UNIVERSIDADE D
COIMBRA

Isabel Cristina do Vale Ferreira

THE ROLE OF DANGER SIGNALS IN ALLERGIC DISEASES TO LOW MOLECULAR WEIGHT CHEMICALS

Tese no âmbito do Doutoramento em Ciências Farmacêuticas, ramo de Farmacologia e Farmacoterapia, orientada pela Professora Doutora Maria Celeste Lopes, Professora Doutora Maria Teresa de Teixeira Cruz Rosete e pelo Professor Doutor Bruno Miguel Rodrigues das Neves e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

abril de 2021

Faculdade de Farmácia
da Universidade de Coimbra

THE ROLE OF DANGER SIGNALS IN ALLERGIC DISEASES TO LOW MOLECULAR WEIGHT CHEMICALS

Isabel Cristina do Vale Ferreira

Dissertação de Doutoramento em Ciências Farmacêuticas, ramo de Farmacologia e Farmacoterapia orientada pela Professora Doutora Maria Celeste Lopes, Professora Doutora Maria Teresa de Teixeira Cruz Rosete e pelo Professor Doutor Bruno Miguel Rodrigues das Neves e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

abril de 2021

1 2 9 0

UNIVERSIDADE D
COIMBRA

This work was performed at CNC — Centre for Neuroscience and Cell Biology, University of Coimbra (Coimbra, Portugal), having been financed by The Johns Hopkins Center for Alternatives to Animal Testing through/under the Project no: 2014-07 and by Portuguese national funds via the Portuguese Foundation for Science and Technology (FCT) under the project UIDB/04539/2020 and the PhD scholarship SFRH/BD/110717/2015.

Este trabalho foi realizado no CNC — Centro de Neurociências e Biologia Celular, Universidade de Coimbra (Coimbra, Portugal), tendo sido financiado pelo The Johns Hopkins Center for Alternatives to Animal Testing através do projecto n.° 2014-07 e por fundos nacionais através da Fundação para a Ciência e Tecnologia (FCT) pelo projeto UIDB/04539/2020 e pela bolsa de doutoramento SFRH/BD/110717/2015.

Aos meus pais,

Agradecimentos

Em primeiro lugar gostaria de agradecer aos meus orientadores Professora Doutora Teresa Cruz, Professor Doutor Bruno Neves e Professora Doutora Celeste Lopes pela oportunidade e confiança depositadas e por me terem proporcionado as condições necessárias à concretização deste trabalho.

À Professora Doutora Teresa Cruz por todo o apoio, confiança e liberdade concedidas para a realização deste trabalho. Pelos momentos de discussão científica, mas também pela amizade, pelas conversas e gargalhadas e sobretudo pelo exemplo, o qual levo para a vida. Exemplo de simplicidade, competência, compreensão e preocupação com alunos e orientandos, a qual ultrapassa o que o dever impõe. Preocupada com a realização do trabalho, mas principalmente com o ser humano. Aprendemos a trabalhar em grupo, a respeitar o próximo e, principalmente, que é muito mais fácil multiplicar quando sabemos dividir. Ao Professor Doutor Bruno Neves, agradeço igualmente pelo apoio, confiança e liberdade concedidas para a realização deste trabalho. Pela disponibilidade, amizade e paciência. Por todas as sugestões, discussões e revisões e principalmente por promover o meu sentido crítico desde 2011*!* Pelo exemplo de capacidade de trabalho, paixão e dedicação à ciência. À Professora Doutora Celeste Lopes, por ter aceite embarcar comigo nesta aventura que é o doutoramento, pela disponibilidade, carinho, ajuda e simpatia ao longo desta etapa.

Agradeço igualmente às Instituições onde foi possível a realização deste trabalho ao longo destes anos, nomeadamente ao Centro de Neurociências e Biologia Celular da Universidade de Coimbra, assim como à Fundação para a Ciência e Tecnologia (FCT) e todas as outras entidades/bolsas/prémios pelo apoio financeiro, sem o qual não teria chegado até aqui e desenvolvido este trabalho.

A todos os colegas de laboratório que, para além da amizade e estímulo, colaboraram de alguma forma na obtenção de dados, nas discussões de resultados ou tão somente, mas não menos importante, na criação de um bom ambiente de trabalho. À Ana Silva, confidente das horas vagas. Pelos desabafos e cumplicidade partilhados ao longo de todos estes anos. Pelas discussões científicas, mas também pelas filosóficas. À Cátia Sousa, "ó filha", pela alegria característica que contagia o laboratório, por ter uma enorme capacidade de memória, a qual lhe permite saber onde está quase tudo no laboratório e

pela disponibilidade em ajudar todos os que a rodeiam, desde ficar até mais tarde no laboratório à ajuda em questões científicas e burocráticas. À Mylene Carrascal pelos ensinamentos e discussão dos resultados de citometria. À Mónica, ao João Calmeiro e ao Gonçalo pelo sentido de humor, discussões sobre ciência e pelos momentos de partilha no *open space*. À Professora Alexandrina, com quem iniciei o meu percurso científico pelos ensinamentos, amizade e disponibilidade ao longo de todos estes anos.

O meu sincero agradecimento à Dona Céu, pela amabilidade e apoio constante que nos proporciona. Sem si, o nosso trabalho seria certamente mais difícil. À Dona Natividade e à Dona Fernanda, por tornarem os começos de dia mais divertidos.

Ao meu pai pelo amor incondicional e todo o apoio, encorajamento e liberdade na tomada de decisões e escolhas. Por me demonstrar que somos responsáveis pela nossa felicidade, pela calma com que aceita o que a vida traz e se reinventa. Obrigada por, juntamente com a mãe, serem modelos de coragem, perseverança e resiliência. Pelo ensino e transmissão de valores importantes que me acompanham até hoje.

Um agradecimento especial ao João, por me por a cabeça em água e por ter construído comigo o que mais prezo na vida. Por fomentar a minha atitude crítica e acima de tudo pelo amor, carinho e paciência.

A todos os meus amigos que de uma forma ou de outra partilharam este período da minha vida e que, por lapso ou por limite de escrita, não foram especificamente referidos, um muito obrigada pela amizade e apoio.

Publications

The contents presented in this dissertation are published or will be submitted for publication in peer-reviewed scientific journals as follows:

1. **Ferreira, I.**, Liberal, J., Martins, J. D., Silva, A., Neves, B. M. & Cruz, M.T. (2017). Inflammasome in dendritic cells immunobiology: implications to diseases and therapeutic strategies. Current Drug Targets, 18(9), 1003-1018. https://doi.org/10.2174/1389450117666160921144830

2. **Ferreira, I.,** Silva, A., Martins, J.D., Neves, B. M. & 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 Biology*, 16, 75-86. https://doi.org/10.1016/j.redox.2018.02.002

3. Brites, G. S., **Ferreira, I.**, Sebastião, A. I., Silva, A., Carrascal, M., Neves, B. M., & Cruz, M. T. (2020). Allergic Contact Dermatitis: from pathophysiology to development of new preventive strategies. *Pharmacological Research*, 162:105282. https://doi.org/10.1016/j.phrs.2020.105282

4. Sebastião, A. I.*, **Ferreira, I.***, Brites, G., Silva, A., Neves, B. M. & Teresa, M. (2020). NLRP3 inflammasome and allergic contact dermatitis: a connection to demystify. Pharmaceutics, 12(9), 867. * equally contributing authors. https://doi.org/10.3390/pharmaceutics12090867.

5. **Ferreira, I.**, Cruz, M.T. & Neves, B. M. Thiol reactive sensitizers activate inflammasome through lysosomal membrane destabilization and consequent cathepsin leakage: a step forward the comprehension of sterile inflammation, Manuscript in preparation

Note: Sections of the review articles 1, 3 and 4 were included in Chapter 1. The results presented in this dissertation, included in Chapters 2 and 3, are formatted according to the style of the journal where the papers were published or submitted for publication, with minor modifications.

Table of contents

List of Figures

List of Supplementary Figures

List of Tables

List of Supplementary Tables

Abbreviations

ACD	Allergic contact dermatitis
ADP	Adenosine diphosphate
AIM-2	Absent in melanoma 2
Akt/PKB	Protein kinase B
ALR	AIM2-like receptor
AMP	Adenosine monophosphate
AOP	Adverse outcome pathway
AP1	Activator protein 1
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein
ATP	Adenosine triphosphate
BC	Benzalkonium chloride
BCR	B cell receptor
BMDCs	Bone marrow-derived dendritic cells
CARD	Caspase activating and recruitment domain
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDCs	Classical/conventional/myeloid dendritic cells
CHS	Contact hypersensitivity
CI	Calcium ionophores
CLA	Cutaneous lymphocyte antigen
CLR	C-type lectin receptor
Cr	Chromium
CTL/Tc	Cytotoxic T cells
CTLA-4	Cytotoxic T-lymphocyte–associated antigen 4
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
Cys	Cysteine
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNCB	2,4-Dinitrochlorobenzene
DNFB	1-Fluoro-2,4-dinitrobenzene/2,4-dinitrofluorobenzene
DNP	2,4-dinitrophenol
DNTB	5,5′-Dithiobis(2-nitrobenzoic acid, Ellman’s reagent
DPRA	Direct peptide reactivity assay
DTT	Dithiothreitol
EC	Epithelial cells
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FCCP	Trifluoromethoxy carbonylcyanide phenylhydrazone
FITC	Fluorescein isothiocyanate
Flt3L	Fms-like tyrosine kinase cytokine-3 ligand
GCLM	Glutamate-cysteine ligase modifier subunit
GM-CSF	Granulocyte-macrophage colony–stimulating factor
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
HA	Hyaluronic acid
HAS	Hyaluronan synthases
HBSS	Hank's Balanced Salt Solution
HDI	Hexamethylene diisocyanate
HLA-DR	MHC class II cell surface receptor
HMGB1	High mobility box 1
HMOX1	Heme oxygenase-1
HMW	High molecular weight
HMWHA	High molecular weight HA
HSC	Hematopoietic stem cell
HSP	Heat shock protein

HYAL	Hyaluronidase
ICAM-1/CD54	Intercellular Adhesion Molecule 1/Cluster of Differentiation 54
ICD	Irritant contact dermatitis
IFN	Interferon
IgE	Immunoglobulin E
IKK	IκB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IPS	Instituto Português do Sangue/Portuguese Blood Institute
JNK	c-Jun N-terminal kinase
K562	Chronic myelogenous leukemia cell line;
KC	Keratinocyte
Keap-1	Kelch-like ECH-associated protein 1
KG-1	Human myelogenous leukemia cell line
KO	Knockout
LA	Lactic acid
LAMP	Lysosome-associated membrane protein
LC	Langerhans cell
LLME	Leu-Leu-OMe
LMW	Low molecular weight
LMWHA	Low molecular weight HA
LN	Lymph node
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
Lys	Lysine
MAPK	Mitogen activated protein kinase
MCI	Methylchloroisothiazolinone
MCP-1/CCL2	Monocyte chemoattractant protein-1
MCP-3/CCL7	Monocyte chemotactic protein-3
MCSF	Macrophage stimulating factor
MHC	Major histocompatibility complex
MI	2-Methyl-4-isothiazolin-3-one
MIE	Molecular Initiating Event

MIP-1	Macrophage inflammatory protein-1
MMP	Metalloproteinases
moDCS	Monocyte-derived dendritic cells
Monomac-6	Human acute monocytic leukemia cell line
MSU	Monosodium urate crystals
mtDNA	Mitochondrial DNA
MUTZ-3	Acute myelomonocytic leukemia cell line
MW	Molecular weight
NAC	N-Acetyl Cysteine
NACHT	Nucleotide-binding oligomerization
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor κB
NK	Natural killer
NLRC4	NLR family CARD domain containing 4
NLRP3	NLR family PYRIN domain containing 3
NLRs	(NOD)-like receptors
NOD	Nucleotide-binding oligomerization domain
NQO1	NAD(P)H: quinone oxidoreductase 1
Nrf2	Nuclear factor E2-related factor 2
OA	Oxonate
OECD	Organisation for Economic Co-operation and Development
OVA	Ovalbumin
OXA	Oxazolone
P2X7R	P2X7 purinergic receptor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-Buffered Saline
PD-1	Programmed death 1
PD-1	Programmed death ligand 1
pDCs	Plasmacytoid dendritic cells
PI3K	Phosphatidylinositide 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
POA	Potassium oxonate

PPD	p-phenylenediamine
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
PYD	Pyrin domain
RAGE	Receptors for advanced glycation end products
RANKL	Receptor activator of NF-κB ligand
RANTES/CCL5	Chemokine (C-C motif) ligand 5
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNAi	RNA interference
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
RS	Respiratory sensitization
RT-PCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SLC	Secondary lymphoid tissue chemokine
SOD	Superoxide dismutase
TAP	Antigen processing complex
TCR	T-cell receptor
TDI	Toluene diisocyanate
TGF	Transforming growth factor
Th	T helper cell
THP-1	Human monocytic leukemia cell line
TLR	Toll-like receptor
TMAC	Trimellitic anhydride chloride
TNCB	Trinitrochlorobenzene/2,4,6-trinitro-1-chlorobenzene/
TNF	Tumor necrosis factor
Treg	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
U-937	Human acute monocytic leukemia cell line
UA	Uric acid

VCAM-1	Vascular cell adhesion protein
VLA	Very late antigen
XO	Xanthine oxidase

Abstract

Allergic diseases resulting from exposure to chemicals represent important occupational health problems, as their prevalence is increasing worldwide, with high levels of morbidity, and significant socio-economic impact. Although they can take a variety of forms, the most relevant are allergic contact dermatitis (ACD) resulting from skin sensitization and asthma and occupational rhinitis caused by sensitization of the respiratory tract. ACD affects about 20% of the European population while occupation asthma accounts for 10-25% of total asthma cases. Although thousands of chemicals have been implicated as contact allergens, only about less than 80 chemicals have been confirmed as respiratory allergens. As expected, the nature of cellular and molecular events leading to skin sensitization is rather well characterized compared to respiratory sensitization (RS). It is well established that both diseases depend on the chemical's ability to covalent modify proteins to trigger sensitization. Concomitantly there's also a release of danger signals by epithelial cells that promote dendritic cell (DC) recruitment, activation and maturation, which in turn will prime naïve T cells. However, the knowledge concerning how, and to what degree, the release of danger signals contributes to the differential priming of T cells observed in ACD (Th1 response) and RS (Th2), remains limited. To fill these gaps and move ahead the state of the art, this work aimed to shed light on the differential danger signals elicited by respiratory and skin allergens, using THP-1 cells as a surrogate for DC.

In a first approach, the nature and kinetics of reactive oxygen species (ROS) production, elicited by 1-fluoro-2,4-dinitrobenzene (DNFB) and trimellitic anhydride chloride (TMAC), two golden standards of skin and chemical respiratory allergy, respectively, were evaluated. To track this goal, time course modifications of ROS production and cellular antioxidant defenses were addressed as well as the modulation of MAPKs signaling pathways and transcription of pathophysiological relevant genes in THP-1 cells. The thiol-reactive sensitizer DNFB was shown to directly react with cytoplasmic glutathione (GSH) causing its rapid and marked depletion which resulted in a general increase in ROS accumulation. In turn, TMAC, which preferentially reacts with amine groups, induced a delayed GSH depletion as a consequence of increased mitochondrial ROS production. These divergences in ROS production seemed to be correlated with the different extension of intracellular signaling pathways activation and, by consequence, with

distinct transcription kinetics of genes such as *HMOX* and *NQO1*. Together, these data suggest that skin sensitizers-induced Th1 polarization may result from a sustained transcription of pro-inflammatory cytokines and co-stimulatory molecules in DCs, while Th2 polarization is characterized by a modest and transitory transcription.

In a second approach, and since several danger signals are also described as inflammasome activators, the mechanisms behind sensitizer-induced inflammasome activation were characterized. Inflammasome activation has been recognized as critical for successful sensitization and activation of T cell responses. Inflammasomes are cytoplasmic caspase-1-activating protein complexes that promote maturation and secretion of the proinflammatory cytokines interleukin (IL)-1ß and IL-18. The most intensively studied inflammasome, (NOD)-like receptor protein 3 (NLRP3) inflammasome, can be activated by a plethora of trigger such as potassium efflux, lysosomal rupture, ROS production, and mitochondrial disruption. Although lysosomal rupture is often associated with crystalline and particulate materials, herein we demonstrate that the skin sensitizer DNFB activates NLRP3 inflammasome through lysosomal destabilization and subsequent cathepsin leakage. Inhibition of cathepsin activity has shown to impair NLRP3 activation and the DNFB-induced expression of the maturation marker CD86, thus disclosing an innate immune mechanism crucial for the development of allergic contact sensitization to LMW chemicals. Furthermore, this new mechanism of inflammasome was observed with other thiol-reactive skin sensitizers, suggesting that this mechanism is shared by sensitizers with high thiol reactivity. A new method to immunoprecipitate DNFB-haptenated proteins was also proposed has a tool not only to comprehend how DNFB induces lysosomal destabilization but also to identify possible therapeutics targets for ACD.

Altogether these results generate important insights regarding danger signals differential involvement in ACD and RS, which may be crucial for Th1 or Th2 cell response development. Furthermore, we describe for the first time a mechanism by which non-particulate structures or chemicals without detergent-like effects, trigger the assembly of NLRP3 inflammasome.

Resumo

As alergias decorrentes da exposição a produtos químicos têm vindo a aumentar a nível mundial, com elevados índices de morbidade e um impacto socioeconómico significativo. Entre as alergias ocupacionais mais comuns, são de destacar a dermatite de contacto alérgica (ACD), resultante da sensibilização da pele, e a asma e rinite ocupacionais, causadas pela sensibilização do trato respiratório. A ACD afeta cerca de 20% da população europeia, enquanto a asma ocupacional é responsável por 10-25% do total de casos de asma. A fisiopatologia da ACD é consideravelmente mais estudada que a sensibilização respiratória, sendo que atualmente milhares de compostos foram identificados como alergénios cutâneos enquanto apenas 80 foram identificados como alergénios respiratórios. Não obstante, é consensual dentro da comunidade científica que ambas as patologias dependem da capacidade dos compostos modificarem proteínas celulares através da formação de ligações covalentes com as mesmas, processo necessário para promoverem uma reação imunológica. Concomitantemente, há a liberação de mediadores moleculares responsáveis pelo recrutamento, diferenciação e maturação das células dendríticas (DC), as quais migram para os nódulos linfáticos onde ativam linfócitos T. Apesar da importância dos mediadores moleculares em ambas as respostas imunológicas, o conhecimento relativo a como e em que grau a sua libertação contribui para o *priming* e polarização diferencial dos linfócitos T em Th1 e Th2, característicos da ACD e sensibilização respiratória, respetivamente, permanece limitado. Com o objetivo de preencher as lacunas existentes e avançar no estado da arte, este trabalho teve como objetivo identificar os diferentes mediadores moleculares diferencialmente modulados por alergénios respiratórios e cutâneos, usando células THP-1 como modelo de células dendríticas.

Numa primeira abordagem, foram avaliadas a natureza e a cinética da produção de espécies reativas de oxigénio (ROS) induzida por 1-fluoro-2,4-dinitrobenzeno (DNFB) e cloreto de anidrido trimelítico (TMAC), compostos referência para a ACD e a sensibilização respiratória, respetivamente. Com este objetivo, foram avaliados os níveis de ROS e defesas antioxidantes, bem como as vias de sinalização MAPKs e a transcrição de genes envolvidos na resposta antioxidante. Os resultados obtidos demonstraram que o alergénio cutâneo DNFB, que reage preferencialmente com grupos tiol, conduz a uma rápida depleção da glutationa citoplasmática, despoletando um aumento nos níveis de

ROS. Por sua vez, o alergénio respiratório TMAC, que reage preferencialmente com grupos amina, induziu uma depleção de glutationa mais tardia como consequência do aumento do ROS mitocondrial. Estes resultados correlacionam-se com a intensidade com que determinadas vias de sinalização são ativadas e, por conseguinte, com a expressão diferencial de genes como *HM0X* e *NQ01*. Em conjunto, estes resultados sugerem que a polarização Th1 induzida por alergénios cutâneos resulta de uma produção de citocinas pró-inflamatórias e expressão de moléculas co-estimuladoras mantidas ao longo do tempo, enquanto a polarização Th2 é caracterizada por uma produção modesta e transitória.

Uma vez que muitos dos mediadores moleculares responsáveis pelo recrutamento, diferenciação e maturação DC, estão descritos como ativadores do inflamassoma, numa segunda abordagem, foram caracterizados os mecanismos de ativação do inflamassoma por alergénios cutâneos e respiratórios. Os inflamassomas são complexos proteicos citoplasmáticos que ativam a caspase-1 e promovem a maturação e secreção de citocinas pró-inflamatórias como a IL-1ß e a IL-18. Atualmente, o inflamassoma mais estudado é o NLRP3 e pode ser ativado por uma variedade de estímulos tais como efluxo de potássio, destabilização do lisossoma, ROS e destabilização da mitocôndria. Apesar da destabilização do lisossoma estar maioritariamente associada a partículas com estrutura cristalina, os resultados apresentados nesta dissertação demonstram que o alergénio DNFB ativa o inflamassoma NLRP3 via destabilização lisossomal e consequente libertação da enzima catepsina B. O pré-tratamento das células com um inibidor da catepsina B diminui a ativação do inflamassoma NLRP3 induzida pelo DNFB, assim como a expressão do marcador de maturação CD86, revelando ser um mecanismo crucial para o desenvolvimento de ACD em resposta a alergénios de baixo peso molecular. Este novo mecanismo de ativação do inflamassoma foi igualmente observado com outros alergénios com elevada reatividade para grupos tiol, sugerindo que este mecanismo pode ser comum a alergénios com elevada reatividade para grupos tiol. Foi também proposto um novo método para imunoprecipitar proteínas haptenizadas pelo DNFB, não só como uma ferramenta para a compreensão dos mecanismos moleculares subjacentes à destabilização do lisossoma pelo DNFB, mas também na perspectiva de identificação de novos alvos terapêuticos para a ACD.

Na sua globalidade, os resultados descritos nesta dissertação fornecem evidências importantes sobre o envolvimento diferencial das alarminas na ACD e sensibilização

respiratória, as quais são cruciais para o desenvolvimento de uma resposta imunológica Th1 ou Th2. Adicionalmente, e tendo em conta o estado da arte, este é o primeiro estudo que reporta a ativação do inflamassoma por um alergénio envolvendo a destabilização do lisossoma.

CHAPTER 1

Introduction

1.1 CHEMICAL ALLERGY

Daily, humans are exposed to several natural and synthetic chemicals that may or may not be recognized as foreign by the human organism (xenobiotics). Usually, the interaction with such xenobiotics often occurs through inhalation, ingestion, or skin exposure, without perceived physiological consequences. Although, some may provoke an immune response and can be divided in two categories: high molecular weight chemicals (HMW, >1000 Dalton) and low molecular weight reactive chemicals (LMW, <1000 Dalton). HMW molecules can directly stimulate the immune system and include environmental or occupational proteins such as flours and cereals, enzymes, plant proteins, pollens, dust mite and cockroach allergens. In turn, LMW chemicals are too small to be recognized by the immune system and therefore must first react with large carrier molecules such as proteins to elicit an immune response. This process is called haptenation and was recognized as a mandatory event for sensitization-induction over 80 years ago by Landsteiner and Jacobs (Landsteiner *et al.*, 1936). While some LMW chemical are intrinsically reactive, others need to be biochemical modified, following exposure to their environment or enzymatically metabolized, to yield reactive compounds. These chemicals can be classified either as pre-haptens, which are transformed abiotically (e.g. by UV light, temperature or oxygen) or as pro-haptens, which are converted via enzymatic transformation (Lepoittevin, 2006; Aptula *et al.*, 2007; Vocanson *et al.*, 2009). From an epidemiologic perspective, the most relevant forms of chemical allergy are allergic contact dermatitis (ACD), resulting from skin sensitisation, and occupational rhinitis and asthma caused by sensitisation of the respiratory tract (Kimber *et al.*, 2011). ACD affects about 20% of the European population (Diepgen *et al.*, 2016) while asthma is one of the most prevalent occupational lung disease. Asthma affects more than 339 million people worldwide (Vos *et al.*, 2017), of which 10-25% suffer from occupational asthma (Cartier *et al.*, 2019). In fact, ACD and respiratory sensitization (RS) prevalence and incidence has been increasing worldwide, not only due to genetic background, but mostly to environmental factors, namely the "modern-lifestyle". These diseases, have high social and economic burdens, with direct and indirect costs, affecting both the worker, the employer and the government entities. ACD and RS are associated with a high rate of prolonged work disruption, job changes and even permanent unemployment and loss of income, which all together, also affect worker's mental health. Furthermore, due to worker's sickness absence and labor turnover, the employer suffers a loss in productivity as well as

an increase in costs associated with compensation and insurance. Ultimately, state's costs include health care, compensation, unemployment support and loss of tax revenues (Baur *et al*., 2012). Although ACD and RS can manifest as occupational diseases, they can also occur outside of work environment. Indeed, the recently increased demand for gel nails and acrylic nails (methacrylate-based gel varnishes) led to a shift of contact allergy to consumers (Muttardi *et al*., 2016).

While many thousands of chemicals have been implicated as contact allergens, only about less than 80 chemicals have been confirmed as respiratory allergens (Kimber *et al*., 2018). Likewise, and although scientific knowledge is far from complete, the nature of cellular and molecular events leading to the skin sensitization is rather well characterized while the mechanisms/events leading to sensitization of the respiratory tract are not clearly understood and some still remain controversial (e.g. route of exposure and requirement of Immunoglobulin E (IgE)). Although these pathologies share some similarities, they have clear mechanistic differences which, under normal circumstances, will culminate in a preferential elicitation of a T helper type 1 (Th1) response for contact allergens, while respiratory allergens will favor the development of Th2 responses (Kimber *et al*., 2014). Therefore, the better-defined processes of skin sensitization to LMW chemicals will be addressed firstly, followed by what is currently known about LMW respiratory sensitization. Similarities and differences between ACD and RS will also be disclosed.

1.2 ALLERGIC DISEASES TO LOW MOLECULAR WEIGHT (LMW) CHEMICALS

1.2.1 Allergic Contact Dermatitis

ACD is the second most common type of contact dermatitis following irritant contact dermatitis (ICD). Although both diseases share some signs and symptoms, their pathophysiological mechanism is quite different. ICD, which accounts for 80% of all contact dermatitis cases, is an inflammatory non-immunologic cutaneous reaction triggered after a single exposure to an irritant or toxic substance (e.g. abrasives, cleaning, oxidizing, and reducing agents) resulting in skin damage and cutaneous inflammation from direct cytotoxic effects (Usatine et al., 2010; Wolff et al., 2017). It can occur as an acute or

chronic disease and typically presents with erythema, blisters, pustules, haemorrhage, crusts, scales and erosions, and also with pruritus or even pain – acute ICD, or diffuse or localized lesions with typically poorly defined erythematous scaly patches and plaques, dryness of skin, lichenification and desquamation – chronic ICD (Novak-Bilić *et al*., 2018). ACD, which accounts for 20% of the cases of contact dermatitis, represents the most prevalent form of immunotoxicity found in humans (Thyssen *et al*., 2007). It is a type IV, T cell–mediated, delayed hypersensitivity reaction that occurs after skin exposure to a specific hapten (e.g. jewelry metals (e.g. nickel), cosmetic products, fragrances and preservatives) in genetically predisposed and previously sensitized individuals. It usually only manifests upon repeated exposures to the sensitizing agent, with intense pruritus, stinging and pain accompanied by well-demarcated erythema and edema, commonly involving the hands, face, or eyelids (Novak-Bilić *et al*., 2018; Owen *et al*., 2018). Despite not being lethal, ACD can cause considerable morbidity. Age, sex, ethnicity, skin barrier function, lifestyle, and occupational exposures also play a role in ACD elicitation. Although ACD can occur in any setting, many cases are related to exposures in the workplace. Metal workers, hairdressers, healthcare workers, cleaners, employees in the food industry, construction workers and painters are the most affected professionals (Fyhrquist *et al*., 2014).

1.2.1.1 Skin sensitizers

To date, more than 4000 chemical substances have been implicated in ACD (Esser *et al*., 2017). Common skin allergens include transition metals (nickel, chromium, and cobalt), medication, preservatives, fragrances, hair dyes, acrylates, rubber chemicals, epoxy resins and are described in more detail in Table 1.1 (Qin *et al*., 2015; Shih *et al*., 2015; Gonçalo *et al*., 2018; Nassau *et al*., 2020; Uter *et al*., 2020).

Table 1.1 Common chemical contact allergens and source of exposure. Adapted from Qin *et al*., 2015; Shih *et al*., 2015; Gonçalo *et al.*, 2018; Nassau *et al*., 2020 and Uter *et al.*, 2020.

