vitro* (Sallusto and Lanzavecchia, 1994). Since monocytes are progenitors for a wide range of cells including MoDC, monocyte-derived LCs and monocyte-derived macrophages, the phenotypic differentiation is dictated by the cellular microenvironment, i.e. the cytokines in the cell culture medium. The classical approach for the generation of MoDCs comprises GM-CSF and IL-4 induced maturation of blood monocytes (Sallusto and Lanzavecchia, 1994). Differentiation to monocyte-derived LCs is induced by minor alterations to the aforementioned protocol by using GM-CSF, IL-4, and additionally transforming growth factor beta 1β (Geissmann et al., 1998). As the differentiation of monocytes is sensitive to bias due to deviation in cytokine concentrations, generated cells thus require extensive characterization, to allow comparable scientific results. Another drawback of MoDC-generation is the restricted availability of the material, since blood samples are limited and can only be handled in laboratories with the respective biosecurity category. Also, donor variances have to be considered (Aiba et al., 1997; Rougier et al., 2000). For fundamental research, MoDCs have frequently been used, however for routine and high throughput testing limited availability and biological variance are exclusion criteria. For this reason, cell lines are frequently used for these applications.

7.3 THP-1 cells as DC model for regulatory testing of skin sensitizers

Skin sensitization is an adverse health effect that chemicals marketed in the EU are routinely tested for. The gold standard for skin sensitizer testing, the LLNA, is an animal experiment (OECD, 2010). The implementation of the 3R principle in scientific research has escalated the demand for non-animal test systems. The overarching goal is to develop and apply NAMs to generate reliable *in vitro* data that are able to picture the *in vivo* situation as precise as possible. One pillar of alternative *in vitro* systems is the use of cell culture systems carefully selected to meet the experimental criteria for the endpoint under investigation. Working with cell lines has some advantages over e.g. primary tissues or cells. Experiments with cell lines can be easily scaled up as sample material is usually not limited. Furthermore, the biological variation is low, which facilitates the identification of biological effects. However, since many cell lines have been genetically modified, e.g. immortalized, to make them easy to handle and multiply in the lab, they may possess alterations in metabolism and phenotype that differ from their primary counterpart (Bosshart and Heinzelmann, 2016).

For the work within this thesis, THP-1 cells were used to investigate allergen-induced proteomic changes. The use of THP-1 cells should be questioned as DC surrogate for the testing of sensitizing chemicals due to their monocytic character and leukemia background. The latter could potentially affect the suitability of THP-1 cells as DC model for assays with metabolic readout. For example, despite pronounced activation of metabolic pathways like pentose phosphate pathway and tricarboxylic acid (TCA) cycle, that are crucial for metabolic reprogramming in DCs, we were unable to detect significant induction of glycolysis in allergen-treated THP-1 cells on proteome level (Höper et al., 2023). Basal glycolytic levels of THP-1 cells were found to be the lowest among eight acute myeloid leukemia cells lines investigated by Erdem et al. (2022). The authors also described a pronounced proteomic upregulation of TCA cycle enzymes in THP-1 cells and glutaminolysis was proposed as major pathway sustaining high TCA activity in the cells (Erdem et al., 2022). Besides, Guak et al. (2018) showed that long-term upregulation of glycolysis is only achieved by strong DC stimuli like LPS. Weak stimuli were shown to induce glycolytic genes only shortly after exposure. Furthermore, DCs seem to sustain a basal OXPHOS activity, which enables switching from glycolysis to OXPHOS in glucose-deprived media. However, OXPHOS alone does not deliver sufficient energy for full maturation of the DCs (Guak et al., 2018). Likewise to DCs, monocytes undergo metabolic reprogramming upon activation, which is also characterized by a switch to glycolysis (Lachmandas et al., 2016). Yet, Lachmandas et al. (2016) demonstrated that also monocyte activation is not uniformly connected to a switch towards glycolysis and was also dependent on the stimuli itself. LPS-treated monocytes induce glycolysis in favor of OXPHOS whereas Pam3CysSK4 (P3C, a TLR2 ligand) was shown to induce both pathways (Lachmandas et al., 2016). This demonstrates that the extent of metabolic reprogramming is strongly dependent on the offending stimuli on the immune cells, making it difficult to determine a fixed signature of regulated proteins after activation of DCs. Despite Ni, the other allergens investigated here have not yet been reported to directly bind to TLRs which might be a reason for insufficient cellular activation and thus no long-term shift towards glycolysis. Although Ni directly binds to TLR4, we could already show in MoDCs that LPS is a more potent inducer of cellular activation compared to Ni (Höper et al., 2021). The different reprogramming regimes of MoDCs and THP-1 cells that support maturation of the cells make it delicate to identify metabolic activation marker that are allergen-specific. It could thus be helpful to test further allergens in MoDCs first and then validate these findings in THP-1 cells or other surrogate cell lines at a later time point.

In addition to different metabolic capacities, DCs and THP-1 cells differ also concerning alterations of cell surface marker expression and cytokine secretion upon treatment with contact allergens (Hitzler et al., 2013). In this comparative study, Hitzler et al. (2013) found significant differences for CD86 and HLA-DR expression and IL-8 secretion by MoDCs, THP-1, and MUTZ-3 cells. Based on the cell surface markers and cytokines analyzed within this study, MoDCs were found to be more sensitive

and predictive for the identification of skin sensitizers than THP-1 and MUTZ-3 cells (Hitzler et al., 2013). The group of van Helden et al. (2008) characterized multiple human and murine cell lines for their applicability as DC model. In this study MUTZ-3 cells clearly outperformed THP-1 cells as their immunological functions such as phenotype, antigen presentation, cytokine secretion, and migration resembled maturated MoDCs most closely. Bocchietto et al. (2007) compared THP-1 cells and MoDCs with regard of their suitability as *in vitro* test system. The expression of the costimulatory molecules CD80 and CD86 on the cell surface after treatment with extreme, strong, and medium sensitizers was assessed. MoDCs and THP-1 cells were found to perform similar and the authors thus concluded that THP-1 cells are a useful tool for the identification of extreme to moderate contact allergens. However, for discriminating weak sensitizers and irritants MoDCs are more sensitive. Other studies compared THP-1 cells with their primary counterparts. THP-1 cells were found to differ significantly on transcriptional and functional level from primary monocytes (Schildberger et al., 2013; Riddy et al., 2018; Liu et al., 2021). In general, THP-1 cells are considered to be easy to culture in a laboratory environment. However, significant performance variances in the hCLAT assay were reported for cells from four different biorepositories (Kosaka et al., 2008). More recently, Noronha et al. (2020) revealed genetic drift and phenotypic discrepancies among THP-1 cells from varying vendors.

In order to improve the predictability of allergens using THP-1 cells, multiple approaches were proposed in the past, e.g. the co-cultivation of THP-1 cells with keratinocytes, which was shown to improve immunological responses of THP-1 cells (Cao et al., 2012; Karri et al., 2021), detection of prohaptens (Hennen et al., 2011; Hennen and Blömeke, 2017) and activation of THP-1 cells by weak and moderate allergens (Galbiati et al., 2020). More recently, THP-1 cell activation was assessed in a co-culture model with human reconstructed epidermis (Schellenberger et al., 2019). Applying the hCLAT assay for the assessment of potential sensitizers relies on augmented CD54 and CD86 expression (Ashikaga et al., 2006a). However, the addition of further cellular markers as readouts, such as IL-1, IL-8 release, or HLA-DR expression, was shown to improve predictability of sensitizing chemicals in THP-1 cells (Takahashi et al., 2011; Corti et al., 2015; Iulini et al., 2020; Terui et al., 2021).

All these efforts were made to enhance the correct predictivity of THP-1 cells in *in vitro* assays for skin sensitization. Yet, none of the proposed ameliorations have been validated and incorporated in official OECD test guidelines. However, in light of genetic drift, vendor-specific biological responses as well as limited ability to correctly mirror DC biology, THP-1 cells should be carefully assessed in the future and be replaced by superior models as soon as available. At the moment, MUTZ-3 cells seem promising. Furthermore, differentiation of the THP-1 monocytes to immature THP-1-DCs before

treatment with skin sensitizers could overcome the known immunologic limitations of the cells. Hölken and Teusch (2023) developed a sophisticated differentiation protocol for the generation of iDCs and mDCs from THP-1 cells that express classical DC markers like CD54, CD86, CD83, and CD11b.

7.4 Cholesterol pathway

Altered lipid metabolism is crucial for DC maturation as it supports the reconstruction of the plasma membrane in order to express cell surface markers that serve immunological signaling. Besides, lipids can also be broken down to provide the cell with energy. Lipid class alterations in mature MoDCs have been characterized by Lühr et al. (2020). Among the lipids, cholesterol and its role during skin sensitization have emerged as interesting target for research. Cholesterol is essential for the organism since it is an integral part of the cell membrane and also serves as precursor for bile acids as well as steroid hormones. Cellular cholesterol levels are finely balanced by sterol response element binding proteins (SREBP) and liver X receptors (LXR). These two families of transcription factors have antithetical effects with SREBPs inducing cholesterol accumulation and LXRs cholesterol efflux. Especially altered cholesterol levels in cell membranes were described to affect DCs during response to xenobiotics leading to impaired cellular differentiation and maturation, antigen presentation, migration as well as priming of T cells (Wang et al., 2012). During DC maturation, the Golgi apparatus and endoplasmic reticulum are expanded to enable increased protein translation for the cellular reconstruction. Cholesterol plays a pivotal role during this process. Cholesterol is known to stabilize cell membranes by reducing their fluidity (Simons and Ikonen, 2000). Furthermore, perturbed cholesterol concentrations strongly influence the functionality of lipid rafts in the cell membrane. Since major immunological signaling receptors such as TLRs or MHCs are located there, receptor induced signaling like NFκB- or IFN-signaling is directly impacted by altered cholesterol levels (Hiltbold et al., 2003; Cheng et al., 2012; Wang et al., 2012; York et al., 2015). In DCs from apolipoprotein E-deficient mice, clearance of cholesterol from the cell membrane was found to be impaired (Bonacina et al., 2018). This resulted in lipid raft accumulation. As these rafts were found to possess higher MHC II contents, DCs showed a higher antigen presentation ability than DCs from wild type animals. In contrast, expression levels of costimulatory molecules like CD86 and CD80 were not altered (Bonacina et al., 2018). When treated with the strong skin sensitizer fluorescein isothiocyanate, apolipoprotein E deficiency resulted in a markedly decreased DC migration to lymph nodes which eventually culminated in diminished contact sensitization (Angeli et al., 2004). Despite its role as cellular building block for membranes, cholesterol also affects the cellular stress and hypoxia response as well as immunological signaling (Hughes et al., 2005). For instance, cholesterol was shown to play an important role in antiviral defense. Low cholesterol levels significantly hamper viral infection and replication in hepatitis C infections (Ye et al., 2003; Kapadia et al., 2007). During viral

defense, the oxysterol Ch25h is upregulated after TLR4 activation leading to increased production and secretion of 25-hydroxycholesterol (25HC) in macrophages. 25HC induces the down-regulation of SREBP2-dependent genes which results in reduced cholesterol biosynthesis. This prevents viral cell-to-cell spreading and inhibits viral replication after the virus has entered the cell (Blanc et al., 2011). Elevated 25HC levels negatively regulate the adaptive immune response through declining IgA production in B cells (Bauman et al., 2009). Oxysterols were also shown to promote a DC-like phenotype of THP-1 cells exposed to 7α-hydroxycholesterol or 27-hydroxycholesterol (Son et al., 2013).

In Höper et al. (2021) a pronounced alteration in cholesterol biosynthesis on protein level after treatment of MoDCs with the contact allergen $NiSO_4$ was described for the first time. Strikingly, these effects were not detected in LPS-treated cells (Höper et al., 2021). On gene level, the induction of selected genes relevant for cholesterol biosynthesis like *HMGCS1*, *DHCR7* and *CYP51A1* was already described to contribute to the discrimination of skin sensitizing chemicals from non-sensitizers and irritants in MUTZ-3 cells (Johansson et al., 2011a). Höper et al. (2021) were able to confirm these proteins and additional proteins linked to the cholesterol biosynthesis to be significantly upregulated in $NiSO_4$-treated MoDCs. Furthermore, cellular cholesterol levels were found to be diminished pointing to a high demand of cholesterol. Depletion of cellular cholesterol levels was also observed by Lühr et al. (2020) in maturated MoDCs. Lindberg et al. (2020) and de Ávila et al. (2022) also found proteins of the cholesterol biosynthetic pathway to be enriched in MUTZ-3 cells which were treated with pesticide formulations that were previously classified as skin sensitizers by the GARD assay. Recently, time-of-flight secondary ion mass spectrometry analysis of human skin biopsies exposed to metal allergens indeed revealed altered lipid composition of the affected skin. Ni^{2+}-induced changes were found to differ from changes induced by Co^{2+} and Cr^{3+} (Knox et al., 2022). In this study, Co^{2+} was reported to increase cholesterol levels in the stratum corneum of affected skin whereas Ni^{2+} seemed to reduce cholesterol in the epidermis (Knox et al., 2022). These differences were linked to the limited skin penetration capabilities of Ni^{2+} compared to the other metal allergens (Knox et al., 2022). Evidence for altered protein expression of cholesterol-related pathways in THP-1 was also found (Höper et al., 2023). For the treatment with $NiSO_4$, the exposure time as well as cell number and the final concentration of 400 µM $NiSO_4$ were adopted from the preliminary work with MoDCs. Yet, the cholesterol biosynthesis was found to be down-regulated in THP-1 cells after treatment with the four contact allergens investigated. Hence, the role of cholesterol biosynthesis during skin sensitization needs to be further investigated to potentially deduce mechanistic information.

The experimental results of this thesis have greatly contributed to the understanding of cellular mechanisms during skin sensitization. The pathway of cholesterol biosynthesis is thus, besides long-

known pathways like Nrf2-mediated oxidative stress response, an interesting target for studying DC activation and possibly identifying predictive protein markers for the *in vitro* assessment of skin sensitizers. The latter could then be implemented into *in vitro* testing guidelines for the determination of skin sensitizing chemicals. Although the scientific evidence for a central role of cholesterol during skin sensitization is substantiating, the fate of cholesterol and the underlying mechanisms are yet unresolved and multiple scientific approaches can be pursued to investigate fundamental mechanisms in more detail. A metabolomic or lipidomic study of the lipid class alterations during the course of skin sensitization would provide quantitative results that could aid mechanistic understanding. Despite cellular signaling pathways that can best be investigated by silencing of selected enzymes or receptors, cholesterol efflux should be confirmed by treatment with other contact allergens and could potentially also be explained by the secretion of extra cellular vesicles. Extracellular vesicles are important for cell-to-cell communication and can contribute to immunological signaling (Vallhov et al., 2015). However, their role during skin sensitization has not been elucidated, yet. Due to their small size and low weight, extracellular vesicles are not pelleted during classical centrifugation protocols for eukaryotic cell cultures e.g. 300 × g (Brennan et al., 2020). Thus, potential vesicles were washed away during preparation of the cells for cholesterol quantification (Höper et al., 2021). Extracellular vesicles can be retrieved by differential ultracentrifugation or other techniques as compiled by Clos-Sansalvador et al. (2022). Membrane marker like CD9, CD63, and CD81 can be used to identify extracellular vesicles by immunoblot or flow cytometry (Campos-Silva et al., 2019). After isolation, an extensive characterization of the extracellular vesicles using flow cytometry and proteomics would be a good starting point, which can later be followed by functional assays.

Overall, the pathway of cholesterol biosynthesis should be further investigated to ameliorate the mechanistic understanding of skin sensitization and optimize existing *in vitro* testing strategies for the assessment of skin sensitizers.

7.5 From Proteomics towards refinement of current *in vitro* testing strategies and beyond

The in chapter 4.6 mentioned animal-free test methods for skin sensitization have been incorporated into testing strategies. These test strategies have proven to largely mirror LLNA data in terms of sensitivity as well as accuracy and even outperform the LLNA in terms of specificity when comparing to a human data set (OECD, 2021). In research as well as regulation, the focus has now been set on advanced *in vitro* models to investigate and predict skin sensitization. Especially 3D skin models using human reconstituted skin are extremely valuable for risk assessment as they also allow to estimate a more realistic exposure scenario. The penetration properties of the chemical under investigation can be analyzed in parallel to the metabolism and the toxicological effects on different skin cells represented in the model (e.g. keratinocytes, fibroblasts, melanocytes). 3D skin models could also be

beneficial for the classification of prohaptens, lipophilic chemicals, mixtures or even nano materials that are difficult to assess or cannot be tested with the current test batteries. Reconstructed human epidermis models have already gained regulatory acceptance for testing the phototoxicity as well as skin corrosion potential of chemicals (OECD, 2019; 2023).

However, these models still have major limitations: the handling is delicate, they are expensive, and so far, there are no immune-competent models commercialized. In most approaches, immune cells like DCs are often co-cultured in the cell culture media of the skin models, while the skin models themselves are cultured on a membrane at the air-liquid interface (Schellenberger et al., 2019). Bock et al. (2018) succeeded in the incorporation of LCs derived from the cell line MUTZ-3 or primary monocytes into a human 3D skin model. After 14 days of cultivation, the models were treated with contact allergens and both Langerhans cell models displayed distinct functions like migration, cytokine release, and expression of relevant marker proteins. Again, the cells derived from primary monocytes outperformed the Langerhans cells with cell lineage background. Another way forward could be the use of immune-competent micro-physiological systems that have already been used to determine chemical toxicity and skin irritation (Ramadan and Ting, 2016; Wufuer et al., 2016; Lee et al., 2022).

Despite of the refinement of existing *in vitro* assays and development of possible therapies, proteomic data can also be used for computer-based prediction models for skin sensitization like, for example, the Bayesian integrated testing strategy proposed by Jaworska et al. (2013). This would require a set of high-quality proteomic data generated from treatment with a significant number of different contact allergens and controls. Based on triggered pathways and proteins, the models could then retrieve structure-related signatures which can be used for read-across for future predictions and assessments of skin sensitizers *in silico* (Alves et al., 2015; Chayawan et al., 2022; Golden et al., 2023). Of particular interest in this context are data from structurally closely related compounds that differ only by minor modifications of e.g. side chains. Kanazawa et al. (1999) and Roberts et al. (1988) have already uncovered, that the length of an alkyl side chain strongly impacts the sensitizing potential of chemicals. Furthermore, various chemical reaction mechanisms, by which haptenation of skin proteins occurs, were identified and investigated (Roberts et al., 2007a; Roberts et al., 2007b). Ferreira et al. (2018) showed that electrophilicity and the reaction mechanism potentially have an impact on the kinetics of cellular activation in allergen-treated THP-1 cells. Cellular ROS and therefore redox imbalance are crucial for activation of the cells upon exposure to contact allergens (Esser et al., 2012b). The highly thiol-reactive skin sensitizer 1-fluoro-2,4-dinitrobenzene induced this imbalance via glutathione-depletion much faster and with a different cellular mechanism than an amine-reactive allergen (Ferreira et al., 2018). Overall chemical reactivity is well reflected in the EC3 values of the LLNA (Roberts et al., 2007b). Yet, until today, it is not fully understood, whether the different reaction

mechanisms are somehow related to the potency of the allergens. Thus, by investigating a large set of contact sensitizers with a preferably multi-omics approach, structure-related information could be elucidated.