Chemical Class	Examples	Use/Occupation/Source
Metals	Nickel sulfate Cobalt chloride Copper sulfate Potassium dichromate	Jewelry, metal items, coins, medical products, tools, musical instruments, office supplies

Medications	Bacitracin, neomycin sulfate Benzocaine, procaine Corticosteroids	Antimicrobials Anesthetics Corticosteroids
Preservatives/ Antiseptics/ Disinfectants	Formaldehyde/formaldehyde releasers Isothiazolinones Parabens Methyldibromo glutaronitrile Thimerosal Iodopropynyl burylcarbamate	Household products, soaps, cleansers, paper, pressboard, fabric, urea-formaldehyde foam insulations, textile finishing treatments, metalworking fluids, personal hygiene products
Fragrances	Balsam of Peru (myroxylon pereirae) d-Limonene	Fragrances, perfume, cosmetics
Hair dyes	p-phenylenediamine	Hair coloring
Rubber accelerators	Carbamates Thiurams Mercaptobenzothiazole	Rubber chemicals – Shoes, gloves, elastic, waistbands, rubberized computer accessories
Adhesives	phenol-formaldehyde resins epoxy resin	Adhesives and glues, laminates, surface coatings, paints and inks, dental bonding agents
Acrylates/ methacrylates	2-hydroxyethyl methacrylate 2-hydroxypropyl methacrylate ethyleneglycol dimethacrylate Ethylene glycol dimethacrylate Triethylene glycol dimethacrylate Methyl methacrylate	Nail cosmetic products, dentistry, bone cement
Vehicles	Propylene glycol	Vehicle in topical medications, personal care/hygiene products, auto care, cosmetics, foods, household cleaners, oral care, industry, sunscreens, wipes, yard care

1.2.1.2 Pathophysiology of Allergic Contact Dermatitis

The current knowledge of the molecular mechanisms and pathophysiology of ACD has been mainly derived from contact hypersensitivity (CHS) animal models, in which skin inflammation is induced by painting mouse ears with strong experimental contact sensitizers such as 2,4-dinitrofluorobenzene (DNFB), dinitrochlorobenzene (DNCB), trinitrochlorobenzene (TNCB) and oxazolone (Vocanson *et al.*, 2009). Skin sensitization involves the activation of both innate and the adaptive arms of the immune system and can be divided into two temporally and spatially distinct phases.

First, the sensitization phase, also referred as afferent or induction phase, in which an inherently susceptible subject is exposed to a threshold or greater concentration of a

contact allergen that causes priming of the immune system. Briefly, the contact allergen gains access across the *stratum corneum* and reaches the viable epidermis and beyond, where it covalently binds to carrier proteins converting them into immunogenic hapten-protein complexes – haptenation. Chemical modification of proteins by contact allergens usually occurs at nucleophilic sites, namely cysteine and lysine residues, but other amino acids are also modified (e.g. methionine, tyrosine and histidine), although with a lesser extent (Vocanson *et al*., 2009). The covalent modification of cellular proteins is associated with a certain level of cytotoxicity resulting in the activation of innate immunity and the production of cytokines, chemokines, reactive oxygen species (ROS), and the release of danger signals and damage-associated molecular patterns (DAMPs), such as hyaluronic acid (HA) fragments and adenosine triphosphate (ATP) (Vocanson *et al*., 2009; Esser *et al*., 2012; Martin, 2012). These signals are then recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) on skin dendritic cells (DCs) such as Langerhans cells (LCs) and dermal DCs, and are involved in their recruitment, migration, and maturation.

ACD requires a close cooperation of different cell types such as antigen-presenting cells (APCs), T, B, NK (natural killer) lymphocytes, keratinocytes, endothelial cells, mast cells, fibroblast and platelets, which communicate through direct contact and by numerous cytokines and chemokines (Tonċić *et al*., 2011; Martin *et al*., 2018). Mast cells, which are an important source of tumor necrosis factor (TNF)-α, are responsible for neutrophil extravasation into inflamed tissue, through localization on the proximity of blood vessels and by secretion of the neutrophil-attracting chemokine (C-X-C motif) ligand 1 (CXCL1) and CXCL2. Furthermore, mast cell deficiency or depletion significantly impairs CHS development, also compromising T-cell recruitment to the skin (Martin *et al*., 2018). Keratinocytes are also important players in ACD. Indeed, besides expressing TLRs and answer to haptens, they also secret interleukin (IL)-1β, IL-1α, IL-8, IL-10, IL-18, IL-23, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-1α, TNF-α and GM-CSF promote E-cadherin downregulation and upregulation of the expression of adhesion molecules (cluster of differentiation (CD)54, α_6 integrin and CD44 variants), facilitating LC and dermal DC migration. Concomitantly, these cells also increase their expression of chemokine receptors such as C-C chemokine receptor type 4 (CCR4), CCR7 and C-X-C chemokine receptor type 4 (CXCR4), and produce matrix metalloproteinases (MMP), such as MMP-9, that facilitate LC migration through the dermis

into the regional lymph nodes (Agner *et al.*, 2011; Peiser *et al.*, 2012; Dhingra *et al.*, 2013; Honda *et al.*, 2013; Koppes *et al.*, 2017; Rustemeyer *et al.*, 2020). CCR7 ligands (chemokine ligand (CCL)19 and secondary lymphoid tissue chemokine (SLC)/CCL21) are produced by both lymphatics and high endothelial cells, making CCR7 particularly important in guiding mature DCs to lymph node paracortical areas, where CCR7-expressing naïve T cells are also found (Agner *et al.*, 2011; Kaplan *et al.*, 2012; Rustemeyer *et al.*, 2020). LCs/DCs maturation process is characterized by morphological, functional and phenotypical changes, namely the up-regulation of costimulatory molecules (CD83, CD86, CD40, CD58 and CD54, also known as intercellular adhesion molecule 1 (ICAM-1)) and antigen-presenting molecules (major histocompatibility complex (MHC)-II) (Banchereau *et al.*, 2000; Agner *et al.*, 2011). In the lymph node, LCs and DCs present the antigen to T lymphocytes through MHC and T cell receptor (TCR) interaction. Antigen bound to MHC-I or MHC-II are recognized by $CD8^+$ and $CD4^+$ T cells, respectively (Agner *et al.*, 2011). For an efficient priming of naïve T lymphocytes, a triple signal coming from skin DCs must be provided: signal 1, binding of MHC-antigen complex to TCR; signal 2, interaction of co-stimulatory molecules on DCs with their ligands on T cells and signal 3, secretion of cytokines and chemokines. In the absence of co-stimulatory signals, MHC/TCR interaction normally leads to anergy or deletion, promoting tolerogenic responses. Upon activation, T-cells produce IL-2, which is a highly potent T-cell growth factor, allowing primed T-cells to proliferate abundantly. Signal 3 - DCs cytokine profile, defines T cell polarization, with IL-12 leading to the formation of effector cells, while IL-10 promotes regulatory T cell generation. This leads to T-cell clonal expansion and activation, culminating in the formation of immunological memory against the presented antigens. T lymphocytes (regulatory $CD4^+$ Th1/Th17 and effectors $CD8^+$ Tc1/Tc17 T cells) then return to blood circulation via the thoracic duct where they will patrol various tissues, including the skin. A microenvironment rich in IL-12p70, and interferon (IFN)-γ promotes the differentiation of Th1 and Tc1 cells, while IL-6, transforming growth factor (TGF)-β, IL-21, IL-23 and IL-1β leads to Th17/22 polarization. In the presence of IL-4, T cells will develop a Th2 phenotype (Koppes et al., 2017). Skin DCs also induce the expression of skin T cell homing receptors (e.g. cutaneous lymphocyte antigen (CLA), CCR4 and CCR10), which preferably direct these lymphocytes to the tissue of origin of the corresponding DCs (Agner *et al.*, 2011; Tonc̈ić *et al.*, 2011; Koppes *et al.*, 2017). These cytokines also attract more immune cells to the challenged site (e.g. skin), strengthening

the immune responses. The outcome is determined by the balance of both sensitizing and tolerizing pathways. In humans, the sensitization phase takes 10-15 days while in the murine contact hypersensitivity model it only takes 5-7 days (Vocanson *et al.* 2009).

Second, the elicitation phase, also known as efferent phase, occurs upon re-exposure of the skin to the same chemical. Haptens diffuse into the skin and are taken up by skin cells that then present MHC classes I and/or II-haptenated peptide complexes, to effector and memory T-lymphocytes developed during the sensitization phase (Figure 1.1). This triggers a cascade of biochemical and cellular responses resulting in cutaneous inflammatory reaction often characterized by erythema, edema, vesicles, oozing, and notably intense pruritus, which are clinically recognized as ACD. Despite the crucial role of DCs in the sensitization phase, they are not required in the elicitation phase. As mentioned before, macrophages, keratinocytes and mast cells may also act as "non-professional" APCs amplifying the activation of hapten-specific effector T cells. Indeed, studies with UVB/topical corticosteroid-induced LC depletion or their selective ablation in experimental models did not result in a reduced allergic response (Agner *et al.*, 2011).

The chemical-induced innate immune response triggers the release of IL-1β, TNF-α, and IL-18 from keratinocytes and LCs. Keratinocytes also secrete T cell-attracting chemokines such as CXCL9/10, CCL17, CCL20, and CCL27. T cells must cross the dermal microvasculature and the dermis to reach the keratinocytes modified by the allergen. Although some antigen-driven T cell-specific migration may occur, most T cells are recruited in a non-antigen specific way, attracted to the skin through the expression of chemokines and adhesion molecules by keratinocytes, DCs, fibroblasts, mast cells and endothelial cells (Goebeler *et al.*, 2001; Agner *et al.*, 2011). Activated T cells present the homing antigen CLA, very late antigen (VLA)-4 as well as multiple chemokine receptors. CLA binds to E-selectin (expressed on stimulated endothelial cells) and VLA-4 binds to endothelial integrin vascular cell adhesion protein (VCAM)-1, initiating diapedesis (Agner *et al.*, 2011). Upon encounter of their specific antigen, specific T cells proliferate *in loco* (Honda *et al.*, 2013). Both $CD4^+$ and $CD8^+$ mediate skin inflammatory reaction, however, $CD8^+$, which enter the skin first, are the main effector cells, whereas $CD4^+$ could assume a pathogenic or regulatory role (Agner *et al.*, 2011; Tonc̈ić *et al.*, 2011). After the chemical challenge, NK T cells are also activated and produce IL-4, resulting in type-1 B lymphocytes activation and specific IgM production, which cleaves complement, thus forming C5a, that in turn promotes the release by mast cells and platelets of vasoactive substances such as

serotonin and TNF-α. (Agner *et al*., 2011; Tonc̈ić *et al*., 2011). Furthermore, C5a also functions as a chemoattract for T cells and macrophages (Tonc̈ić *et al*., 2011). Th1 cells, carrying the receptors CXCR3 or CCR5 are mostly attracted by CXCL10 and CXCL9 together with CCL2 and CCL5, respectively. On the other hand, chemokines CCL20 and CCL27 preferentially attract $CCR6^+$ and $CCR10^+$ T cells, including Th1, and particularly Th17 and Th22. CCL17 mostly attract Th2 cells expressing high levels of CCR4. IL-8/CXCL8 produced in response to IL-17 and IL-22 will further attract neutrophils. The relative predominance of these sub-phenotypes of effector T cells in the area of contact with the sensitizer may account for the clinical and histologic variations in the ACD patterns. In the skin, infiltrating T cells release INF-γ, IL-4, IL-17 and TNF-α. In response to INF-γ, keratinocytes upregulate adhesion molecules and cytokines/chemokines, which further increases the recruitment of T-cells, NK cells, macrophages, mast cells and/or eosinophils to the challenged site (Koppes *et al*., 2017). An inflammatory response is then mounted to eliminate antigen-modified keratinocytes, which undergo apoptosis, resulting in loss of cell cohesion, tissue destruction and desquamation. Desquamation removes the antigen and the inflammatory process decreases (Agner *et al*., 2011). Furthermore, IL-17 makes keratinocytes particularly sensitive to the T cell killing by Th1 cells (Pennino *et al*., 2010).

The inflammatory reaction persists for several days and then decreases following the activation of down-regulatory mechanisms (Vocanson *et al*., 2009). The resolution phase of ACD involves several different mechanisms and cells. In the presence of inflammatory cytokines such as IFN-**γ**, TNF-α and IL-1, keratinocytes upregulate MHC class I and II molecules. Although, they do not upregulate the costimulatory molecules (e.g. CD80 or CD86), which are required for T cell activation, thus resulting in $CD4^+$ T cell clonal anergy. Anergic $CD4^+$ cells express high levels of IL-2 receptors, consequently competing with fully activated T cells for this critical growth factor, limiting the amplitude and duration of ACD. Furthermore, keratinocytes also secret the anti-inflammatory cytokine IL-10 and initiate T regulatory cell (Treg) activation through receptor activator of NF-κB ligand (RANKL) overexpression (Gober *et al*., 2008; Kaplan *et al*., 2012). Evidence also suggests that $CD4^+$ Treg cells control both the priming/expansion of specific $CD8^+$ T cells in lymphoid organs and the activation of $CD8^+$ T cells in the skin, although their mode of action still remains to be disclosed (Vocanson *et al*., 2009).

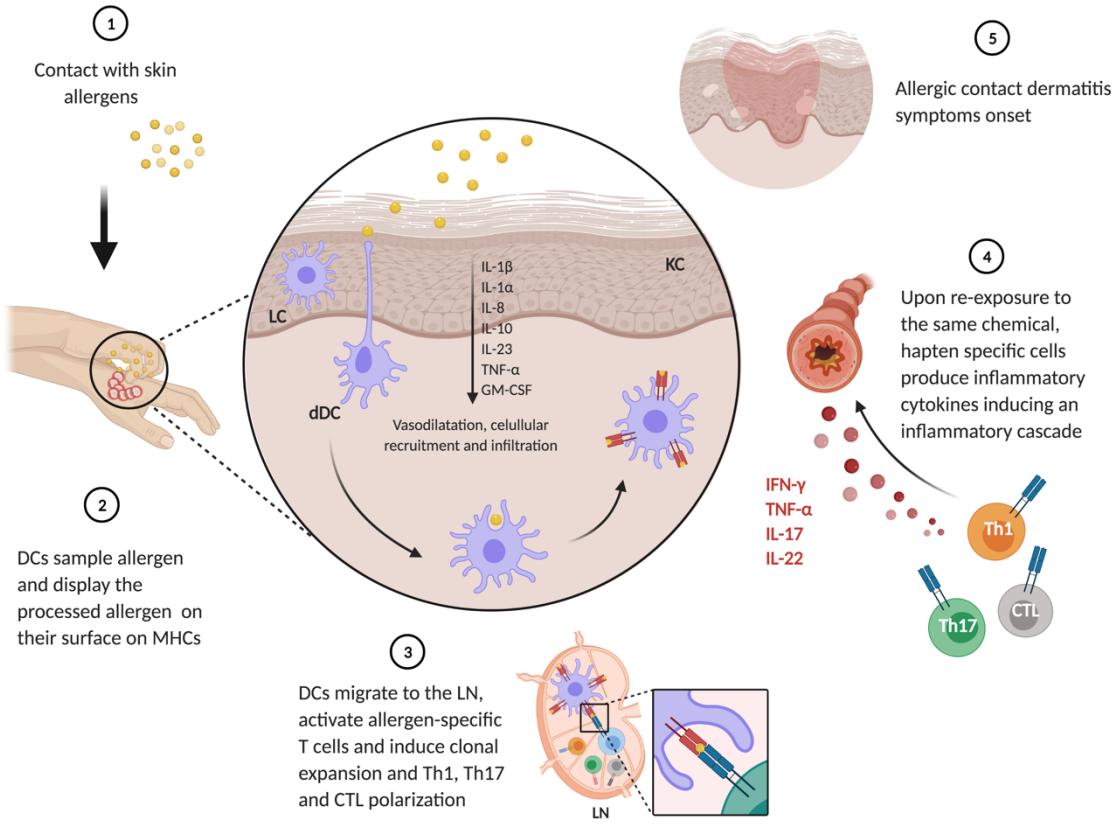


Figure 1.1 Pathophysiology of allergic contact dermatitis. Sensitization phase (1-3): Following skin contact with an hapten, it rapidly forms protein-hapten complexes and promotes the release of danger signals from keratinocytes (KCs) triggering the activation of Langerhans cells (LCs) and dermal dendritic cells (dDCs), dDCs and LCs then leave the skin and migrate via afferent lymphatics to the draining lymph nodes where they effectively present antigens to naïveT-cells. Hapten-specific T cells now expand abundantly and generate effector and memory cells, which are released via the efferent lymphatics into the circulation. Elicitation phase (1,4,5): Following re-exposure of the skin to the same contact allergen, hapten-specific cytotoxic $CD8^+$ T lymphocytes (CTLs) release inflammatory cytokines causing disease-specific local skin injuries. Th, T helper cell; CTL, cytotoxic T cell. Adapted from Dhingra *et al.*, 2013. Adapted from "Allergic Airway Sensitization", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

Over the last decades, a great progress has been made in the comprehension of the mechanisms behind ACD pathophysiology, resulting in the publication of the first "Adverse Outcome Pathway" (AOP), proposed in 2012, (OECD, 2012 a). The AOP presents detailed information about the different levels of biological organization (molecular, cellular, organ and organism) leading to an adverse health outcome while providing clear mechanistic representation of the toxicological effects. Four different key events have been identified and associated with the development of skin sensitization: *Key event 1*, also known as *Molecular Initiating Event*: covalent binding of chemicals to cellular proteins, forming hapten-protein complexes; *Key event 2*: epidermal inflammatory

response, with release of danger signals and co-factors by keratinocytes, which promote and support adaptive immune responses; *Key event 3*: DC activation and *key event 4*: T lymphocytes activation, division and differentiation (OECD, 2012 a) (Figure 1.2).

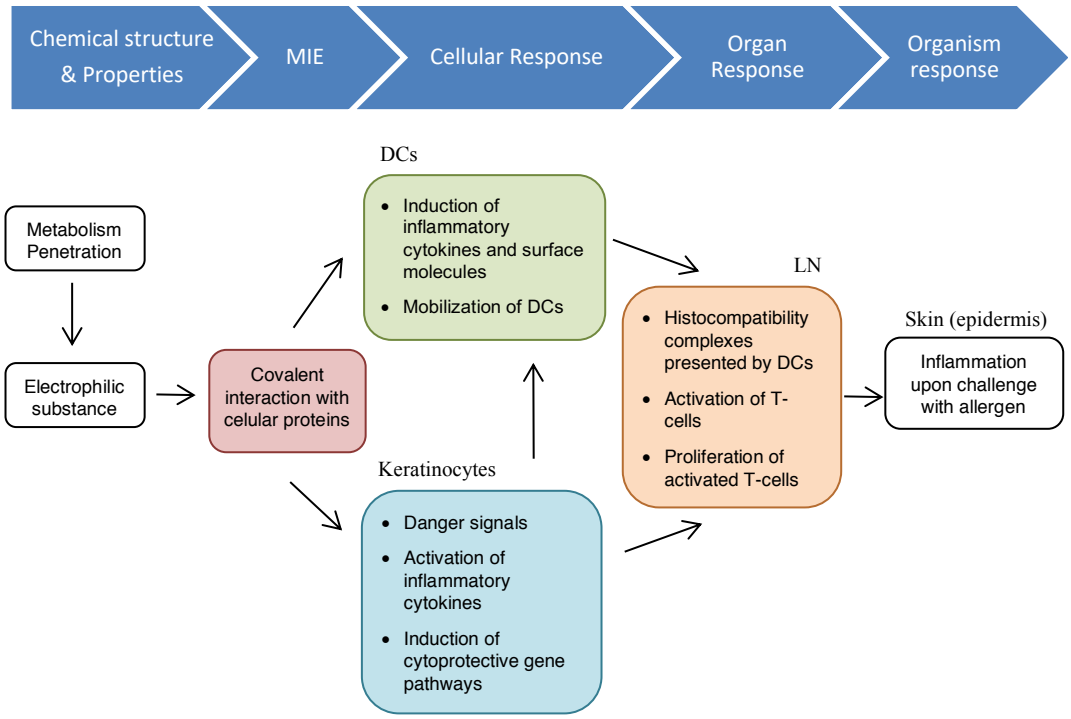


Figure 1.2 Adverse Outcome Pathway for Allergic Contact Dermatitis. The molecular initiating event (MIE) or key event 1 (red) is the covalent binding of chemical haptens to cell proteins, which serves as a trigger to the release of danger signals by keratinocytes (key event 2 - blue) and activation/maturation of DC (key event 3 - green). Once activated, DCs migrate to the local lymph nodes (LN) where they prime T-cells (key event 4 - organ response). LN, lymph node, DCs, dendritic cells.

1.2.1.3 Management and therapeutic strategies in Allergic Contact Dermatitis

The only definitive treatment for ACD relies on the identification and elimination of the culprit agent (Kostner *et al.*, 2017; Martin *et al.*, 2018; Nassau *et al.*, 2020). If avoidance is not possible, the rash may become chronic, disabling and lead to a major impairment in quality of life. In these cases, protective measures are encouraged, namely carefully instruction about protective arrangements, such as the use of appropriate clothes (e.g. gloves, masks), barrier creams and ointments. Due to ACD heterogenic clinical presentation of ACD, the available treatment options often require the use of different

therapeutic approaches. The first line of treatment for ACD usually includes topical application of emollients along with topical steroids (class II-III), calcineurin inhibitors such as pimecrolimus and tacrolimus. This typically results in rapidly lesion healing, although, in severe cases, ultraviolet irradiation (narrow-band UV-B or psoralen plus UV-A), systemic steroids or systemic immunosuppressants such as methotrexate, cyclosporine, and azathioprine may be necessary to control inflammation (Kostner *et al.*, 2017; Martin *et al.*, 2018; Nassau *et al.*, 2020). However, systemic steroids should be avoided due to risk of osteoporosis and type 2 diabetes development (Martin *et al.*, 2018). The use of biologic therapies already licensed for other eczematous and immunoinflammatory skin conditions (e.g. monoclonal antibodies inhibiting TNF-α, IL-12, IL-17, IL-23, IL-4, and IgE has gained momentum, although, evidence pointing to their efficacy remains limited (Martin *et al.*, 2018; Sung *et al.*, 2019; Bhatia *et al.*, 2020). Furthermore, the use of barrier creams and ointments may help reducing the skin contact with contaminants, facilitate their removal as well as repair and restore skin barrier.

1.2.2 Respiratory Sensitization

Respiratory allergy is a hypersensitivity reaction of the upper and lower respiratory tract to HMW or LMW xenobiotics. The most typical phenotypes include asthma, allergic rhinitis, rhino-conjunctivitis and sinusitis. Usually, this reaction is immediate and characterized by wheezing, breathlessness, tightness in the chest, bronchoconstriction and/or nasal congestions, which can appear within minutes to hours after chemical exposure (Cochrane *et al.*, 2015). The most frequent respiratory allergens are HMW chemicals, namely environmental protein such as pollen, dust mite and cockroach allergens, flours, cereals and enzymes (Willart *et al.*, 2010; Cartier *et al.*, 2019). However, in occupation/industrial settings, respiratory sensitization can also be mediated by LMW. Risk factors include genetic, epigenetic and environmental factors. Nevertheless, the predisposition to mount IgE antibody responses (atopy), gender and pre-existing non-specific bronchial hypersensitivity do not show an association with chemical respiratory allergy development (Cochrane *et al.*, 2015).

1.2.2.1 Respiratory sensitizers

The most common classes of industrial chemicals implicated in respiratory allergies include diisocyanates (e. g. toluene diisocyanate, diphenylmethane diisocyanate and hexamethylene diisocyanate), acid anhydrides (e.g. phthalic anhydride, tetrachlorophthalic anhydride, trimellitic anhydride, hexahydrophthalic anhydride, methyl hexahydrophthalic anhydride, methyltetrahy-drophthalic anhydride, and maleic anhydride) reactive dyes, chloroplatinate slats and glutaraldehyde (Kimber *et al.*, 1997). Some of these chemicals as well as examples of their use and potential occupational exposures are outlined in Table 1.2 (van Amsterdam *et al.*, 2011; Cochrane *et al.*, 2015).

Table 1.2 Common chemical respiratory allergens and source of exposure. Adapted from van Amsterdam *et al.*, 2011 and Cochrane *et al.*, 2015.

Chemical Class	Examples	Use/Occupation/Source
Diisocyanates	Toluene diisocyanate Hexamethylene diisocyanate Diphenylmethane diisocyanate	Spray painters, polyurethane industry, manufacturers of plastics, rubber
Acid anhydrides	Phthalic anhydride Trimellitic anhydride Tetrachlorophtalic anhydride	Manufacturers of plastics, epoxy resin, adhesives, floor polishes
Amines	2-ethanoldiamine Piperazine	Cleaners, photography, resins, solvents
Metal and their compounds	Chromium (VI) salts Cobalt sulfate Nickel sulfate Chloroplatinate salts Tungsten carbide	Electroplaters, welders, hard metal industry, tanning
Medicines	Hydralazine Penicillamine	Pharmaceutical industry, pharmacists
Reactive dyes (hair dyes, textile dyes)	Basic blue 99 p-phenylene diamine Azo dyes	Textile workers, hairdressers
Persulfate salts	Ammonium persulfate Sodium persulfate Potassium persulfate	Hairdressers
Other	Glutaraldehyde Colophony Chloramine-T	Health care workers Electronic workers Cleaners

1.2.2.2 Pathophysiology of Respiratory Sensitization

In a similar way to contact sensitizers, respiratory sensitizers are too small and must first react with proteins in order to induce an immune response (the so-called

molecular initiating event). The current knowledge about the biological processes behind the sensitization of the respiratory tract is much more limited than for skin sensitization, which leaves room to a variety of significant toxicological challenges. Indeed, there is still no consensus about the route(s) of exposure and the relevance of/and requirement for IgE antibodies. Thus, contrary to what happens with skin sensitization, there are currently no validated *in vivo* or *in vitro* methods for the assessment of respiratory sensitizing potential of chemicals. Indeed, skin exposure to chemical sensitizers is also a strong route to prime the respiratory sensitization, both for chemical respiratory sensitizers (van Triel *et al.*, 2011) as well as for contact allergens, which can cause asthma by inhalation or dermal exposures (Kimber *et al.*, 2005). Accordingly, the chemical respiratory sensitizer glutaraldehyde was reported to also cause skin sensitization (Kimber *et al.*, 2011).

The epithelium of the respiratory tract is constantly exposed to potentially threatening chemicals. Due to its large surface area, thinness and presence of immune cells, the alveolar region is of the uttermost importance for studying the pathophysiology of respiratory sensitization. Indeed, it represents the first line of defense against exogenous material and regulates the recruitment of effective immune cells through secretion of signaling molecules. Besides removing foreign material by phagocytosis, alveolar macrophages also modulate DCs. Upon antigen uptake and/or autocrine or paracrine secretion, DCs upregulate the expression of adhesion molecules, such as ICAM-1/CD54, co-stimulatory molecules, such as CD40, CD80 and CD86 and receptors for chemotactic factors such as CCR7 on their cell surface. In the lymph nodes, activation of naïve T-cells and development of acquired immunity is then achieved with a triple signal as described above for ACD. Interestingly, similar to what happens with keratinocytes in skin sensitization, interactions with airway epithelial cells (ECs) are crucial for DC activation (Lambrecht and Hammad 2010). Briefly, upon reaching the alveolar barrier, sensitizers can be eliminated by macrophages or can activate ECs. Once activated, ECs secrete monocyte chemotactic protein-3 (MCP-3) and macrophage inflammatory protein-1 (MIP-1) as well as cytokines (e.g. GM-CSF, thymic stromal lymphopoietin (TSLP), IL-33 and IL-25) and danger signals/DAMPs (ATP, uric acid), which contribute to the recruitment and maturation/migration of DCs. After reaching the lymphoid organs, activated DCs present the processed antigen to naïve T cells through interaction of MHC-II with TCR, leading to differentiation and clonal expansion of memory T-cells and effector Th2 cells (Holgate, 2012; Chary *et al.*, 2018) (Figure 1.3).

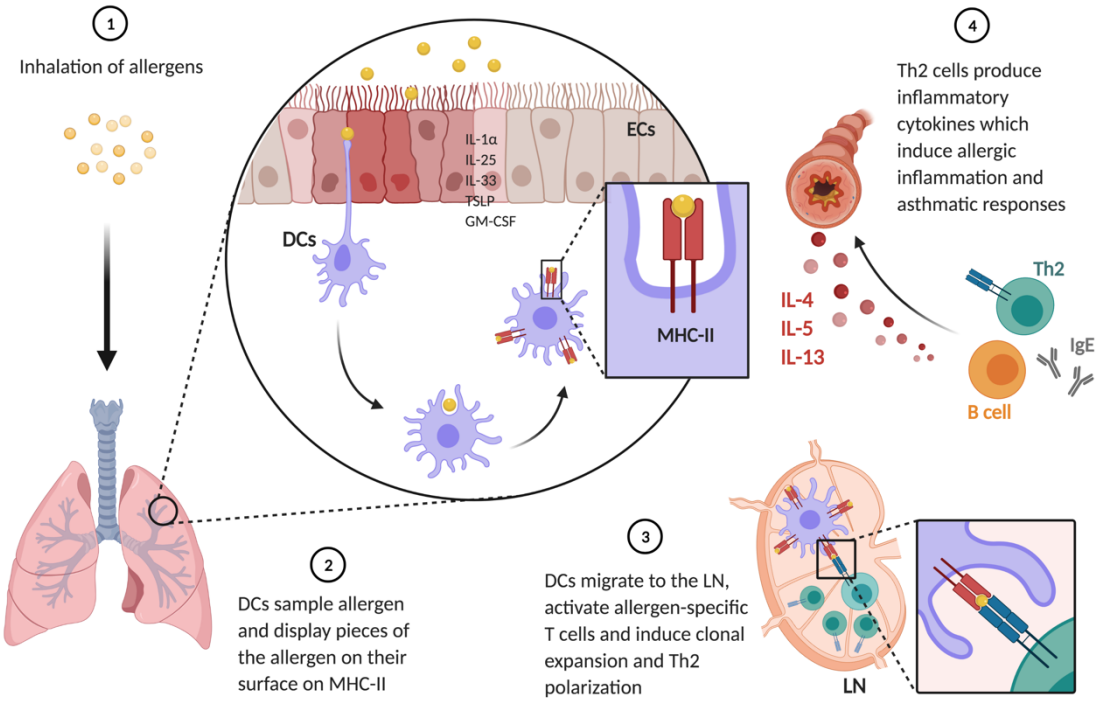


Figure 1.3 Pathophysiology of respiratory sensitization. Respiratory sensitizers interact with epithelial cells at the air-blood interface and form hapten-protein complexes. Activated epithelial cells produce cytokines (e.g. IL-1α, GM-CSF, IL-25, IL-33 and TSLP) as well as danger signals/DAMPs (ATP and uric acid) that contribute to DCs maturation and migration to local lymph nodes upon antigen capture. Once in the lymph nodes, DCs prime naïve T-lymphocytes, stimulating the differentiation and clonal expansion of memory and effector T-helper cells 2 (Th2). Th2 cells secrete interleukins 4, 5 and 13, which in turn trigger IgE production by B cells. B cells can also be directly activated by sensitizers through their B-cell receptor (BCR) (not shown). GM-CSF, Granulocyte-macrophage colony-stimulating factor; EC, epithelial cell; TSLP, Thymic stromal lymphopoietin. Adapted from "Allergic Airway Sensitization", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

Traditionally, chemical sensitizers are classified as skin or respiratory sensitizers based on their route of exposure (skin or respiratory tract) and their ability to induce Th1 or Th2 cell dominant responses, respectively. Although, it is important to note that some chemicals may not be clearly classified based in this dichotomic immune response since they induce production of both Th1 and Th2 (e.g. IL-4, IL-5 and IL-13) cytokines.

1.2.2.3 Management and therapeutic strategies in Allergic rhinitis and allergic asthma

As for ACD, complete avoidance of the sensitizer is the principal strategy used to reduce signs and symptoms. Allergic rhinitis and allergic asthma share not only a common

biochemical onset, but also some therapeutic approaches. Currently, available options for the treatment of symptoms of allergic asthma are generally classified as either controllers, taken daily for the reduction of symptoms and disease control or "rapid relievers," which are taken on demand for rapid relief of severe symptoms. Therapeutics used to treat allergic inflammation include oral or intranasal administration of corticosteroids, long-acting bronchodilators (formoterol and salmeterol), leukotriene inhibitors, anticholinergics (ipatropium), decongestants, theophylline, anti-IgE monoclonal antibodies, antihistamines and mast cell stabilizers (e.g. chromones). Furthermore, for rapid relief of asthma exacerbation, the recommended therapeutics include the administration of rapid-acting β-agonists (for instance salbutamol), combination of corticosteroid with formoterol and the use of systemic corticosteroids. Nasal saline is also recommended for allergic rhinitis treatment, particularly during pregnancy and also in children and run down patients (Molinari *et al*., 2014; Hossenbaccus *et al*., 2020).

1.3 DENDRITIC CELLS AT A GLANCE

Dendritic cells, namely epidermal dendritic cells (Langerhans cells) were first identified by Paul Langerhans in 1868 (Langerhans, 1868). However, only over a century later, by the hands of Steinman and Cohn, a full systematic description of the phenotypic and functional characteristics of DC in mouse lymphoid organs was published (Steinman *et al*., 1973, 1974). In these pioneer studies they showed that DCs are powerful APCs, playing a critical role in the interplay between innate and adaptive immune systems. Typically, DCs are found in an immature state at areas of the body that are close to the outside environment, which includes skin and the mucosa of respiratory and gastrointestinal tracts. Upon exposure to a potential threat, DCs capture and process it, displaying the resultant antigens on MHC-I or MHC-II molecules. Simultaneously, DCs start to mature and migrate towards the draining lymph nodes where they present the processed antigens to naïve T cells, initiating a specific immune response. Furthermore, depending on their activation/maturation status, DCs have the capacity to polarize naïve T lymphocytes into their different effector (Th1, Th2, Th17 and cytotoxic T cells (CTL)) or regulator subpopulations (adaptive regulatory T cells: Tr1 and Th3) (Figure 1.7).

1.3.1 Dendritic cells origin and heterogeneity

DCs are a heterogeneous family of innate immune cells with frequently overlapping characteristics, making it hard to define a clear ontogeny and classification. Although, in 2014, Guilliams and colleagues, proposed a new classification based on a two level system: first, based on their ontogeny (level one) and secondly on their function, location and/or phenotype (level two) (Guilliams *et al.*, 2014). DCs are derived from a common hematopoietic $CD34^+$ stem cell (HSC) precursor at the bone marrow that originates multiple intermediate precursors, which further give rise to several DCs subsets. This process is highly dependent on hematopoietic cytokines and growth factors. Due to DC's enormous heterogeneity, their classification is rather complex and has been a constant challenge. According to Guilliams classification, DCs residing in lymphoid and non-lymphoid tissues are roughly classified into plasmacytoid DCs (pDCs) and 'classical' or 'myeloid' DCs (cDCs) respectively. The authors also propose cDCs subdivision into two subsets, cDC1 and cDC2 based on their expression of CD141 and CD1c, respectively (Guilliams *et al.*, 2014). Later in 2018, Collin and colleagues proposed an update to human DC subsets, where they characterize the tree main population of DCs based on their expression of specific markers (Collin *et al.*, 2018). A selection of different surface markers specific for cDCs, pDCs and Langerhans cells is summarized in Table 1.3 (Humeniuk *et al.*, 2017; Collin *et al.*, 2018).

cDCs1 are mainly found in the blood, lymph nodes, tonsil, spleen, bone marrow and non-lymphoid tissues such as skin, lung, intestine, and liver. These cells have a high intrinsic capacity to cross-present antigens via MHC class I to $CD8^+$ T(Jongbloed *et al.*, 2010) cells and to, through IL-12p70 release, promote Th1 and natural killer responses (Nizzoli *et al.*, 2013). Due to their expression of TLR3, TLR9 and TLR10, cDCs1 are particularly specialized in the recognition of viral and intracellular antigens. Also, they are the major producers of type III interferons IFNλ1-3, which are known to play an important role in viral clearance (Lauterbach *et al.*, 2010).

cDCs2 are 10 times more frequent than cDCs1 and represent the major population of myeloid cDCs in human blood, tissues, and lymphoid organs. These cells are equipped with a wide range of PPRs namely TLRs (2,4,5,6 and 8), (NOD-like receptors (NOD2, NLRP1, NLRP3 and NAIP) and lectin receptors (CLEC4A, CLEC6A, CLEC7A CLEC10A, CLEC12A) and the asialoglycoprotein receptor (Calmeiro *et al.*, 2018). Once activated, they produce IL-12p70 and can function as cross presenting cells. They also

secret IL-23, IL-1, TNF-α, IL-8 and IL-10, although the secreted levels of type III interferon are consistently low. These cells are able to polarize naïve T cells towards Th1, Th2, Th17, Th22 and CTL effector populations, which gives them the ability to respond to a plethora of immune activators (Calmeiro *et al*., 2018; Collin *et al*., 2018).

Table 1.3 Comparative functional analysis of human DCs and their specific surface markers. cDC: classic (or conventional) DC; IFN: interferon; TNF: tumor necrosis factor. Adapted from Humeniuk *et al*., 2017 and Collin *et al*., 2018.

DC Subtype	Conventional Markers	Extended Markers	Cellular function
cDC1	CD141	CLEC9A CADM1 XCR1 BTLA CD26 DNAM-1	Potent cross-presenting cells; IL-12 and type III IFN- production; Expression of TLR3, TLR 9 and TLR10; Induce Th1/Th2 responses.
cDC2	CD1c CD11c CD11b	CD2 FCER1 SIRPA ILT1 DCIR CLEC10A	IL-1, IL-6, IL-8, IL-10, IL-23 and TNF-α production; Expression of TLRs (2,4,5,6 and 8); Induce Th1/Th2/Th17/Th22 and CTL response.
pDC	CD123 CD303 CD304	FCER1 ILT3 ILT7 DR6	Anti-viral responses TNF-α, IL-6, IFN type I and III production Expression of TLR7 and TLR 9.
LC	CD207 CD1A E-cadherin	EpCAM TROP2	Maintain epidermal integrity Induce Tregs and Th17, Th22.

pDCs are mainly found in the blood, thymus, bone marrow, and secondary lymphoid tissue, albeit at a very low frequency at the steady state (Soumelis *et al*., 2006). Due to their high expression of TLR-7 and TLR-9, which recognize single-stranded RNA and double-stranded DNA, respectively, pDCs are critical mediators of antiviral immune responses (Kadowaki *et al*., 2001). pDCs also have the ability to rapidly produce high quantities of type I and III interferons as well as TNF, IL-6 and granzyme B. pDCs efficiently perform cross presentation of viral antigens to $CD8^+$ T lymphocytes (Hoeffel *et al*., 2007; Lui *et al*., 2009). Additionally, pDCs have an enormous functional plasticity since they are able to regulate T cell responses through the polarization of naïve T-cells into Th1, Th2 and Treg (Ito *et al*., 2007).

Finally, LCs, reside on basal epidermis and other stratified squamous epithelia where they maintain epidermal health and tolerance to commensals while preserving the ability to respond to intracellular pathogens and viruses (Kashem *et al.*, 2015). This particular sub-set of DCs are derived from embryonic precursors and can self-renew locally (Kanitakis *et al.*, 2011). LCs in the skin are in close interaction with surrounding keratinocytes through E-cadherin. When the skin becomes inflamed, LCs downregulate E-cadherin expression, detach from keratinocytes and migrate across the basement membrane, reaching the dermis, where they enter the dermal lymphatics and proceed to the draining lymph nodes (Kissenpfennig *et al.*, 2005).

1.3.2 Antigen recognition, processing, and presentation

DCs have an outstanding ability for antigen capture and processing. These features were initially attributed to immature DCs, however, Platt and colleagues showed that mature DCs also internalize and process antigens efficiently (Platt *et al.*, 2010). DCs are endowed with an array of PRRs able to recognize a variety of common molecular motifs derived from 1) microbes, the so-called "pathogen associated molecular patterns" (PAMPs), and from 2) damaged host cells during stress states, the so-called DAMPs (Liu *et al.*, 2013). These evolutionary conserved receptors survey the extracellular and intracellular environment for pathogenic elements and injury and include TLRs, NLRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), absent in melanoma 2 (AIM2)-like receptors (ALR) and C-type lectin receptors (CLRs) (Palm NW *et al.*, 2009). TLRs are located on the cell surface and endosomal compartments where they can recognize extracellular or phagocytosed pathogens. CLRs exist both in membrane-bound and secreted forms and bind carbohydrate-based PAMPs and DAMPs. RLRs, ALRs, and other nucleic acid sensing PRRs, along with NLRs are located exclusively in the cytosol and nucleus where they sense pathogens or danger signals that entered the cell (Palm NW *et al.*, 2009; Liu *et al.*, 2013).

DCs antigen uptake and processing can occur by three mechanisms: (1) receptor-mediated endocytosis, where DCs internalize macromolecules by invagination and subsequent inclusion in clathrin coated vesicles; (2) phagocytosis, that is a form of endocytosis where DCs internalize large antigen particles, apoptotic and necrotic cells, and opsonized pathogens (Groves *et al.*, 2008); and (3) macropinocytosis, through the

internalization of large volumes of extracellular fluid, providing an efficient and non-selective route for internalization of soluble antigens (Sallusto *et al.*, 1995; Liu *et al.*, 2015).

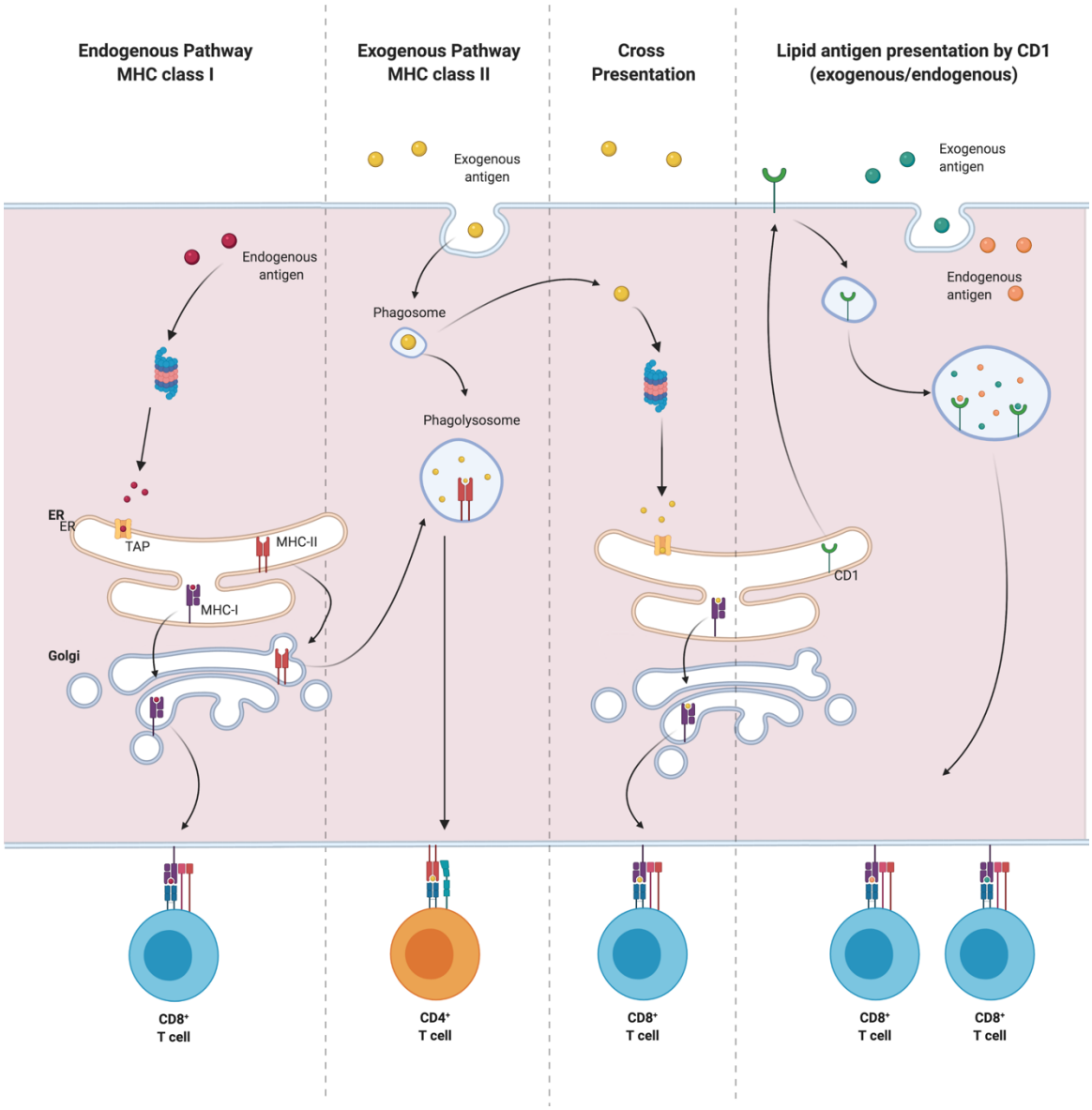


Figure 1.4 Antigen recognition, processing, and presentation in dendritic cells. Endogenous or MHC- I pathway: Endogenous antigens are processed on the proteasome and the resulting peptide fragments (epitopes) are imported into the endoplasmic reticulum (ER), by the transporter associated with antigen-processing complex (TAP), where they bind to MHC-I molecules. These complexes are then transported to the cell membrane where they interact with $CD8^+$ T cells. Exogenous or MHC- II pathway: Exogenous antigens are taken up by endocytosis or phagocytosis, entering the endocytic pathway where they are proteolyzed and denatured, and where they encounter MHC-II molecules. These complexes are then transported to the cell membrane where they interact with $CD4^+$ T cells. Cross presentation: DCs can present exogenous antigens to $CD8^+$ cells through MHC-I molecules. The most studied cross-presentation pathway is endosome-to-cytosol pathway, where antigens are transported into the cytosol for proteasomal degradation. Afterwards, antigen-derived peptides are transported back into the endosomes or into the ER, via TAP, where they are loaded into MHC-I molecules. Lipid antigen presentation by CD1: Upon assembly and correct folding in the endoplasmic reticulum, CD1 molecules travel through the secretory pathway directly to the plasma membrane.

Once expressed, they are internalized into lysosomal compartments where they can encounter self and bacterial lipid antigens before recycling back to the plasma membrane for recognition by T cells. Created with BioRender.com.

Antigen processing depends on the origin (endogenous or exogenous) and biochemical nature (protein or lipid) of the antigen (Vyas *et al*., 2008). As a result, three processing and presentation pathways have been described: (1) the exogenous (or endosomal) pathway, where the antigenic peptides are bound to MHC-II molecules in phagolysosomes and then transported in exocytic vesicles to the cell surface to be presented to $CD4^+$ T cells; (2) the endogenous or proteasome pathway, in which endogenous cellular antigens are degraded by the proteasome and the resulting peptides attached to MHC-I molecules in the endoplasmic reticulum (ER) and quickly carried through the trans-Golgi network to the plasma membrane where they are presented to $CD8^+$ T lymphocytes; and (3) a third mechanism where lipid antigens are coupled to MHC-class-I-like molecules of the CD1 family in the ER and presented to $CD8^+$ T cells, γ/δ T cells or NK T cells (Adams, 2014).

Exogenous antigens can also be loaded on MHC-I molecules, a process named cross-presentation, by two main cross-presentation pathways: the vacuolar pathway and the cytosol pathway. In the vacuolar pathway, upon internalization, antigens are degraded by lysosomal proteases and antigen-derived peptides are loaded onto MHC-I molecules there. In cytosol pathway, internalized proteins escape to the cytosol and are degraded in the proteasome. The resulting antigenic peptides are coupled to MHC-I molecules in the ER and subsequently presented to $CD8^+$ T cells (Amigorena *et al*., 2010) (Figure 1.4). Therefore, cross-presentation can be involved in immunogenic responses (cross-priming) or tolerance (cross-tolerance).

1.3.3 Maturation and Migration of Dendritic Cells

DCs normally reside in their environment in the so-called immature state, characterized by an active endocytosis and antigen processing, but weak antigen presenting functions. In response to a spectrum of environmental and endogenous stimuli, immature DC mature and acquire several fundamental skills, namely antigen processing and presentation, migration and T-cell co-stimulation.

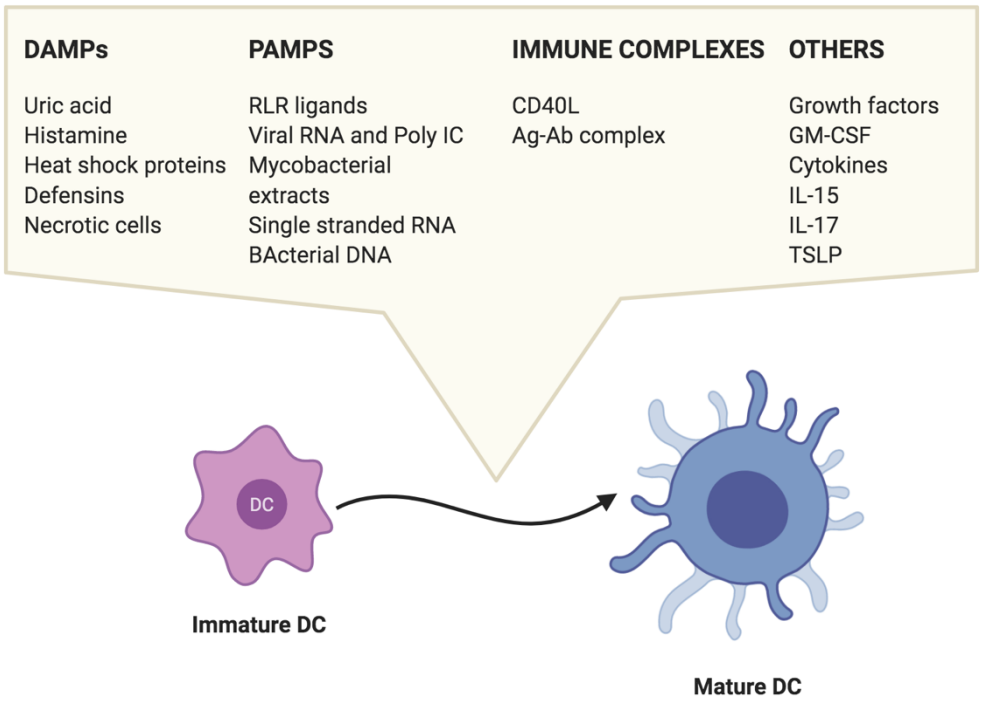


Figure 1.5 Dendritic cell maturation signals. Adapted from Aiba, 2007. Created with BioRender.com. Ag-Ab complex, antigen-antibody complex; CD40L, CD40 ligand; DC, Dendritic cell; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IL, interleukin; TSLP, Thymic stromal lymphopoietin.

The stimuli shown to trigger DCs maturation can be roughly classified into small reactive chemicals (haptens), inflammatory cytokines (TNF, IL-1β, IL-6, IFNs and TSLP), PAMPs (lipopolysaccharides, bacterial DNA and double-stranded RNA) DAMPs (uric acid, histamine, heat shock proteins, defensins and ATP) and immune complexes (CD40-CD40 ligand interactions, antigen-antibody complexes and Fc receptors) (Banchereau *et al.*, 2000; Aiba, 2007; Steinman *et al.*, 2007) (Figure 1.5). After antigen uptake in the peripheral tissues, DCs migrate to local draining lymph nodes where they present antigens to naïve T lymphocytes (Figure 1.6).

DCs maturation process, is characterized by an initial decrease in the expression of endocytic receptors and a stabilization of class II-peptide complexes. In addition, DCs undergo a significant upregulation of co-stimulatory molecules, namely CD40, CD80, CD83 and CD86, and adhesion molecules such as CD54, which are crucial for the adequate stimulation of T cells during antigen presentation (Banchereau *et al.*, 2000). Furthermore, DCs maturation also promotes a shift in the profile of secreted cytokines and chemokines, which dependent on the stimulus that triggers the maturation process.

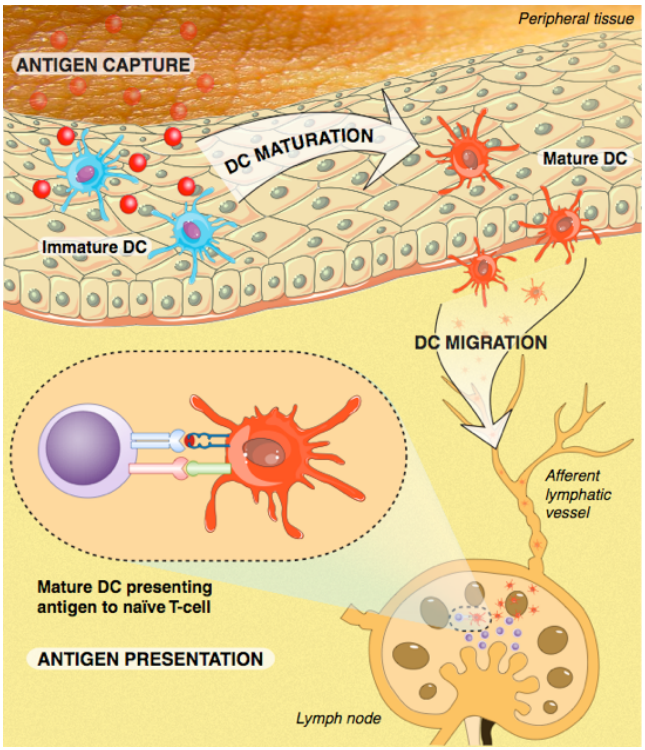


Figure 1.6 Dendritic cell activation, maturation, migration to the lymph nodes and antigen presentation to naïve T-cells. After antigen uptake in the peripheral tissues, DCs experience a phenotypic and morphological modification that leads to a loss of phagocytic ability and an increased migratory capacity - maturation. Activated DCs then start to migrate via the afferent lymphatics to the draining lymph nodes where they present antigens to naïve T lymphocytes (Ferreira *et al.*, 2018).

Overall, DCs maturation increases the expression of the cytokines TNF-α, IL-10, IL-1α/β, IL-12p70, IFN-γ, IL-8, IL-6 and IL-23 (Blanco *et al.*, 2008) and transiently promotes the release of chemokines, such as chemokine CCL2, CCL3, CCL4, CCL5, CCL8, and CXCL8, which are essential for the recruitment of monocytes and neutrophils to the site of infection/inflammation. In an advanced stage of maturation DCs increase the expression of lymphoid chemokines, such as CCL17, CCL18, CCL19, CCL22, and CXCL10, which attract T and B lymphocytes and thus facilitate DCs interaction with them (Lukacs-Kornek *et al.*, 2008). These changes are also accompanied by alterations in the expression of chemokines receptors. While immature DCs express CCR1, CCR2, CCR5, CCR6, CXCR1, and CXCR2, mature DCs upregulate CXCR4, CCR4, CCR5 and CCR7 that efficiently promote the migration of DCs to the lymphoid organs (Alvarez *et al.*, 2008).

1.3.4 Dendritic cells – T cell interactions

After capturing and processing antigens, and upon maturation, DCs upregulate the expression of specific molecules, providing three signals that drive the activation and polarization of naïve T cells into their effector and regulatory populations (Reis e Sousa, 2006) (Figure 1.7).

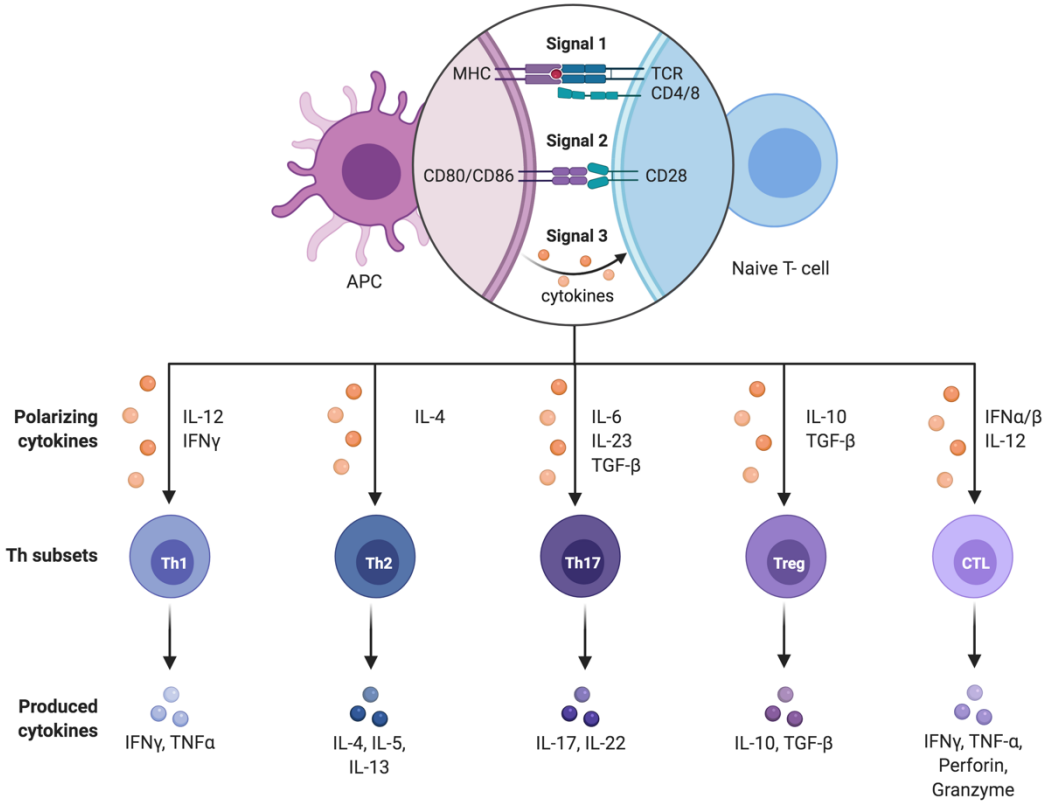


Figure 1.7 Interaction between dendritic cells and naive T-cells. After capturing and processing antigens, and upon maturation, DCs upregulate the expression of specific molecules, providing three signals that drive the activation and polarization of naïve T cells into their effector and regulatory populations. Signal 1: stimulation of the T lymphocyte receptor (TCR) by MHC/peptide complexes. Signal 2: interactions between co-stimulatory ligands on the APC (e.g. CD80 and CD86) and CD28 on T cell's surface. Signal 3: secretion of T-cell-polarizing signals by APCs (e.g. cytokines and chemokines). CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; PRRs, pattern recognition receptors; TCR, T cell receptor; Th, T-helper lymphocyte; Treg, regulatory T lymphocyte. Adapted from "T cell activation and differentiation", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

Signal 1 is triggered by the binding of MHC-antigen complexes presented by DCs to the TCR and determines the antigen-specificity of the response. This signal alone is insufficient to induce T cell activation and normally leads to anergy or deletion, promoting tolerogenic responses.