The greatest potential of the application of proteomics in ACD research may indeed be the use as diagnostic tool. Patients that report to doctors with unknown cause of skin eczema could be tested for ACD as can already be done for the differentiation of psoriasis and eczema based on NOS2 and CCL27 gene regulation (Quaranta et al., 2014b). Skin biopsies or blood samples can be analyzed and relevant data from the different cell types involved can be obtained by a targeted proteomic approach. However, this requires intensified research on biomarker identification. Once established, this biomarker screening could also be useful to discriminate between different skin diseases like atopic dermatitis, psoriasis, urticaria, and irritant and allergic contact dermatitis in one analytical measurement (Spiewak, 2023). For atopic dermatitis for example, biomarker signatures were identified by immunological methods which are always limited in the number of identified proteins and biased by antibody selection (Wang et al., 2017; Pavel et al., 2020). These signatures can be expanded with data derived from modern LC-MS/MS-based proteomic techniques as done by Winget et al. (2016), Leung et al. (2019) and Morelli et al. (2021).

Furthermore, proteomic data and omics data in general are extremely valuable for the development of precision medicine. The generation of extensive data sets are useful both for identification of molecular targets as well as for accompanying drug development and clinical studies (Ogulur et al., 2021). Proteomics was shown to harbor a great potential for identification of individual drug-development, especially in cancer therapy (Correa Rojo et al., 2021; Su et al., 2021). Precision medicine is a fast-emerging research field which has led to novel therapeutics for immunological disorders. Often, specific antibodies are developed after molecular targets were identified. Atopic dermatitis, for example, is a chronic inflammation of the skin. It manifests with severe itch and flares that can also weep. In patients with severe symptoms, administration of antibodies is now commonly employed to manage the disorder if conventional therapeutics like e.g. glucocorticoids or cyclosporine failed to substantially reduce the symptoms. These antibodies inhibit pathways and cytokines that are important for inflammatory T cell response like for example OX40, IL-4, and IL-13 and thereby alleviate clinical symptoms of atopic dermatitis (Kraft and Worm, 2017; Duggan, 2021; Guttman-Yassky et al., 2023). Whilst private people can easily manage ACD by simply reducing or avoiding to be exposed to the culprit chemical, occupational ACD may lead to repeated sick notifications and eventually unemployability. Occupational contact dermatitis (including ICD and ACD) accounts for approximately 80% of all occupational skin diseases which bears great economic impact on the industry (Chu et al.,

2020). Thus, development of proteomic based precision medicine entails relief for affected individuals as well as economic impact for employers.

In summary, the implementation of proteomics into regulatory testing for skin sensitization is at present unlikely in the near future as existing methods were designed to be simple and fast, thereby outperforming proteomics. Most OECD validated TGs focus on a single key event by testing in suitable cell models for specific gene induction or protein regulation. However, the implementation of advanced models for skin sensitization such as immunocompetent 3D skin models poses a great potential for the application of proteomics. This would enable researchers to assess changes in keratinocytes, DCs, and maybe even T cells at the same time. By doing so, regulatory testing for skin sensitizing chemicals would gain more mechanistic complexity and thereby also more significance.

8 Conclusion and outlook

The ability to distinguish sensitizers from non-sensitizing chemicals such as irritants or microbial activators is central for development and refinement of *in vitro* test procedures. The need for proteomic experiments is substantiated by limitations in current methods and the few available publications in the field, as identified by a systematic literature research (Höper et al., 2017). Proteomics are considered a suitable tool to complement transcriptomic data. Here, in a two-tiered approach, proteomic data from primary human cells exposed to the contact allergen Ni were mapped against data derived from cell lines exposed to Ni and three other allergens (BQ, DNCB, NBB).

The first study presented showed that the proteome of MoDCs activated with bacterial LPS significantly differed from contact allergen ($NiSO_4$) activation although agonistic binding through the TLR4 is involved in both cases (Höper et al., 2021). In contrast to LPS, $NiSO_4$ strongly induced HIF1α and Nrf2 signaling as well as cholesterol biosynthesis in human MoDCs. The latter has recently also emerged in other studies as an interesting target for mechanistic research to shed light on the underlying cellular mode of action in the context of skin sensitization. $NiSO_4$-treatment induced over 40 Nrf2-regulated proteins, confirming Nrf2 as a key player during skin sensitization. Therefore, Nrf2 activation should be considered as a hallmark during skin sensitization not only in keratinocytes but also in DCs.

To circumvent intricate sample acquisition and preparation for MoDCs, the MS-based methodology was then applied to the THP-1 cell line. In order to validate the MoDC findings in THP-1 cells, the latter were treated with $NiSO_4$ or three other potent sensitizers (Höper et al., 2023). THP-1 cells also reacted to the treatment with skin sensitizers with upregulation of the Nrf2-dependent stress response. Conversely, cholesterol biosynthesis was down-regulated and the TCA-cycle was strongly induced in THP-1. Overall, regulated proteins and pathways differed markedly between the two cell models but superordinate diseases and functions were triggered likewise. THP-1 cells were testified to be a limited model for MoDCs on global proteome level.

Based on the proteomic studies presented in this thesis (Höper et al., 2021; Höper et al., 2023) and already published transcriptomic data in the field (Johansson et al., 2013), it can be concluded that the search for single protein-based biomarkers for skin sensitization seems to be more challenging than the search for a biomarker signature. In order to identify suitable candidates for this signature, more chemicals have to be screened. This should include a larger number of skin sensitizers with different applicability domains as well as skin irritating and non-adverse controls. Since irritants fail to induce the adaptive immune response, regulated proteins in DCs that are linked to adaptive immunity are of particular interest as biomarkers for contact allergy. These markers can only be identified upon

detailed comparison of allergens with non-allergens. To ensure identification of biologically meaningful marker proteins, the biomarkers could furthermore be validated in skin biopsies from allergic patients. However, it is more important that an *in vitro* biomarker for skin sensitization serves the prediction of the *in vivo* situation accurately rather than verifying the marker *in vivo*.

Shotgun proteomics is an adequate tool to complement other omics-approaches for the in-depth characterization of tissues, cells and organisms. Furthermore, untargeted proteomics, as applied here, allows the elucidation of biological mechanisms by screening thousands of proteins at the same time. However, in the context of skin sensitization and enhancement of existing and future predictive regulatory *in vitro* assays, shotgun proteomics may not be feasible for routine screening of larger sets of chemicals. Hence, after identification of suitable proteome-based markers it would thus be eligible to establish targeted methods for routine screening. When setting-up an assay for specific proteins, a straight-forward option is to work antibody-based. With the help of specific antibodies, ELISA or flow cytometry assays can be designed. Especially flow cytometry-based readouts can easily be integrated into existing assay workflows like the hCLAT protocol. Another option is the establishment of targeted proteomics workflows. These assays may comprise immunoprecipitation of target proteins with antibodies. The antibodies are typically bound to magnetic beads which allows removal of interfering matrix proteins through washing steps. After tryptic proteolysis of the proteins, peptides are quantified with the help of stable isotope-labeled standards. Due to the enrichment of the target proteins and reduction of matrix signals, the separation of the peptides via liquid chromatography can be conducted using significantly higher flow rates and shorter gradients compared to the commonly used nano flow liquid chromatography for shotgun proteomics. With regard to instrumentation, shotgun proteomics are mostly performed on high resolution mass spectrometers like Orbitraps. However, targeted proteomics are also well suited for other mass spectrometers like triple quadrupole devices. Targeted proteomics significantly reduce the number of recorded spectra and thus facilitates data analysis work flows. In summary, mass spectrometry-based proteomics harbor a great potential for the elucidation of cellular mode of actions as well as regulated protein biomarkers involved in skin sensitization and targeted testing strategies for routine assessment of skin sensitizers.

The OCED's AOP on skin sensitization and the available test guidelines are astonishingly well able to predict most skin sensitizers *in vitro*. Despite this high correlation with the *in vivo* situation, several challenges remain including limited applicability domains, missing procedures for the assessment of products, mixtures and novel nano- or advanced materials. To complement existing methods and for a better understanding of pathomechanisms, proteomic-based approaches offer new and promising solutions, as outlined in this thesis.

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10 References

Adamik, J., Munson, P.V., Hartmann, F.J., Combes, A.J., Pierre, P., Krummel, M.F., Bendall, S.C., Argüello, R.J., and Butterfield, L.H. (2022). Distinct metabolic states guide maturation of inflammatory and tolerogenic dendritic cells. *Nat Commun* 13**,** 5184.

Ade, N., Antonios, D., Kerdine-Romer, S., Boisleve, F., Rousset, F., and Pallardy, M. (2007). NF-kappaB plays a major role in the maturation of human dendritic cells induced by NiSO(4) but not by DNCB. *Toxicol Sci* 99**,** 488-501.

Ade, N., Leon, F., Pallardy, M., Peiffer, J.L., Kerdine-Romer, S., Tissier, M.H., Bonnet, P.A., Fabre, I., and Ourlin, J.C. (2009). HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: Role of the Keap1/Nrf2 pathway. *Toxicological Sciences* 107**,** 451-460.

Agner, T., Johansen, J.D., Overgaard, L., Vølund, A., Basketter, D., and Menné, T. (2002). Combined effects of irritants and allergens. Synergistic effects of nickel and sodium lauryl sulfate in nickel- sensitized individuals. *Contact Dermatitis* 47**,** 21-26.

Ahlstrom, M.G., Thyssen, J.P., Menne, T., and Johansen, J.D. (2017). Prevalence of nickel allergy in Europe following the EU Nickel Directive - a review. *Contact Dermatitis* 77**,** 193-200.

Ahrens, S., Zelenay, S., Sancho, D., Hanč, P., Kjær, S., Feest, C., Fletcher, G., Durkin, C., Postigo, A., Skehel, M., Batista, F., Thompson, B., Way, M., Reis e Sousa, C., and Schulz, O. (2012). F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. *Immunity* 36**,** 635-645.

Aiba, S., Manome, H., Nakagawa, S., Mollah, Z.U., Mizuashi, M., Ohtani, T., Yoshino, Y., and Tagami, H. (2003). p38 Mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, NiCl2 and 2,4-dinitrochlorobenzene. *J Invest Dermatol* 120**,** 390-399.

Aiba, S., Terunuma, A., Manome, H., and Tagami, H. (1997). Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *European Journal of Immunology* 27**,** 3031-3038.

Aleksic, M., Pease, C.K., Basketter, D.A., Panico, M., Morris, H.R., and Dell, A. (2007). Investigating protein haptenation mechanisms of skin sensitisers using human serum albumin as a model protein. *Toxicology in Vitro* 21**,** 723-733.

Alinaghi, F., Bennike, N.H., Egeberg, A., Thyssen, J.P., and Johansen, J.D. (2019). Prevalence of contact allergy in the general population: A systematic review and meta-analysis. *Contact Dermatitis* 80**,** 77-85.

Alves, V.M., Muratov, E., Fourches, D., Strickland, J., Kleinstreuer, N., Andrade, C.H., and Tropsha, A. (2015). Predicting chemically-induced skin reactions. Part II: QSAR models of skin permeability and the relationships between skin permeability and skin sensitization. *Toxicol Appl Pharmacol* 284**,** 273-280.

Anderson, S.E., Long, C., and Dotson, G.S. (2017). OCCUPATIONAL ALLERGY. *European Medical Journal (Chelmsford, England)* 2**,** 65-71.

Angeli, V., Llodra, J., Rong, J.X., Satoh, K., Ishii, S., Shimizu, T., Fisher, E.A., and Randolph, G.J. (2004). Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. *Immunity* 21**,** 561-574.

Aparicio-Soto, M., Riedel, F., Leddermann, M., Bacher, P., Scheffold, A., Kuhl, H., Timmermann, B., Chudakov, D.M., Molin, S., Worm, M., Heine, G., Thierse, H.J., Luch,

A., and Siewert, K. (2020). TCRs with segment TRAV9-2 or a CDR3 histidine are overrepresented among nickel-specific CD4+ T cells. *Allergy* 75**,** 2574-2586.

Api, A.M., Parakhia, R., O'Brien, D., and Basketter, D.A. (2017). Fragrances Categorized According to Relative Human Skin Sensitization Potency. *Dermatitis* 28**,** 299-307.

Ashikaga, T., Sakaguchi, H., Sono, S., Kosaka, N., Ishikawa, M., Nukada, Y., Miyazawa, M., Ito, Y., Nishiyama, N., and Itagaki, H. (2010). A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). *Altern Lab Anim* 38**,** 275-284.

Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama, K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., and Toyoda, H. (2006a). Development of an in vitro skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol. *Toxicol In Vitro* 20**,** 767-773.

Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama, K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., and Toyoda, H. (2006b). Development of an in vitro skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol. *Toxicol In Vitro* 20.

Azam, P., Peiffer, J.L., Chamousset, D., Tissier, M.H., Bonnet, P.A., Vian, L., Fabre, I., and Ourlin, J.C. (2006). The cytokine-dependent MUTZ-3 cell line as an in vitro model for the screening of contact sensitizers. *Toxicol Appl Pharmacol* 212**,** 14-23.

Babelova, A., Moreth, K., Tsalastra-Greul, W., Zeng-Brouwers, J., Eickelberg, O., Young, M.F., Bruckner, P., Pfeilschifter, J., Schaefer, R.M., Gröne, H.J., and Schaefer, L. (2009). Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. *J Biol Chem* 284**,** 24035-24048.

Balato, A., Balato, N., Di Costanzo, L., and Ayala, F. (2008). Contact sensitization of older patients in an academic department in Naples, Italy. *Dermatitis* 19**,** 209-212.

Bangsgaard, N., Zachariae, C., Menné, T., and Skov, L. (2011). Lack of effect of ustekinumab in treatment of allergic contact dermatitis. *Contact Dermatitis* 65**,** 227-230.

Basit, F., Mathan, T., Sancho, D., and de Vries, I.J.M. (2018). Human Dendritic Cell Subsets Undergo Distinct Metabolic Reprogramming for Immune Response. *Frontiers in Immunology* 9.

Basketter, D.A., Scholes, E.W., and Kimber, I. (1994). The performance of the local lymph node assay with chemicals identified as contact allergens in the human maximization test. *Food and Chemical Toxicology* 32**,** 543-547.

Bauman, D.R., Bitmansour, A.D., McDonald, J.G., Thompson, B.M., Liang, G., and Russell, D.W. (2009). 25-Hydroxycholesterol secreted by macrophages in response to Toll-like receptor activation suppresses immunoglobulin A production. *Proc Natl Acad Sci U S A* 106**,** 16764-16769.

Belsito, D., Wilson, D.C., Warshaw, E., Fowler, J., Ehrlich, A., Anderson, B., Strober, B.E., Willetts, J., and Rutledge, E.S. (2006). A prospective randomized clinical trial of 0.1% tacrolimus ointment in a model of chronic allergic contact dermatitis. *J Am Acad Dermatol* 55**,** 40-46.

Bennett, M.F., Lowney, A.C., and Bourke, J.F. (2016). A study of occupational contact dermatitis in the pharmaceutical industry. *Br J Dermatol* 174**,** 654-656.

Bergal, M., Puginier, M., Gerbeix, C., Groux, H., Roso, A., Cottrez, F., and Milius, A. (2020). In vitro testing strategy for assessing the skin sensitizing potential of "difficult to test" cosmetic ingredients. *Toxicol In Vitro* 65**,** 104781.

Bergström, M.A., Ott, H., Carlsson, A., Neis, M., Zwadlo-Klarwasser, G., Jonsson, C.A., Merk, H.F., Karlberg, A.T., and Baron, J.M. (2007). A skin-like cytochrome P450 cocktail

activates prohaptens to contact allergenic metabolites. *J Invest Dermatol* 127**,** 1145-1153.

Blanc, M., Hsieh, W.Y., Robertson, K.A., Watterson, S., Shui, G., Lacaze, P., Khondoker, M., Dickinson, P., Sing, G., Rodriguez-Martin, S., Phelan, P., Forster, T., Strobl, B., Muller, M., Riemersma, R., Osborne, T., Wenk, M.R., Angulo, A., and Ghazal, P. (2011). Host defense against viral infection involves interferon mediated down-regulation of sterol biosynthesis. *PLoS Biol* 9**,** e1000598.

Bocchietto, E., Paolucci, C., Breda, D., Sabbioni, E., and Burastero, S.E. (2007). Human monocytoid THP-1 cell line versus monocyte-derived human immature dendritic cells as in vitro models for predicting the sensitising potential of chemicals. *Int J Immunopathol Pharmacol* 20**,** 259-265.

Bock, S., Said, A., Müller, G., Schäfer-Korting, M., Zoschke, C., and Weindl, G. (2018). Characterization of reconstructed human skin containing Langerhans cells to monitor molecular events in skin sensitization. *Toxicology in Vitro* 46**,** 77-85.

Bonacina, F., Coe, D., Wang, G., Longhi, M.P., Baragetti, A., Moregola, A., Garlaschelli, K., Uboldi, P., Pellegatta, F., Grigore, L., Da Dalt, L., Annoni, A., Gregori, S., Xiao, Q., Caruso, D., Mitro, N., Catapano, A.L., Marelli-Berg, F.M., and Norata, G.D. (2018). Myeloid apolipoprotein E controls dendritic cell antigen presentation and T cell activation. *Nat Commun* 9**,** 3083.

Bordignon, V., Palamara, F., Altomonte, G., Sperduti, I., Pietravalle, M., Cavallotti, C., Cordiali-Fei, P., Fuggetta, M.P., Cristaudo, A., and Ensoli, F. (2015). A laboratory test based on determination of cytokine profiles: a promising assay to identify exposition to contact allergens and predict the clinical outcome in occupational allergic contact dermatitis. *BMC Immunol* 16**,** 4.

Bordignon, V., Palamara, F., Cordiali-Fei, P., Vento, A., Aiello, A., Picardo, M., Ensoli, F., and Cristaudo, A. (2008). Nickel, palladium and rhodium induced IFN-gamma and IL-10 production as assessed by in vitro ELISpot-analysis in contact dermatitis patients. *BMC Immunol* 9**,** 19.