Signal 2 (co-stimulation) is required for the initiation of protective immunity and results from the interaction of co-stimulatory molecules expressed by DCs with the respective ligands on T cells' surface. This signal is mainly mediated by interaction of CD28 expressed in lymphocytes with CD80 and CD86 expressed by mature DCs after PRRs activation. Together, TCR engagement and co-stimulation, mainly promote T-cell survival, proliferation and metabolic competence also stabilizing cytokine production (Kapsenberg, 2003), although, they can also negative regulate T-cell immune function, promoting tolerance. Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) engagement in lymphocytes by CD80 and programmed death ligand 1 (PD-L1) on dendritic cells functions as an immune checkpoint pathway, negatively regulating T-cell immune function (Kong *et al.*, 2019).

Finally, signal 3 is the T-cell-polarizing signal mediated by various soluble or membrane-bound factors such as cytokines and chemokines. These factors drive the differentiation of $CD8^+$ T cells into CTLs (Curtsinger *et al.*, 2003) and the polarization of $CD4^+$ T cells into their various effectors (Th1, Th2, and Th17) (Kaiko *et al.*, 2008) or regulatory cells (Treg, Tr1, and Th3) (Kastenmuller *et al.*, 2011).

1.3.5 Dendritic cell models

DCs may be isolated or generated from human blood mononuclear cells, although, mature DCs normally account for only ~ 0.2% of human blood mononuclear cells (Nair *et al.*, 2012). In the past, the inability to isolate and purify large numbers of their different subpopulations hampered the study of DCs immunobiology. Nonetheless, several protocols for *in vitro* differentiation of DCs, mainly from human cord blood $CD34^+$ hematopoietic precursors or peripheral blood $CD14^+$ monocytes, have been developed (Nair *et al.*, 2012). These precursors are isolated and cultivated with appropriate cocktails of cytokine/growth factors, leading to generation of multiple DC subtypes such as immature interstitial DCs (using GM-CSF and IL-4) or LCs (using GM-CSF, IL-4 and TGF-β). However, and due to the inherent variability among human donors, the difficulty to access cell sources and the time-consuming and expensive cell culture procedures, surrogates to primary DC were actively pursued in the last decades. Myeloid and lymphoid DC-like cell lines, although presenting some unmatching phenotypical

characteristics, have proven to be of great value for mechanistic and functional studies. In 2008, Santegoets and colleagues comprehensively reviewed the available human DC models for DC differentiation and vaccination. They described that functional DCs can be differentiated from leukemia-derived cell lines, particularly those originated from the myelogenous or monocytic lineage, namely THP-1, HL-60, KG-1 and MUTZ-3 (Santegoets *et al*., 2008). The key DC differentiation properties of these common DC-like cell lines are outlined in Table 1.4.

Table 1.4 Common DC-like cell lines and their main characteristics. Adapted from Santegoets *et al*., 2008.

Cell Line	**Characteristics**
THP-1	Human monocytic leukemia cell line; Monocytic characteristics: lysozyme production and phagocytosis capacity; Very low DC differentiation capabilities fewer than 5% of THP-1 cells express the classic myeloid DC marker CD1a following differentiation; Acquire DC properties upon stimulation with cytokines; Addition of calcium ionophores (CI) to maturation cocktail results in complete differentiation and maturation; Upon maturation: -express high levels of CD80, CD86, CD40 and CD83 -high allogenic T cell-stimulatory capacity -decreased receptor-mediated endocytosis
KG-1	Human myelogenous leukemia cell line; Develop monocyte/macrophage features (pseudopodia, phagocytosis, nonspecific esterase and alysozyme-secreting activity) upon treatment with phorbol esters; Acquire DC-like properties upon stimulation with cytokines or phorbol 12-myristate 13-acetate (PMA) ± CI; Very low DC differentiation capabilities fewer than 10% express the classic myeloid DC marker CD1a following differentiation; Upon maturation: -express intermediate to high levels of CD86, CD83 and HLA-DR -enhanced allogenic T cell-stimulatory capacity Incomplete differentiation/maturation, without a loss of antigen uptake and increase in migratory capacities.

HL-60	Acute promyelocytic leukemia cell line Upon exposure to different chemicals can differentiate into granulocytes (such as DMSO and retinoic acid), monocytes-macrophages (phorbol esters and 1,25-dihydroxyvitamin D3) or eosinophilic granulocytes (mild alkaline conditions) Limited capacity to be differentiated into DC-like cells even after cytokine exposure Addition of CI results in some cells differentiating into functional DCs: -up-regulate CD86 -rapidly up-regulate CD83, CD80 and CD54 -delayed CD1a expression -enhanced allogenic T cell-stimulatory capacity -fail to express MHC II and downregulate MHC I
Monomac-6	Human acute monocytic leukemia cell line; Monocyte phenotype with phagocytosis activity; Resemble human blood monocytes; Express NaF-sensitive nonspecific esterases and the mature monocyte marker CD14; In response to LPS: -produce IL-1β, IL-6, and TNF-α -Capable of migration toward β-chemokines such as MIP-1α and -β, RANTES and MCP-1 Unable to differentiate into DCs.
U-937	Human acute monocytic leukemia cell line; Monocytic characteristics; Monoblast morphology; Lysozyme production and esterase activity; Do not display phagocytosis activity; Upon stimulation with PMA, they acquire mature, monocytic-like morphology and phenotype; Unable to differentiate into DCs.
K562	Chronic myelogenous leukemia cell line; Multipotent (able to differentiate into megakaryocytic, erythroid, and monocytic pathways) In response to PMA/TNF-α: -Develop cytoplasmic projections -Expression of CD86, CD40 and CD83 remains low Unresponsive to cytokine-induced DC differentiation;

	Responsive to protein kinase C signaling Unable to differentiate into DCs.
MUTZ-3	Acute myelomonocytic leukemia cell line; Monocytic characteristics (expression of monocyte-specific esterase and myeloperoxidase enzymes and the expression of monocytic marker CD14); Cytokine-dependent proliferation and survival; Characteristics of $CD34^+$ derived DC precursors and downregulation of CD14 in response to GM-CSF and IL-4 Sensitive to cytokine-driven DC differentiation; High DC differentiation capacity (60% for interstitial DC and 90% for LC); -expression of intermediate to high levels of co-stimulatory, adhesion, and MHC class I and II molecules (true DC phenotype) -expression of CD86, CD40 and CD83 remains low -CCR7 expression and migration capacity toward lymph-node homing chemokines (CCL19 and CCL21) Relatively stable induced mature DC phenotype (~ 20% even after 3 days without maturation cocktail)

Given the crucial role of DCs in skin and respiratory sensitization, the experimental work addressed on this thesis was performed with the DC-surrogate human leukemic cell line THP-1. This cell line was chosen attending to the fact that it has been used in several studies to identify chemical skin sensitizers (Ashikaga *et al.*, 2002; Lambrechts *et al.*, 2009; Arkusz *et al.*, 2010; Takahashi *et al.*, 2011). Furthermore, OECD already approved two *in vitro* test methods to address skin sensitization, h-CLAT and IL-8 Luc assay, using THP-1 or THP-1-derived cells, respectively (OECD, 2018 a). Also, cell cultures are relatively easy to maintain and rather inexpensive, showing reproducible results when cells are cultured for periods of time shorter than 2 months.

1.4 MOLECULAR MECHANISMS ELICITED BY SENSITIZERS

As stated earlier, the precise molecular mechanism by which sensitizers trigger and shape DCs maturation are complex and remain incompletely understood. Even though, it is important to acknowledge that these modifications, both at genomic and proteomic levels, result from the coordination of different intracellular events. Indeed, several studies addressed the release and/or generation of danger signals and DAMPs accompanied by

the activation of intracellular signal transduction pathways as well as assembly of multimeric protein complexes such as the NLRP3 inflammasome.

1.4.1 Danger signals

Danger signals were first postulated in 1994 by Polly Matzinger as part of a model of immunity suggesting that the immune system responds to substances that cause damage, rather than exclusively to those that are foreign. The spur for this hypothesis was the observation that in autoimmune diseases, transplants and malignancies, strong immune responses can be mounted in the apparent absence of microbial infection. Indeed, DCs may be activated by endogenous signals, even without any foreign substances. These signals are normally absent in healthy cells or their local environment but are released or exposed in response to cellular stress, damage, or necrotic cell death. Once released, they activate innate immune cells through PRRs, such as TLRs, and other receptors. DAMPs can initiate, amplify and sustain an inflammatory response even in the absence of external pathogens, triggering the so-called sterile inflammation. As mentioned above, these molecules recruit innate inflammatory cells, contributing to inflammation induction. Remarkably, most chemical allergens deliver both antigenic and danger signals. The irritant capacity of these chemicals results in cell and tissue trauma leading to the release of DAMPs, which are key events for successful contact hypersensitivity. The actual repertoire of DAMPs can be of cytosol, nuclear, mitochondria, endoplasmic reticulum or extracellular origin. Common DAMPs include uric acid, mitochondrial DNA (mtDNA), ATP, heat shock proteins (HSPs), ROS, oxidized phospholipids, high mobility box 1 (HMGB1) and hyaluronic acid.

1.4.1.1 Reactive Oxygen Species

ROS are key players in host defense against bacterial infection, skin aging, cancer and many chronic diseases. Under homeostatic conditions, cellular redox status is maintained by a dynamic equilibrium of processes that produce and eliminate ROS. Known sources of cellular ROS include Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the mitochondrial respiratory cycle and xanthine oxidase, which generates superoxide through the oxidation of hypoxanthine to uric acid. To manage these deleterious oxidative molecules, cells are equipped with a variety of antioxidants that can

be enzymatic, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, and non-enzymatic, such as glutathione (GSH) (Birben *et al*., 2012). Although not completely understood, it is widely accepted that sensitizers trigger and shape DCs maturation through generation of ROS. Indeed, ROS production leading to protein oxidation is an early danger signal occurring in the sensitization phase of chemical-induced allergy (Mizuashi *et al*., 2005; Byamba *et al*., 2010; Esser *et al*., 2012). Pre-treatment with specific inhibitors of the three main cellular sources of ROS, namely the mitochondrial electron transport (rotenone), xanthine oxidase (allopurinol), NADPH and nitric oxide synthase (diphenylene iodonium), lead to a decrease in allergen-induced IL-18 production in human keratinocytes (Galbiati *et al*., 2014), a cytokine that favors the Th1 type immune response and has been pointed as a marker for contact sensitizers identification (Corsini *et al*., 2009). Byamba and colleagues demonstrated that DNCB-treated monocyte-derived dendritic cells (moDC) showed an increased ROS production, protein carbonylation and upregulation of CD86 and HLA-DR, which were both inhibited when cells were pre-treated with the antioxidant N-acetyl cysteine (NAC) (Byamba *et al*., 2010). Indeed, multiple signaling pathways involved in DC maturation are known to be redox-sensitive, including transcription factors such as nuclear factor nuclear kappa B (NF-κB) and activator protein (AP-1), mitogen-activated protein kinases (MAPKs), and several phosphatases and proteins directly involved in oxidative/electrophilic stress detection such as Keap-1 (Kelch-like ECH-associated protein 1)/Nrf2, hypoxia inducible factor-1 and thioredoxin (Cosentino-Gomes *et al*., 2012). Topical pre-treatment of the murine ear skin with antioxidants (NAC, Trolox or α-Tocopherol) before sensitization results in inhibition of the TNCB-induced CHS. In addition, antioxidant application after challenged of sensitized mice also reduced CHS, indicating that antioxidants can be used both in the sensitization as well as in the elicitation phase (Esser *et al*., 2012). Providing further support for a role for oxidative stress in contact allergy, both DNCB and nickel reduce GSH/GSSG (reduced/oxidized glutathione) ratio in moDCs, accompanied with an increase in p38MAPK phosphorylation. In line with this results, treatment with NAC increased the GSH/GSSG ratio and suppressed the phosphorylation of p38 MAPK as well as the augmentation of CD86 expression elicited by sensitizers (Mizuashi *et al*., 2005).

Furthermore, a disturbed redox homeostasis may lead to thiol modification in proteins, which can change the conformational structure of molecules and inherently their function. Contact sensitizers were shown to induce oxidation of cell surface thiols, which

further contributes to DC maturation (Kagatani *et al.*, 2010). Regarding contact allergy to metals, ROS has also been implicated in metal-induced contact dermatitis. Indeed, Cr(VI) induces innate immune responses via mitochondrial ROS (mtROS) accumulation, causing K^+ efflux and leading to NLRP3 inflammasome activation in THP-1 cells (Adam *et al.*, 2017). This was further supported by Wang and colleagues who showed that in the HaCaT keratinocyte cell line Cr(VI) leads to increased ROS formation, increased activation of Akt, NF-kB and MAPK pathways, *TNF* expression, and the release of IL-1α (Wang *et al.*, 2010). Additionally, $NiCl_2$ was shown to induce mtROS accumulation as well as the release of mtDNA, thus activating NLRP3 inflammasome pathway (Guo *et al.*, 2019).

ROS has also been implicated in diisocyanate-induced occupational asthma (Silva *et al.*, 2014; Choi *et al.*, 2018). Our group demonstrated that the respiratory sensitizer hexamethylene diisocyanate (HDI) induces O_2^- increase in THP-1 cells through enzymatic inhibition of cytoplasmic superoxide dismutase 1, which might reduce mitochondrial membrane potential, further leading to mitochondrial O_2^- production. The increased O_2^- levels induced ERK activation as well as the expression of Nrf2 dependent genes and the DC maturation marker CD83 (Silva *et al.*, 2014). Another respiratory sensitizer, toluene-2, 4-diisocyanate (TDI), has reported to increase both systemic, respiratory airways and lung tissue oxidative stress (Muti *et al.*, 2016). Furthermore, in lung inflammation, ROS has also been implicated in hyaluronan degradation through p38 MAPK dependent up-regulation of hyaluronidase (Monzon *et al.*, 2010).

1.4.1.2 High-mobility group box 1

HMGB1 are highly conserved non-histone nuclear proteins that act as architectural chromatin-binding factors. Under normal conditions, HMGB1 resides mostly in the nucleus, where it acts as a DNA chaperon and contributes to gene transcription and DNA repair. Upon injury or stress, HMGB1 is released into the extracellular space acting as proinflammatory mediator, mainly through receptors for advanced glycation end products (RAGE) and TLR2, TLR4 and TLR9 signaling (reviewed in Vénéreau, Ceriotti, and Bianchi 2015). HMGB1 has been implicated in the pathogenesis of several chronic inflammatory diseases, including rheumatoid arthritis, atherosclerosis, Parkinson's disease, Alzheimer's disease, multiple sclerosis, cancer (reviewed in Paudel *et al.* 2018 and Musumeci, Roviello, and Montesarchio 2014), allergic airway inflammation (Ullah *et al.*, 2014) and allergic

contact dermatitis (Galbiati *et al*., 2014). Interestingly, upon activation, DCs actively release HMGB1, which is necessary for both sustaining DCs maturation (via upregulation of CD80, CD83 and CD86) and to the cross-talk with naïve T cells (Dumitriu *et al*., 2005). Extracellular HMGB1 can act both as a chemoattractant (when fully reduced) for leukocytes as well as a proinflammatory mediator (when fully oxidized), promoting the release of TNF-α, IL-1, IL-6, and other cytokines from recruited leukocytes and resident immune cells (Venereau *et al*., 2012). Furthermore, HMGB1 is required for T cell survival, proliferation, and functional Th1 polarization (via IL-12 production) (Dumitriu *et al*., 2005). Galbiati and co-workers demonstrated the ability of the contact allergens PPD (p-phenylenediamine) and DNCB to induce the release of HMGB1, which triggers TLR4-dependent synthesis of IL-18 in keratinocytes. Additionally, allergen-induced IL-18 and IL-1α production, but not IL-8, was reduced in the presence of glycirrizic acid, an HMGB1 inhibitor (Galbiati *et al*., 2014). In the context of respiratory allergies, HMGB1 sputum levels are correlated with asthma severity. Indeed, TDI-induced murine asthma model show increased pulmonary HMGB1 levels through PI3K/caspase-1 activation (Liang *et al*., 2015). More recently, Imbalzano and colleagues, extensively reviewed the literature and confirmed HMGB-1 involvement in pulmonary pathologies. Indeed, exogenous HMGB1 induces a dominance of Th2 cells, a Th17-type response, and an attenuated Th1-type response. On the other hand, these effects were reversed in the presence of drugs known to lower HMGB-1 levels or with anti-HMGB-1 antibodies (Imbalzano *et al*. 2017).

1.4.1.3 Hyaluronic Acid

HA is a free unbranched glycosaminoglucan ubiquitously distributed in the extracellular matrix with a key role in dermis metabolism. It also plays an important role in wound healing and tissue repair processes, mainly due to its ability to maintain a humid environment, which favors the stimulation of growth factors, cellular constituents, and the migration of various cells essential for healing. HA is synthesized by three isoforms of hyaluronan synthases (HAS 1-3), degraded by hyaluronidases (HYAL 1 and 2) and exerts its biological function through several receptors such as TLR2/TLR4 and CD44 (Stern *et al*., 2006; Lee-Sayer *et al*., 2015). HA immunomodulatory properties strictly depend on HA size. High molecular weight HA (HMWHA; < $1x10^6$ kDa) is anti-angiogenic, anti-inflammatory, and immunosuppressive. Instead, HA breakdown products (1.2 - 500 kDa)

are immunostimulatory, presumably via TLR2 and/or TLR4 engagement in immune cells such as macrophages or DCs. These low molecular weight HA (LMWHA) are generated during inflammation or tissue damage either enzymatically through hyaluronidases (HYAL1 or 2) or non-enzymatically by oxidative de-polymerization induced by ROS. Contact sensitizers induce skin inflammation through ROS production with concomitant HA breakdown. Studies by Esser and colleagues demonstrated the role of HA on CHS. They showed that blocking HA function with a peptide inhibitor prior to sensitization reduced CHS response to TNCB in NMR1 mice (Martin *et al*., 2008 a). Indeed, ROS increased HYAL12 expression and activity in human bronchial/tracheal epithelial cells via p38MAPK signaling (Monzon *et al*. 2010). Also, contact sensitizers induce ROS-dependent degradation of HMWHA in the skin. Blocking ROS production and HA degradation with antioxidants, a hyaluronidase-inhibitor or a p38MAPK inhibitor prevented both sensitization as well as elicitation of CHS (Esser *et al*., 2012). DNCB-induced skin inflammation can be linked to its effects on hyaluronidase activity and ROS-induced HA degradation (Heo *et al*., 2018). Indeed, the action of hyaluronidase, or an increase of small fragments of HA, activates cutaneous DCs, modulating the capacity to induce contact allergy (Muto *et al*., 2014). Interestingly it is reported that keratinocyte contact allergen-dependent sensitization is partially mediated through LMWHA/TLR4/NF-κB signaling axis. Treatment of keratinocytes with PPD and DNCB leads to an increased TLR4, HAS1-3, HYAL1 expression and NF-κB activity. Moreover, TLR4 blockage with a neutralizing antibody before allergen treatment, decreases both HYAL1, HAS1 and 3 expression as well as NF-κB activity, suggesting a correlation between HA synthesis and TLR4 downstream effects. (Nikitovic *et al*., 2015; Kavasi *et al*., 2019). Interestingly, HA receptor CD44 has also been implicated in the development of airway inflammation in a murine model of allergic asthma (Katoh *et al*., 2003).

1.4.1.4 Adenosine Triphosphate

ATP was first discovered in 1929 and has been universally seen as the energy exchange factor between energy-producing and energy-demanding processes through formation, hydrolysis, or transfer of its terminal phosphate group. Under physiological conditions, intracellular ATP concentration ranges between 3 and 10 mM while the extracellular concentrations is relatively low (10 nM). Upon stress or damage, dying cells

release ATP which interacts with plasma membrane P2 purinergic receptors of neighbor cells. These receptors can be divided into ligand-gated cation channels (P2X receptors) and G protein-coupled receptors (P2Y receptors), being responsible for binding extracellular nucleotides as well as their degradation products adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine. Indeed, several studies show that chemical injured keratinocytes release ATP into the extracellular space through pannexin channels (Mizumoto *et al*., 2003; Onami *et al*., 2014). There, it binds to P2X7 receptors in DCs surface, promoting Ca^{2+} influx and/or K^{+} efflux, which ultimately leads to NLRP3 inflammasome activation and IL-1β secretion. Indeed, in response to ATP, DCs show a transiently increase in endocytosis, followed by up-regulation of CD86, CD54, and MHC-II, IL-12p70 secretion and improved T cell stimulatory activity (Schnurr *et al*., 2000). Indeed, ATP has been implicated in allergen-driven lung inflammation and contact hypersensitivity reactions (Idzko *et al*., 2007; Willart *et al*., 2009; Weber *et al*., 2010 a). Supporting the importance of ATP in airway inflammation, studies by Idzko and colleagues showed increased ATP levels in bronchoalveolar lavage fluid of both asthmatic humans and mice challenged with allergens (Idzko *et al*., 2007). Recently, Li and colleagues demonstrated that ATP/P2X7 axis and NLRP3 inflammasome modulate airway inflammation, mucus production in the lung tissue, airway hyper-responsiveness and differentiation of Th2, Th17 in OVA-sensitized and challenged asthma model (Li *et al*., 2018). Interestingly, the administration of the ATP hydrolysing enzyme apyrase, or the broad-range inhibitor of P2Rs suramine, reduced airway inflammation accompanied by a decrease in IL-4, IL-5 and IL-13 (Idzko *et al*., 2007). Weber and colleagues also observed a similar response in mice treated with the contact allergen TNCB. The authors showed that mice lacking purinergic receptor P2X7 failed to develop CHS in response to contact allergens and that P2X7 was crucial for the sensitization but not for the effector phase of CHS (Weber *et al*., 2010 a). Indeed, ATP exerts its function through P2X7 receptor, which indirectly stimulates NLRP3 inflammasome and IL-1β secretion (Ferrari *et al*., 2006; Di Virgilio, 2007) that has a crucial role in CHS. More recently, our group showed that DNFB-induced THP-1 maturation was impaired after purinergic signaling inhibition, and that the transcription of the purinergic metabotropic receptors P2Y2 and P2Y11 was modulated by DNFB (Martins *et al*., 2016). Interestingly, although ATP is known as an inhibitor of Th1 immune responses and a promoter of Th2-type cytokines producing cells (la Sala *et al*., 2001; La Sala *et al*., 2002), as stated above, several studies report the

importance of ATP in ACD reactions, typically a Th1 immune response. Indeed, null deficient mice in the ectonucleosides CD39 and CD73, which catalyze the degradation of ATP and ADP to AMP, show impaired CHS (Ring *et al.*, 2009; Neuberger *et al.*, 2017).

1.4.1.5 Uric Acid

Uric acid is a breakdown product of purine metabolism, released by dying cells or cells exposed to oxidative stress and has long been associated with gouty arthritis (gout) and more recently to vascular diseases. Soluble uric acid is a potent antioxidant and free radical scavenger, although, at higher concentrations it can precipitate into monosodium urate crystals (MSU), a well-known inflammasome activator. Although studies addressing the modulation of MSU production by contact allergen are scarce, several works support the role of MSU as an adjuvant. Shi *et al.* have shown that MSU drives the maturation of DCs *in vitro*, leading to an increase in the expression of the costimulatory molecules CD80 and CD86 (Shi *et al.*, 2003). Indeed, mice sensitized with TNCB in the presence of MSU showed a delayed recovery in ear swelling. Furthermore, pre-treatment with potassium oxonate (POA), an uricase inhibitor, significantly increased CHS response both in magnitude and duration. Consistent with observations of Shi *et al.*, POA mice sensitized with TNCB in the presence of MSU show an increased CD86 expression as well as a slightly elevation on $CD40^+$ cells (Liu *et al.*, 2007). Uric acid levels are also increased in bronchoalveolar lavage fluid and pulmonary tissue homogenate of asthmatic patients or animal models. Indeed, house dust mite-induced uric acid promotes Th2 sensitization by increasing epithelial production of TSLP, GM-CSF, IL-25 in airway epithelial cells. Furthermore, the degradation of uric acid by uricase completely prevents allergic sensitization (Kool *et al.*, 2011). MSU has also been associated with increased production of ROS through NADPH oxidase activation as well as inflammasomes and NF-κB and MAPK pathways activation (Reviewed in So and Thorens 2010; Martinon *et al.* 2006), which are known to be involved in the pathophysiology of inflammatory diseases such as ACD and RS.

1.4.2 Signal transduction pathways

So far, studies indicate that the most common signal transduction pathways preferentially modulated by skin sensitizers include tyrosine phosphorylation, protein kinase C (PKC), MAPKs, NF-kB and Nrf2 (Figure 1.8).

1.4.2.1 Tyrosine phosphorylation, PKC and MAPK

In 1998, Kuhn and colleagues were the first ones demonstrating that strong sensitizers, contrary to weak sensitizers and irritants, could induce tyrosine phosphorylation in human moDCs, suggesting that tyrosine phosphorylation plays an important role in the activation of moDCs by haptens (Kühn *et al.*, 1998). This work was further confirmed by Neisius and colleagues, who, in murine LCs, observed a significant increase in tyrosine phosphorylation in response to the strong sensitisers TNCB and MCI/MI, but not after cell treatment with the irritants sodium dodecyl sulfate (SDS) and benzoic acid (Neisius *et al.*, 1999). PKC involvement on LC migration was firstly demonstrated by the hands of Halliday and Lucas in 1993 (Halliday *et al.*, 1993). The authors showed that application of a diacylglycerol analogue, the physiological activator of PKC, considerably reduced LCs density on the epidermis 24 h following chemicals application, suggesting their migration. Furthermore, PKC inhibition resulted in blockage of TNCB-induced LC migration, although it not inhibited contact sensitivity skin characteristics.

Activation of MAPKs and NF-kB play an important role in DC maturation and were also reported in multiple studies with chemical sensitizers (Arrighi *et al.*, 2001; Matos *et al.*, 2005; Ade *et al.*, 2007; Neves *et al.*, 2009; Silva *et al.*, 2014; Ferreira *et al.*, 2018). MAPKs constitute a family of kinases that include extracellular signal-regulated kinases (ERKs), c-Jun N-terminal or stressed activated protein kinase (JNK/SAPK) and p38 MAPK. While ERKs are the MAPK prototype stimulated by multiple mitogenic stimuli as well as differential signals, p38 and JNK are activated in response to cellular stress such as UV radiation, osmotic shock, genotoxic agents, pro-inflammatory cytokines and oxidations (Reviewed in Stępnik and Arkusz 2003; Neves *et al.* 2011). These kinases are activated by phosphorylation on both threonine and tyrosine residues by distinct upstream dual-specific MAPK kinases. Activated MAPKs are then translocated to the nucleus where they

phosphorylate their respective substrates (transcription factors) on serine or threonine residues, resulting in their activation and further gene transcription.

In 2001, Arrighi and colleagues firstly reported that the sensitizers DNFB and nickel, contrary to the irritants SDS and benzalkonium chloride (BC), induced p38 MAPK phosphorylation. Furthermore, up-regulation of the DC maturation markers CD80, CD86 and CD83 was inhibited by SB203580, a specific p38 MAPK inhibitor. Indeed, the authors postulated that this differentially p38 MAPK activation by contact sensitizers and irritants in DCs could represent a useful tool for identifying contact sensitizers (Arrighi *et al*., 2001). p38 and ERK1/2 activation by contact sensitizers was also reported by Bruchhausen and Aiba (Aiba *et al*., 2003; Bruchhausen *et al*., 2003). The authors showed that DNCB triggered p38 MAPK and JNK1/2 phosphorylation, while nickel induced p38 MAPK, JNK1/2 and ERK1/2 phosphorylation in moDCs. Similarly, to the results described by Arrighi, the authors also reported no activation of these transduction pathways by irritants BC and SDS. Moreover, pre-treatment with p38 MAPKs inhibitor SB203580 abrogated DNCB-induced up-regulation of CD86, HLA-DR, CD83 and IL-8 and also the nickel-induced increase of CD83 and IL-12 p40 while ERK inhibitor PD98059 supressed the nickel-induced IL-1β and tumor necrosis factor (TNF)-α expression and production (Aiba *et al*., 2003). Indeed, Neves and colleagues extensively reviewed the literature and concluded that while studies addressing ERK and JNK activation are scarce and not always concordant, p38 is consistently reported to be activated by sensitizers and not by irritants (Neves *et al*., 2011). Although the results for p38 MAPK activation are concordant, the different cell models used in the experiments could account for the reported differences in ERK and JNK activation. The same authors also developed an *in vitro* DC-based test for skin sensitizer identification in the mouse fetal skin-derived dendritic cell line (FSDC), where they show selective and marked increase in phospho-JNK1/2 levels following exposure to sensitizers. Indeed, JNK was revealed to be selectively modulated by sensitizers even more robustly than p38 MAPK and was therefore considered as a good predictor variable in this test (Neves *et al*., 2013). More recently, our group showed the involvement of ERK signaling on HDI-induced (respiratory sensitizer) up-regulation of CD86 and CD83 (Silva *et al*., 2014). Indeed, different signaling pathways have different roles in DCs maturation process (expression of co-stimulatory molecules, cytokines, and the capacity to prime T-lymphocytes). p38 MAPK is reported to be involved in the up-

regulation of the co-stimulatory molecules CD80, CD83, CD86, CD54, CCR7, CD40, HLA-DR and cytokines IL-18, IL-12, IL-6, IL-8 (reviewed in Neves *et al.* 2011).

1.4.2.2 Nrf2/ARE genes

As stated previously, redox homeostasis shapes the innate immune response to chemical sensitizers, protecting against inflammation. Nrf2/ARE pathway is one of the major signaling pathways involved in the regulation of cellular oxidative stress, mainly by promoting detoxification and excretion of both organic xenobiotics and toxic metals. In steady state, Nrf2 is sequestrated in the cytoplasm by the Keap1, which promotes Nrf2 ubiquitination followed by its degradation in the proteasome. Exposure to oxidants or electrophilic stress promotes Keap1 conformational changes which in turn block Nrf2 ubiquitination, favoring its accumulation. Nrf2 then rapidly translocates into the nucleus where it binds to antioxidant-responsive elements (AREs) in gene promoters such as NAD(P)H: quinone oxidoreductase 1 (*NQO1*), heme oxygenase-1 (*HMOX1*), glutathione S-transferase (*GST*), catalase, superoxide dismutase (*SOD*) and glutamate-cysteine ligase modifier subunit (*GCLM*). Besides functioning as an oxidative stress sensor, Keap1 is a known cysteine-rich protein, accounting for more than 25 cysteine residues, which makes it an attractive target for skin sensitizers preferentially reacting with thiol residues (Reviewed in Helou *et al.* 2019). Indeed, in 2008, Natsch and Emter were the first ones proposing activation of Nrf2 pathway as a method to predict skin sensitization *in vitro* (Natsch *et al.*, 2008). Indeed, the threshold for sensitization to contact allergens in mice lacking Nrf2 is much lower, which then reflects on an increased manifestation of CHS, most likely due to the increased inflammatory status and loss of redox homeostasis (van der Veen *et al.*, 2013). Furthermore, Nrf2 KO mice can even mount CHS responses to weak contact sensitizers which fail to do so in wildtype mice (El Ali *et al.*, 2013). Treatment of primary DCs or the monocytic human cell line THP-1 with several sensitizers induced the expression of typical Nrf2 target genes like *NQO1* or *HMOX1* (Ade *et al.*, 2009). Moreover, loss of Nrf2 leads to enhanced expression of co-stimulatory molecules on the surface of DCs (Yeang *et al.*, 2012).

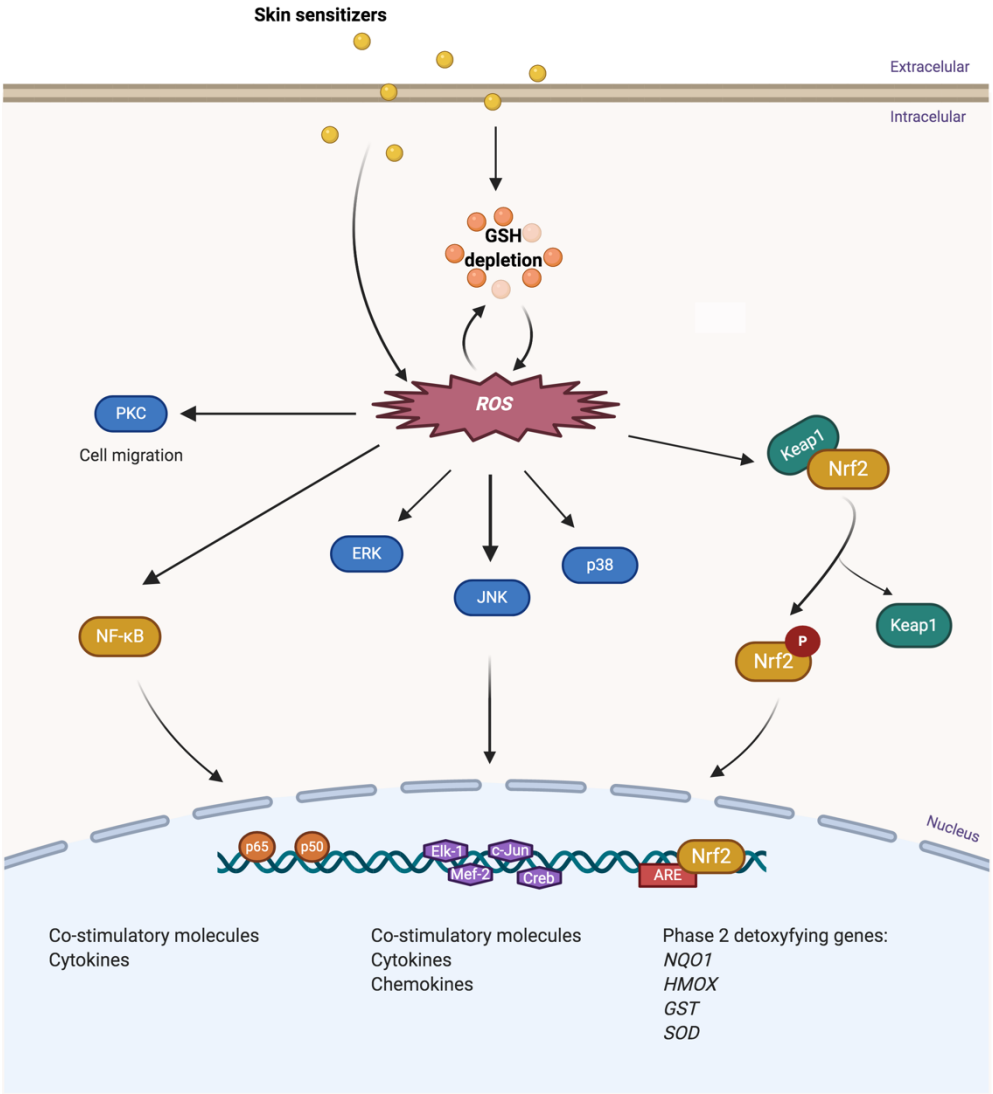


Figure 1.8. Signaling pathways modulated by contact sensitizers in dendritic cells. LMW chemicals are electrophilic compounds able to covalently bind to proteins mainly through lysine and cysteine residues. These compounds induce oxidative/electrophilic stress, through directly interaction with GSH and subsequent depletion or by the increase of oxidative/electrophilic stress promoted by the hapten-protein complexes. The most common pathways involved in DCs activation by contact sensitizers include protein kinase C (PKC), mitogen-activated protein kinases (MAPK) and the transcription factors nuclear factor kappa-B (NF-kB) and NRf2. Activated MAPKs (p38, JNK and ERK) can translocate into the nucleus where they phosphorylate substrates such as transcription factors (Elk-1, Mef-2, Creb, c-Jun). The most important class of MAPK and transcription factor involved in DCs activation by sensitizers are p38 MAPK and Nrf2. Adapted from Neves *et al.* 2011. Created with BioRender.com.

Although numerous studies report that Nrf2 regulates inflammation mostly through inhibitory crosstalk with many redox sensitive inflammatory pathways including NF-κB and inflammasomes (Reviewed in Helou *et al.* 2019), other antioxidant-independent mechanisms were also involved. Indeed, besides regulating DNCB-induced transcription of antioxidant genes *(Hmox1*, *Gclc*, *Nqo1*) and chemokines (*Ccl2*, *Ccl4*, *Ccl11*), Nrf2 is also involved in polymorphonuclear neutrophils clearance through CD36 upregulation in skin-

resident macrophages (Helou *et al*., 2019 b). Currently, one of the OECD's test guidelines approved for the *in vitro* identification of contact allergens (OECD 442D) is based on a luciferase reporter of Nrf2 activation in the human keratinocyte cell line HaCaT (OECD, 2018 b).

Nrf2 deletion was also shown to result in high susceptibility and severity of insults in various models of respiratory diseases such as respiratory infections, acute respiratory distress syndrome, asthma, idiopathic pulmonary fibrosis and lung cancer. Indeed respiratory sensitizers also induce upregulation of Nrf2-mediated oxidative stress response genes (Remy *et al*., 2014; Silva *et al*., 2014), although this pathway may not be used as a biomarker to discriminate between skin and respiratory sensitizers.

1.4.2.3 Nf-kB

The transcription factor NF-*κ*B is considered a central regulator of both the innate and adaptive immune responses and has been demonstrated to play a cardinal role in inflammatory and allergic diseases. NF-κB family comprises five related proteins: p50, p52, RelA (also known as p65), c-Rel, and RelB that can homo- and heterodimerize. Its activity is tightly controlled by the inhibitory protein, I*κ*Bα, which sequesters NF-*κ*B in the cytoplasm, promoting low basal transcriptional activity in unstimulated cells. Upon cellular stimulation, the I*κ*B kinase phosphorylates I*κ*B*α*, which is then ubiquitinated and degraded through the 26S proteasome pathway, allowing the exposure of NF-*κ*B nuclear localization sequence, promoting its translocation to the nucleus, where it stimulates gene transcription. Several phenotypical and functional characteristics of DCs, such as expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules, are positively regulated by NF-kB. (Arrighi *et al*., 2001; Aiba *et al*., 2003; Neves *et al*., 2009). Though, the involvement of NF-kB in contact sensitization remains controversial. Studies with metallic haptens showed that $NiSO_4$ and $CoCl_2$, but not $K_2Cr_2O_7$ induce NF-kB activation (Antonios *et al*., 2009, 2010) on human DCs, although, Cr(VI) was reported to induce NF-kB activation in HaCaT cells (Wang *et al*., 2010). In the mouse FSDC line, no NF-kB activation was observed upon treatment with skin sensitizers $NiSO_4$, DNFB, oxazolone, PPD (Neves *et al*., 2013). More recently Kavasi and colleagues reported an increase in NF-kB phosphorylated levels as well as NF-κB translocation to the nucleus upon keratinocytes treatment with PPD and DNCB for 2 h. This activation was

partially mediated through TLR4 engagement by LMWHA (Kavasi *et al*., 2019). Since the expression of most cytokines and chemokines requires the activation of NK-kB, we can speculate that in DCs, other signaling pathways and transcription factors, such as MAPKs and NRF2, as well as a non-canonical pathway of NF-kB activation could be activated by the sensitizers.

Interestingly, NF-kB activation has been reported to positively regulate Th2 cytokines (Neves *et al*., 2009). Indeed, moderate asthmatic patients peripheral blood mononuclear cells (PBMCs) show higher NF-κB p65 protein levels, IκB phosphorylation and IKK-β protein levels (Gagliardo *et al*., 2003). Furthermore, studies by Das and colleagues showed that p50 knockout mice are unable to produce IL-4, IL-5 and IL-13, therefore failing to mount airway eosinophilic inflammation. Blockage of NF-κB nuclear translocation, concomitantly inhibited GATA-3 expression (uniquely expressed on Th2 cells) and IL-5 and IL-13 production in splenic $CD4^+$ T cells, but not in committed Th2 cells (Das *et al*., 2001).

1.4.3 Inflammasome

Inflammasomes are multimeric protein complexes that function as intracellular innate immune sensors and are crucial for host defense to infection and endogenous danger signals. Indeed, they recognize and respond to a diverse range of PAMPs, DAMPs and danger signals (e.g. ROS, uric acid crystals and ATP), promoting the secretion of the pro-inflammatory cytokines IL-1β and IL-18, also being involved in the rapid and pro-inflammatory form of cell death called pyroptosis. IL-1β is critical for Th17 cell differentiation, maintain the production of Th17-associated cytokines, and facilitate allergic responses. IL-18, on the other hand, stimulates mast cells, T cells, and basophils to secrete Th2 cytokines, such as IL-13 and IL-4. Interestingly, IL-18 can also induce Th1 responses (Xiao *et al*., 2018).

NLRP3 inflammasome complexes, the most intensively studied, have been shown to participate in the pathogenesis of many diseases, including allergic reactions, autoimmune diseases, neurodegenerative and metabolic diseases (Xiao *et al*., 2018). Interestingly, they are triggered by a large variety of activators that do not share any structural similarities (Davis *et al*., 2011).

1.4.3.1 Inflammasome structure

After discovery of transmembrane TLRs, three new classes of microbial molecules' cytosolic sensors were described: RLRs and ALRs, which are nucleic acid sensing PRRs, and NLRs. NLRs respond to a wide variety of PAMPs and DAMPs as well as intracellular and extracellular signals generated by cells of the innate and adaptive immune system (Liu *et al.*, 2013). They play crucial roles in numerous aspects of immune and inflammatory responses, ranging from antimicrobial mechanisms to the control of adaptive responses. Following NLR activation, multiple signaling pathways initiate or shape a suitable inflammatory response and, mostly through the action of DCs, activate T and B lymphocytes.

After recognition of PAMPs or DAMPs, NLRs (NLRP1, NLRP3, NLRP6, NLRP7 (NOD12), NLRP12, NLRC4, NLRC5, NAIP2 or NAIP5) form multi-protein complexes called inflammasomes. Several inflammasomes have been described so far although containing NLRP3 (formerly known as Nalp3) or NLRC4 (formerly known as IPAF) are the most studied. Besides the core component sensor molecule NLR, the inflammasome platform also integrates the adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 (Figure 1.9).

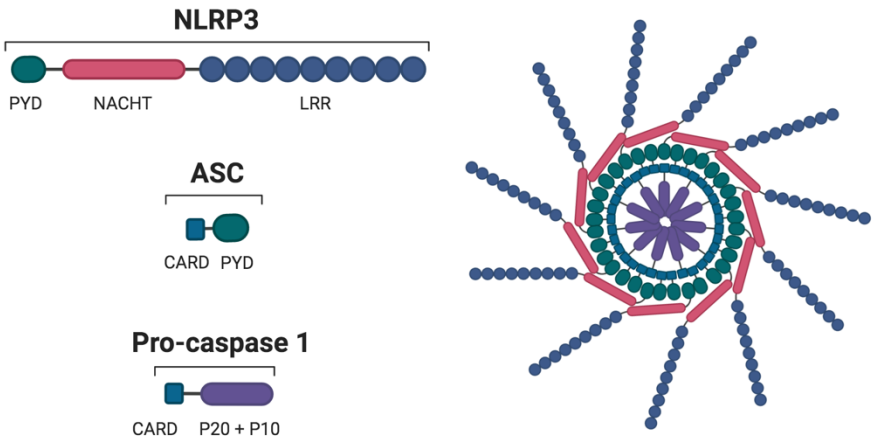


Figure 1.9. Inflammasome structure. Upon activation, NLR binds to ASC via pyrin–pyrin domain interaction and ASC recruits pro-caspase-1 via a CARD–CARD interaction, leading to caspase-1 activation. LRR, leucine-rich-repeat; NACHT, Nucleotide-binding oligomerization; PYD, pyrin domain; CARD, caspase activating and recruitment domain. Created with BioRender.com.

ASC consists both on a pyrin and a CARD domain, enabling it to interact with the pyrin domain of NLRs and the CARD domain of pro-caspase-1. This interaction initiates

the self-cleavage of pro-caspase-1 and the formation of the active heterotetrameric caspase-1 after inflammasome assembly. Active caspase-1 proteolytically cleaves pro-IL-1β and pro-IL-18 that are subsequently secreted from the cell. In addition to the production of the pro-inflammatory cytokines IL-1β and IL-18, inflammasome/caspase-1 complexes are responsible for a rapid and highly pro-inflammatory form of cell death termed pyroptosis (Guo *et al.*, 2015; Swanson *et al.*, 2019).

Since no NLR inflammasome structure has yet been solved, there is still a debate on the exact composition of inflammasomes (Liu *et al.*, 2013). Indeed, NLRC4 and NLRP1 do not need ASC to form inflammasomes, although when ASC is recruited, the production of cytokines following NLRC4 signaling is much more efficient (Latz *et al.*, 2013).

1.4.3.2 Inflammasome activation/assembly

A wide range of substances that emerge during infections, tissue damage or metabolic imbalances triggers the assembly of inflammasomes. A two-signal hypothesis was proposed to explain NLRP3 inflammasome activation (Yuk *et al.*, 2013). The initial priming signal is provided by TLR engagement and consequent activation of NF-κB leading to the expression of inflammasome components and cytokines precursors, including pro-IL-1β and pro-IL-18. This first signal is prompted by several pathogens or their components, including muramyl dipeptide moiety of peptidoglycans, lipopolysaccharides, influenza virus, among others. The second signal directly triggers assembly of the NLRP3 inflammasome, thus inducing caspase-1 to cleave pro-IL-1β and is started by endogenous sterile DAMP signals. These signals include intracellular calcium fluxes, potassium efflux, protein kinase R (PKR) activation, increased ROS production or the release of contents from phagolysosomes upon frustrated phagocytosis of silica, asbestos, aluminium salts, amyloid deposits and cholesterol crystals (Franchi *et al.*, 2009; Jo *et al.*, 2016). Potassium efflux through membrane pores is triggered by ATP binding to its receptor P2X7R, which results in NLRP3 inflammasome assembly (Yuk *et al.*, 2013; Tsuchiya *et al.*, 2014). The diversity of NLRP3-activating stimuli suggests that these compounds trigger cellular intermediates that couple to NLRP3. Mitochondrial ROS were suggested to be upstream activators of the NLRP3 inflammasome and numerous recent studies point mitochondrial-derived triggers as important regulators of the NLRP3 activation. Indeed, mitochondrial dysfunction, concomitantly with loss of mitochondrial membrane potential and release of

mitochondrial DNA, produces mtROS that in turn activate inflammasome through extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (Harijith *et al.*, 2014). However, mitochondrial injury is not a requisite for NLRP3 inflammasome activation (Allam *et al.*, 2014).

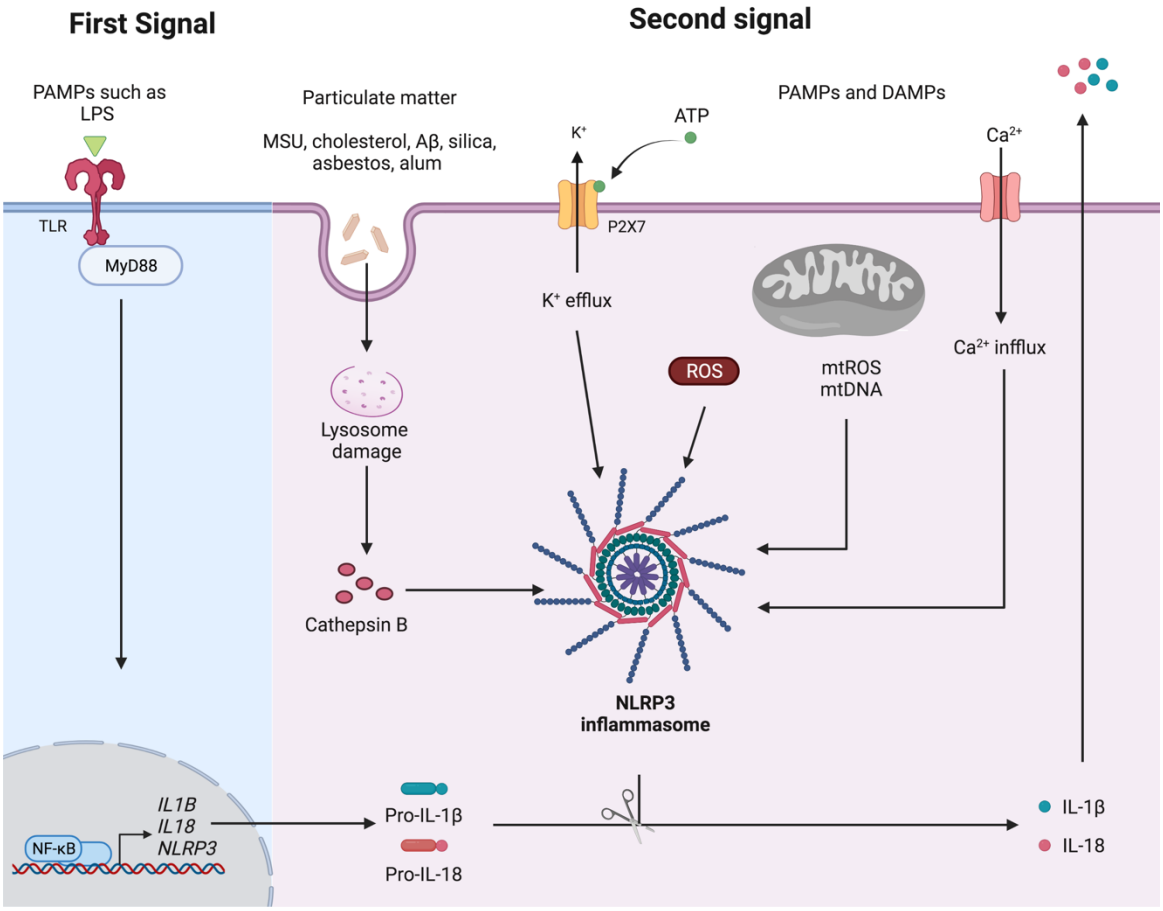


Figure 1.10 NLRP3 inflammasome activation. Inflammasome canonical activation is a two-step process. The first signal (priming step) is triggered by TLR ligands such as pathogen-associated molecular patterns (PAMPs) and involves the activation of nuclear factor-kappa B (NF-κB) or activator protein 1 (AP-1). NF-κB promotes the transcription of NLRP3, pro-IL-1β, and pro-IL-18, which remain in the cytoplasm in inactive forms. The second signal (activation step) activates NLRP3 inflammasome by facilitating the oligomerization of inactive NLRP3, ASC and pro-caspase-1. The assembly of this multimeric complex catalyses the conversion of procaspase-1 to caspase-1, which contributes to the production and secretion of the mature IL-1β and IL-18. The second signal is provided by diverse stimuli and three models have been suggested: (1) Extracellular ATP that, through P2X7-dependent pore induces K^+ efflux. Calcium influx is also involved in this process. (2) ROS production triggered by PAMPs or DAMPs. (3) Release of cathepsins into the cytosol after lysosomal destabilization. Though, other physiological events have also been implicated, including mitochondrial damage and autophagic dysfunction. Adapted from Swanson *et al.*, 2019. Created with BioRender.com.

1.4.3.3 Inflammasome activation in Allergic Contact Dermatitis and Respiratory Sensitization

A crucial role of inflammasome activation and IL-1β in allergic contact dermatitis has been demonstrated elsewhere (Shornick *et al*., 1996; Watanabe *et al*., 2007, 2008). Both keratinocytes and monocyte-derived cells contain molecular components required for inflammasome assembly. Moreover, mice lacking NLRP3 or the adaptor protein ASC show impaired CHS responses to TNCB and DNFB (Watanabe *et al*., 2007, 2008). Caspase-1 or IL-1R deficiency as well as treatment with the IL-1R antagonist anakinra, were also shown to prevent CHS (Antonopoulos *et al*., 2001; Watanabe *et al*., 2007, 2008; Weber *et al*., 2010 a). TNCB and oxazolone treatment induce the release of ATP, a well-known inflammasome activator, from skin cells *in vivo*. Studies by Weber and colleagues showed that mice lacking P2X7, or WT mice treated with the P2X7 antagonist KN-62, or the ATP-degrading enzyme apyrase show impaired CHS. The authors also showed, by cell transfer experiments, that TNCB-modified P2X7-deficient BMDCs failed to sensitize both wild-type and P2X7-deficient recipients, while similarly modified wild-type BMDCs were able to sensitize both wild-type and P2X7- deficient recipients. Interestingly, treatment with alum, a potent P2X7-independent activator of the NLRP3 inflammasome, restored the sensitization capacity of P2X7-deficient BMDCs. Although, in ASC- and NLRP3-deficient BMDCs aluminium pre-treatment was unable to restore sensitization capacity, demonstrating that aluminium and ATP-mediated effects were dependent on NLRP3 inflammasome (Weber *et al*., 2010 a). The crucial role of the inflammasome in sensitization was deciphered by the use of the weak sensitizer dinitrothiocyanobenzene (DNTB), that in combination with IL-1β was able to induce a strong sensitization response (Watanabe *et al*., 2008). Nickel, one of the most common causes of ACD and the first contact allergen identified to directly activate PPRs, namely TLR4, was also shown to activate NLRP3 inflammasome (Li *et al*., 2014). In addition to NLRP3, NLRP12 has also been implicated in CHS. Arthur and colleagues demonstrated that NLRP12$^{-/-}$ mice showed severely attenuated CHS responses to oxazolone and fluorescein isothiocyanate (FITC) as well as reduced neutrophil infiltration and impaired lymph node homing of DCs (Arthur *et al*., 2010).

A growing body of evidence suggest a role of the inflammasome-linked cytokines IL-1β and IL-18 in asthma. Despite several studies have showing that both IL-1β, IL-18 and caspase 1 levels were increased in asthmatic patients, compared to healthy subjects, the

role of inflammasomes on respiratory sensitization to low molecular weight chemicals remains incomplete. Many studies highlight the central role for caspase-1 in the pathogenesis of asthma. Indeed, mice treated with TDI show increased levels of caspase-1 as well as increased amounts of neutrophils and eosinophils and up-regulation of IL-1β (Liang *et al*., 2015). Blockage of IL-1β activity through deletion of the IL-1 receptor type I or administration of neutralizing antibody revokes the progression of TDI-induced asthma (Johnson *et al*., 2005). In line with these observations, IL-18 deficient mice also show decreased neutrophilic airway inflammation and remodeling in OVA-induced asthma (Yamagata *et al*., 2008). Furthermore, pre-treatment of TDI-asthmatic mice with caspase or NLRP3 inhibitors leads to a dramatic reduction in airway hyperresponsiveness, airway inflammation and remodeling as well as a decreased Th2 response and lower levels of IL-1β and IL-18 (Chen *et al*., 2019).

Although being an important inflammasome component, it is unclear whether NLRP3 is involved in other immune functions. Bruchard *et al*. showed that NLRP3 is expressed during the differentiation of $CD4^+$ T cells and is specifically involved in the polarization of Th2 cells. Although naive T cells did not express NLRP3, $CD4^+$ T cells polarized into Th0, Th1 and Th2 cells present a modest, though detectable, amounts of NLRP3 protein. The authors also demonstrated that $Nlrp3^{-/-}$ Th2 cells secreted less IL-4 than did wild-type Th2 cells, whereas $Nlrp3^{-/-}$ and WT Th1 cells secreted similar amounts of IFN-γ. Furthermore, Th2 response was impaired in Nlrp3-deficient mice, but not in Casp-1 or Asc-deficient mice, suggesting that NLRP3 was linked to Th2 response independently of inflammasome activation. Indeed, in Th2 cells, NLRP3 localizes in the nucleus, functioning as a transcription factor, where it binds to promoter regions of Th2 cell–related genes (Bruchard *et al*., 2015).

Despite the well-established role of inflammasome activation in CHS development, the clarification of the axis skin allergens-danger signals-NLRP3 inflammasome activation remains to be elucidated.

1.5 THESIS AIMS

Although the pathophysiology of ACD is well characterized, there is still some controversy about RS. Nevertheless, some similarities are shared between ACD and RS, namely the need of the chemical allergens to covalent modify proteins in order to trigger sensitization. This covalent modification leads to the activation of stress response pathways and cellular danger signals, namely oxidative stress, cytokines, and chemokines released by epithelial and other cells, which ultimately leads to DC maturation and migration to the draining lymph nodes. However, the knowledge remains limited concerning how, and to what degree, the release of DAMPs contributes to the differential priming of T cells observed in ACD (Th1 response) and RS (Th2).

Therefore, the general objective of this thesis is to identify relevant signaling pathways evoked by contact and respiratory allergens on dendritic cells. More specifically, the origin and kinetics of danger signal elicited by these two types of chemicals will be disclosed and concomitantly their impact on DC maturation process will be also unveiled.

To tackle this goal and moving ahead the state of the art, in a first approach, this work aimed to:

- Quantify and identify the origin and kinetics of redox imbalance elicited by the skin sensitizer DNFB and the respiratory sensitizer TMAC, two golden standards of skin and chemical respiratory allergy, respectively;
- Investigate the role of each chemical in the mechanisms associated with stress responses and inflammation, such as MAPKs intracellular signaling pathways;
- Evaluate the transcription of genes involved in DC maturation, namely cytokines and genes containing antioxidant response elements.

Furthermore, and given the role of IL-1β and IL-18 in in the pathophysiology of allergic diseases, in a second approach, we aimed to:

- Evaluate the inflammasome activation by thiol-reactive sensitizers;
- Decipher how thiol-reactive sensitizers trigger inflammasome activation.

Altogether, this study aims to shed light on the differential danger signals elicited by respiratory and skin allergens. Importantly, the detailed identification of the relevant signaling pathways and the mechanisms of their activation by allergens will most likely lead to more targeted therapeutic approaches by interference with these pathways. Moreover,

this will help to refine existing, and to fuel the development of new *in vitro* assays for the identification of contact and respiratory allergens, an important step to replace animal testing e.g. for ingredients of cosmetics which has been prohibited now by EU legislation.