Bosshart, H., and Heinzelmann, M. (2016). THP-1 cells as a model for human monocytes. *Ann Transl Med* 4**,** 438.

Brans, R., Jakasa, I., Goc, S., John, S.M., and Kezic, S. (2021). Stratum corneum levels of inflammatory mediators and natural moisturizing factor in patch test reactions to thiurams and fragrances and their possible role in discrimination between irritant and allergic reactions to hapten mixtures. *Contact Dermatitis* 84**,** 299-307.

Brennan, K., Martin, K., FitzGerald, S.P., O'Sullivan, J., Wu, Y., Blanco, A., Richardson, C., and Mc Gee, M.M. (2020). A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. *Scientific Reports* 10**,** 1039.

Buckley, D.A., Rycroft, R.J., White, I.R., and McFadden, J.P. (2003). The frequency of fragrance allergy in patch-tested patients increases with their age. *Br J Dermatol* 149**,** 986-989.

Campos-Silva, C., Suárez, H., Jara-Acevedo, R., Linares-Espinós, E., Martinez-Piñeiro, L., Yáñez-Mó, M., and Valés-Gómez, M. (2019). High sensitivity detection of extracellular vesicles immune-captured from urine by conventional flow cytometry. *Scientific Reports* 9**,** 2042.

Cao, Y.-p., Ma, P.-c., Liu, W.-d., Zhou, W.-q., Tao, Y., Zhang, M.-l., Li, L.-j., and Chen, Z.-y. (2012). Evaluation of the skin sensitization potential of chemicals in THP-1/keratinocyte co-cultures. *Immunopharmacology and Immunotoxicology* 34**,** 196-204.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Durand, I., Cella, M., Lanzavecchia, A., and Banchereau, J. (1997). CD34+ Hematopoietic Progenitors From Human Cord Blood Differentiate Along Two Independent Dendritic Cell Pathways in Response to Granulocyte-Macrophage Colony-Stimulating Factor Plus Tumor Necrosis Factor α: II. Functional Analysis. *Blood* 90**,** 1458-1470.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., and Banchereau, J. (1994). Activation of human dendritic cells through CD40 cross-linking. *J Exp Med* 180**,** 1263-1272.

Chayawan, Selvestrel, G., Baderna, D., Toma, C., Caballero Alfonso, A.Y., Gamba, A., and Benfenati, E. (2022). Skin sensitization quantitative QSAR models based on mechanistic structural alerts. *Toxicology* 468**,** 153111.

Cheng, A.M., Handa, P., Tateya, S., Schwartz, J., Tang, C., Mitra, P., Oram, J.F., Chait, A., and Kim, F. (2012). Apolipoprotein A-I attenuates palmitate-mediated NF-kappaB activation by reducing Toll-like receptor-4 recruitment into lipid rafts. *PLoS One* 7**,** e33917.

Cheng, J., and Zug, K.A. (2014). Fragrance allergic contact dermatitis. *Dermatitis* 25**,** 232-245.

Chipalkatti, N., Lee, N., Zancanaro, P., Dumont, N., Kachuk, C., and Rosmarin, D. (2019). A retrospective review of dupilumab for atopic dermatitis patients with allergic contact dermatitis. *J Am Acad Dermatol* 80**,** 1166-1167.

Choi, Y.J., Byun, J.Y., Choi, Y.W., Roh, J.Y., and Choi, H.Y. (2023). Analysis of Positive Patch Test Allergens in Allergic Contact Dermatitis Patients with Atopic Dermatitis. *Ann Dermatol* 35**,** 303-312.

Chu, C., Marks, J.G., Jr., and Flamm, A. (2020). Occupational Contact Dermatitis: Common Occupational Allergens. *Dermatol Clin* 38**,** 339-349.

Clos-Sansalvador, M., Monguió-Tortajada, M., Roura, S., Franquesa, M., and Borràs, F.E. (2022). Commonly used methods for extracellular vesicles' enrichment: Implications in downstream analyses and use. *European Journal of Cell Biology* 101**,** 151227.

Collier, T.S., Sarkar, P., Franck, W.L., Rao, B.M., Dean, R.A., and Muddiman, D.C. (2010). Direct Comparison of Stable Isotope Labeling by Amino Acids in Cell Culture and Spectral Counting for Quantitative Proteomics. *Analytical Chemistry* 82**,** 8696-8702.

Correa Rojo, A., Heylen, D., Aerts, J., Thas, O., Hooyberghs, J., Ertaylan, G., and Valkenborg, D. (2021). Towards Building a Quantitative Proteomics Toolbox in Precision Medicine: A Mini-Review. *Frontiers in Physiology* 12.

Corti, D., Galbiati, V., Gatti, N., Marinovich, M., Galli, C.L., and Corsini, E. (2015). Optimization of the THP-1 activation assay to detect pharmaceuticals with potential to cause immune mediated drug reactions. *Toxicology in Vitro* 29**,** 1339-1349.

Cottrez, F., Boitel, E., Auriault, C., Aeby, P., and Groux, H. (2015). Genes specifically modulated in sensitized skins allow the detection of sensitizers in a reconstructed human skin model. Development of the SENS-IS assay. *Toxicology in Vitro* 29**,** 787-802.

Coutant, K.D., De Fraissinette, A.B.D., Cordier, A., and Ulrich, P. (1999). Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. *Toxicological Sciences* 52**,** 189-198.

Coyle, A.J., and Gutierrez-Ramos, J.-C. (2001). The expanding B7 superfamily: Increasing complexity in costimulatory signals regulating T cell function. *Nature Immunology* 2**,** 203-209.

Cumberbatch, M., Scott, R.C., Basketter, D.A., Scholes, E.W., Hilton, J., Dearman, R.J., and Kimber, I. (1993). Influence of sodium lauryl sulphate on 2,4-dinitrochlorobenzene-induced lymph node activation. *Toxicology* 77**,** 181-191.

Curato, C., Aparicio-Soto, M., Riedel, F., Wehl, I., Basaran, A., Abbas, A., Thierse, H.J., Luch, A., and Siewert, K. (2022). Frequencies and TCR Repertoires of Human 2,4,6-Trinitrobenzenesulfonic Acid-specific T Cells. *Front Toxicol* 4**,** 827109.

de Ávila, R.I., Carreira Santos, S., Siino, V., Levander, F., Lindstedt, M., and Zeller, K.S. (2022). Adjuvants in fungicide formulations can be skin sensitizers and cause different types of cell stress responses. *Toxicol Rep* 9**,** 2030-2041.

De Rentiis, A.M.A., Pink, M., Verma, N., and Schmitz-Spanke, S. (2021). Assessment of the different skin sensitization potentials of irritants and allergens as single substances and in combination using the KeratinoSens assay. *Contact Dermatitis* 84**,** 317-325.

De Smedt, A.C.A., Van Den Heuvel, R.L., Zwi Berneman, N., and Schoeters, G.E.R. (2001). Modulation of phenotype, cytokine production and stimulatory function of CD34+-derived DC by NiCl2 and SDS. *Toxicology in Vitro* 15**,** 319-325.

Denburg, M.R., Hoofnagle, A.N., Sayed, S., Gupta, J., de Boer, I.H., Appel, L.J., Durazo-Arvizu, R., Whitehead, K., Feldman, H.I., and Leonard, M.B. (2016). Comparison of Two ELISA Methods and Mass Spectrometry for Measurement of Vitamin D-Binding Protein: Implications for the Assessment of Bioavailable Vitamin D Concentrations Across Genotypes. *J Bone Miner Res* 31**,** 1128-1136.

Deutsch, E.W., Orchard, S., Binz, P.-A., Bittremieux, W., Eisenacher, M., Hermjakob, H., Kawano, S., Lam, H., Mayer, G., Menschaert, G., Perez-Riverol, Y., Salek, R.M., Tabb, D.L., Tenzer, S., Vizcaíno, J.A., Walzer, M., and Jones, A.R. (2017). Proteomics Standards Initiative: Fifteen Years of Progress and Future Work. *Journal of Proteome Research* 16**,** 4288-4298.

Deza, G., and Gimenez-Arnau, A.M. (2017). Allergic contact dermatitis in preservatives: current standing and future options. *Curr Opin Allergy Clin Immunol* 17**,** 263-268.

Dhingra, N., Shemer, A., Correa da Rosa, J., Rozenblit, M., Fuentes-Duculan, J., Gittler, J.K., Finney, R., Czarnowicki, T., Zheng, X., Xu, H., Estrada, Y.D., Cardinale, I., Suárez-Fariñas, M., Krueger, J.G., and Guttman-Yassky, E. (2014). Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response. *J Allergy Clin Immunol* 134**,** 362-372.

Dickel, H., and Mahler, V. (2020). Leitliniengerechte Diagnostik der Kontaktallergie in der Praxis. *Der Hautarzt* 71**,** 182-189.

Diepgen, T.L., Naldi, L., Bruze, M., Cazzaniga, S., Schuttelaar, M.-L., Elsner, P., Goncalo, M., Ofenloch, R., and Svensson, Å. (2016a). Prevalence of Contact Allergy to p-Phenylenediamine in the European General Population. *Journal of Investigative Dermatology* 136**,** 409-415.

Diepgen, T.L., Ofenloch, R.F., Bruze, M., Bertuccio, P., Cazzaniga, S., Coenraads, P.J., Elsner, P., Goncalo, M., Svensson, A., and Naldi, L. (2016b). Prevalence of contact allergy in the general population in different European regions. *Br J Dermatol* 174**,** 319-329.

Dingess, K.A., van Dam, P., Zhu, J., Mank, M., Knipping, K., Heck, A.J.R., and Stahl, B. (2021). Optimization of a human milk-directed quantitative sIgA ELISA method substantiated by mass spectrometry. *Anal Bioanal Chem* 413**,** 5037-5049.

Divkovic, M., Pease, C.K., Gerberick, G.F., and Basketter, D.A. (2005). Hapten-protein binding: from theory to practical application in the in vitro prediction of skin sensitization. *Contact Dermatitis* 53**,** 189-200.

Duggan, S. (2021). Tralokinumab: First Approval. *Drugs* 81**,** 1657-1663.

Dyring-Andersen, B., Skov, L., Løvendorf, M.B., Bzorek, M., Søndergaard, K., Lauritsen, J.P., Dabelsteen, S., Geisler, C., and Bonefeld, C.M. (2013). CD4(+) T cells producing interleukin (IL)-17, IL-22 and interferon-γ are major effector T cells in nickel allergy. *Contact Dermatitis* 68**,** 339-347.

EC (2009). Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *Official Journal of the European Union* L 342/59-209.

El Ali, Z., Gerbeix, C., Hemon, P., Esser, P.R., Martin, S.F., Pallardy, M., and Kerdine-Römer, S. (2013). Allergic skin inflammation induced by chemical sensitizers is controlled by the transcription factor Nrf2. *Toxicol Sci* 134**,** 39-48.

EMA (2015). Guideline on non-clinical local tolerance testing of medicinal products. EMA/CHMP/SWP/2145/2000 Rev. 1, Corr. 1*.

Emter, R., Ellis, G., and Natsch, A. (2010). Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. *Toxicology and Applied Pharmacology* 245**,** 281-290.

Erdem, A., Marin, S., Pereira-Martins, D.A., Cortés, R., Cunningham, A., Pruis, M.G., de Boer, B., van den Heuvel, F.A.J., Geugien, M., Wierenga, A.T.J., Brouwers-Vos, A.Z., Rego, E.M., Huls, G., Cascante, M., and Schuringa, J.J. (2022). The Glycolytic Gatekeeper PDK1 defines different metabolic states between genetically distinct subtypes of human acute myeloid leukemia. *Nat Commun* 13**,** 1105.

Esser, P.R., Kimber, I., and Martin, S.F. (2014). "Correlation of Contact Sensitizer Potency with T Cell Frequency and TCR Repertoire Diversity," in *T Lymphocytes as Tools in Diagnostics and Immunotoxicology,* ed. S.F. Martin. (Basel: Springer Basel), 101-114.

Esser, P.R., Wölfle, U., Dürr, C., von Loewenich, F.D., Schempp, C.M., Freudenberg, M.A., Jakob, T., and Martin, S.F. (2012a). Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PloS one* 7**,** e41340-e41340.

Esser, P.R., Wölfle, U., Dürr, C., von Loewenich, F.D., Schempp, C.M., Freudenberg, M.A., Jakob, T., and Martin, S.F. (2012b). Contact Sensitizers Induce Skin Inflammation via ROS Production and Hyaluronic Acid Degradation. *PLOS ONE* 7**,** e41340.

European Commission (2006). Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. *Official Journal of the European Union* L 396/1–849.

European Commission (2008). Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. *Official Journal of the European Union* L 53/1–1355.

European Commission (2020). Report on the statistics on the use of animals for scientific purposes in the Member States of the European Union in 2015-2017. *Official Journal of the European Union* COM/2020/16 final.

European Commission (2021). Summary Report on the statistics on the use of animals for scientific purposes in the Member States of the European Union and Norway in 2018. *Official Journal of the European Union* SWD(2021)204.

Everts, B., Amiel, E., Huang, S.C., Smith, A.M., Chang, C.H., Lam, W.Y., Redmann, V., Freitas, T.C., Blagih, J., van der Windt, G.J., Artyomov, M.N., Jones, R.G., Pearce,

E.L., and Pearce, E.J. (2014). TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. *Nat Immunol* 15**,** 323-332.

Everts, B., Amiel, E., van der Windt, G.J., Freitas, T.C., Chott, R., Yarasheski, K.E., Pearce, E.L., and Pearce, E.J. (2012). Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood* 120**,** 1422-1431.

Faupel-Badger, J.M., Fuhrman, B.J., Xu, X., Falk, R.T., Keefer, L.K., Veenstra, T.D., Hoover, R.N., and Ziegler, R.G. (2010). Comparison of liquid chromatography-tandem mass spectrometry, RIA, and ELISA methods for measurement of urinary estrogens. *Cancer Epidemiol Biomarkers Prev* 19**,** 292-300.

Ferreira, G.B., Vanherwegen, A.S., Eelen, G., Gutiérrez, A.C.F., Van Lommel, L., Marchal, K., Verlinden, L., Verstuyf, A., Nogueira, T., Georgiadou, M., Schuit, F., Eizirik, D.L., Gysemans, C., Carmeliet, P., Overbergh, L., and Mathieu, C. (2015). Vitamin D3 Induces Tolerance in Human Dendritic Cells by Activation of Intracellular Metabolic Pathways. *Cell Rep* 10**,** 711-725.

Ferreira, I., Silva, A., Martins, J.D., Neves, B.M., and Cruz, M.T. (2018). Nature and kinetics of redox imbalance triggered by respiratory and skin chemical sensitizers on the human monocytic cell line THP-1. *Redox Biol* 16**,** 75-86.

Fitzpatrick, J.M., Roberts, D.W., and Patlewicz, G. (2017). What determines skin sensitization potency: Myths, maybes and realities. The 500 molecular weight cut-off: An updated analysis. *J Appl Toxicol* 37**,** 105-116.

Fortin, T., Salvador, A., Charrier, J.P., Lenz, C., Lacoux, X., Morla, A., Choquet-Kastylevsky, G., and Lemoine, J. (2009). Clinical quantitation of prostate-specific antigen biomarker in the low nanogram/milliliter range by conventional bore liquid chromatography-tandem mass spectrometry (multiple reaction monitoring) coupling and correlation with ELISA tests. *Mol Cell Proteomics* 8**,** 1006-1015.

Fortino, V., Wisgrill, L., Werner, P., Suomela, S., Linder, N., Jalonen, E., Suomalainen, A., Marwah, V., Kero, M., Pesonen, M., Lundin, J., Lauerma, A., Aalto-Korte, K., Greco, D., Alenius, H., and Fyhrquist, N. (2020). Machine-learning-driven biomarker discovery for the discrimination between allergic and irritant contact dermatitis. *Proc Natl Acad Sci U S A* 117**,** 33474-33485.

Fransen, M., Overgaard, L.E.K., Johansen, J.D., and Thyssen, J.P. (2018). Contact allergy to lanolin: temporal changes in prevalence and association with atopic dermatitis. *Contact Dermatitis* 78**,** 70-75.

Freudenberg, M.A., Esser, P.R., Jakob, T., Galanos, C., and Martin, S.F. (2009). Innate and adaptive immune responses in contact dermatitis: analogy with infections. *G Ital Dermatol Venereol* 144.

Fuchs, S.M., Krajewski, K., Baker, R.W., Miller, V.L., and Strahl, B.D. (2011). Influence of Combinatorial Histone Modifications on Antibody and Effector Protein Recognition. *Current Biology* 21**,** 53-58.

Funch, A.B., Mraz, V., Gadsbøll, A.-S.Ø., Jee, M.H., Weber, J.F., Ødum, N., Woetmann, A., Johansen, J.D., Geisler, C., and Bonefeld, C.M. (2022). CD8+ tissue-resident memory T cells recruit neutrophils that are essential for flare-ups in contact dermatitis. *Allergy* 77**,** 513-524.

Gaglia, J.L., Greenfield, E.A., Mattoo, A., Sharpe, A.H., Freeman, G.J., and Kuchroo, V.K. (2000). Intercellular Adhesion Molecule 1 Is Critical for Activation of CD28-Deficient T Cells1. *The Journal of Immunology* 165**,** 6091-6098.

Galbiati, V., Maddalon, A., Iulini, M., Marinovich, M., and Corsini, E. (2020). Human keratinocytes and monocytes co-culture cell system: An important contribution for the study of moderate and weak sensitizers. *Toxicology in Vitro* 68**,** 104929.

Gamerdinger, K., Moulon, C., Karp, D.R., Van Bergen, J., Koning, F., Wild, D., Pflugfelder, U., and Weltzien, H.U. (2003). A new type of metal recognition by human T cells: contact residues for peptide-independent bridging of T cell receptor and major histocompatibility complex by nickel. *J Exp Med* 197**,** 1345-1353.

Geissmann, F., Prost, C., Monnet, J.P., Dy, M., Brousse, N., and Hermine, O. (1998). Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J Exp Med* 187**,** 961-966.

Gendrisch, F., Völkel, L., Fluck, M., Apostolova, P., Zeiser, R., Jakob, T., Martin, S.F., and Esser, P.R. (2022). IRE1 and PERK signaling regulates inflammatory responses in a murine model of contact hypersensitivity. *Allergy* 77**,** 966-978.

Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W., and Lepoittevin, J.P. (2004). Development of a peptide reactivity assay for screening contact allergens. *Toxicological Sciences* 81**,** 332-343.

Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., Quintal, L., Sekut, L., Talanian, R., Paskind, M., Wong, W., Kamen, R., Tracey, D., and Allen, H. (1997). Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. *Nature* 386**,** 619-623.

Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X.-M., Stanley, E.R., Randolph, G.J., and Merad, M. (2006). Langerhans cells arise from monocytes in vivo. *Nature Immunology* 7**,** 265-273.

Golden, E., Ukaegbu, D.C., Ranslow, P., Brown, R.H., Hartung, T., and Maertens, A. (2023). The Good, The Bad, and The Perplexing: Structural Alerts and Read-Across for Predicting Skin Sensitization Using Human Data. *Chemical Research in Toxicology* 36**,** 734-746.

Goossens, A., and Hulst, K.V. (2011). Occupational contact dermatitis in the pharmaceutical industry. *Clin Dermatol* 29**,** 662-668.

Gregorio, J., Meller, S., Conrad, C., Di Nardo, A., Homey, B., Lauerma, A., Arai, N., Gallo, R.L., Digiovanni, J., and Gilliet, M. (2010). Plasmacytoid dendritic cells sense skin injury and promote wound healing through type I interferons. *J Exp Med* 207**,** 2921-2930.

Guak, H., Al Habyan, S., Ma, E.H., Aldossary, H., Al-Masri, M., Won, S.Y., Ying, T., Fixman, E.D., Jones, R.G., McCaffrey, L.M., and Krawczyk, C.M. (2018). Glycolytic metabolism is essential for CCR7 oligomerization and dendritic cell migration. *Nature Communications* 9**,** 2463.

Guedes, S., Neves, B., Vitorino, R., Domingues, R., Cruz, M.T., and Domingues, P. (2016). Contact dermatitis: in pursuit of sensitizer's molecular targets through proteomics. *Archives of Toxicology***,** 1-15.

Gurung, P., Li, B., Subbarao Malireddi, R.K., Lamkanfi, M., Geiger, T.L., and Kanneganti, T.D. (2015). Chronic TLR Stimulation Controls NLRP3 Inflammasome Activation through IL-10 Mediated Regulation of NLRP3 Expression and Caspase-8 Activation. *Sci Rep* 5**,** 14488.

Guttman-Yassky, E., Simpson, E.L., Reich, K., Kabashima, K., Igawa, K., Suzuki, T., Mano, H., Matsui, T., Esfandiari, E., and Furue, M. (2023). An anti-OX40 antibody to treat moderate-to-severe atopic dermatitis: a multicentre, double-blind, placebo-controlled phase 2b study. *The Lancet* 401**,** 204-214.

Hagvall, L., Bäcktorp, C., Svensson, S., Nyman, G., Börje, A., and Karlberg, A.T. (2007). Fragrance compound geraniol forms contact allergens on air exposure. Identification and quantification of oxidation products and effect on skin sensitization. *Chem Res Toxicol* 20**,** 807-814.

Hardonnière, K., Szely, N., El Ali, Z., Pallardy, M., and Kerdine-Römer, S. (2022). Models of Dendritic Cells to Assess Skin Sensitization. *Front Toxicol* 4**,** 851017.

Harrill, J.A., Viant, M.R., Yauk, C.L., Sachana, M., Gant, T.W., Auerbach, S.S., Beger, R.D., Bouhifd, M., O'Brien, J., Burgoon, L., Caiment, F., Carpi, D., Chen, T., Chorley, B.N., Colbourne, J., Corvi, R., Debrauwer, L., O'Donovan, C., Ebbels, T.M.D., Ekman, D.R., Faulhammer, F., Gribaldo, L., Hilton, G.M., Jones, S.P., Kende, A., Lawson, T.N., Leite, S.B., Leonards, P.E.G., Luijten, M., Martin, A., Moussa, L., Rudaz, S., Schmitz, O., Sobanski, T., Strauss, V., Vaccari, M., Vijay, V., Weber, R.J.M., Williams, A.J., Williams, A., Thomas, R.S., and Whelan, M. (2021). Progress towards an OECD reporting framework for transcriptomics and metabolomics in regulatory toxicology. *Regul Toxicol Pharmacol* 125**,** 105020.

Hennen, J., Aeby, P., Goebel, C., Schettgen, T., Oberli, A., Kalmes, M., and Blömeke, B. (2011). Cross Talk between Keratinocytes and Dendritic Cells: Impact on the Prediction of Sensitization. *Toxicological Sciences* 123**,** 501-510.

Hennen, J., and Blömeke, B. (2017). Keratinocytes improve prediction of sensitization potential and potency of chemicals with THP-1 cells. *Altex* 34**,** 279-288.

Hiltbold, E.M., Poloso, N.J., and Roche, P.A. (2003). MHC class II-peptide complexes and APC lipid rafts accumulate at the immunological synapse. *J Immunol* 170**,** 1329-1338.

Hitzler, M., Bergert, A., Luch, A., and Peiser, M. (2013). Evaluation of selected biomarkers for the detection of chemical sensitization in human skin: a comparative study applying THP-1, MUTZ-3 and primary dendritic cells in culture. *Toxicol In Vitro* 27**,** 1659-1669.

Hoeffel, G., Wang, Y., Greter, M., See, P., Teo, P., Malleret, B., Leboeuf, M., Low, D., Oller, G., Almeida, F., Choy, S.H., Grisotto, M., Renia, L., Conway, S.J., Stanley, E.R., Chan, J.K., Ng, L.G., Samokhvalov, I.M., Merad, M., and Ginhoux, F. (2012). Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med* 209**,** 1167-1181.

Hölken, J.M., and Teusch, N. (2023). The Monocytic Cell Line THP-1 as a Validated and Robust Surrogate Model for Human Dendritic Cells. *International Journal of Molecular Sciences* 24**,** 1452.

Holzhauser, T., Johnson, P., Hindley, J.P., O'Connor, G., Chan, C.-H., Costa, J., Fæste, C.K., Hirst, B.J., Lambertini, F., Miani, M., Robert, M.-C., Röder, M., Ronsmans, S., Bugyi, Z., Tömösközi, S., and Flanagan, S.D. (2020). Are current analytical methods suitable to verify VITAL® 2.0/3.0 allergen reference doses for EU allergens in foods? *Food and Chemical Toxicology* 145**,** 111709.

Honda, T., Egawa, G., Grabbe, S., and Kabashima, K. (2013). Update of Immune Events in the Murine Contact Hypersensitivity Model: Toward the Understanding of Allergic Contact Dermatitis. *Journal of Investigative Dermatology* 133**,** 303-315.

Hooyberghs, J., Schoeters, E., Lambrechts, N., Nelissen, I., Witters, H., Schoeters, G., and Van Den Heuvel, R. (2008). A cell-based in vitro alternative to identify skin sensitizers by gene expression. *Toxicology and Applied Pharmacology* 231**,** 103-111.

Höper, T., Karkossa, I., Dumit, V.I., von Bergen, M., Schubert, K., and Haase, A. (2023). A comparative proteomics analysis of four contact allergens in THP-1 cells shows distinct alterations in key metabolic pathways. *Toxicology and Applied Pharmacology* 475**,** 116650.

Höper, T., Mussotter, F., Haase, A., Luch, A., and Tralau, T. (2017). Application of proteomics in the elucidation of chemical-mediated allergic contact dermatitis. *Toxicology Research* 6**,** 595-610.

Höper, T., Siewert, K., Dumit, V.I., von Bergen, M., Schubert, K., and Haase, A. (2021). The Contact Allergen NiSO4 Triggers a Distinct Molecular Response in Primary Human Dendritic Cells Compared to Bacterial LPS. *Frontiers in Immunology* 12.

Hu, Z.B., Ma, W., Zaborski, M., MacLeod, R., Quentmeier, H., and Drexler, H.G. (1996). Establishment and characterization of two novel cytokine-responsive acute myeloid and monocytic leukemia cell lines, MUTZ-2 and MUTZ-3. *Leukemia* 10**,** 1025-1040.

Hughes, A.L., Todd, B.L., and Espenshade, P.J. (2005). SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast. *Cell* 120**,** 831-842.

Irizar, A., Bender, H., Griem, P., Natsch, A., Vey, M., and Kimber, I. (2022). Reference Chemical Potency List (RCPL): A new tool for evaluating the accuracy of skin sensitisation potency measurements by New Approach Methodologies (NAMs). *Regul Toxicol Pharmacol* 134**,** 105244.

Iulini, M., Maddalon, A., Galbiati, V., Marinovich, M., and Corsini, E. (2020). In vitro identification of drugs inducing systemic hypersensitivity reactions known in vivo to be associated with specific HLA genotypes. *Toxicology in Vitro* 68**,** 104953.

Jakob, A., Mussotter, F., Ohnesorge, S., Dietz, L., Pardo, J., Haidl, I.D., and Thierse, H.J. (2017). Immunoproteomic identification and characterization of Ni(2+)-regulated proteins implicates Ni(2+) in the induction of monocyte cell death. *Cell Death Dis* 8**,** e2684.

Jaworska, J., Dancik, Y., Kern, P., Gerberick, F., and Natsch, A. (2013). Bayesian integrated testing strategy to assess skin sensitization potency: from theory to practice. *Journal of Applied Toxicology* 33**,** 1353-1364.

Johansson, H., Albrekt, A.S., Borrebaeck, C.A., and Lindstedt, M. (2013). The GARD assay for assessment of chemical skin sensitizers. *Toxicol In Vitro* 27**,** 1163-1169.

Johansson, H., Gradin, R., Johansson, A., Adriaens, E., Edwards, A., Zuckerstätter, V., Jerre, A., Burleson, F., Gehrke, H., and Roggen, E.L. (2019). Validation of the GARD™skin Assay for Assessment of Chemical Skin Sensitizers: Ring Trial Results of Predictive Performance and Reproducibility. *Toxicological Sciences* 170**,** 374-381.

Johansson, H., Lindstedt, M., Albrekt, A.-S., and Borrebaeck, C.A. (2011a). A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. *BMC Genomics* 12**,** 399.

Johansson, H., Lindstedt, M., Albrekt, A.S., and Borrebaeck, C.A. (2011b). A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. *BMC Genomics* 12**,** 399.

Kanazawa, Y., Yoshida, T., and Kojima, K. (1999). Structure-activity relationships in allergic contact dermatitis induced by methacrylates. Studies of the influence of side-chain length of methacrylates. *Contact Dermatitis* 40**,** 19-23.

Kapadia, S.B., Barth, H., Baumert, T., McKeating, J.A., and Chisari, F.V. (2007). Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. *J Virol* 81**,** 374-383.

Kaplan, D.H., Igyarto, B.Z., and Gaspari, A.A. (2012). Early immune events in the induction of allergic contact dermatitis. *Nature Reviews Immunology* 12**,** 114-124.

Karri, V., Lidén, C., Fyhrquist, N., Högberg, J., and Karlsson, H.L. (2021). Impact of mono-culture vs. Co-culture of keratinocytes and monocytes on cytokine responses induced by important skin sensitizers. *J Immunotoxicol* 18**,** 74-84.

Kiama, S.G., Cochand, L., Karlsson, L., Nicod, L.P., and Gehr, P. (2001). Evaluation of phagocytic activity in human monocyte-derived dendritic cells. *J Aerosol Med* 14**,** 289-299.

Kim, M.K., and Kim, J. (2019). Properties of immature and mature dendritic cells: phenotype, morphology, phagocytosis, and migration. *RSC Advances* 9**,** 11230-11238.

Kinbara, M., Sato, N., Kuroishi, T., Takano-Yamamoto, T., Sugawara, S., and Endo, Y. (2011). Allergy-inducing nickel concentration is lowered by lipopolysaccharide at both the sensitization and elicitation steps in a murine model. *Br J Dermatol* 164**,** 356-362.

Kligman, A.M. (1966a). The identification of contact allergens by human assay. 3. The maximization test: a procedure for screening and rating contact sensitizers. *J Invest Dermatol* 47.

Kligman, A.M. (1966b). The identification of contact allergens by human assay. II. Factors influencing the induction and measurement of allergic contact dermatitis. *J Invest Dermatol* 47**,** 375-392.

Knox, S., Hagvall, L., Malmberg, P., and O'Boyle, N.M. (2022). Topical Application of Metal Allergens Induces Changes to Lipid Composition of Human Skin. *Frontiers in Toxicology* 4.

Kosaka, N., Okamoto, K., Mizuno, M., Yamada, T., Yoshida, M., Kodama, T., Sono, S., Ashikaga, T., Sato, J., Ota, N., Hasegawa, S., Okamoto, Y., Kuwahara, H., Sakaguchi, H., and Ohno, Y. (2008). A Study of the Criteria for Selection of THP-1 Cells in the Human Cell Line Activation Test (h-CLAT): Results of 2nd Japanese Inter-laboratory Study. *Alternatives to Animal Testing and Experimentation* 13**,** 55-62.

Kraft, M., and Worm, M. (2017). Dupilumab in the treatment of moderate-to-severe atopic dermatitis. *Expert Rev Clin Immunol* 13**,** 301-310.

Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J.R., Jung, E., Thompson, C.B., Jones, R.G., and Pearce, E.J. (2010). Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* 115**,** 4742-4749.

Kwangsukstith, C., and Maibach, H.I. (1995). Effect of age and sex on the induction and elicitation of allergic contact dermatitis. *Contact Dermatitis* 33**,** 289-298.

Lachmandas, E., Boutens, L., Ratter, J.M., Hijmans, A., Hooiveld, G.J., Joosten, L.A.B., Rodenburg, R.J., Fransen, J.A.M., Houtkooper, R.H., van Crevel, R., Netea, M.G., and Stienstra, R. (2016). Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. *Nature Microbiology* 2**,** 16246.

Lagrelius, M., Wahlgren, C.F., Bradley, M., Melén, E., Kull, I., Bergström, A., and Lidén, C. (2020). Filaggrin gene mutations in relation to contact allergy and hand eczema in adolescence. *Contact Dermatitis* 82**,** 147-152.

Lambrechts, N., Nelissen, I., Van Tendeloo, V., Witters, H., Van Den Heuvel, R., Hooyberghs, J., and Schoeters, G. (2011). Functionality and specificity of gene markers for skin sensitization in dendritic cells. *Toxicology Letters* 203**,** 106-110.

Larsson, K., Lindstedt, M., and Borrebaeck, C.A. (2006). Functional and transcriptional profiling of MUTZ-3, a myeloid cell line acting as a model for dendritic cells. *Immunology* 117.

Lee, J.S., Kim, J., Cui, B., Kim, S.K., Cho, S.-A., An, S., and Cho, S.-W. (2022). Hybrid skin chips for toxicological evaluation of chemical drugs and cosmetic compounds. *Lab on a Chip* 22**,** 343-353.

Lefevre, M.-A., Nosbaum, A., Rozieres, A., Lenief, V., Mosnier, A., Cortial, A., Prieux, M., De Bernard, S., Nourikyan, J., Jouve, P.-E., Buffat, L., Hacard, F., Ferrier-Lebouedec, M.-

C., Pralong, P., Dzviga, C., Herman, A., Baeck, M., Nicolas, J.-F., and Vocanson, M. (2021). Unique molecular signatures typify skin inflammation induced by chemical allergens and irritants. *Allergy* 76**,** 3697-3712.

Leung, D.Y.M., Calatroni, A., Zaramela, L.S., LeBeau, P.K., Dyjack, N., Brar, K., David, G., Johnson, K., Leung, S., Ramirez-Gama, M., Liang, B., Rios, C., Montgomery, M.T., Richers, B.N., Hall, C.F., Norquest, K.A., Jung, J., Bronova, I., Kreimer, S., Conover Talbot, C., Crumrine, D., Cole, R.N., Elias, P., Zengler, K., Seibold, M.A., Berdyshev, E., and Goleva, E. (2019). The nonlesional skin surface distinguishes atopic dermatitis with food allergy as a unique endotype. *Science Translational Medicine* 11**,** eaav2685.

Li, Y., and Li, L. (2021). Contact Dermatitis: Classifications and Management. *Clinical Reviews in Allergy & Immunology* 61**,** 245-281.

Li, Z., Adams, R.M., Chourey, K., Hurst, G.B., Hettich, R.L., and Pan, C. (2012). Systematic Comparison of Label-Free, Metabolic Labeling, and Isobaric Chemical Labeling for Quantitative Proteomics on LTQ Orbitrap Velos. *Journal of Proteome Research* 11**,** 1582-1590.

Liden, C., Yazar, K., Johansen, J.D., Karlberg, A.T., Uter, W., and White, I.R. (2016). Comparative sensitizing potencies of fragrances, preservatives, and hair dyes. *Contact Dermatitis* 75**,** 265-275.

Lindberg, T., de Ávila, R.I., Zeller, K.S., Levander, F., Eriksson, D., Chawade, A., and Lindstedt, M. (2020). An integrated transcriptomic- and proteomic-based approach to evaluate the human skin sensitization potential of glyphosate and its commercial agrochemical formulations. *Journal of Proteomics* 217**,** 103647.

Lisi, P., and Hansel, K. (1998). Is benzoquinone the prohapten in cross-sensitivity among aminobenzene compounds? *Contact Dermatitis* 39**,** 304-306.

Liu, Y., Li, H., Czajkowsky, D.M., and Shao, Z. (2021). Monocytic THP-1 cells diverge significantly from their primary counterparts: a comparative examination of the chromosomal conformations and transcriptomes. *Hereditas* 158**,** 43.

Lühr, J.J., Alex, N., Amon, L., Kräter, M., Kubánková, M., Sezgin, E., Lehmann, C.H.K., Heger, L., Heidkamp, G.F., Smith, A.S., Zaburdaev, V., Böckmann, R.A., Levental, I., Dustin, M.L., Eggeling, C., Guck, J., and Dudziak, D. (2020). Maturation of Monocyte-Derived DCs Leads to Increased Cellular Stiffness, Higher Membrane Fluidity, and Changed Lipid Composition. *Front Immunol* 11**,** 590121.

Luo, X.-L., and Dalod, M. (2020). The quest for faithful in vitro models of human dendritic cells types. *Molecular Immunology* 123**,** 40-59.

Martin, S., Delattre, V., Leicht, C., Weltzien, H.U., and Simon, J.C. (2003). A high frequency of allergen-specific CD8+ Tc1 cells is associated with the murine immune response to the contact sensitizer trinitrophenyl. *Exp Dermatol* 12**,** 78-85.

Martin, S.F. (2012). Contact dermatitis: from pathomechanisms to immunotoxicology. *Experimental Dermatology* 21**,** 382-389.

Martin, S.F., Dudda, J.C., Bachtanian, E., Lembo, A., Liller, S., Dürr, C., Heimesaat, M.M., Bereswill, S., Fejer, G., Vassileva, R., Jakob, T., Freudenberg, N., Termeer, C.C., Johner, C., Galanos, C., and Freudenberg, M.A. (2008). Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. *J Exp Med* 205**,** 2151-2162.

Martin, S.F., Esser, P.R., Weber, F.C., Jakob, T., Freudenberg, M.A., Schmidt, M., and Goebeler, M. (2011). Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 66**,** 1152-1163.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10**,** 417-426.

Mass, E., Ballesteros, I., Farlik, M., Halbritter, F., Günther, P., Crozet, L., Jacome-Galarza, C.E., Händler, K., Klughammer, J., Kobayashi, Y., Gomez-Perdiguero, E., Schultze, J.L., Beyer, M., Bock, C., and Geissmann, F. (2016). Specification of tissue-resident macrophages during organogenesis. *Science* 353.

Matsue, H., Edelbaum, D., Shalhevet, D., Mizumoto, N., Yang, C., Mummert, M.E., Oeda, J., Masayasu, H., and Takashima, A. (2003). Generation and Function of Reactive Oxygen Species in Dendritic Cells During Antigen Presentation. *The Journal of Immunology* 171**,** 3010-3018.

Matthews, K., Chung, N.P., Klasse, P.J., Moore, J.P., and Sanders, R.W. (2012). Potent induction of antibody-secreting B cells by human dermal-derived CD14+ dendritic cells triggered by dual TLR ligation. *J Immunol* 189**,** 5729-5744.

McDonagh, A.J.G., Wright, A.L., Cork, M.J., and Gawkrodger, D.J. (1992). Nickel sensitivity: the influence of ear piercing and atopy. *British Journal of Dermatology* 126**,** 16-18.

McGovern, N., Schlitzer, A., Gunawan, M., Jardine, L., Shin, A., Poyner, E., Green, K., Dickinson, R., Wang, X.N., Low, D., Best, K., Covins, S., Milne, P., Pagan, S., Aljefri, K., Windebank, M., Miranda-Saavedra, D., Larbi, A., Wasan, P.S., Duan, K., Poidinger, M., Bigley, V., Ginhoux, F., Collin, M., and Haniffa, M. (2014). Human dermal $CD14^+$ cells are a transient population of monocyte-derived macrophages. *Immunity* 41**,** 465-477.

McSweeney, S.M., White, I.R., Kimber, I., McFadden, J.P., and Tziotzios, C. (2020). Contact allergy across the human lifespan. *Journal of Allergy and Clinical Immunology* 145**,** 1352-1354.

Megherbi, R., Kiorpelidou, E., Foster, B., Rowe, C., Naisbitt, D.J., Goldring, C.E., and Park, B.K. (2009). Role of protein haptenation in triggering maturation events in the dendritic cell surrogate cell line THP-1. *Toxicology and Applied Pharmacology* 238**,** 120-132.

Merad, M., and Manz, M.G. (2009). Dendritic cell homeostasis. *Blood* 113**,** 3418-3427.

Miller, J.C., Brown, B.D., Shay, T., Gautier, E.L., Jojic, V., Cohain, A., Pandey, G., Leboeuf, M., Elpek, K.G., Helft, J., Hashimoto, D., Chow, A., Price, J., Greter, M., Bogunovic, M., Bellemare-Pelletier, A., Frenette, P.S., Randolph, G.J., Turley, S.J., Merad, M., Gautier, E.L., Jakubzick, C., Randolph, G.J., Best, A.J., Knell, J., Goldrath, A., Miller, J., Brown, B., Merad, M., Jojic, V., Koller, D., Cohen, N., Brennan, P., Brenner, M., Shay, T., Regev, A., Fletcher, A., Elpek, K., Bellemare-Pelletier, A., Malhotra, D., Turley, S., Jianu, R., Laidlaw, D., Collins, J., Narayan, K., Sylvia, K., Kang, J., Gazit, R., Rossi, D.J., Kim, F., Rao, T.N., Wagers, A., Shinton, S.A., Hardy, R.R., Monach, P., Bezman, N.A., Sun, J.C., Kim, C.C., Lanier, L.L., Heng, T., Kreslavsky, T., Painter, M., Ericson, J., Davis, S., Mathis, D., Benoist, C., and the Immunological Genome, C. (2012). Deciphering the transcriptional network of the dendritic cell lineage. *Nature Immunology* 13**,** 888-899.

Minang, J.T., Areström, I., Troye-Blomberg, M., Lundeberg, L., and Ahlborg, N. (2006). Nickel, cobalt, chromium, palladium and gold induce a mixed Th1- and Th2-type cytokine response in vitro in subjects with contact allergy to the respective metals. *Clinical and Experimental Immunology* 146**,** 417-426.

Miyazawa, M., Ito, Y., Yoshida, Y., Sakaguchi, H., and Suzuki, H. (2007). Phenotypic alterations and cytokine production in THP-1 cells in response to allergens. *Toxicology in Vitro* 21**,** 428-437.

Mizuashi, M., Ohtani, T., Nakagawa, S., and Aiba, S. (2005). Redox imbalance induced by contact sensitizers triggers the maturation of dendritic cells. *J Invest Dermatol* 124.

Monzon, M.E., Fregien, N., Schmid, N., Falcon, N.S., Campos, M., Casalino-Matsuda, S.M., and Forteza, R.M. (2010). Reactive oxygen species and hyaluronidase 2 regulate airway epithelial hyaluronan fragmentation. *J Biol Chem* 285**,** 26126-26134.

Moon, J.J., Chu, H.H., Pepper, M., McSorley, S.J., Jameson, S.C., Kedl, R.M., and Jenkins, M.K. (2007). Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* 27**,** 203-213.

Morelli, P., Gaspari, M., Gabriele, C., Dastoli, S., Bennardo, L., Pavel, A.B., Patruno, C., Del Duca, E., and Nisticò, S.P. (2021). Proteomic analysis from skin swabs reveals a new set of proteins identifying skin impairment in atopic dermatitis. *Exp Dermatol* 30**,** 811-819.

Murata, A., and Hayashi, S.-I. (2020). CD4+ Resident Memory T Cells Mediate Long-Term Local Skin Immune Memory of Contact Hypersensitivity in BALB/c Mice. *Frontiers in Immunology* 11.

Mussotter, F., Potratz, S., Budczies, J., Luch, A., and Haase, A. (2018). A multi-omics analysis reveals metabolic reprogramming in THP-1 cells upon treatment with the contact allergen DNCB. *Toxicol Appl Pharmacol* 340**,** 21-29.

Mussotter, F., Tomm, J.M., El Ali, Z., Pallardy, M., Kerdine-Romer, S., Gotz, M., von Bergen, M., Haase, A., and Luch, A. (2016). Proteomics analysis of dendritic cell activation by contact allergens reveals possible biomarkers regulated by Nrf2. *Toxicol Appl Pharmacol* 313**,** 170-179.

Natsch, A., and Gerberick, G.F. (2022). Integrated skin sensitization assessment based on OECD methods (I): Deriving a point of departure for risk assessment. *ALTEX - Alternatives to animal experimentation*.

Natsch, A., Ryan, C.A., Foertsch, L., Emter, R., Jaworska, J., Gerberick, F., and Kern, P. (2013). A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. *Journal of Applied Toxicology* 33**,** 1337-1352.

Ndreu, L., Erber, L.N., Törnqvist, M., Tretyakova, N.Y., and Karlsson, I. (2020). Characterizing Adduct Formation of Electrophilic Skin Allergens with Human Serum Albumin and Hemoglobin. *Chemical Research in Toxicology* 33**,** 2623-2636.

Nguyen, C.D.L., Malchow, S., Reich, S., Steltgens, S., Shuvaev, K.V., Loroch, S., Lorenz, C., Sickmann, A., Knobbe-Thomsen, C.B., Tews, B., Medenbach, J., and Ahrends, R. (2019). A sensitive and simple targeted proteomics approach to quantify transcription factor and membrane proteins of the unfolded protein response pathway in glioblastoma cells. *Scientific Reports* 9**,** 8836.

Nielsen, N.H., and Menné, T. (1993). Nickel sensitization and ear piercing in an unselected Danish population. Glostrup Allergy Study. *Contact dermatitis* 29**,** 16-21.

Noronha, N., Ehx, G., Meunier, M.-C., Laverdure, J.-P., Thériault, C., and Perreault, C. (2020). Major multilevel molecular divergence between THP-1 cells from different biorepositories. *International Journal of Cancer* 147**,** 2000-2006.

Nosbaum, A., Vocanson, M., Rozieres, A., Hennino, A., and Nicolas, J.F. (2009). Allergic and irritant contact dermatitis. *Eur J Dermatol* 19**,** 325-332.

Nukada, Y., Miyazawa, M., Kosaka, N., Ito, Y., Sakaguchi, H., and Nishiyama, N. (2008). Production of IL-8 in THP-1 cells following contact allergen stimulation via mitogen-activated protein kinase activation or tumor necrosis factor-alpha production. *J Toxicol Sci* 33**,** 175-185.

Nyman, G.S.A., Giménez-Arnau, A.M., Grigaitiene, J., Malinauskiene, L., Paulsen, E., and Hagvall, L. (2021). Patch Testing with Propolis of Different Geographical Origins in a Baseline Series. *Acta Derm Venereol* 101**,** adv00591.

OECD (2010). *Test No. 429: Skin Sensitisation: Local Lymph Node Assay.*

OECD (2014). *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins.*
OECD (2018). *Test No. 442E: In Vitro Skin Sensitisation.*
OECD (2019). *Test No. 431: In vitro skin corrosion: reconstructed human epidermis (RHE) test method.*
OECD (2021). *Guideline No. 497: Defined Approaches on Skin Sensitisation.*
OECD (2022a). *Omics technologies in chemical testing* [Online]. Available: https://www.oecd.org/chemicalsafety/testing/omics.htm [Accessed].
OECD (2022b). *Test No. 442E: In Vitro Skin Sensitisation.*
OECD (2023). *Test No. 498: In vitro Phototoxicity - Reconstructed Human Epidermis Phototoxicity test method.*
Ogulur, I., Pat, Y., Ardicli, O., Barletta, E., Cevhertas, L., Fernandez-Santamaria, R., Huang, M., Bel Imam, M., Koch, J., Ma, S., Maurer, D.J., Mitamura, Y., Peng, Y., Radzikowska, U., Rinaldi, A.O., Rodriguez-Coira, J., Satitsuksanoa, P., Schneider, S.R., Wallimann, A., Zhakparov, D., Ziadlou, R., Brüggen, M.C., van de Veen, W., Sokolowska, M., Baerenfaller, K., Zhang, L., Akdis, M., and Akdis, C.A. (2021). Advances and highlights in biomarkers of allergic diseases. *Allergy* 76**,** 3659-3686.
Ohl, L., Mohaupt, M., Czeloth, N., Hintzen, G., Kiafard, Z., Zwirner, J., Blankenstein, T., Henning, G., and Förster, R. (2004). CCR7 Governs Skin Dendritic Cell Migration under Inflammatory and Steady-State Conditions. *Immunity* 21**,** 279-288.
Orchard, S., Hermjakob, H., and Apweiler, R. (2003). The Proteomics Standards Initiative. *PROTEOMICS* 3**,** 1374-1376.
Orsini, G., Legitimo, A., Failli, A., Massei, F., Biver, P., and Consolini, R. (2012). Enumeration of human peripheral blood dendritic cells throughout the life. *International Immunology* 24**,** 347-356.
Pambor, M., and Wiesner, M. (1990). Allergic contact dermatitis due to p-nitrobenzyl bromide. *Contact Dermatitis* 23**,** 201-202.
Pantel, A., Teixeira, A., Haddad, E., Wood, E.G., Steinman, R.M., and Longhi, M.P. (2014). Direct Type I IFN but Not MDA5/TLR3 Activation of Dendritic Cells Is Required for Maturation and Metabolic Shift to Glycolysis after Poly IC Stimulation. *PLOS Biology* 12**,** e1001759.
Parkinson, E., Aleksic, M., Arthur, R., Regufe Da Mota, S., Cubberley, R., and Skipp, P.J. (2020a). Proteomic analysis of haptenation by skin sensitisers: Diphencyprone and ethyl acrylate. *Toxicology in Vitro* 62**,** 104697.
Parkinson, E., Aleksic, M., Kukic, P., Bailey, A., Cubberley, R., and Skipp, P. (2020b). Proteomic analysis of the cellular response to a potent sensitiser unveils the dynamics of haptenation in living cells. *Toxicology* 445**,** 152603.
Patlewicz, G., Casati, S., Basketter, D.A., Asturiol, D., Roberts, D.W., Lepoittevin, J.-P., Worth, A.P., and Aschberger, K. (2016). Can currently available non-animal methods detect pre and pro-haptens relevant for skin sensitization? *Regulatory Toxicology and Pharmacology* 82**,** 147-155.
Pavel, A.B., Zhou, L., Diaz, A., Ungar, B., Dan, J., He, H., Estrada, Y.D., Xu, H., Fernandes, M., Renert-Yuval, Y., Krueger, J.G., and Guttman-Yassky, E. (2020). The proteomic skin profile of moderate-to-severe atopic dermatitis patients shows an inflammatory signature. *Journal of the American Academy of Dermatology* 82**,** 690-699.
Pearce, E.J., and Everts, B. (2015). Dendritic cell metabolism. *Nat Rev Immunol* 15**,** 18-29.
Pedersen, L.K., Johansen, J.D., Held, E., and Agner, T. (2004). Augmentation of skin response by exposure to a combination of allergens and irritants - a review. *Contact Dermatitis* 50**,** 265-273.

Perrin-Cocon, L., Aublin-Gex, A., Diaz, O., Ramiere, C., Peri, F., Andre, P., and Lotteau, V. (2018). Toll-like Receptor 4-Induced Glycolytic Burst in Human Monocyte-Derived Dendritic Cells Results from p38-Dependent Stabilization of HIF-1alpha and Increased Hexokinase II Expression. *J Immunol* 201**,** 1510-1521.

Petersen, T.H., Jee, M.H., Gadsbøll, A., Schmidt, J.D., Sloth, J.J., Sonnenberg, G.F., Geisler, C., Thyssen, J.P., and Bonefeld, C.M. (2019). Mice with epidermal filaggrin deficiency show increased immune reactivity to nickel. *Contact Dermatitis* 80**,** 139-148.

Politano, V.T., and Api, A.M. (2008). The Research Institute for Fragrance Materials' human repeated insult patch test protocol. *Regul Toxicol Pharmacol* 52**,** 35-38.

Potts, R.O., and Guy, R.H. (1992). Predicting skin permeability. *Pharm Res* 9**,** 663-669.

Prechtel, A.T., and Steinkasserer, A. (2007). CD83: an update on functions and prospects of the maturation marker of dendritic cells. *Arch Dermatol Res* 299**,** 59-69.

Proksch, E., and Brasch, J. (2012). Abnormal epidermal barrier in the pathogenesis of contact dermatitis. *Clinics in Dermatology* 30**,** 335-344.

Przybilla, B., Burg, G., and Thieme, C. (1983). Evaluation of the immune status in vivo by the 2,4-dinitro-1-chlorobenzene contact allergy time (DNCB-CAT). *Dermatologica* 167**,** 1-5.

Python, F., Goebel, C., and Aeby, P. (2007). Assessment of the U937 cell line for the detection of contact allergens. *Toxicology and Applied Pharmacology* 220**,** 113-124.

Quaranta, M., Knapp, B., Garzorz, N., Mattii, M., Pullabhatla, V., Pennino, D., Andres, C., Traidl-Hoffmann, C., Cavani, A., Theis, F.J., Ring, J., Schmidt-Weber, C.B., Eyerich, S., and Eyerich, K. (2014a). Intraindividual genome expression analysis reveals a specific molecular signature of psoriasis and eczema. *Sci Transl Med* 6**,** 244ra290.

Quaranta, M., Knapp, B., Garzorz, N., Mattii, M., Pullabhatla, V., Pennino, D., Andres, C., Traidl-Hoffmann, C., Cavani, A., Theis, F.J., Ring, J., Schmidt-Weber, C.B., Eyerich, S., and Eyerich, K. (2014b). Intraindividual genome expression analysis reveals a specific molecular signature of psoriasis and eczema. *Science Translational Medicine* 6**,** 244ra290-244ra290.

Ramadan, Q., and Ting, F.C. (2016). In vitro micro-physiological immune-competent model of the human skin. *Lab Chip* 16**,** 1899-1908.

Richter, A., Schmucker, S.S., Esser, P.R., Traska, V., Weber, V., Dietz, L., Thierse, H.J., Pennino, D., Cavani, A., and Martin, S.F. (2013). Human T cell priming assay (hTCPA) for the identification of contact allergens based on naive T cells and DC--IFN-gamma and TNF-alpha readout. *Toxicol In Vitro* 27**,** 1180-1185.

Riddy, D.M., Goy, E., Delerive, P., Summers, R.J., Sexton, P.M., and Langmead, C.J. (2018). Comparative genotypic and phenotypic analysis of human peripheral blood monocytes and surrogate monocyte-like cell lines commonly used in metabolic disease research. *PLOS ONE* 13**,** e0197177.

Riedel, F., Aparicio-Soto, M., Curato, C., Münch, L., Abbas, A., Thierse, H.J., Peitsch, W.K., Luch, A., and Siewert, K. (2022). Unique and common TCR repertoire features of Ni(2+) -, Co(2+) -, and Pd(2+) -specific human CD154 + CD4+ T cells. *Allergy*.

Roberts, D.W., Aptula, A.O., and Patlewicz, G. (2007a). Electrophilic chemistry related to skin sensitization. Reaction mechanistic applicability domain classification for a published data set of 106 chemicals tested in the mouse local lymph node assay. *Chemical Research in Toxicology* 20**,** 44-60.

Roberts, D.W., Goodwin, B.F., and Basketter, D. (1988). Methyl groups as antigenic determinants in skin sensitisation. *Contact Dermatitis* 18**,** 219-225.

Roberts, D.W., Mekenyan, O.G., Dimitrov, S.D., and Dimitrova, G.D. (2013). What determines skin sensitization potency–myths, maybes and realities. Part 1. The 500 molecular weight cut-off. *Contact Dermatitis* 68**,** 32-41.

Roberts, D.W., Patlewicz, G., Kern, P.S., Gerberick, F., Kimber, I., Dearman, R.J., Ryan, C.A., Basketter, D.A., and Aptula, A.O. (2007b). Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. *Chem Res Toxicol* 20.

Romita, P., Foti, C., Stingeni, L., Hansel, K., Magrone, T., Belsito, D.V., and Bonamonte, D. (2019). Contact Allergy in Children with Atopic Dermatitis: A Retrospective Study. *Endocr Metab Immune Disord Drug Targets* 19**,** 1083-1087.