CHAPTER 2

Nature and kinetics of redox imbalance triggered by respiratory and skin chemical sensitizers on the human monocytic cell line THP-1

Isabel Ferreira, Ana Silva, João Demétrio Martins, Bruno Miguel Neves, Maria Teresa Cruz

Redox Biology, 2018

DOI: 10.1016/j.redox.2018.02.002

Abstract

Low molecular weight reactive chemicals causing skin and respiratory allergies are known to activate dendritic cells (DC), an event considered to be a key step in both pathologies. Although generation of reactive oxygen species (ROS) is considered a major danger signal responsible for DC maturation, the mechanisms leading to cellular redox imbalance remain poorly understood. Therefore, the aim of this study was to unveil the origin and kinetics of redox imbalance elicited by 1-fluoro-2,4-dinitrobenzene (DNFB) and trimellitic anhydride chloride (TMAC), two golden standards of skin and chemical respiratory allergy, respectively. To track this goal, we addressed the time course modifications of ROS production and cellular antioxidant defenses as well as the modulation of MAPKs signaling pathways and transcription of pathophysiological relevant genes in THP-1 cells. Our data shows that the thiol-reactive sensitizer DNFB directly reacts with cytoplasmic glutathione (GSH) causing its rapid and marked depletion which results in a general increase in ROS accumulation. In turn, TMAC, which preferentially reacts with amine groups, induces a delayed GSH depletion as a consequence of increased mitochondrial ROS production. These divergences in ROS production seem to be correlated with the different extension of intracellular signaling pathways activation and, by consequence, with distinct transcription kinetics of genes such as *HMOX*, *IL8*, *IL1B* and *CD86*. Ultimately, our observations may help explain the distinct DC phenotype and T-cell polarizing profile triggered by skin and respiratory sensitizers.

Keywords: ROS; Oxidative stress; Glutathione; Allergic contact dermatitis; Chemical respiratory allergy; Dendritic cells maturation.

Highlights

- Distinctive ROS origin and kinetics elicited by skin and respiratory sensitizers.
- ROS production elicited by DNFB results primarily from direct GSH haptenation.
- Distinct expression of genes involved in DC maturation and T-cell polarizing capacity.

2.1 INTRODUCTION

Contact and respiratory allergies to low molecular weight (LMW) chemicals are growing among general population in result of an increased exposure to environmental and industrial compounds present in toiletry and household products. Studies focusing in the physiopathology of allergy have pointed out common key molecular events triggered by contact and respiratory allergens that are crucial for the development of the so called adverse outcome pathway (OECD, 2012 a; b). The first assumption is that low molecular weight chemicals (LMW; < 1000 Da) are too small to be recognized by the immune system and must first react with a protein (Chipinda *et al.*, 2011; Lalko *et al.*, 2011). Such chemicals behave as haptens and are either naturally protein-reactive or are rapidly metabolized into protein-reactive compounds. Covalent binding of an hapten to a protein is believed to be a relevant mechanism for immune recognition and further development of antigenic LMW-induced chemical allergies (Landsteiner *et al.*, 1936; Chipinda *et al.*, 2011). The second assumption is that protein-hapten conjugates induce stress responses and xenoinflammation through release of damage-associated molecular patterns (DAMPs), such as reactive oxygen species (ROS), uric acid, hyaluronic acid fragments and extracellular ATP/ADP. These DAMPs are required for the activation of pattern recognition receptors (PRRs) and intracellular signaling pathways in antigen presenting cells such as dendritic cells (DCs), leading to their maturation. Then, and as third assumption, DCs process the conjugates and subsequently migrate to the draining lymph nodes where they prime naive T lymphocytes. T-cells become activated and expand into allergen-specific effector T-cells that disseminate systemically and elicit a strong inflammatory reaction upon later contact with the same chemical (Dearman *et al.*, 2003). Respiratory tract sensitization has been associated with the development of a Th2 response (promoting immediate-type allergic hypersensitivity), which is consistent with the secretion of interleukin (IL)-4, IL-5, IL-6, IL-10, IL-13 and, for most chemicals, the production of IgE by B lymphocytes (Dearman *et al.*, 2003; Kimber *et al.*, 2005, 2011). In contrast, skin sensitization is a cell-mediated, delayed type hypersensitivity reaction, involving a preferential polarization of Th1 and cytotoxic T cells with the secretion of interferon-gamma (IFN-γ), IL-2 and tumor necrosis factor β (TNF-β) (Dearman *et al.*, 2003). An important clue to be further deciphered consists in the identification of the molecular mechanisms that first trigger these qualitatively distinct immunotoxic responses,

although it has been shown that depending on DCs maturation state and cytokine/chemokine profiles, they are able to polarize naive T cells into distinct effector populations (Steinman *et al*., 2007).

As stated previously, ROS (superoxide, O2• − and hydrogen peroxide, H_2O_2) as well as protein oxidation play an important role in allergen-induced sensitization (Okayama, 2005; Byamba *et al*., 2010). Indeed, there is growing evidence that redox equilibrium influences DCs ability to trigger T-cell activation and to regulate the polarity of the immune response (Matsue *et al*., 2003). Multiple signaling pathways involved in DC maturation are known to be redox-sensitive, including transcription factors such as NF-κB and AP-1, mitogen-activated protein kinases (MAPKs), and several phosphatases and proteins directly involved in oxidative stress detection such as Keap-1 [Kelch-like ECH-associated protein 1]/Nrf2], hypoxia inducible factor-1 and thioredoxin (Cosentino-Gomes *et al*., 2012). Despite the importance attributed to ROS in allergen-induced sensitization, little is known about their nature and formation kinetics. We hypothesize that different intracellular toxicity pathways evoked by respiratory and contact allergens may trigger divergent immune responses. This rational prompted us to investigate the potential sites of ROS generation triggered by respiratory and skin chemical sensitizers on the human monocytic cell line THP-1, as well as the activation of MAPKs signaling pathways (e.g., ERK, JNK and p38 MAPK) and the modulation of relevant genes such as *HMOX*, *NQO1, MDR1, IL1B, IL8, IL12B, IL18* and *CD86*. To accomplish this goal, we used 1-fluoro-2,4-dinitrobenzene (DNFB) and trimellitic anhydride chloride (TMAC), two golden standards of contact and respiratory allergies, respectively, that possess an equivalent immunogenic potential. Methyl salicylate (MeSA) was used as a respiratory and contact irritant (negative control) and bacterial lipopolysaccharide (LPS) as a non-allergen immunogenic compound.

2.2 MATERIALS AND METHODS

2.2.1 Materials

The chemicals L-Lysine, L-Cysteine hydrochloride, TMAC, DNFB, MeSA, LPS from *Escherichia coli* (serotype 026:B6), Dibromobimane (34025) and SOD determination Kit (19160) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). The

tetramethyl-rhodamine ethyl ester (TMRE) mitochondrial membrane potential assay kit (ab113852) was obtained from Abcam (Cambridge, UK). Amplex Red Xanthine/Xanthine Oxidase Assay Kit (a22182), hoechst 3342 (H3570), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; D399) for oxidative stress detection and MitoSOX (M36008) red mitochondrial superoxide indicator were obtained from Molecular Probes (Eugene, OR, USA). Phospho-p44/p42 MAPK (ERK1/ ERK2) (9101S), phospho-p38 MAPK (9211S), phospho-SAPK/JNK (4668S), total p44/p42 MAPK (ERK1/ ERK2) (9102S), p38 MAPK (9212S) and SAPK/JNK (9252S) were from Cell Signaling Technologies (Danvers, MA, USA). The polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corp (Bedford, MA, USA). Alkaline phosphatase-conjugated secondary antibodies were purchased from GE Healthcare (Chalfont St. Giles, UK). Protease and phosphatase inhibitor cocktails were from Roche (Mannheim, Germany). TRIzol reagent was purchased from Invitrogen (Barcelona, Spain) and RNA Storage Solution was from Ambion (Foster City, CA, USA). The NZY First-Strand cDNA Synthesis Kit was obtained from NZYTech (Lisbon, Portugal) and custom oligonucleotide primers were from Eurofins MWG Operon (Ebersberg, Germany).

2.2.2 Cell Culture and treatment

The THP-1 human monocytic cell line (ATCC TIB-202, American Type Culture Collection, Manassas, VA, USA) was cultured and maintained at a cell density between 0.2 x 10^6 and 1 x 10^6 cells/mL in RPMI 1640 supplemented with 10% inactivated fetal bovine serum, 25 mM glucose, 10 mM Hepes, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.05 mM 2-mercaptoethanol. Cells were subcultured every 3 or 4 days and kept in culture for a maximum of 2 months.

2.2.3 Chemical exposure

Since a certain level of cytotoxicity is essential for effective DC maturation (Hulette *et al*., 2005), the concentrations of chemicals inducing up to 30% decrease in cell viability (EC_{30} value) were determined through the resazurin assay (Supplementary data, Figure S1.1). In all subsequent experiments' cells were exposed for the indicated times to the EC_{30} concentration of each chemical, corresponding to 7 μM for DNFB, 400 μM for TMAC

and 600 µM for MeSA. In certain experiments, cysteine (Cys) or lysine (Lys) were pre-incubated *in chemico* with sensitizers. More specifically, we mixed Cys/Lys with sensitizers on microcentrifuge tubes (*in chemico* reaction) and allowed them to react for 1 h at 37 °C. After that, we stimulated THP-1 cells with the mixture (Cys/Lys + sensitizer) for the indicated times. The final concentration for Cys/Lys was 10 mM and for DNFB and TMAC, 7 µM and 400 µM respectively. Cells were also exposed to LPS (1 µg/mL) as a control for a non-allergen DC maturation inducer.

2.2.4 Oxidative stress evaluation

Chemical-induced ROS formation was assayed with ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Briefly, 0.5 x 10^6 cells/mL were plated in a 12-well plate, exposed to chemicals during indicated times, washed with PBS and then loaded with 5 µM H_2DCFDA and 0.5 µg/mL Hoechst in HBSS (in mM: 1.3 CaCl2, 0.5 $MgCl_2$, 5.3 KCl, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 138 NaCl, 0.34 Na_2HPO_4 and 5,5 Glucose, pH 7.4) for 30 min at 37 °C in the dark. Cells were then washed with PBS, transferred to µ-slides 8-well ibidiTreat (ibidi GmbH, München, Germany) for observation. Images were obtained using an Axio Observer.Z1 inverted microscope (Zeiss) and analyzed with Fiji software from ImageJ (http://fiji.sc/Fiji).

2.2.5 Mitochondrial membrane potential (MMP) integrity

The MMP integrity was evaluated by the TMRE mitochondrial membrane potential assay kit according to the manufacturer's instructions. Briefly, 1 x 10^6 cells/mL were plated in a 48-well plate and exposed to chemicals for 6 h. Cells were also incubated for 10 min, with 50 µM FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), a protonophore that collapses the MMP, as a negative control. TMRE (1 mM) was then added for 30 min and cells were further collected, washed and TMRE fluorescence was read (λ_{exc} = 549 nm; λ_{em} = 575 nm).

2.2.6 Mitochondrial superoxide anion measurement

Mitochondrial O^{2-} generation was determined using MitoSOX according to the manufacturer's instructions. Briefly, 0.5 x 10^6 cells/mL were plated in a 12-well plate, exposed to chemicals for the indicated times, washed with PBS and then loaded with 5 µM MitoSOX and 0.5 µg/mL Hoechst in HBSS for 10 min at 37 °C in the dark. Cells were then washed with PBS and transferred to µ-slides 8-well ibidiTreat (ibidi GmbH, München, Germany) for observation. Images were obtained using an Axio Observer.Z1 inverted microscope (Zeiss) and analyzed with Fiji software from ImageJ (http://fiji.sc/Fiji).

2.2.7 Determination of superoxide dismutase activity

THP-1 cells were plated at a density of 1 x 10^6 cells/mL, in a 6 well plate and treated as previously described (see Chemical exposure). Cells were further collected, centrifuged (300 g for 5 min, at 4 °C) and washed in ice-cold PBS. After a second centrifugation, the pellet was incubated in RIPA lysis buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) for 30 min on ice. The nuclei and the insoluble cell debris were removed by centrifugation (10,000 g for 15 min, at 4 °C) and the supernatant collected and used as total cell lysates. Total SOD activity was then determined using the SOD Determination Kit, according to the manufacturer's instructions, with some modifications. Cell extract duplicates were also incubated with KCN (2 mM) to inhibit SOD 1, thus allowing the measurement of mitochondrial SOD 2. SOD 1 activity was then calculated by subtraction of SOD 2 from total SOD.

2.2.8 Quantification of xanthine oxidase or hypoxanthine

Xantine or hypoxantine levels were determined using the Amplex Red Xanthine/Xanthine Oxidase Assay Kit according to the manufacturer's instructions. Briefly, 1 x 10^6 cells/mL were plated in a 6-well plate and exposed to chemicals for 6 h. Briefly, 50 µL of cell extracts and controls were added to separate wells of a microplate and incubated with equal volume of Amplex Red reagent/HRP/xanthine oxidase (100 µM/0.4 U/mL/40 mU/mL) or Amplex Red reagent/HRP/hypoxantine working solution (100

μM/0.4 U/mL/200 μM) for 48 h at 37 °C in the dark. Fluorescence was then measured in a microplate reader using (λ_{exc} = 530 nm; λ_{em} = 590 nm).

2.2.9 Glutathione (GSH) depletion assay

The effect of chemicals on cell GSH content was determined visualized by fluorescence microscopy. Briefly, 0.5 x 106 cells/mL were plated in a 12-well plate, exposed to chemicals during indicated times, washed with PBS and then loaded with 30 μM dibromobimane for 30 min at 37 °C in the dark. Cells were then washed with PBS and transferred to μ-slides 8-well ibidiTreat (ibidi GmbH, München, Germany) for observation. Images were obtained using an Axio Observer.Z1 inverted microscope (Zeiss) and analyzed with Fiji software from ImageJ (http://fiji.sc/Fiji).

2.2.10 Analysis of gene transcription by quantitative real-time PCR

Total RNA was isolated from cells with TRIzol reagent according to the manufacturer's instructions. RNA concentration was determined by OD_{260} measurement using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and samples stored in RNA Storage Solution at -80 °C until use. Briefly, 1 μg of total RNA was reverse-transcribed using the NZY First-Strand cDNA Synthesis Kit and quantitative real-time PCR (qPCR) reactions were performed, in duplicate for each sample, on a Bio-Rad MyCycler iQ5 as previously described (Neves *et al*., 2013). After amplification, a threshold was set for each gene and C_t values were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software. The results were normalized using *HPRT1* as reference gene. Primer sequences were designed using Beacon Designer software version 7.7 (Premier Biosoft International, Palo Alto, CA, USA) (Supplementary data, Table S1.1) and thoroughly tested.

2.2.11 Cell lysates and Western Blot analysis

Cells were plated at a density of 0.8 x 10^6 cell/mL, in a six-well plate with a final volume of 3 mL and treated as previously described (see Chemical exposure). After

incubation with chemicals, for the indicated times, cells were collected, centrifuged (300 g, 5 min at 4 °C), and washed in ice-cold PBS. After a second centrifugation, the pellet was incubated in RIPA lysis buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA), freshly supplemented with 1 mM dithiothreitol (DTT) and protease and phosphatase inhibitor cocktails, for 30 min in ice. The nuclei and the insoluble cell debris were removed by centrifugation (12,000 g for 10 min, at 4 °C). The post-nuclear extracts were collected and used as total cell lysates. Protein concentration was determined using the bicinchoninic acid method, and the cell lysates were denatured at 95 °C, for 5 min, in sample buffer (0.125 mM Tris, pH 6.8; 2% w/v SDS; 100 mM DTT; 10% glycerol; and bromophenol blue) for subsequent use in Western blot analysis. Briefly, 25 μg of protein were electrophoretically separated on a 4-10% (v/v) sodium dodecyl sulfate – polyacrylamide gel and transferred to a PVDF membrane. The membranes were blocked with 5% (w/v) fat-free dry milk in Tris- buffered saline containing 0.1% (v/v) Tween 20 (TBS-T), for 1 h, at room temperature. Blots were then incubated overnight at 4 °C with the primary antibodies against phospho-p44/p42 MAPK (ERK1/ ERK2) (1:1000), phospho-p38 MAPK (1:1000) and phospho-SAPK/JNK(1:1000). The membranes were then washed for 30 min with TBS-T and incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit antibody (1:20,000). The immune complexes were detected by membrane exposure to the enhanced chemifluorescence reagent for 5 min, followed by scanning for blue excited fluorescence on the Typhoon imager (GE Healthcare). The generated signals were analyzed using TotalLab TL120. To test whether similar amounts of protein were loaded for each sample, the membranes were stripped and reprobed with antibodies to total ERK1/ ERK2, SAPK/JNK and p38 MAPK. The blots were then developed with alkaline phosphatase-conjugated secondary antibodies and visualized by enhanced chemifluorescence. Phosphorylated protein levels were calculated relative to total protein levels (p-ERK1/2/total ERK, p-p38/total p38 and p-JNK/total JNK).

2.2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 for Mac OS X (GraphPad Software, San Diego, CA, USA; www.graphpad.com). For each experimental condition, the results are presented as the mean value ± SEM of at least 3 independent experiments.

Comparisons between two groups were made by the two-tailed unpaired Student *t*-test and multiple group comparisons by one-way ANOVA analysis, with a Dunnett´s multiple comparison post-test. Significance levels are as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

2.3 RESULTS

In this study, we aimed to further elucidate the mechanisms involved in skin and respiratory sensitization evoked by LMW chemicals, focusing on the oxidative stress toxicity pathways of THP-1 cells. The THP-1 cell line is frequently used as a DC surrogate in *in vitro* skin sensitization tests since, upon stimulation, cells display activation markers, such as increase in phosphotyrosine levels, up-regulation of cell surface co-stimulatory molecules and increase in cytokine and chemokine production (Miyazawa *et al*., 2007; dos Santos *et al*., 2009). Because a certain level of cytotoxicity is essential for effective DCs activation (Hulette *et al*., 2005), THP-1 cells were exposed to allergens DNFB and TMAC and the irritant MeSA in concentrations that induced up to 30% cytotoxicity (EC_{30}). Cells were also exposed to the non-sensitizing but immunogenic LPS, an immunostimulatory molecule from gram-negative bacteria cell wall that induces the maturation of DCs by binding to the transmembrane TLR4, as a control for DC maturation induction by a non-allergen.

2.3.1 Both DNFB and TMAC induce ROS production, yet with different origins and kinetics

ROS production and protein oxidation are referred as early molecular events triggered during allergen-induced sensitization. Therefore, we attempted to decipher whether chemicals directly induce ROS production or interfere with the antioxidant defenses of the cell. Thus, we evaluated general ROS formation, mitochondrial membrane potential (MMP) integrity and mitochondrial superoxide levels at different time points. ROS production was addressed using the cell-permeant probe, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Fig. 2.1). H_2DCFDA is non-fluorescent in its reduced state being converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) upon cleavage of the acetate groups by intracellular esterases and oxidation. The

results demonstrate that only DNFB significantly increased ROS production at 1 h, which decreased at 6 h, although to values still above the control condition (Fig. 2.1).

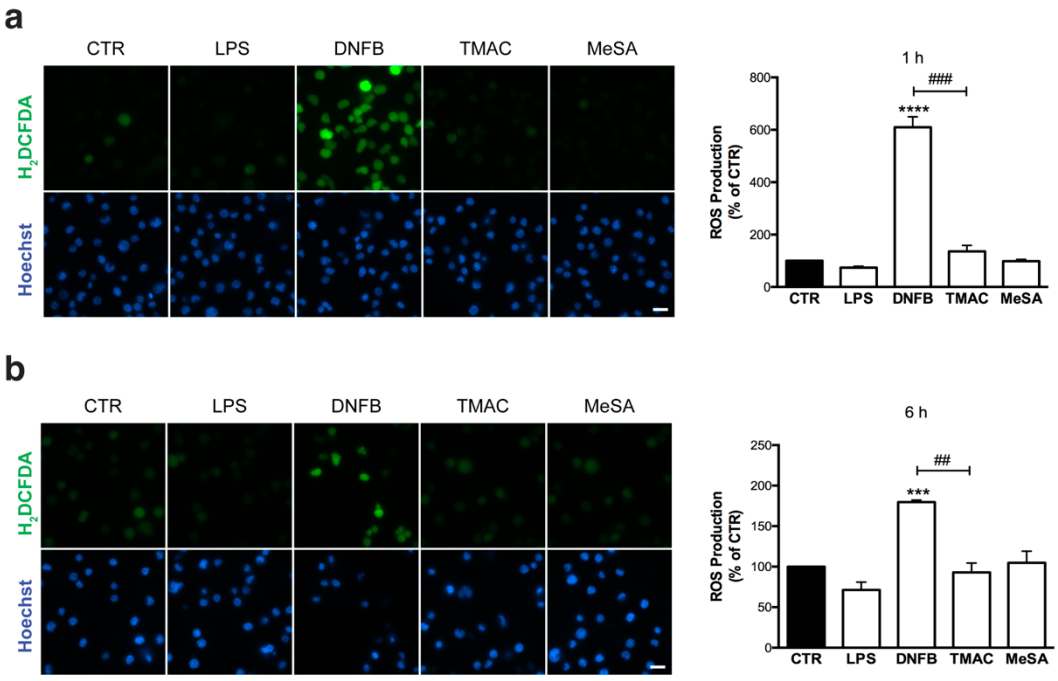


Figure 2.1. Chemical-induced reactive oxygen species production. Human THP-1 cells were exposed to LPS, DNFB, TMAC and MeSA for 1 h (a) and 6 h (b), and ROS production was evaluated by fluorescence microscopy using the cell-permeant dye H_2DCFDA. Hoechst 3342 was used as a fluorescent marker for the nucleus. Images shown are representative of three independent experiments. Magnification: 63×; Scale bar = 20 µm. Results are presented as the means ± SEM of cellular fluorescent intensity of at least 50 cells per experiment. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to untreated cells; *t*-test, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

Next, we evaluated the activity of the cytoplasmatic enzyme xanthine oxidase (XO), which generates superoxide through the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. All the stimuli tested increased the activity of xanthine oxidase, although only the irritant MeSA and the respiratory allergen TMAC reached statistical significance (p = 0.0219 and p = 0.002, respectively) (Figure 2.2a). The increase in XO activity is accompanied by a concomitant consumption of its substrate hypoxanthine, as seen in Figure 2.2b.

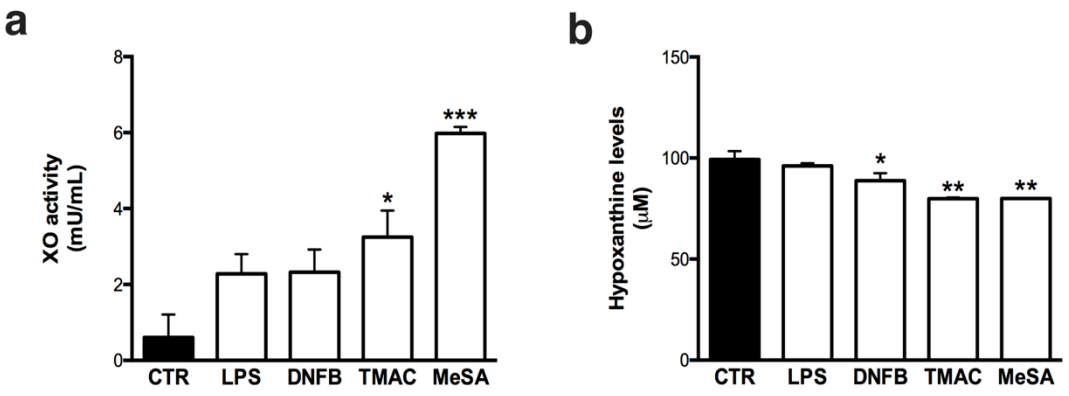


Figure 2.2 Xanthine oxidase activity (a) and hypoxantine levels (b) after exposure to different stimuli. Human THP-1 cells were exposed to LPS, DNFB, TMAC, and MeSA for 6 h. Xanthine oxidase activity and hypoxanthine levels were further evaluated with the Xanthine/Xanthine Oxidase Assay Kit. The bars in the graphs correspond to the means ± SEM of three independent experiments. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to untreated cells.

Since mitochondrial respiratory chain is a major ROS source and ROS production is often associated with mitochondrial dysfunction, we proceeded to evaluate mitochondrial function using MitoSOX and TMRE (Figure 2.3). MitoSOX red reagent is a fluorogenic dye specifically targeted to mitochondria in live cells. Once in the mitochondria, it is rapidly oxidized by superoxide but not by other ROS or reactive nitrogen species (RNS). The oxidized product is highly fluorescent upon binding to nucleic acid. TMRE is a cell permeant, positively charged, red-orange dye that readily accumulates in active mitochondria due to their relative negatively charged matrix. Depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE. FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) was used as a positive control of depolarized mitochondria, since it is an ionophore uncoupler of oxidative phosphorylation able to mitigate mitochondrial membrane potential and TMRE staining. After 6 h of incubation with chemicals, mitochondrial superoxide levels were similar to those observed in untreated cells except for TMAC, which showed a 2.5-fold increase (Figure 2.3b). MMP was not significantly altered by cell exposure to any of the chemicals tested (Figure 2.3c).

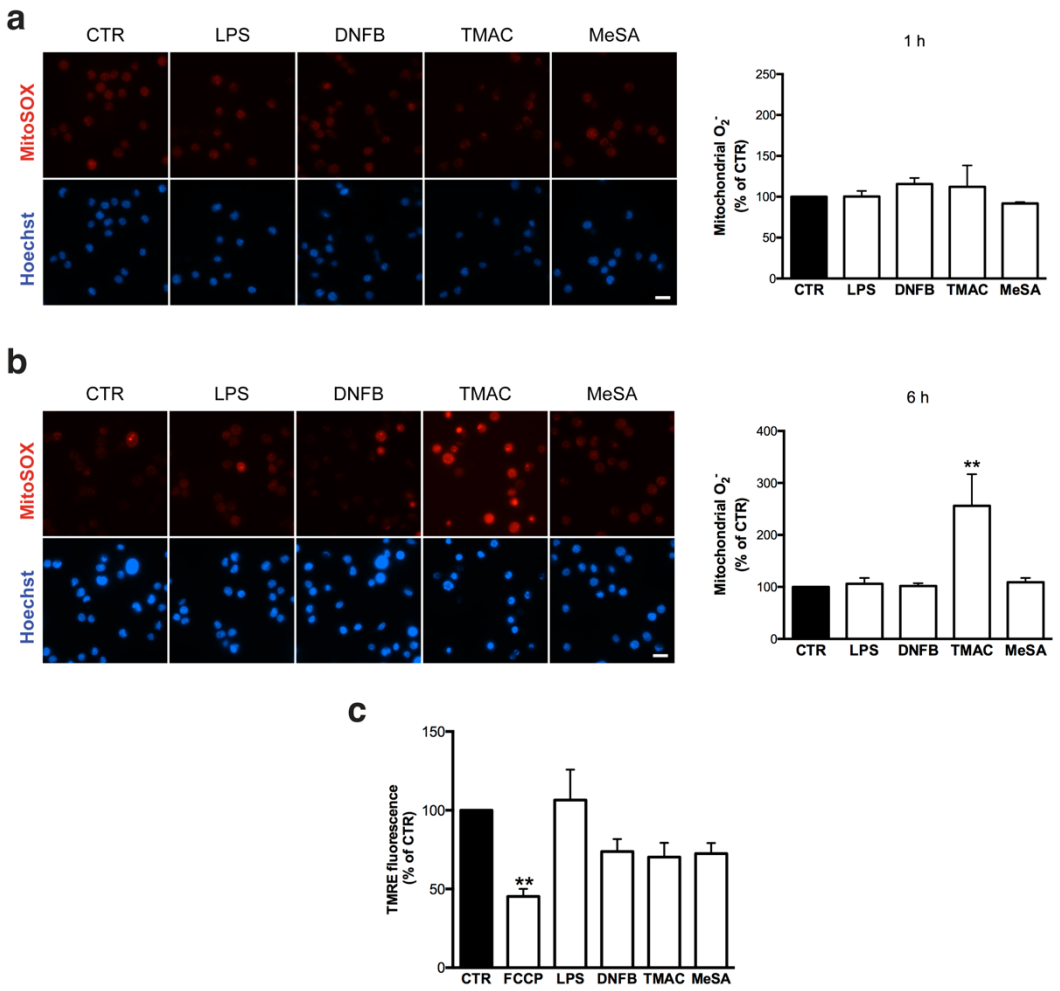


Figure 2.3 Effect of stimuli over mitochondrial membrane potential and superoxide production. Human THP-1 cells were exposed to LPS, DNFB, TMAC and MeSA for 1 h (a) and 6 h (b and c). Mitochondrial O_2^- formation was evaluated by fluorescence microscopy using the MitoSOX superoxide indicator (a and b). Hoechst 3342 was used as a fluorescent marker for the nucleus. Magnification: 63×; Scale bar = 20 µm. Results are presented as the means ± SEM of cellular fluorescent intensity of at least 50 cells per experiment and the images shown are representative of three independent experiments. MMP alterations due to chemical exposure were determined by TMRE fluorescence (c). FCCP (50 µM), a protonophore that collapses mitochondrial membrane potential, was used as a positive control. The bars in the graph correspond to means ± SEM of at least three independent experiments. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test; $^{**}p < 0.01$ compared to untreated cells.

2.3.2 DNFB and TMAC deplete intracellular glutathione with different kinetics

We proceeded to investigate the effect of chemicals on major antioxidant defense systems, namely the soluble antioxidant glutathione (GSH) and the antioxidant enzymes superoxide dismutase (SOD) 1 and 2. Glutatione (GSH) is the most common source of thiol groups present in the cell, with concentrations reaching millimolar levels (1-10 mM). Traditional methods for measuring GSH usually rely on the reaction of compounds, such

as dibromobimane, with GSH. Dibromobimane is a cross-linking reagent essentially nonfluorescent, that emits fluorescence when conjugated with several low molecular weight thiols, including glutathione. Indeed, this probe is broadly used to assess changes in GSH (Hedley *et al.*, 1994; Cox *et al.*, 2007; Yakubu *et al.*, 2011).