Rougier, N., Redziniak, G., Mougin, D., Schmitt, D., and Vincent, C. (2000). In vitro evaluation of the sensitization potential of weak contact allergens using langerhans-like dendritic cells and autologous T cells. *Toxicology* 145**,** 73-82.

Rustemeyer, T., Von Blomberg, B.M.E., Van Hoogstraten, I.M.W., Bruynzeel, D.P., and Scheper, R.J. (2004). Analysis of effector and regulatory immune reactivity to nickel. *Clinical & Experimental Allergy* 34**,** 1458-1466.

Saito, M., Arakaki, R., Yamada, A., Tsunematsu, T., Kudo, Y., and Ishimaru, N. (2016). Molecular Mechanisms of Nickel Allergy. *International Journal of Molecular Sciences* 17**,** 8.

Sakaguchi, H., Ryan, C., Ovigne, J.M., Schroeder, K.R., and Ashikaga, T. (2010). Predicting skin sensitization potential and inter-laboratory reproducibility of a human Cell Line Activation Test (h-CLAT) in the European Cosmetics Association (COLIPA) ring trials. *Toxicology in Vitro* 24**,** 1810-1820.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *Journal of Experimental Medicine* 179**,** 1109-1118.

Samelko, L., Landgraeber, S., McAllister, K., Jacobs, J., and Hallab, N.J. (2016). Cobalt Alloy Implant Debris Induces Inflammation and Bone Loss Primarily through Danger Signaling, Not TLR4 Activation: Implications for DAMP-ening Implant Related Inflammation. *PloS one* 11**,** e0160141-e0160141.

Sansom, D.M. (2000). CD28, CTLA-4 and their ligands: who does what and to whom? *Immunology* 101**,** 169-177.

Santegoets, S.J.A.M., Bontkes, H.J., Stam, A.G.M., Bhoelan, F., Ruizendaal, J.J., van den Eertwegh, A.J.M., Hooijberg, E., Scheper, R.J., and de Gruijl, T.D. (2008). Inducing Antitumor T Cell Immunity: Comparative Functional Analysis of Interstitial Versus Langerhans Dendritic Cells in a Human Cell Line Model. *The Journal of Immunology* 180**,** 4540.

Scheib, N., Tiemann, J., Becker, C., Probst, H.C., Raker, V.K., and Steinbrink, K. (2022). The Dendritic Cell Dilemma in the Skin: Between Tolerance and Immunity. *Front Immunol* 13**,** 929000.

Scheibner, K.A., Lutz, M.A., Boodoo, S., Fenton, M.J., Powell, J.D., and Horton, M.R. (2006). Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J Immunol* 177**,** 1272-1281.

Scheinman, P.L., Vocanson, M., Thyssen, J.P., Johansen, J.D., Nixon, R.L., Dear, K., Botto, N.C., Morot, J., and Goldminz, A.M. (2021). Contact dermatitis. *Nature Reviews Disease Primers* 7**,** 38.

Schellenberger, M.T., Bock, U., Hennen, J., Groeber-Becker, F., Walles, H., and Blömeke, B. (2019). A coculture system composed of THP-1 cells and 3D reconstructed human

epidermis to assess activation of dendritic cells by sensitizing chemicals after topical exposure. *Toxicology in Vitro* 57**,** 62-66.

Schildberger, A., Rossmanith, E., Eichhorn, T., Strassl, K., and Weber, V. (2013). Monocytes, Peripheral Blood Mononuclear Cells, and THP-1 Cells Exhibit Different Cytokine Expression Patterns following Stimulation with Lipopolysaccharide. *Mediators of Inflammation* 2013**,** 697972.

Schmidt, J.D., Ahlström, M.G., Johansen, J.D., Dyring-Andersen, B., Agerbeck, C., Nielsen, M.M., Poulsen, S.S., Woetmann, A., Ødum, N., Thomsen, A.R., Geisler, C., and Bonefeld, C.M. (2017). Rapid allergen-induced interleukin-17 and interferon-γ secretion by skin-resident memory CD8+ T cells. *Contact Dermatitis* 76**,** 218-227.

Schmidt, M., Raghavan, B., Muller, V., Vogl, T., Fejer, G., Tchaptchet, S., Keck, S., Kalis, C., Nielsen, P.J., Galanos, C., Roth, J., Skerra, A., Martin, S.F., Freudenberg, M.A., and Goebeler, M. (2010). Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat Immunol* 11**,** 814-819.

Schnuch, A., Westphal, G., Mössner, R., Uter, W., and Reich, K. (2011). Genetic factors in contact allergy—review and future goals. *Contact Dermatitis* 64**,** 2-23.

Sebastião, A.I., Ferreira, I., Brites, G., Silva, A., Neves, B.M., and Teresa Cruz, M. (2020). NLRP3 Inflammasome and Allergic Contact Dermatitis: A Connection to Demystify. *Pharmaceutics* 12.

Segura, E., and Amigorena, S. (2013). Inflammatory dendritic cells in mice and humans. *Trends Immunol* 34**,** 440-445.

Segura, E., Touzot, M., Bohineust, A., Cappuccio, A., Chiocchia, G., Hosmalin, A., Dalod, M., Soumelis, V., and Amigorena, S. (2013). Human Inflammatory Dendritic Cells Induce Th17 Cell Differentiation. *Immunity* 38**,** 336-348.

Sim, W.J., Ahl, P.J., and Connolly, J.E. (2016). Metabolism Is Central to Tolerogenic Dendritic Cell Function. *Mediators Inflamm* 2016**,** 2636701.

Simons, K., and Ikonen, E. (2000). How cells handle cholesterol. *Science* 290**,** 1721-1726.

Sköld, M., Hagvall, L., and Karlberg, A.T. (2008). Autoxidation of linalyl acetate, the main component of lavender oil, creates potent contact allergens. *Contact Dermatitis* 58**,** 9-14.

Smith, C.K., Moore, C.A., Elahi, E.N., Smart, A.T., and Hotchkiss, S.A. (2000). Human skin absorption and metabolism of the contact allergens, cinnamic aldehyde, and cinnamic alcohol. *Toxicol Appl Pharmacol* 168**,** 189-199.

Smith, D.R. (2009). The continuing rise of contact dermatitis, Part 1: The academic discipline. *Contact Dermatitis* 61**,** 189-193.

Soltés, L., Mendichi, R., Kogan, G., Schiller, J., Stankovska, M., and Arnhold, J. (2006). Degradative action of reactive oxygen species on hyaluronan. *Biomacromolecules* 7**,** 659-668.

Son, Y., Kim, S.-M., Lee, S.-A., Eo, S.-K., and Kim, K. (2013). Oxysterols induce transition of monocytic cells to phenotypically mature dendritic cell-like cells. *Biochemical and Biophysical Research Communications* 438**,** 161-168.

Spiewak, R. (2023). Diseases from the Spectrum of Dermatitis and Eczema: Can "Omics" Sciences Help with Better Systematics and More Accurate Differential Diagnosis? *Int J Mol Sci* 24.

Stejskal, V.D., Forsbeck, M., and Nilsson, R. (1990). Lymphocyte transformation test for diagnosis of isothiazolinone allergy in man. *J Invest Dermatol* 94**,** 798-802.

Strasser, L., Dang, H.H., Schwarz, H., Asam, C., Ferreira, F., Horejs-Hoeck, J., and Huber, C.G. (2017). Unbiased Quantitative Proteomics Reveals a Crucial Role of the Allergen Context for the Activation of Human Dendritic Cells. *Sci Rep* 7**,** 16638.

Streilein, W.J. (1989). Antigen-Presenting Cells in the Induction of Contact Hypersensitivity in Mice: Evidence that Langerhans Cells Are Sufficient but not Required. *Journal of Investigative Dermatology* 93**,** 443-448.

Su, M., Zhang, Z., Zhou, L., Han, C., Huang, C., and Nice, E.C. (2021). Proteomics, Personalized Medicine and Cancer. *Cancers* 13**,** 2512.

Sundström, C., and Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* 17**,** 565-577.

Takahashi, T., Kimura, Y., Saito, R., Nakajima, Y., Ohmiya, Y., Yamasaki, K., and Aiba, S. (2011). An In Vitro Test to Screen Skin Sensitizers Using a Stable THP-1–Derived IL-8 Reporter Cell Line, THP-G8. *Toxicological Sciences* 124**,** 359-369.

Tam, I., Hill, K.R., Park, J.M., and Yu, J. (2021). Skin tape stripping identifies gene transcript signature associated with allergic contact dermatitis. *Contact Dermatitis* 84**,** 308-316.

Tamoutounour, S., Guilliams, M., Montanana Sanchis, F., Liu, H., Terhorst, D., Malosse, C., Pollet, E., Ardouin, L., Luche, H., Sanchez, C., Dalod, M., Malissen, B., and Henri, S. (2013). Origins and Functional Specialization of Macrophages and of Conventional and Monocyte-Derived Dendritic Cells in Mouse Skin. *Immunity* 39**,** 925-938.

Termeer, C., Benedix, F., Sleeman, J., Fieber, C., Voith, U., Ahrens, T., Miyake, K., Freudenberg, M., Galanos, C., and Simon, J.C. (2002). Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med* 195**,** 99-111.

Terui, H., Kimura, Y., Fujimura, C., and Aiba, S. (2021). The IL-1 promoter-driven luciferase reporter cell line THP-G1b can efficiently predict skin-sensitising chemicals. *Arch Toxicol* 95**,** 1647-1657.

Thierse, H.J., Moulon, C., Allespach, Y., Zimmermann, B., Doetze, A., Kuppig, S., Wild, D., Herberg, F., and Weltzien, H.U. (2004). Metal-protein complex-mediated transport and delivery of Ni2+ to TCR/MHC contact sites in nickel-specific human T cell activation. *J Immunol* 172**,** 1926-1934.

Thwe, P.M., and Amiel, E. (2018). The role of nitric oxide in metabolic regulation of Dendritic cell immune function. *Cancer Lett* 412**,** 236-242.

Thwe, P.M., Pelgrom, L., Cooper, R., Beauchamp, S., Reisz, J.A., D'Alessandro, A., Everts, B., and Amiel, E. (2017). Cell-Intrinsic Glycogen Metabolism Supports Early Glycolytic Reprogramming Required for Dendritic Cell Immune Responses. *Cell Metabolism* 26**,** 558-567.e555.

Thyssen, J.P., Johansen, J.D., Linneberg, A., Menné, T., Nielsen, N.H., Meldgaard, M., Szecsi, P.B., Stender, S., and Carlsen, B.C. (2010). The association between null mutations in the filaggrin gene and contact sensitization to nickel and other chemicals in the general population. *Br J Dermatol* 162**,** 1278-1285.

Thyssen, J.P., Johansen, J.D., and Menné, T. (2007). Contact allergy epidemics and their controls. *Contact Dermatitis* 56**,** 185-195.

Toebak, M.J., Gibbs, S., Bruynzeel, D.P., Scheper, R.J., and Rustemeyer, T. (2009). Dendritic cells: biology of the skin. *Contact Dermatitis* 60**,** 2-20.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer* 26**,** 171-176.

UN (2021). Globally harmonized system of classification and labelling of chemicals (GHS). *United Nations: New York, NY, USA*.

Union, E. (1994). "European Parliament and Council Directive 94/27/EC of 30 June 1994 amending for the 12th time Directive 76/769/EEC on the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations".).

UniProt Consortium, T. (2017). UniProt: the universal protein knowledgebase. *Nucleic Acids Res* 45**,** D158-d169.

Uter, W., Aalto-Korte, K., Agner, T., Andersen, K.E., Bircher, A.J., Brans, R., Bruze, M., Diepgen, T.L., Foti, C., Giménez Arnau, A., Gonçalo, M., Goossens, A., McFadden, J., Paulsen, E., Svedman, C., Rustemeyer, T., White, I.R., Wilkinson, M., Johansen, J.D., and Group, o.b.o.t.E.E.C.D.R. (2020a). The epidemic of methylisothiazolinone contact allergy in Europe: follow-up on changing exposures. *Journal of the European Academy of Dermatology and Venereology* 34**,** 333-339.

Uter, W., Gefeller, O., Mahler, V., and Geier, J. (2020b). Trends and current spectrum of contact allergy in Central Europe: results of the Information Network of Departments of Dermatology (IVDK) 2007-2018. *Br J Dermatol* 183**,** 857-865.

Uter, W., and Schnuch, A. (2004). Fragrance allergy increases with age. *Br J Dermatol* 150**,** 1218-1220; author reply 1220.

Vallhov, H., Gutzeit, C., Hultenby, K., Valenta, R., Gronlund, H., and Scheynius, A. (2015). Dendritic cell-derived exosomes carry the major cat allergen Fel d 1 and induce an allergic immune response. *Allergy* 70**,** 1651-1655.

van Helden, S.F.G., van Leeuwen, F.N., and Figdor, C.G. (2008). Human and murine model cell lines for dendritic cell biology evaluated. *Immunology Letters* 117**,** 191-197.

Vennegaard, M.T., Dyring-Andersen, B., Skov, L., Nielsen, M.M., Schmidt, J.D., Bzorek, M., Poulsen, S.S., Thomsen, A.R., Woetmann, A., Thyssen, J.P., Johansen, J.D., Ødum, N., Menné, T., Geisler, C., and Bonefeld, C.M. (2014). Epicutaneous exposure to nickel induces nickel allergy in mice via a MyD88-dependent and interleukin-1-dependent pathway. *Contact Dermatitis* 71**,** 224-232.

Virgens, A.R., Goes, H.F.O., de Carvalho, G.C., Pietrobon, A.J., C. C. Branco, A.C., Ramos, Y.A.L., Pereira, N.V., Orfali, R.L., Aoki, V., da Silva, L.F.F., Sotto, M.N., dos Reis, V.M.S., and Sato, M.N. (2022). Perivascular clusters of Th2 cells and M2 macrophages in allergic contact dermatitis to methylchloroisothiazolinone and methylisothiazolinone. *Experimental Dermatology* 31**,** 191-201.

Vocanson, M., Hennino, A., Rozieres, A., Poyet, G., and Nicolas, J.F. (2009). Effector and regulatory mechanisms in allergic contact dermatitis. *Allergy* 64**,** 1699-1714.

Wang, J., Suárez-Fariñas, M., Estrada, Y., Parker, M.L., Greenlees, L., Stephens, G., Krueger, J., Guttman-Yassky, E., and Howell, M.D. (2017). Identification of unique proteomic signatures in allergic and non-allergic skin disease. *Clinical & Experimental Allergy* 47**,** 1456-1467.

Wang, S.H., Yuan, S.G., Peng, D.Q., and Zhao, S.P. (2012). HDL and ApoA-I inhibit antigen presentation-mediated T cell activation by disrupting lipid rafts in antigen presenting cells. *Atherosclerosis* 225**,** 105-114.

Warbrick, E.V., Dearman, R.J., Lea, L.J., Basketter, D.A., and Kimber, I. (1999). Local lymph node assay responses to paraphenylenediamine: intra- and inter-laboratory evaluations. *J Appl Toxicol* 19**,** 255-260.

Weber, F.C., Németh, T., Csepregi, J.Z., Dudeck, A., Roers, A., Ozsvári, B., Oswald, E., Puskás, L.G., Jakob, T., Mócsai, A., and Martin, S.F. (2015). Neutrophils are required for both the sensitization and elicitation phase of contact hypersensitivity. *J Exp Med* 212**,** 15-22.

Werner, P., Wisgrill, L., Riskumäki, M., Jalonen, E., Vendelin, J., Suomela, S., Lauerma, A., Alenius, H., and Fyhrquist, N. (2021). Identification of novel miRNA-mRNA regulatory networks in contact dermatitis by integrated microarray analysis. *Allergy* 76**,** 1257-1261.

Wilkinson, S.M., Gonçalo, M., Aerts, O., Badulici, S., Dickel, H., Gallo, R., Garcia-Abujeta, J.L., Giménez-Arnau, A.M., Hamman, C., Hervella, M., Isaksson, M., Johansen, J.D., Mahler, V., Niklasson, B., Pigatto, P., Ponyai, G., Rustemeyer, T., Schuttelaar, M.L.A., Spiewak, R., Stingeni, L., Thyssen, J.P., and Uter, W. (2023). The European baseline series and recommended additions: 2023. *Contact Dermatitis* 88**,** 87-92.

Winget, J.M., Finlay, D., Mills, K.J., Huggins, T., Bascom, C., Isfort, R.J., and Moritz, R.L. (2016). Quantitative Proteomic Analysis of Stratum Corneum Dysfunction in Adult Chronic Atopic Dermatitis. *J Invest Dermatol* 136**,** 1732-1735.

Wishart, D.S., Guo, A., Oler, E., Wang, F., Anjum, A., Peters, H., Dizon, R., Sayeeda, Z., Tian, S., Lee, B.L., Berjanskii, M., Mah, R., Yamamoto, M., Jovel, J., Torres-Calzada, C., Hiebert-Giesbrecht, M., Lui, V.W., Varshavi, D., Varshavi, D., Allen, D., Arndt, D., Khetarpal, N., Sivakumaran, A., Harford, K., Sanford, S., Yee, K., Cao, X., Budinski, Z., Liigand, J., Zhang, L., Zheng, J., Mandal, R., Karu, N., Dambrova, M., Schiöth, H.B., Greiner, R., and Gautam, V. (2022). HMDB 5.0: the Human Metabolome Database for 2022. *Nucleic Acids Res* 50**,** D622-d631.

Wright, R.C., and Goldman, L. (1979). Contact Dermatitis: A Historical Perspective. *International Journal of Dermatology* 18**,** 665-668.

Wufuer, M., Lee, G., Hur, W., Jeon, B., Kim, B.J., Choi, T.H., and Lee, S. (2016). Skin-on-a-chip model simulating inflammation, edema and drug-based treatment. *Sci Rep* 6**,** 37471.

Ye, J., Wang, C., Sumpter, R., Jr., Brown, M.S., Goldstein, J.L., and Gale, M., Jr. (2003). Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci U S A* 100**,** 15865-15870.

York, A.G., Williams, K.J., Argus, J.P., Zhou, Q.D., Brar, G., Vergnes, L., Gray, E.E., Zhen, A., Wu, N.C., Yamada, D.H., Cunningham, C.R., Tarling, E.J., Wilks, M.Q., Casero, D., Gray, D.H., Yu, A.K., Wang, E.S., Brooks, D.G., Sun, R., Kitchen, S.G., Wu, T.T., Reue, K., Stetson, D.B., and Bensinger, S.J. (2015). Limiting Cholesterol Biosynthetic Flux Spontaneously Engages Type I IFN Signaling. *Cell* 163**,** 1716-1729.

Zhang, J.G., Czabotar, P.E., Policheni, A.N., Caminschi, I., Wan, S.S., Kitsoulis, S., Tullett, K.M., Robin, A.Y., Brammananth, R., van Delft, M.F., Lu, J., O'Reilly, L.A., Josefsson, E.C., Kile, B.T., Chin, W.J., Mintern, J.D., Olshina, M.A., Wong, W., Baum, J., Wright, M.D., Huang, D.C., Mohandas, N., Coppel, R.L., Colman, P.M., Nicola, N.A., Shortman, K., and Lahoud, M.H. (2012). The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments. *Immunity* 36**,** 646-657.

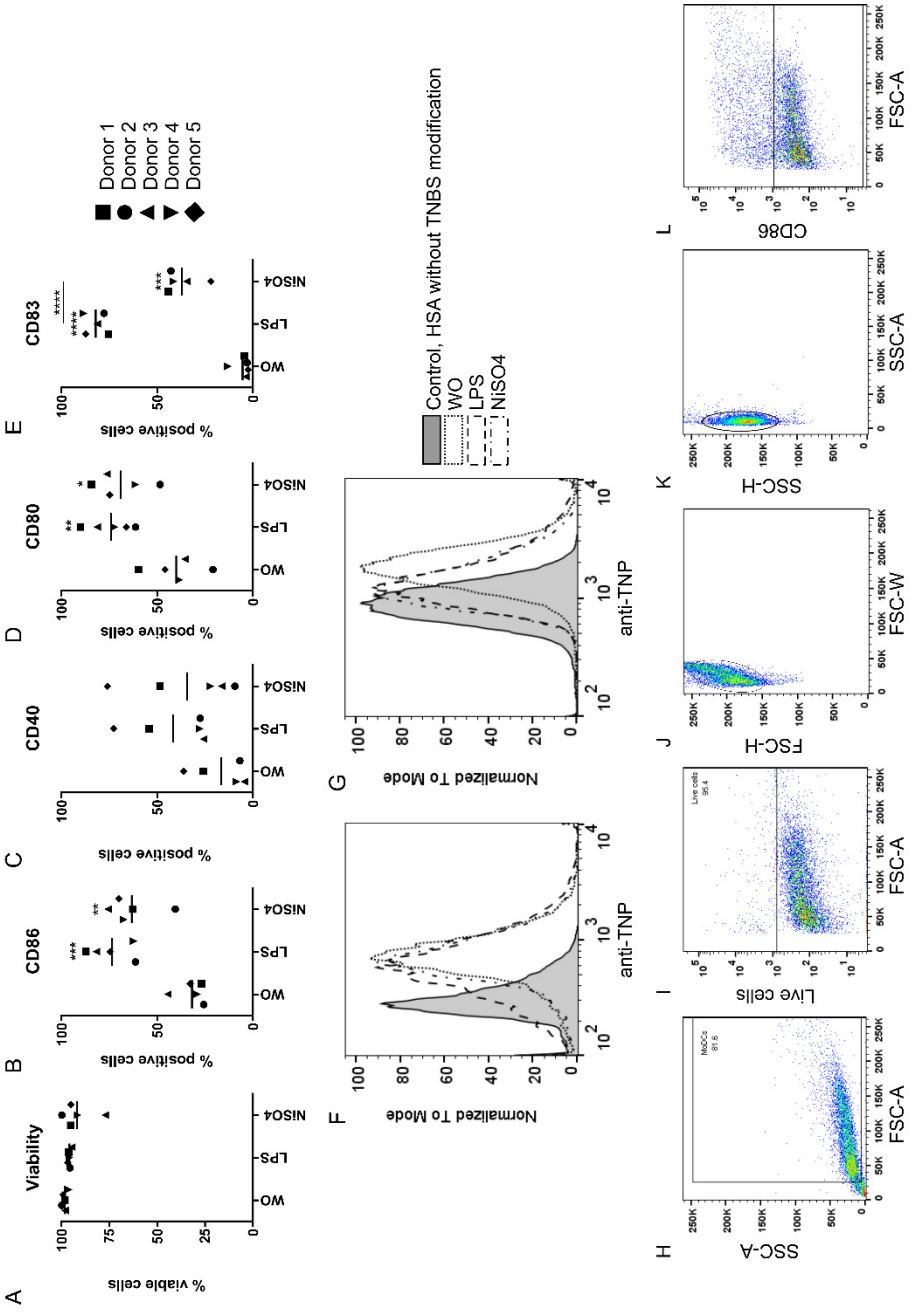
Zhang, Y., Hosoki, M., Oshima, M., Tajima, T., Miyagi, M., Raman, S., Raju, R., and Matsuka, Y. (2023). Identification of microRNA Signatures in Peripheral Blood of Young Women as Potential Biomarkers for Metal Allergy. *Biomedicines* 11.

Zhang, Y., and Wilcox, D.E. (2002). Thermodynamic and spectroscopic study of Cu(II) and Ni(II) binding to bovine serum albumin. *J Biol Inorg Chem* 7**,** 327-337.

Zinkevičienė, A., Kainov, D., Lastauskienė, E., Kvedarienė, V., Bychkov, D., Byrne, M., and Girkontaitė, I. (2015). Serum Biomarkers of Allergic Contact Dermatitis: A Pilot Study. *Int Arch Allergy Immunol* 168**,** 161-164.

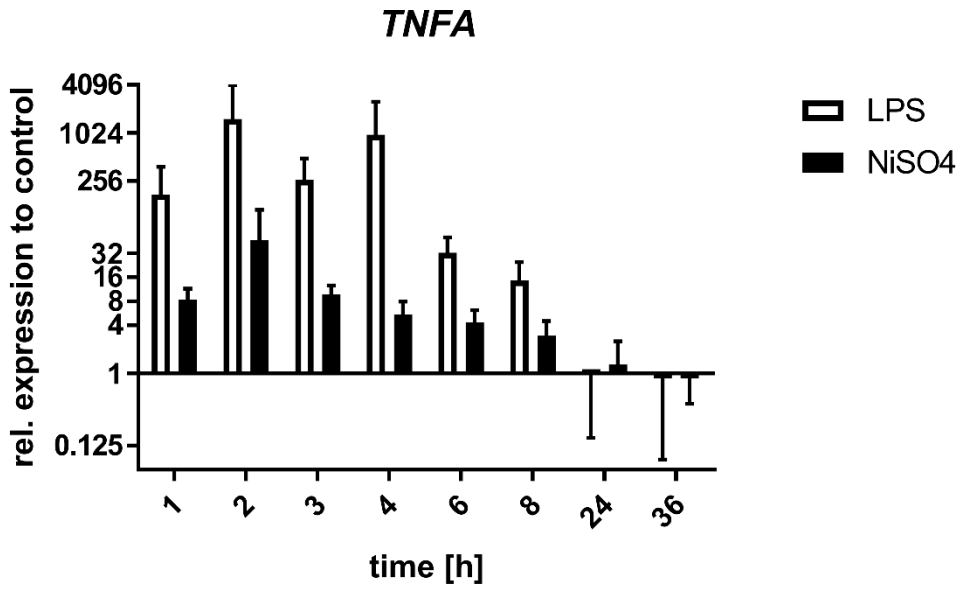
11 Annex I: Supplementary material for chapter 6.2

Supplementary Material

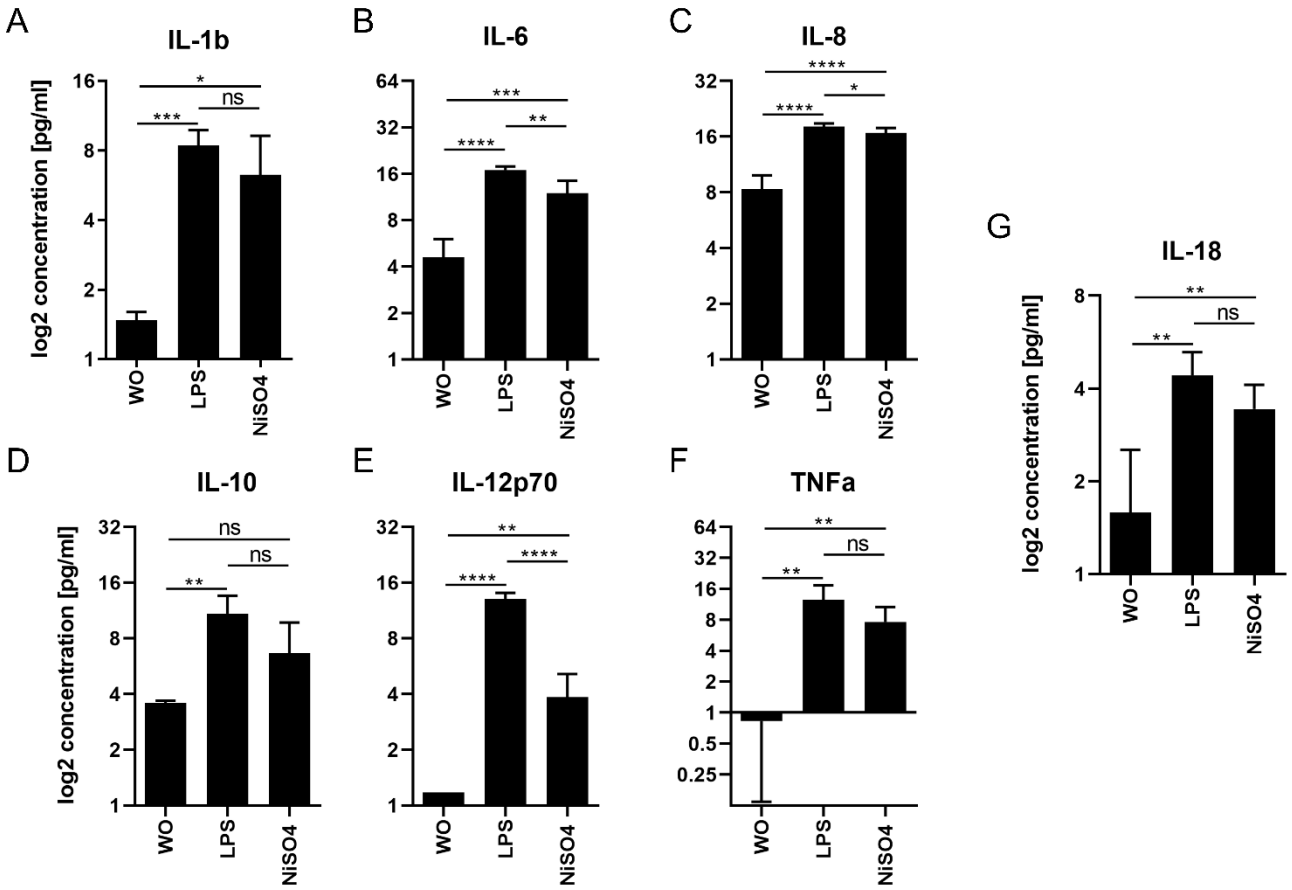


Supplementary Figure 1: Surface marker expression and protein uptake of LPS- and $NiSO_4$-treated MoDCs. Immature MoDCs were treated for 24 h with 400 µM $NiSO_4$ or 2.5 µg/ml LPS. (A) Cell viability was assessed by flow cytometry using near-IR dead cell stain. Induced expression of the activation markers CD86 (B), CD40 (C), CD80 (D), CD83 (E) on the live cells depicted in (A).

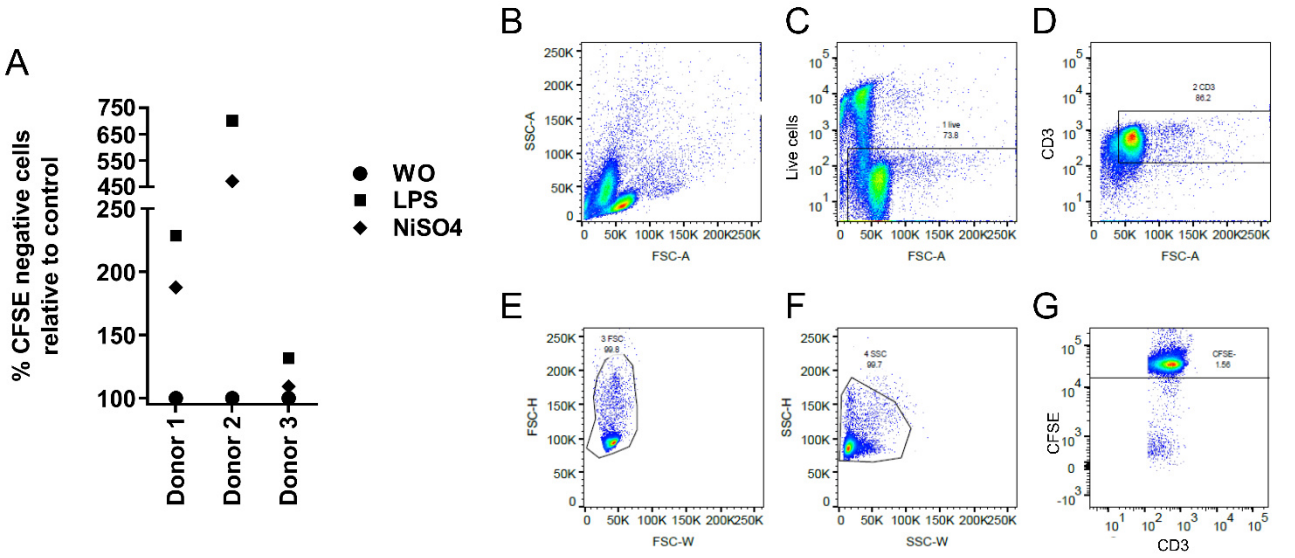
Depicted are the means as well as individual percent values of live cells or positive cells, respectivley (n = 5 donors). Significance to medium control was calculated using Student's t-test (*: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001). (F, G) Phagocytic activity was assessed by HSA-TNP uptake of MoDCs treated with $NiSO_4$ or LPS for 3 h (F) or 24 h (G). One representative experiment out of three is shown. (H-L) Gating strategy for MoDCs: Cells were gated based on size (H), live cells (I), FSC (J) and SSC (K) single cells and the marker of interest (L).



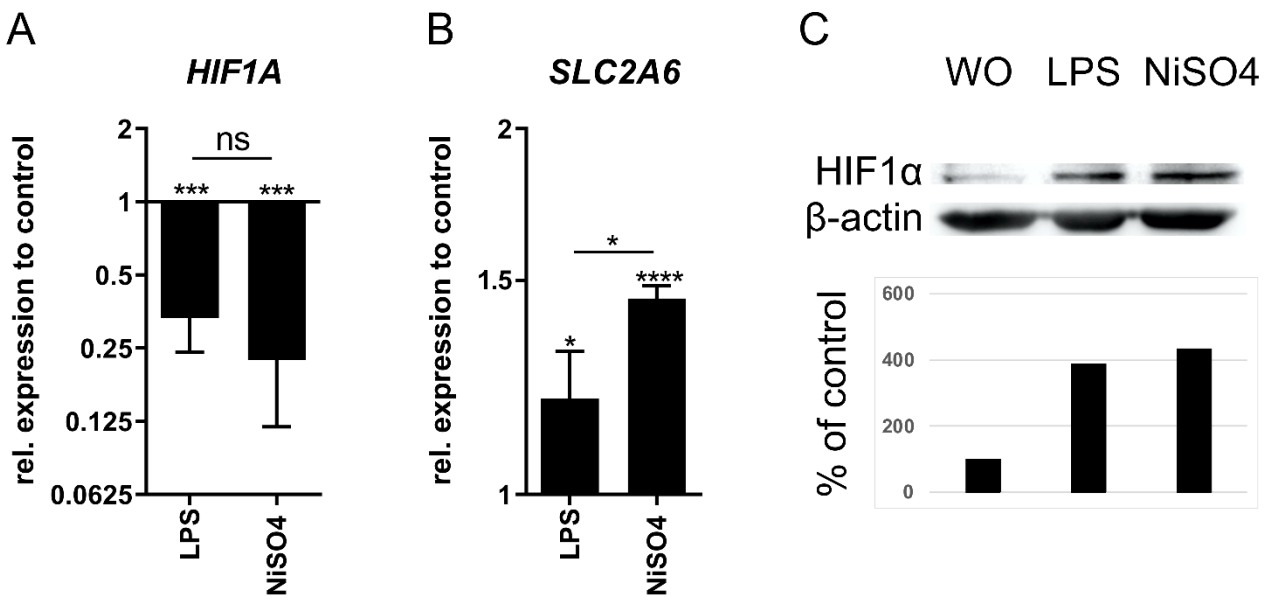
Supplementary Figure 2: Relative TNFA gene expression after treatment with 2.5 µg/ml LPS or 400 µM $NiSO_4$ at the indicated time points. Gene expression was normalized to the gene expression of HPRT (housekeeping gene) and medium control. Means and SDs of the normalized data are shown (n = 3 donors).



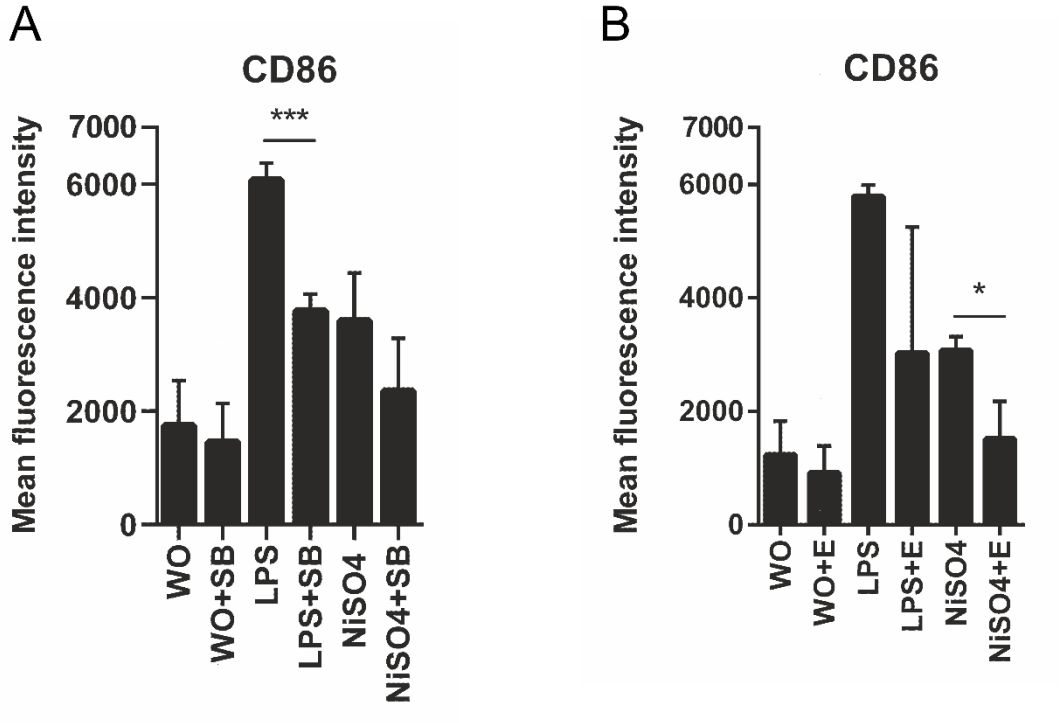
Supplementary Figure 3: Quantification of secreted cytokines in the cell culture supernatant after 24 h treatment of MoDCs with 400 µM $NiSO_4$, 2,5 µg/ml LPS or medium only (WO). IL-1β, IL-6 and IL-8, IL10, IL12p70, IL-18 and TNFα were quantified using a bead-based assay. Shown are means and SDs of the log2 concentrations (n = 5 donors). Significance to medium control was calculated using Student's t-test (ns: not significant; *: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001; ****: p-value < 0.0001).