A significant depletion of GSH was observed in cells exposed to DNFB for 1 h (Fig. 2.4a and b). However, GSH levels recovered to basal values when DNFB treatment was prolonged to 6 h (Fig. 2.4a and c). These results have a similar trend to those observed for ROS production in cells exposed to DNFB (Figure 2.1). In contrast, TMAC only significantly depleted GSH at 6 h (Figure 2.4a and c), which is also in accordance with the observed increase in mitochondrial superoxide levels following 6 h of treatment (Figure 2.3b). Next, we evaluated SOD activity and, as shown in Fig. 2.4d and e, only LPS significantly affected this enzymatic system.

2.3.3 Incubation with Cys or Lys blocks DNFB induced ROS production, GSH depletion and MAPK activation

It is well established that sensitizers are naturally highly reactive or are rapidly metabolized into compounds that react with thiol or primary amine groups present on proteins (Chipinda *et al.*, 2011). Given the rapid and extensive changes evoked by DNFB on cellular ROS and GSH levels we next evaluated whether pre-incubation of the sensitizer with cysteine or lysine blocked the observed effects. DNFB and its analogue DNCB were reported to have a mixed reactivity with both thiol and amine groups (Gerberick *et al.*, 2007; Lalko *et al.*, 2013). Indeed, when DNFB was pre-incubated *in chemico* for 1 h with cysteine or lysine before addition to THP-1 cells, we did not observe an increase in ROS production (Figure 2.5a) or GSH depletion (Figure 2.5b). To assess MAPK activation, we first evaluated p38, JNK and ERK phosphorylation in THP-1 cells stimulated with chemicals for different periods of time by western blot analysis (Supplementary data, Figure S2.2). Results indicate that only p38 and JNK were phosphorylated at all times tested, with the peak occurring at 1 h. We then proceeded with 1 h of incubation for further experiments. Similarly to what was observed for ROS production and GSH depletion in cells treated with DNFB, MAPKs activation was also suppressed if DNFB and TMAC were previously incubated with lysine or cysteine (Fig. 2.5c and d). Regardless of previous studies reporting TMAC reactivity only towards lysine

residues (Gerberick *et al.*, 2007), our results suggest that TMAC reacts with both cysteine and lysine. Indeed, JNK and p38 phosphorylation was significantly lower when TMAC was pre-incubated with cysteine.

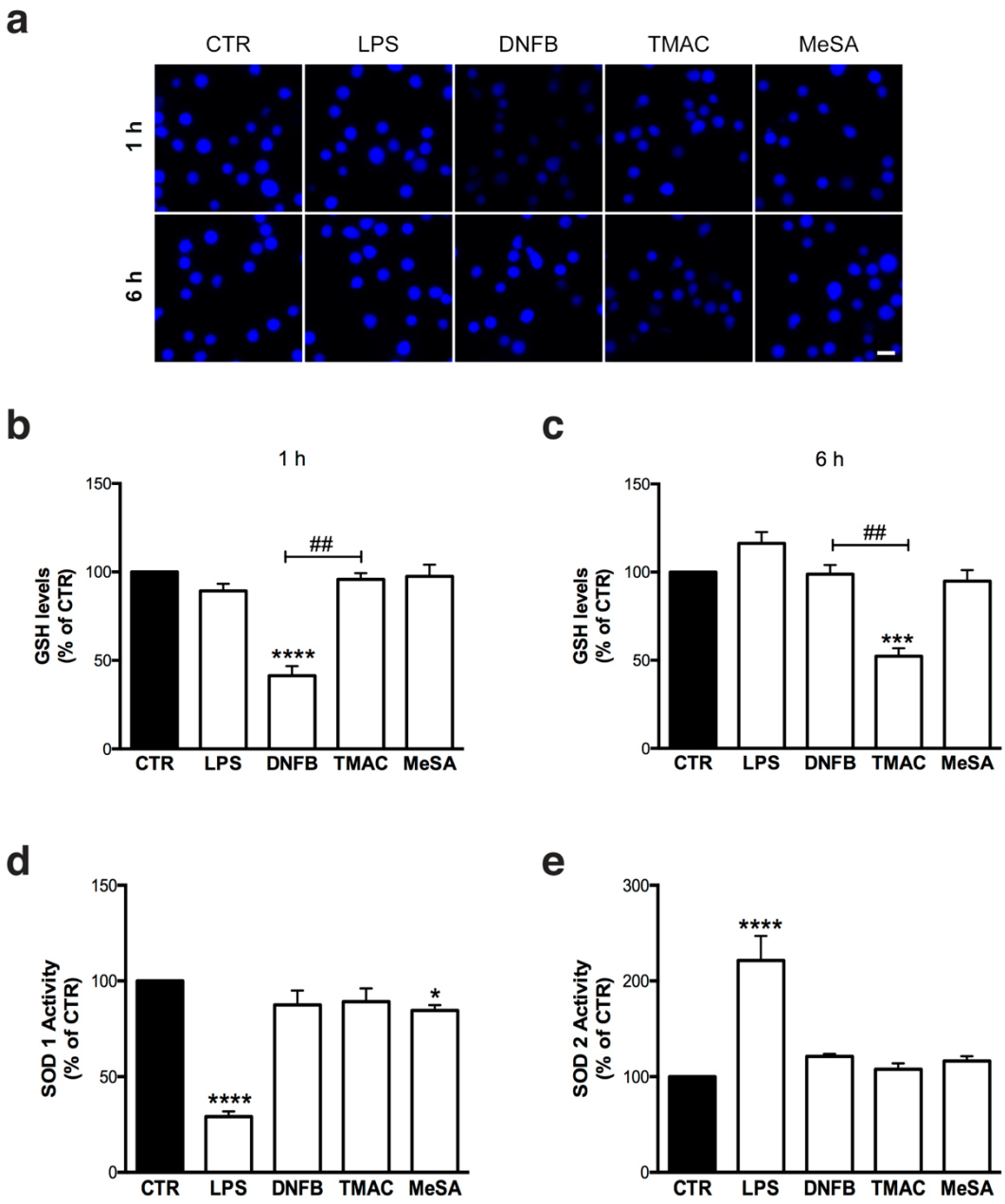


Figure 2.4 Effect of stimuli on cellular antioxidant defenses. THP-1 cells were exposed to LPS, DNFB, TMAC and MeSA for 1 h (a and b) and 6 h (a, c, d and e). GSH depletion (a, b and c) was determined by fluorescence microscopy using the thiol reactive protein cross-linking reagent dibromobimane. Magnification: 63×.; Scale bar = 20 µm. Results in the graphs are presented as the means ± SEM of cellular fluorescent intensity of at least 50 cells per experiment. SOD1 (d) and SOD2 (e) activities were evaluated with SOD determination Kit. The bars in the graphs correspond to the means ± SEM of at least three independent experiments. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test, *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ compared to untreated cells; *t*-test, ##$p < 0.01$. Pictures shown (a) are representative of at least three independent experiments.

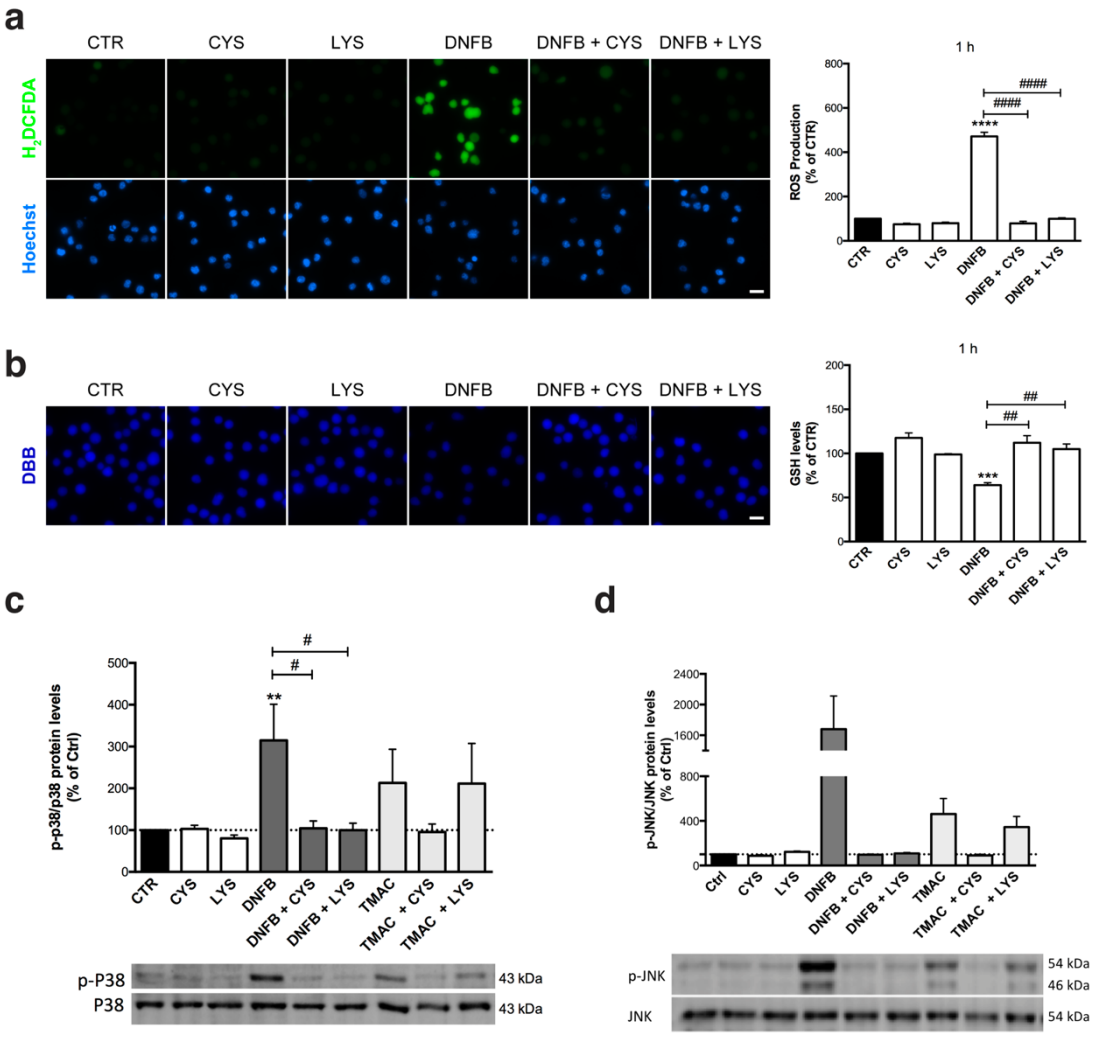


Figure 2.5 Effect of the pre-incubation of sensitizers with cysteine (CYS) or lysine (LYS) on ROS production, GSH depletion and MAPK activation. DNFB and TMAC were pre-incubated *in chemico* for 1 h with cysteine or lysine and later added to THP-1 cells for 1 h. ROS production (a) and GSH depletion (b) were evaluated by fluorescence microscopy, using the cell-permeant dye H2DCFDA and the thiol reactive protein cross-linking reagent dibromobimane, respectively. Hoechst 3342 was used as a fluorescent marker for the nucleus. Magnification: 63×.; Scale bar = 20 µm. Evaluation of the JNK (c) and p38 (d) signaling pathways activation was performed by western blotting of total cell extracts. Data correspond to the means ± SEM of at least three independent experiments and is expressed as % relatively to untreated cells (CTR). Statistical analysis: one-way ANOVA with Dunnett's multiple comparison test: $^{**}p < 0.01$, $^{***}p < 0.001$ $^{****}p < 0.0001$, compared to CTR; t-test: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$. Images shown (a and b) are representative of three independent experiments.

2.3.4 Gene modulation by contact and respiratory chemicals

We further investigated the transcription of genes related to DCs functions on the physiopathology of chemical allergy: genes containing antioxidant response elements (ARE), namely heme oxigenase 1 (*HMOX1*) and NADPH quinone oxidoredutase 1 (*NQO1*); multidrug resistance protein 1 (*MDR1*), an ATP-dependent drug efflux pump for

xenobiotic compounds which is involved in DC migration; cytokines well-known to be modulated upon hapten stimulation (*IL1B*, *IL8*, *IL12B*, *IL18*, and *IL8*) (De Smedt *et al*., 2001; Aiba *et al*., 2003); and CD86, a DC maturation marker.

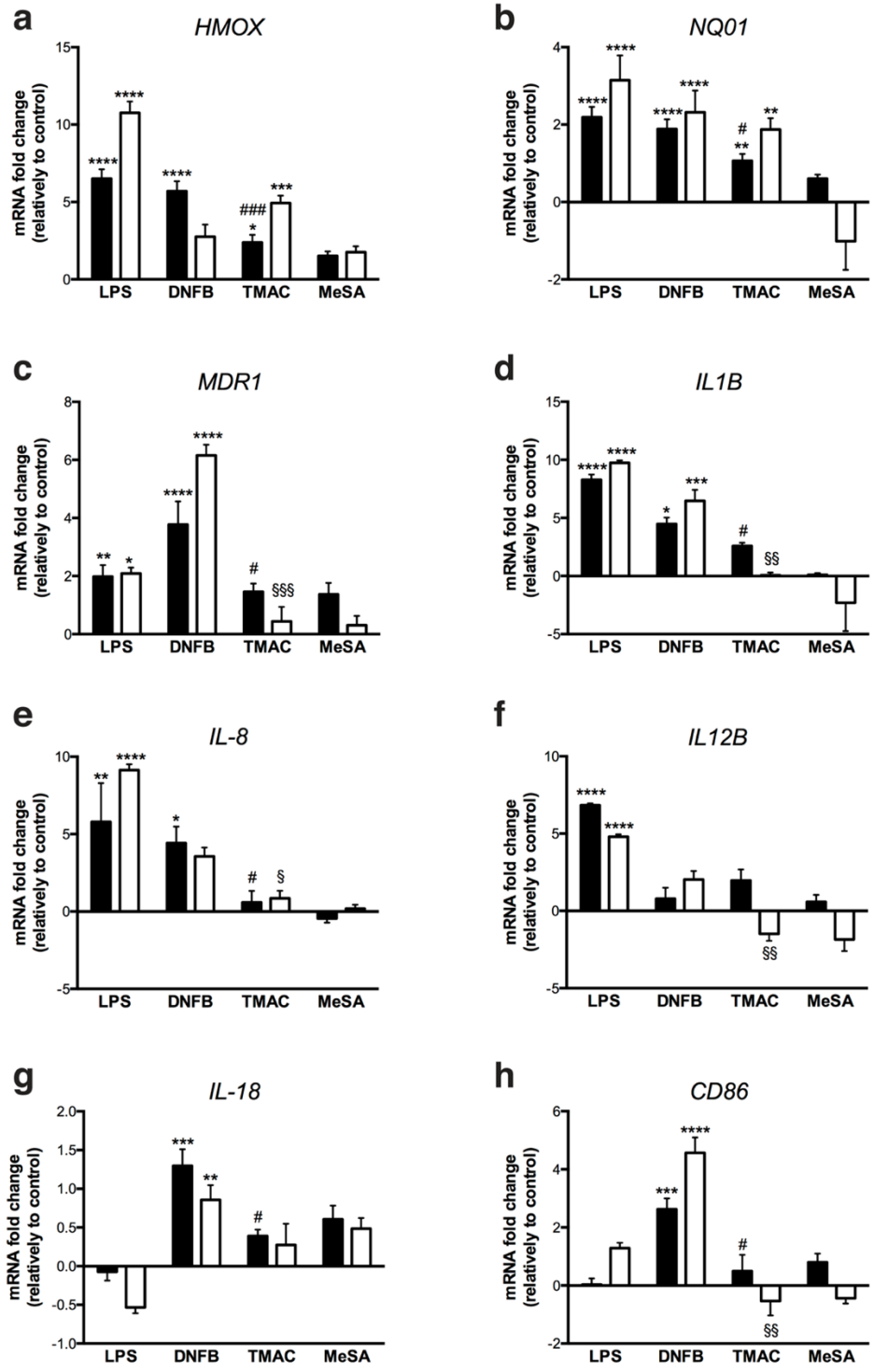


Figure 2.6 Chemical-induced gene transcription. THP-1 cells were exposed to chemicals for 6 h (black bars) and 24 h (white bars) and mRNA levels of *HMOX1* (a), *NQO1* (b), *MDR1* (c), *IL1B* (d), *IL8* (e), *IL12B* (f) *IL18* (g) and *CD86*

(h) were determined by qPCR. Data are represented as log2 of fold transcription levels normalized to control cells (log2(1)=0) of the respective timeline studied. Data depicted in the graphs correspond to the means ± SEM of at least three independent experiments. Statistical analysis: one-way ANOVA with Dunnett's multiple comparison **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 compared to untreated cells; *t*-test (DNFB 6 h vs TMAC 6 h), #p < 0.05, ###p < 0.001, (DNFB 24 h vs TMAC 24 h), §p < 0.05, §§p < 0.01, §§§p < 0.001.

The effect of chemicals on the gene transcription was evaluated by quantitative real-time PCR (qPCR) at 6 and 24 h post-treatment. We observed that the transcription of ARE-dependent genes was markedly induced by the contact allergen DNFB early after exposure, while TMAC elicited a delayed response (Fig. 2.6a and b). These results are in accordance with the observed rapid induction of oxidative stress by DNFB while TMAC seems to activate stress related toxicity pathways with a later profile. Previous studies demonstrated that skin DCs and T cells express MDR1, which has been described as being required for efficient DC maturation and T cell migration (Randolph *et al*., 1998; Pendse *et al*., 2006). Indeed, DNFB significantly increased MDR1 gene transcription compared to untreated cells and cells treated with TMAC at both time points tested (Fig. 2.6c). Concerning the transcription of cytokines, none of the chemicals tested significantly interfere with *IL12B* mRNA levels (Fig. 2.6f). On the other hand, there was an increase in *IL1B* and *IL8* gene transcription in the presence of DNFB (Fig. 2.6d and e), which is consistent with the literature since *IL8* expression is regulated by the transcription factor Nrf2 (Zhang *et al*., 2005). Among the compounds tested only DNFB increased the transcription of *CD86* ($p < 0.001$ and $p < 0.0001$ for 6 h and 24 h respectively). According to our results, none of the genes studied were significantly modulated by the irritant MeSA at both time points tested.

2.4 DISCUSSION

ROS production and protein oxidation are early danger signals occurring in the sensitization phase of chemical-induced allergy (Mizuashi *et al*., 2005; Mokra *et al*., 2012). Indeed, several studies using monocyte-derived dendritic cells and other DC-cell models have shown that exposure to skin sensitizers rapidly induces oxidative stress (Matos *et al*., 2005; Mizuashi *et al*., 2005; Esser *et al*., 2012) and that this event is important for DCs activation and maturation. Although the recognized role, the nature, origin and kinetics of ROS induced by chemical sensitizers remain elusive. To address this question, we analyzed in THP-1 cells the mechanisms of redox imbalance elicited by DNFB and TMAC, two

golden standards of skin and chemical respiratory allergy, respectively. We found that both sensitizers increased ROS production, although with distinct origins and timings. This results in a different extent at which intracellular signaling pathways are activated and, if the results in THP-1 cells are confirmed in normal DCs, may be in part responsible for the distinct T-cell polarizing abilities attributed to DCs in skin or respiratory chemical allergies.

Under homeostatic conditions, cellular redox status is maintained by a dynamic equilibrium of processes that produce and eliminate ROS. Indeed, there are several known sources of cellular ROS, including NADPH oxidase, the mitochondrial respiratory cycle and xanthine oxidase, which generates superoxide through the oxidation of hypoxanthine to xanthine. To manage these deleterious oxidative molecules cells are equipped with a variety of antioxidants that can be enzymatic, such as SODs, catalase and glutathione peroxidase, and non-enzymatic, such as GSH (Birben *et al*., 2012). We found that both the skin sensitizer DNFB and the respiratory sensitizer TMAC induce oxidative stress, though with temporal and intensity differences. DNFB, highly electrophilic and therefore reactive with thiol groups, rapidly reacts with GSH, inactivating it and subsequently leading to an increase in cytoplasmatic ROS. In contrast, for TMAC our data indicates that GSH depletion at later time points may be in part a consequence of the later increase in mitochondrial ROS and increased xanthine oxidase activity. Accordingly, Silva and colleagues showed that another respiratory sensitizer, hexamethylene diisocyanate, which also predominantly reacts with amine groups, increased mitochondrial ROS accumulation, which was relevant for further inducing the expression of cytoprotective genes and DC maturation markers (Silva *et al*., 2014). Besides TMAC, the irritant MeSA also significantly increased xanthine oxidase activity, a result that is corroborated by the decreased hypoxanthine levels observed for the two compounds.

Regarding the modulation of cellular antioxidant defenses, none of the chemicals tested affected SOD1 or SOD2 activity. Although, in LPS-treated cells, we observed a decrease in SOD 1 activity and an increase in SOD 2 activity. Interestingly, several authors have shown that LPS potently increases the activity and the mRNA levels of MnSOD (SOD 2) but did not change or decreases those of Cu/ZnSOD (SOD 1). Studies by Frank and colleagues in rat renal mesangial cells and whole kidney homogenates from LPS-treated rats showed that induction of SOD 1 was clearly dependent on nitric oxide, as none of the many growth factors and inflammatory cytokines tested were able to induce SOD 1.

By contrast, SOD 2 expression was clearly induced by LPS, TNF-α, and IL-1β in mesangial cells *in vitro* (Frank *et al*., 1999). Also, several studies show that in contrast to mouse macrophages, human monocytes stimulated with cytokines or LPS fail to release NO (Zembala *et al*., 1994; Arias *et al*., 1997).These evidences could account for the decrease SOD 1 activity and increased SOD 2 activity observed in cells treated with LPS.

We then proceeded to evaluate the contribution of the major cellular non-enzymatic soluble antioxidant GSH. GSH, present in millimolar concentrations in virtually all cells, donates electrons to H_2O_2 reducing it to H_2O and O_2 while being oxidized to GSSG. It is widely believed that strong contact sensitizers covalently bind to thiol or amino protein groups, with several studies reporting the maturation of DCs by 2,4-dinitrochlorobenzene (DNCB), a structural analogue of DNFB, as a consequence of glutathione depletion (Becker *et al*., 2003; Bruchhausen *et al*., 2003; Mizuashi *et al*., 2005). Recently, several works reported that DNCB rapidly and extensively reacts with GSH, cysteine and SH-containing peptides (Gerberick *et al*., 2007; Pickard *et al*., 2007; Megherbi *et al*., 2009; Jacquoilleot *et al*., 2015). In accordance to this, our results show that DNFB rapidly depletes GSH, which is coincident with the observed increased oxidative status 1 hour after cells treatment. Given that major cellular sources of ROS are not affected by the chemical, we hypothesize that the observed oxidative stress is an event resulting from the direct haptenation of GSH leading to its incapacity to neutralize constitutive ROS production. Supporting our hypothesis, DNCB was shown to cause a decrease of almost 45% of intracellular GSH just 15 min after exposure of human peripheral blood mononuclear cells (Pickard *et al*., 2007). Authors suggested that DNCB primarily depletes intracellular GSH, being the reminiscent chemical free to haptenate cellular proteins. In the case of TMAC we found that it causes a decrease of GSH levels only 6 hours after cell exposure. Studies addressing TMAC reactivity are contradictory, with some works reporting reactivity towards lysine residues and GSH (Gerberick *et al*., 2007; Lalko *et al*., 2012) and others reporting a preferential reactivity with cysteine peptides (Jaworska *et al*., 2011). Our results indicate that delayed GSH depletion caused by TMAC may be the consequence of GSH consumption in the detoxification of H_2O_2 produced from SOD activity due to high levels of $O_2^{\cdot-}$. Therefore, chemicals that preferentially react with thiol groups, as DNFB, will rapidly induce redox imbalance in consequence of direct GSH depletion, while chemicals that react more extensively with primary amines, such as TMAC, will cause a more delayed oxidative stress.

We then proceeded to evaluate the activation of MAPKs, intracellular signaling pathways known to be involved in sensitizers induced DC maturation (Arrighi *et al*., 2001; Boislève *et al*., 2005; Nakahara *et al*., 2006). Only sensitizers and LPS were able to modulate THP-1 MAPK signaling, whereas the non-sensitizer MeSA had no significant effect, as previous described in the literature (Trompezinski *et al*., 2008). ERK activation was not induced by the chemicals tested, while SAPK/JNK and p38 MAPKs were strongly modulated by the sensitizers and LPS, with an increased activation at 1 h post-treatment that fall over time but remained above basal values at 6 h. Accordingly, several studies reported the selective activation of p38 MAPK by contact sensitizers (DNFB, NiSO4) and not by irritants. The authors demonstrated that activation of p38 MAPK is involved in DNFB-induced DC up-regulation of CD86, IL-1ß and IL-8 (Arrighi *et al*., 2001; Brand *et al*., 2002; Nukada *et al*., 2008; Mitjans *et al*., 2010). Regarding the effects of chemicals on JNK pathway, we observed a selective and marked increase in phospho-JNK levels following exposure to sensitizers, even more robustly than the activation observed for p38 MAPK. These results are in line with previous studies reporting a sustained phosphorylation of p38 MAPK and JNK following the treatment of the mouse fetal skin-derived dendritic cell line FSDC with the sensitizers DNFB, oxazolone, 1,4-phenylenediamine and $NiSO_4$ but not with irritants sodium dodecyl sulfate (SDS) and benzalkonium chloride (BC) (Neves *et al*., 2013). Interestingly, a similar activation pattern was also observed in murine and human skin explants as well as in reconstituted skin models EST-100 and AST-200 (Koeper *et al*., 2007). Although the role of JNK in the immunobiology of DC remains less studied than that of p38 MAPK, several authors pointed out that its activation is implicated in the expression of CD83, CD86 and CCR7 (Boislève *et al*., 2005).

Curiously, although TMAC and DNFB elicit different kinetics of ROS production, they share similar activation profiles for p38 and JNK MAPKs. This indicates that rather than ROS themselves, direct interaction of chemical sensitizers with cellular proteins would evoke the intracellular signaling events involved in DC maturation. To clarify this, we evaluated if pre-incubation of sensitizers with cysteine or lysine would hamper their capacity to activate THP-1 intracellular signaling events. Indeed, when sensitizers were pre-incubated with cysteine or lysine, we did not observe GSH depletion, ROS production or MAPKs activation. Accordingly, several studies emphasize the relationship between sensitizers reactivity with specific amino acid residues from critical proteins and the

modulation of signaling pathways involved in DC maturation. Bruchhausen et. al, demonstrated that the thiol antioxidant N-acetyl-cysteine (NAC) revokes trinitrochlorobenzene-induced tyrosine phosphorylation and p38 MAPK activation in human moDCs by preventing the binding of the sensitizer to proteins (Bruchhausen *et al*., 2003). These results, together with the inability of radical scavengers to prevent tyrosine phosphorylation, led them to hypothesize that ROS may not be essential for DCs activation by sensitizers (Bruchhausen *et al*., 2003). Reinforcing this hypothesis, our group recently identified, though a proteomics–based approach, several intracellular proteins that are directly targeted by FITC, a sensitizer that preferentially reacts towards primary amines. Among these proteins, we found that FITC directly haptenizes mixed-lineage protein kinase kinase kinase in THP-1 cells, directly modulating the activation state of p38 and JNK pathways (Guedes *et al*., 2017).

Finally, we analyzed the effects of chemicals on the transcription of several genes related to cytoprotection, DC maturation and T-cell polarizing capacities. We found that both allergens induce the transcription of *HMOX* and *NQO1* detoxifying genes but with different kinetics. DNFB caused an early and marked transcription while TMAC a delayed one. These genes are under regulation of the Nrf2-Keap1-ARE pathway, a signaling cascade that functions primarily as a sensor for electrophilic stress and that has been explored for the identification of cysteine-reactive skin sensitizers *in vitro* (Natsch *et al*., 2008; Natsch, 2009; Emter *et al*., 2010). Briefly, in a steady state, Keap1 which contains highly reactive Cys residues, targets Nrf2 for Cul3-mediated ubiquitinylation and proteolytic degradation in the proteasome. Covalent modification of the reactive Cys residues of Keap1 leads to its dissociation from the transcriptional regulator Nrf2, which then accumulates in the nucleus and activates genes having an ARE domain in their promoter sequence (Wakabayashi *et al*., 2004; Dinkova-Kostova *et al*., 2005). Besides electrophilic stress, oxidative stress was also shown to activate Nrf2-Keap1-ARE pathway (Kobayashi *et al*., 2006). This may explain the different activation kinetics observed for DNFB and TMAC. While early induction by DNFB results from its strong and direct reactivity toward the Cys residues on Keap1, later induction by TMAC is probably caused by cellular oxidative stress.

Regarding the transcription of pro-inflammatory cytokines, we observed that *IL1B*, *IL8* and *IL18* are rapidly and robustly induced by the skin sensitizer DNFB and this effect is maintained over time. In turn, the respiratory sensitizer TMAC caused a modest

induction in *IL1B* at 6 h, which is decreased to basal levels after 24 h. These cytokines, namely IL-12 and IL-18, play an important role in the DC-induced polarization of T-cells into Th1 type subset (Tominaga *et al.*, 2000; Nakanishi *et al.*, 2001). In fact, IL-18 was shown to play an important role in allergic contact sensitization, favoring a Th1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF-α, IL-8 and IFN-γ (Cumberbatch *et al.*, 2001). Moreover, contact sensitizers, including pro-haptens, but not irritants or respiratory sensitizers, were shown to induce *IL18* expression in the human keratinocyte cell line NCTC2455 (Corsini *et al.*, 2009). Major differences were also found in the transcription of the co-stimulatory molecule CD86 and MDR1, a membrane transporter with important roles in DC maturation and migration. Therefore, we may hypothesize that skin sensitizers such as DNFB (preferentially thiol-reactive) evoke a sustained transcription of pro-inflammatory cytokines and co-stimulatory molecules in DCs promoting a Th1 polarization, while modest and transitory transcription caused by respiratory sensitizers such as TMAC (preferentially amine-reactive) lead to Th2 responses.