Supplementary Figure 4: T cell proliferation was assessed in a mixed leukocyte reaction via CFSE dilution. (A) % CFSE negative cells on day 4 of the mixed leukocyte reaction relative to medium control (set to 100 %). Allogenic T cells were stimulated by MoDCs treated for 24 h with 2.5 µg/ml LPS, 400 µM $NiSO_4$ or medium only (n = 3 donor pairs). (B-G) Dot plots of CFSE-negative and $CD3^+$ T cells on day four of the mixed leukocyte reaction. Cells were gated for live cells (C), $CD3^+$ cells (D), FSC (E) and SSC single cell gate (F) and CFSE (G).



Supplementary Figure 5: Relative gene expression as well as protein levels of proteins involved in glucose metabolism. Relative gene expression of hypoxia-inducible factor 1-α (HIF1α, A) and solute carrier family 2 member 6 (SLC2A6, B). Gene expression was measured by qRT-PCR and normalized to the gene expression of HPRT (housekeeping gene) and medium control. Means and SDs of the normalized data are depicted (n = 3 donors). MoDCs were treated with medium only, 2.5 µg/ml LPS or 400 µM $NiSO_4$ for 24 hours. Significance to medium control was calculated using Student's t-test (ns: not significant; *: p-value ≤ 0.05; **: p-value ≤ 0.01; ***: p-value ≤ 0.001; ****: p-value < 0.0001). (C) HIF1α immunoblot of LPS- and $NiSO_4$-treated MoDCs. Cells were harvested after 24 h of treatment. HIF1α was quantified by standardizing the specific band to the respective β-actin loading control. These ratios were normalized to medium only as control (WO). One representative immunoblot is shown (n = 3 donors).



Supplementary Figure 6: Effects of p38 inhibition (A) and HIF1α inhibition (B) on the mean fluorescence intensity of CD86. 10^5 cells were incubated with 20 µM SB203580 or 0.5 nM echinomycin and medium only as control (WO). After 30 min, cells were additionally stimulated with medium only, 2.5 µg/ml LPS or 400 µM $NiSO_4$ for 24 h. CD86 expression was monitored by flow cytometry. Means and SDs are plotted (n = 3 donors). Significance was calculated using Student's t-test with *: $p \leq 0.05$; **: p-value ≤ 0.01 and ***: p-value ≤ 0.001.

12 Annex II: Supplementary material for chapter 6.3

SUPPLEMENTARY INFORMATION

A comparative proteomics analysis of four contact allergens in THP-1 cells shows distinct alterations in key metabolic pathways

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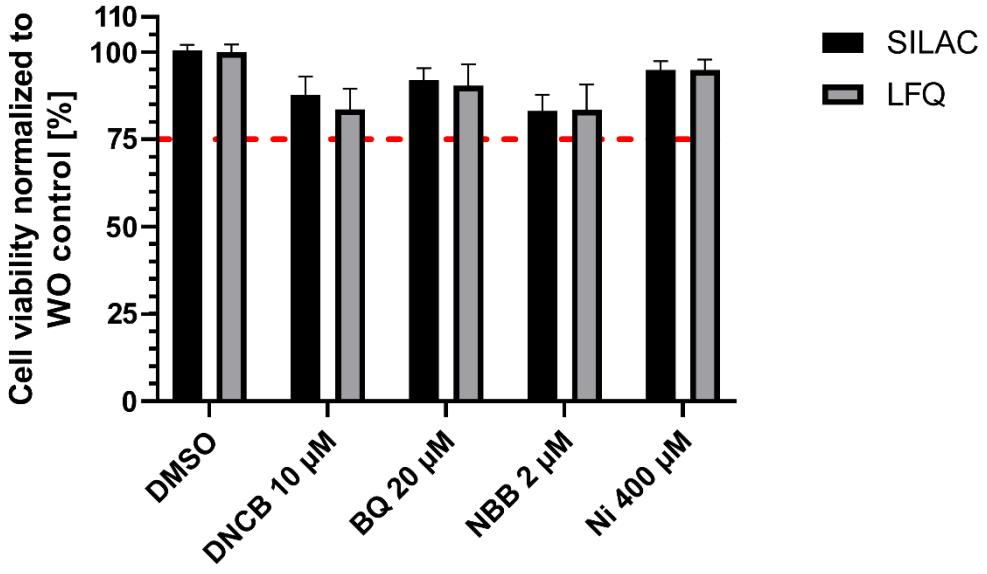

Figure 1 Cell viability normalized to medium control cells. Viability of the cells was determined for each biological replicate used for the proteomic analysis. Data were normalized to corresponding medium control cells. Mean and SD are depicted (n=5). The dashed red line indicates the CV75 value.

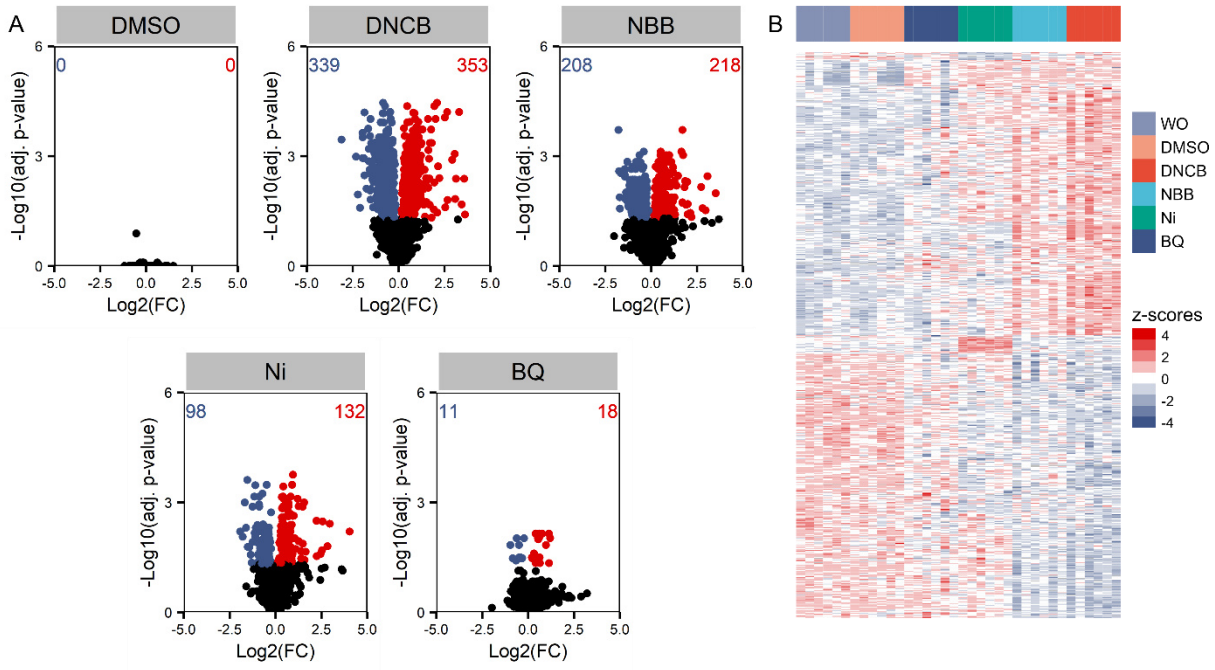

Figure 2 LFQ-based quantitative proteomic analysis in THP-1 cells treated with skin sensitizers. Volcano plots showing Log2(FCs) and –Log10(adjusted p-values) of allergen-treated THP-1 cells indicate changes induced by the allergens tested here. Numbers of regulated proteins (adjusted p-value ≤ 0.05; up: log2(FC) > 0, red; down: log2(FC) < 0, blue) are provided in the corners (A). Clustering of regulated proteins was investigated using z-scored replicate data (B).

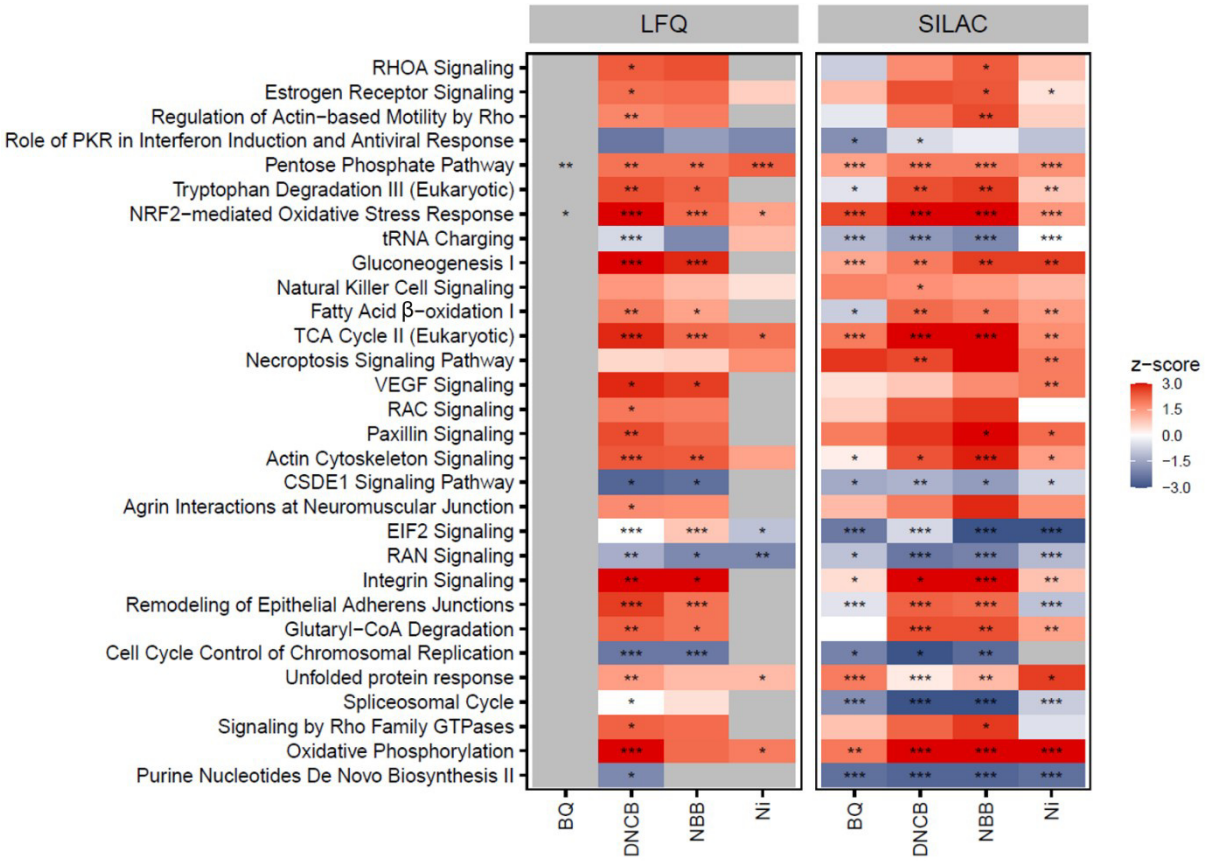

Figure 3 TOP 30 significantly enriched IPA pathways for all treatments for SILAC and LFQ data. Regulated proteins (adjusted p-value ≤ 0.05) were subjected to the enrichment of canonical pathways using IPA, resulting in adjusted p-values (*adjusted p-value ≤ 0.05; **adjusted p-value ≤ 0.01; ***adjusted p-value ≤ 0.001) describing the significance of the enrichment and z-scores reflecting the direction of the regulation (activation (red): z-score > 0, inhibition (blue): z-score < 0). The TOP 30 pathways were extracted based on the adjusted p-values across all treatments.

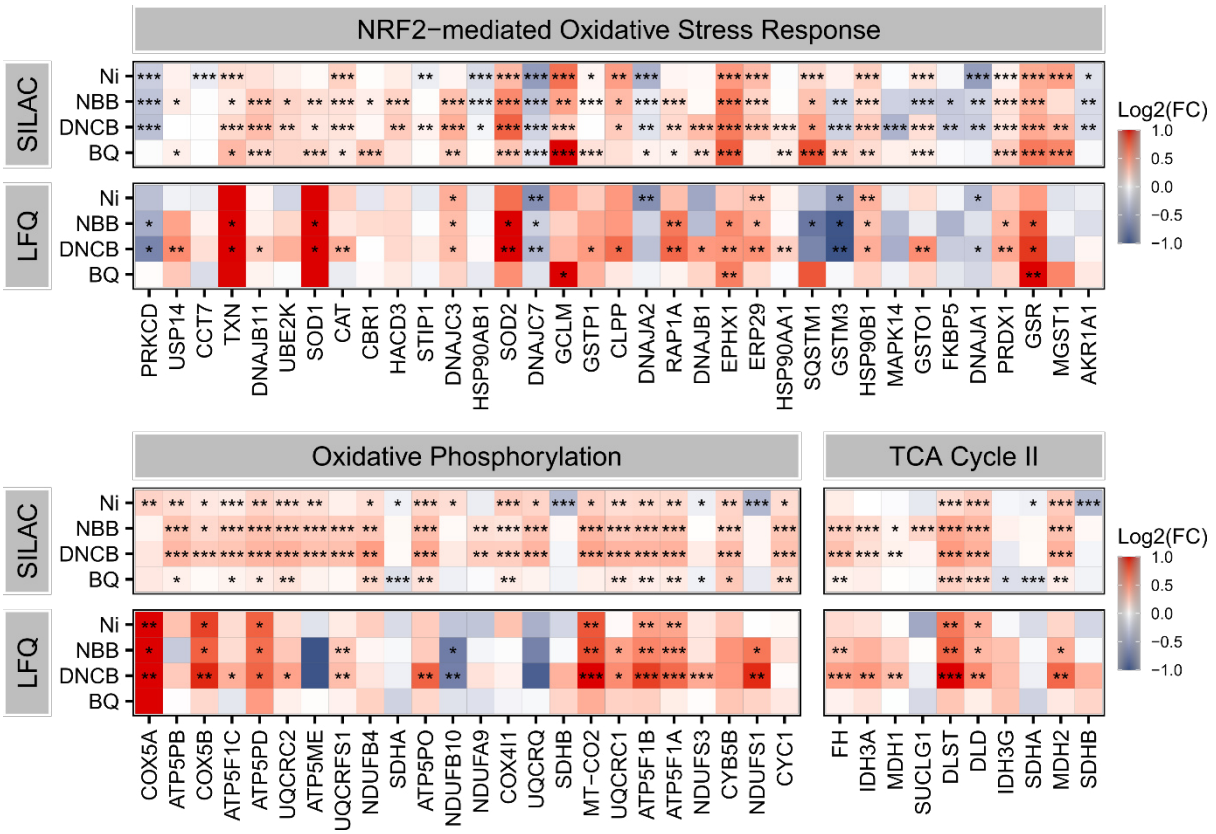

Figure 4 Regulated proteins in selected IPA pathways in THP-1 cells. Proteins assigned to IPA's Nrf2-mediated oxidative stress response, oxidative phosphorylation and TCA cycle II are depicted. Upregulation (red, log2(FC) > 0) and downregulation (blue, log2(FC) < 0) are reflected by colors. Significance is indicated by asterisks: * adjusted p-value ≤ 0.05; ** adjusted p-value ≤ 0.01; *** adjusted p-value ≤ 0.001.

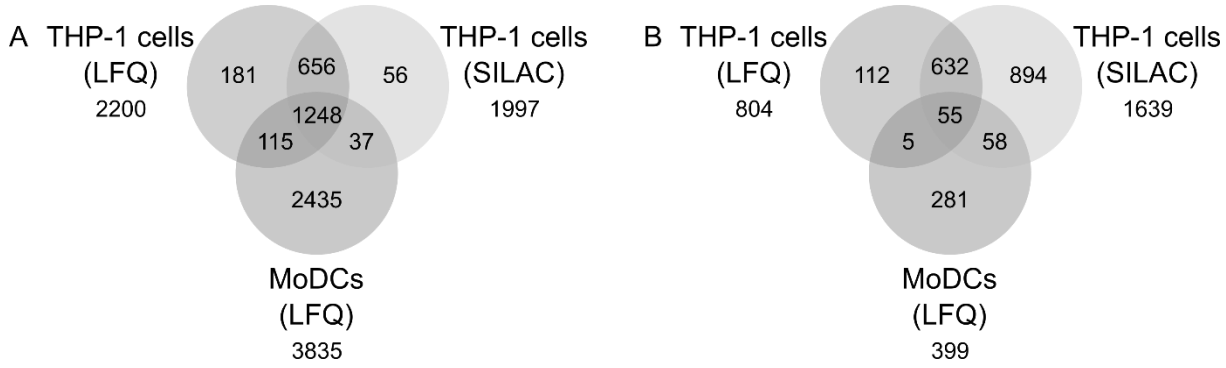

Figure 5 Overlaps of identified and regulated proteins in THP-1 cells investigated with SILAC and LFQ compared to proteins obtained with LFQ in MoDCs. Shown are the overlaps of all reliably identified proteins (A) and the proteins regulated (THP-1 cells: adjusted p-value ≤ 0.05, MoDCs: raw p-value ≤ 0.05) in at least one treatment. MoDC data were extracted from a previously published study of our lab (Höper et al., 2021).

Höper, T., Siewert, K., Dumit, V.I., Von Bergen, M., Schubert, K., and Haase, A. (2021). The Contact Allergen NiSO4 Triggers a Distinct Molecular Response in Primary Human Dendritic Cells Compared to Bacterial LPS. *Frontiers in Immunology* 12.