2.5 CONCLUSIONS

Overall, the present study brought new insights about the origin, nature, kinetics and role of redox imbalance triggered by respiratory and skin sensitizers in the human monocytic cell line THP-1.

According to our data, DNFB, a preferentially thiol-reactive skin sensitizer, induces an early depletion of GSH with a concomitant increase in general ROS levels, while TMAC, a preferentially amine-reactive respiratory sensitizer, induces a delayed GSH depletion in consequence of increased mitochondrial ROS production. Our results indicate that the preferential reactivity of sensitizers over thiol or primary amine groups determines the quickness and extent at which danger signals are generated, conditioning the transcription kinetics of genes such as *HMOX*, *IL1B, IL8*, *IL18* and *CD86* (Fig. 2.7). Ultimately, these events may account for the distinct DC phenotypes and T-cell polarizing profiles triggered by skin and respiratory sensitizers.

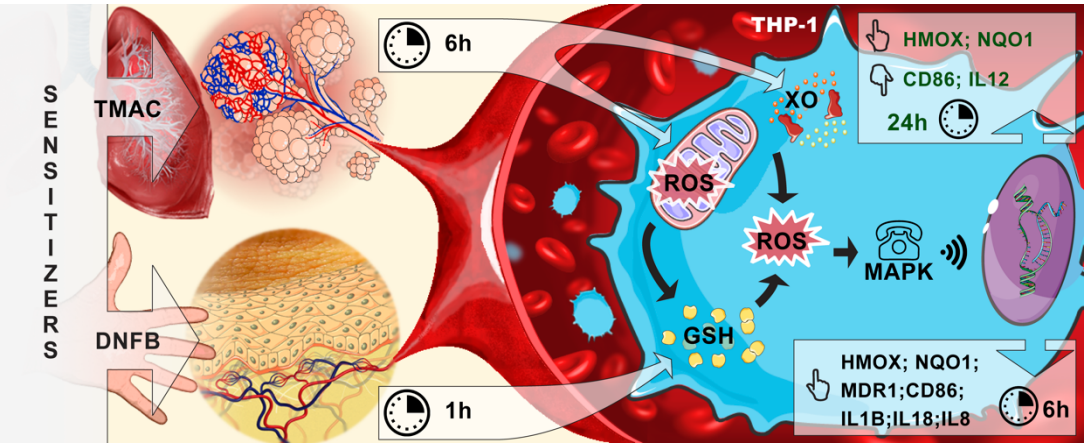


Figure 2.7. Origin, nature, kinetics and role of redox imbalance triggered by respiratory and skin sensitizers in the human monocytic cell line THP-1. Our data shows that the thiol-reactive sensitizer DNFB directly reacts with cytoplasmic glutathione (GSH) causing its rapid and marked depletion which results in a general increase in reactive oxygen species (ROS) accumulation. In turn, TMAC, which preferentially reacts with amine groups, induces a delayed GSH depletion as a consequence of increased mitochondrial ROS production. These divergences in ROS production seem to be correlated with the different extension of intracellular signaling pathways activation observed and, by consequence, with distinct transcription kinetics of genes such as HMOX, IL8, IL1B and CD86.

ACKNOWLEDGMENTS:

This work was financed by The Johns Hopkins Center for Alternatives to Animal Testing, project n°: 2014-07, by the European Regional Development Fund (ERDF), through the Centro 2020 Regional Operational Programme: project CENTRO-01-0145-FEDER-000012-HealthyAging2020, the COMPETE 2020 - Operational Programme for Competitiveness and Internationalisation, and the Portuguese national funds via FCT – Fundação para a Ciência e a Tecnologia, I.P.: project POCI-01-0145-FEDER-007440. Isabel Ferreira and João D. Martins were funded through FCT PhD fellowships refs: SFRH/BD/110717/2015 and SFRH/BD/73065/2010, respectively.

SUPPLEMENTARY DATA

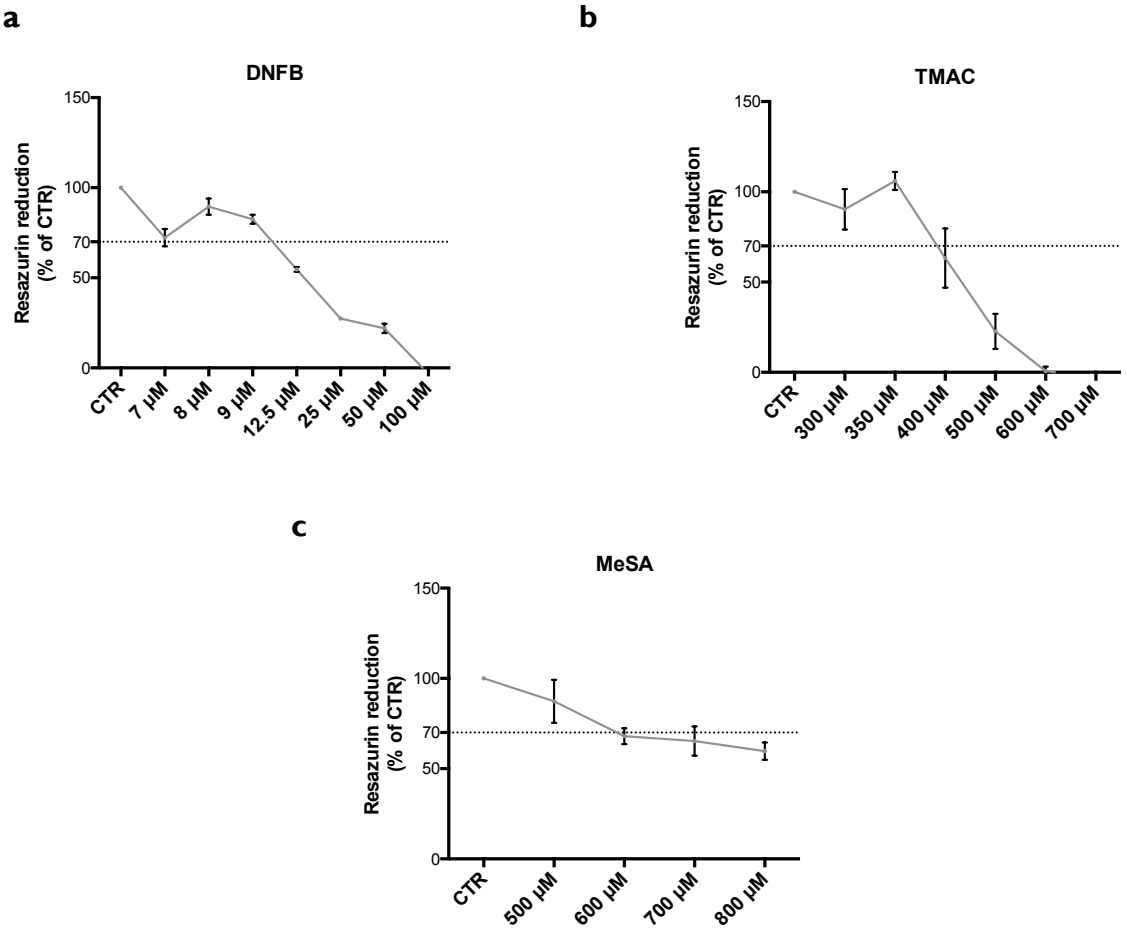


Figure S2.1 Cell viability of THP-1 cells upon treatment with several chemicals. Human DC-like THP-1 cells were exposed to DNFB (a), TMAC (b) and MeSA (c) for 24 h and cell viability addressed by quantification of resazurin reduction. Data correspond to the means ± SEM of at least three independent experiments and are represented as % of control cells (CTR)

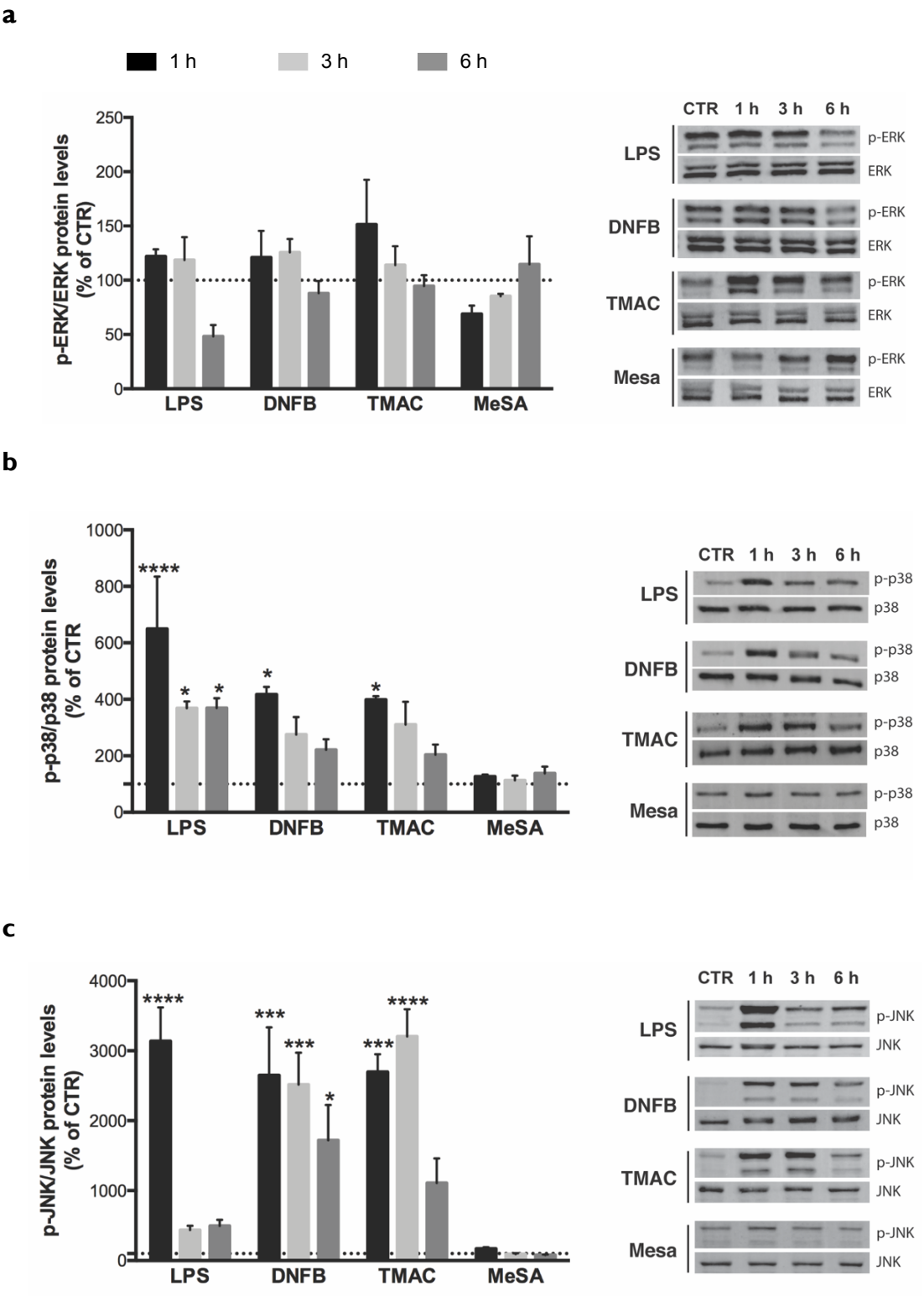


Figure S2.2 Chemicals-induced MAPKs activation. THP-1 cells were exposed to LPS, DNFB, TMAC and MeSA for 1, 3, and 6 h. ERK (a), p38 (b) and JNK (c) signaling pathway activation was further determined by Western blotting of total cell extracts. Data correspond to the means ± SEM of three independent experiments and are represented as % of control cells (CTR). Statistical analysis: One-way ANOVA followed by Dunnett's multiple comparison test. *p < 0.05, ***p < 0.001, ****p < 0.0001, compared to CTR.

Table S2.1. Primer sequences used to amplify target cDNAs

Gene	Primer Forward	Primer reverse
HMOX	CCT GAG TTT CAA GTA TCC	AAC AAC AGA ACA CAA CAA
NQO1	GAG TCT GTT CTG GCT TAT	AAC TGG AAT ATC ACA AGG T
MDR1	AGA GAC ATC ATC AAG TGG AGA G	AGC AAG GCA GTC AGT TAC A
IL1B	GCT TGG TGA TGT CTG GTC	GCT GTA GAG TGG GCT TAT C
IL8	CTT TCA GAG ACA GCA GAG	CTA AGT TCT TTA GCA CTC C
IL12B	TGT CGT AGA ATT GGA TTG GTA TC	AAC CTC GCC TCC TTT GTG
IL18	AGT CAG CAA GGA ATT GTC TC	AGG AAG CGA TCT GGA AGG
CD86	GAA CCT AAG AAG ATG AGT	TCC AGA ATA CAG AAG ATG

CHAPTER 3

Thiol reactive allergens activate inflammasome through lysosomal membrane destabilization and cathepsin leakage: a step forward the comprehension of sterile inflammation

---

Isabel Ferreira, Maria Teresa Cruz, Bruno Miguel Neves

Manuscript in preparation

ABSTRACT

On daily basis, we are exposed to several synthetic and natural products foreign to the organism (xenobiotics). Many of these chemicals induce inflammation as a result of chemically induced injury, typically in the absence of any microorganisms – the so-called "sterile inflammation". Several studies show that these chemicals mimic infection by triggering innate immune responses via pattern recognition receptors (PRRs) and endogenous danger signals. Indeed, inflammasome activation has been recognized as critical for successful chemicals sensitization and for the elicitation of the T cell response. Inflammasomes are cytoplasmic caspase-1-activating protein complexes that promote maturation and secretion of the proinflammatory cytokines interleukin (IL)-1ß and IL-18. The most intensively studied inflammasome, NLRP3 inflammasome, can be activated by many molecular triggers such as potassium efflux, lysosomal rupture, reactive oxygen species (ROS) production, and mitochondrial disruption. However, the molecular events activated by chemicals sensitizers that trigger NLRP3 inflammasome activation remain to be elucidated, constituting the main goal of this work. Although lysosomal rupture is often associated with crystalline and particulate materials, here we demonstrate that the thiol-reactive skin sensitizer 2,4-dinitroflurobenzene (DNFB) activates NLRP3 inflammasome through transient lysosomal destabilization and subsequent cathepsin leakage. Inhibition of cathepsin activity in dendritic-like cells impaired NLRP3 activation and the DNFB-induced expression of the maturation marker CD86, thus disclosing an innate immune mechanism crucial for the development of allergic contact sensitization to LMW chemicals. Furthermore, this new mechanism of inflammasome is observed with other thiol-reactive skin sensitizers, suggesting that this mechanism is shared by sensitizers with high thiol reactivity.

Keywords: thiol-reactive sensitizers, NLRP3 inflammasome, allergic contact dermatitis, respiratory sensitization, DNFB, lysosomal destabilization, cathepsin, dendritic cells

3.1 INTRODUCTION

On daily basis, we are exposed to several synthetic and natural products foreign to the organism (xenobiotics). The interaction with such xenobiotics often occurs through inhalation, ingestion or skin exposure, but without an obvious response from the organism. Even though, some low molecular weight chemicals (LMW, <1000 Dalton) interact with host cells, influencing and evoking pathophysiologic processes. Many of these chemicals induce inflammation as a result of chemically induced injury, typically in the absence of any microorganisms – the so-called "sterile inflammation". The most common are metal ions such as nickel, cobalt and chromate, fragrances, dyes and preservatives (Martin *et al.*, 2011) and have been reported to be involved in the development of occupational diseases, such as allergic contact dermatitis (ACD) and occupational rhinitis and asthma. ACD is a Type IV [delayed-type] hypersensitivity response that is characterized by excessive inflammation and manifests as local skin rash, itchiness, redness, swelling, and lesion. It affects about 20% of the European population (Diepgen *et al.*, 2016) while asthma is one of the most prevalent occupational lung disease, affecting more than 339 million people worldwide (Vos *et al.*, 2017), of which 10-25% suffer from occupational asthma (Cartier *et al.*, 2019).

Understanding how sensitizers promote ACD and sensitization of the respiratory tract is imperative, as well as the precise roles of each cell type involved (i.e., keratinocytes, epithelial cells, dendritic cells (DCs) and T cells). These pathologies share some similarities, although, they have mechanistic differences which, under normal circumstances, will culminate in a preferential elicitation of a T helper type 1 (Th1) response for contact allergens, while respiratory allergens favor the development of Th2 responses. Briefly, sensitizers are too small to be recognized by the immune system and therefore must covalently bind to carrier proteins converting them into immunogenic hapten-protein complexes – haptenation (Chipinda *et al.*, 2011; Lalko *et al.*, 2011). Chemical modification of proteins by sensitizers usually occurs at nucleophilic sites, preferably at cysteine residues (thiol groups) for contact sensitizers and lysine residues (amine groups) for respiratory sensitizers. Although, it is important to bear in mind that this amino acid selectivity may not be applicable to all the classes of skin and respiratory sensitizers (Kimber *et al.*, 2018). The covalent modification of cellular proteins is associated with a certain level of cytotoxicity resulting in the activation of innate immunity and the production of cytokines, chemokines, reactive oxygen species (ROS), and the release of

danger signals and damage-associated molecular patterns (DAMPs), such as hyaluronic acid (HA) fragments and adenosine triphosphate (ATP) (Vocanson *et al*., 2009; Esser *et al*., 2012; Martin, 2012). These signals are then recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) on skin dendritic cells (DCs) such as Langerhans cells (LCs) and dermal DCs. The activation of these innate immune receptors is essential for DC maturation and migration to the draining lymph nodes where they prime naïve T cells. T-cells become activated and expand into allergen-specific effector T-cells that disseminate systemically and elicit a strong inflammatory reaction upon later contact with the same chemical. Indeed, DCs represent the most important initiators and regulators of immune responses, representing a bridge between the immune and adaptive immune system. In ACD, activation of DCs has been shown to rely on several innate pro-inflammatory effector molecules, namely IL-1β, IL-18 and TNF-α. (Martin *et al*., 2008 b; Willart *et al*., 2009; Ainscough *et al*., 2013). Upon contact with skin sensitizers, keratinocytes secrete IL-1β and IL-18 that, in combination with other cytokines, are responsible for inducing vasodilatation, cellular recruitment and infiltration, and DC maturation. (Galbiati *et al*., 2014). In the skin, IL-1β and IL-18, are constitutively expressed by keratinocytes as pre-forms, requiring proteolytic maturation by the cysteine protease caspase-1, which must first be activated by the inflammasome (Watanabe *et al*., 2007, 2008; Li *et al*., 2014; Galbiati *et al*., 2019). Inflammasomes, which are multiprotein intracellular complexes whose assembly is triggered by chemically unrelated pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), are responsible for the processing and activation of the inflammatory cytokines IL-1β and IL-18. They are composed of an upstream sensor protein of the NLR family, an adaptor protein ASC, and the downstream effector caspase-1. The current model of Inflammasome activation postulates that it requires two signals (transcriptional and posttranslational). The first priming signal is provided by the TLR signaling pathway activation and results in the activation of the transcription factor Nuclear factor kappa B (NF-κB), which is crucial for upregulating the transcription of pro-IL-1β, pro-IL-18 as well as the components of the inflammasome itself. The second signal triggers the assembly of inflammasome complex and the release of mature cytokines. NLRP3 inflammasome, the most intensively studied inflammasome, can be activated by both exogenous (tissue damage, infection and metabolic dysregulation) and endogenous molecules such as reactive oxygen species

(ROS), extracellular adenosine triphosphate (ATP; through its cell surface receptor P2X7R), amyloid-β, hyaluronan, and cholesterol crystals (Guo *et al*., 2015). To sum up, so far, three primarily mechanisms concerning the activation of the NLRP3 inflammasome, have been described: potassium efflux, ROS production and lysosomal rupture (Liston & Masters, 2017). Lysosomal damage may occur through internalization of particulate matter (e.g. monosodium urate, silica, asbestos, amyloid deposits, and aluminum salts) by phago-lysosomes leading to cathepsin B leakage, which activates NLRP3 inflammasome (Hornung *et al*., 2008; Willingham *et al*., 2008). Although many different substances have been shown to activate NLRP3, a direct ligand for this inflammasome has yet to be identified. Interestingly, evidence suggests that NLRP3 activation is critical for the development of sensitization. Indeed, Watanabe *et al*., showed that chemical haptens such as trinitrochlorobenzene (TNCB), 2,4-dinitroflurobenzene (DNFB) and dinitro-1-chlorobenzene (DNCB) induce the activation of the NLRP3 inflammasome in primary human keratinocytes and murine models (Watanabe *et al*., 2007) by an indirect pathway involving ROS and ATP. Also, one previous study from our group showed that DNFB induced lysosomal destabilization (Luís *et al*., 2014). Herein, we investigated the molecular mechanisms behind inflammasome activation evoked by molecules without a crystalline/particulate structure in DC surrogate cell line. Our studies were conducted on the THP-1 cell line which has been shown to respond to sensitizers and non-sensitizers in a similar manner to primary DCs (Tietze *et al*., 2008) and is also used in one of the approved OECD *in vitro* tests for skin sensitization (OECD, 2018 a). We demonstrated, for the first time, that NLRP3 inflammasome activation by DNFB was dependent on transient lysosomal membrane destabilization and subsequent leakage of cysteine protease cathepsin B. Our results also showed that activation of NLRP3 inflammasome triggered by lysosomal destabilization was a common pathway shared by low molecular weight chemicals with high reactivity towards cysteines compared to non-sensitizers or chemicals reacting mainly with lysine residues. These findings pointed out the leakage of lysosomal cathepsin B and NLRP3 inflammasome activation as a link between cysteine reactive chemicals and DC maturation, a key event for the development of skin sensitization, thus opening new avenues for the management of ACD.

3.2 MATERIALS AND METHODS

3.2.1 Materials

The chemicals DNFB (D1529), TMAC (T68020), Methylisothiazolinone (73569), Lactic Acid (69785), 4-NB (N13054), Oxazolone (OXA) (E0753), Hexamethylene diisocyanate (HDI) (52649), LPS from *Escherichia coli* (serotype 026:B6) and RPMI-1640 (R6504) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). DMEM (31800-063) was purchased from Gibco™ Invitrogen Corporation (Carlsbad, CA, USA) Cathepsin B (CA-074 methyl ester, 23249) and Cathepsin L (23249) inhibitors were purchased from Cayman Chemical (Ann Harbor, MI, USA). The human IL-1 β ELISA Kit (437007) was obtained from Biolegend (San Diego, CA, USA), CV- Cathepsin B detection Kit (BML-AK125) was obtained from Enzo Life Sciences (Farmingdale, NY, USA), Lysosome Enrichment Kit for Tissue and Cultured Cells was obtained from Thermo Scientific (Pierce Biotechnology, Rockford, IL, USA) and Protein G Sepharose® 4 Fast Flow was obtained from Sigma (St. Louis, MO, USA). Acridine Orange (A6014) and Hoechst 3342 (H3570), were obtained from Sigma (St. Louis, MO, USA) and Molecular Probes (Eugene, OR, USA) respectively. The polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corp (Bedford, MA, USA). Alkaline phosphatase-conjugated secondary antibodies were purchased from GE Healthcare (Chalfont St. Giles, UK). Protease and phosphatase inhibitor cocktails were from Roche (Mannheim, Germany). RNA Storage Solution was from Ambion (Foster City, CA, USA). The NZYol and NZY First-Strand cDNA Synthesis Kit were obtained from NZYTech (Lisbon, Portugal) and custom oligonucleotide primers were from Eurofins MWG Operon (Ebersberg, Germany) and NZYTech (Lisbon, Portugal).

3.2.2 Chemical exposure

Since a certain level of cytotoxicity is essential for effective DCs maturation (Hulette *et al*., 2005), the concentrations of chemicals inducing up to 30% decrease in cell viability (EC30 value) were determined through the resazurin assay (Supplementary data, Fig. S1). In all subsequent experiments, cells were exposed for the indicated times to the EC30 concentration of each chemical, corresponding to 8 μM for DNFB, 17 μM for 4-NB,

130 μM for OXA, 97 μM for MI, 11.5 μM for HDI and 7.5 mM for LA. To evaluate if DNFB activates inflammasome through physical lysosomal membrane destabilization following frustrated phagocytosis of particulate hapten-protein complexes we did a pre-incubation *in chemico* of BSA (2.3 g/L) with DNFB (16 μM) for 1 h at 37 °C. We then concentrated the solution with Vivaspin 6 centrifugal concentrator MWCO 5 kDa (Vivascience AG, Hannover, Germany), according to manufacturer instructions, in order to remove the unbound DNFB. Cells were then treated with the DNFB-protein complexes.

3.2.3 Cell Culture

NLRP3-deficient (THP-1-defNLRP3), ASC-deficient (THP-1-defASC) and control THP-1 (THP-1-Null) cells were purchased from InvivoGen (InvivoGen, Toulouse, France). The cells were cultured and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (30 min at 56 °C), 3.7 g of sodium bicarbonate, 25 mM glucose, 10 mM Hepes, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were sub-cultured every 3 or 4 days and kept in culture for a maximum of 2 months. THP-1 cells were seeded at a density of $0.8x10^6$ cells/mL. When experiments were conducted in the control and knockout cell lines, growth medium was supplemented with the selective antibiotic Hygromycin B Gold (200 μg/mL) following evert other passage. When experiments were conducted in macrophages, THP-1null cells were seeded at a density of $0.75x10^5$ cells/per slide on μ-slides 8-well ibidiTreat (ibidi GmbH, München, Germany) and induced to differentiate with 50 ng/mL 4α-Phorbol 12-myristate 13-acetate (PMA) for 48 h followed by an additional 48 h in fresh medium prior to treatment with chemicals.

The human keratinocyte cell line HaCaT was acquired from CLS (CLS, Cell Lines Service, Eppelheim, Germany). HaCaT cells are spontaneously transformed immortalized human epithelial cells, obtained from adult skin. This cell line maintains full epidermal differentiation capacity (Boukamp *et al.*, 1988). The cells were used after reaching 70–80% confluence, which occurs approximately every 3 days. The cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum (30 min at 56 °C), 3.7 g of sodium bicarbonate, 25 mM glucose, 100 U/mL penicillin and 100 μg/mL streptomycin. To perform the experiments, cells were seeded at a density of $0.80x10^5$ cells/slide on μ-slides 8-well ibidiTreat and allowed to stabilize overnight.

3.2.4 Human peripheral blood mononuclear cells isolation and differentiation into dendritic cells

To obtain primary cultures of human monocytes, peripheral blood mononuclear cells (PBMCs) were firstly isolated by Ficoll-Paque gradient centrifugation from buffy coats of healthy volunteers provided by the Portuguese Blood Institute (IPS). Then, monocytes were isolated by positive selection using CD14 antibody coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), as described by the manufacturer. Monocytes were cultured at a concentration of $1x10^6$ cells/mL in RPMI medium supplemented with 250 U/ml of IL-4 and 400 U/ml GM-CSF to differentiate them into immature moDCs. Half the medium was refreshed every two days and moDCs maturation was induced at day 6, by the addition of several concentrations of the skin sensitizer DNFB.

3.2.5 Western Blot and ELISA

THP-1 cells (silenced and/or wild-type control) were plated at a density of 0.8 x 10^6 cell/mL, in a twelve-well plate with a final volume of 1.5 mL, pre-treated with LPS (1 μg/mL) for 24 h and then treated with various stimuli for 6 h. After incubation with chemicals, the supernatants were collected for ELISA analysis. Cells were washed with ice cold PBS and lysed with RIPA lysis buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA). Up to 25 μg of total protein were subjected to SDS-PAGE (10%) and blotted onto a PVDF membrane. The membrane was blocked with 5% non-fat milk for 1 h at room temperature. The blots were then incubated with primary antibodies: IL-1ß (1:500, sc-7884, (Santa Cruz, Dallas, TX, USA), LAMP-2 (1:1000, sc-18822, Santa Cruz, Dallas, TX, USA), NLRP3 (1:1000, 13158, Cell Signaling, Danvers, MA, USA), ASC (1:1000, 13833, Cell Signaling, Danvers, MA, USA), β-Tubulin I (1:20000, T8716, Sigma, St. Louis, MO, USA), Actin (1:5000, MAB1501, Sigma, St. Louis, MO, USA), GRP78 (1:1000, BD Biosciences, San Jose, CA, USA), Dinitrophenyl-KLH (1:1000, Invitrogen, Darmstadt, Germany) and DNP (1:1000, Bethyl Laboratories, Betyl Laboratories, Montgomery, TX, USA) at 4°C overnight (Supplementary data, Table S1), followed by incubation with alkaline phosphatase conjugated secondary antibodies for 1 h at room temperature and the signal detected by enhanced chemifluorescence with ECF (RPN5785, GE Healthcare). IL-1ß concentration in the supernatants was analyzed

with Legend Max™ ELISA kit (#437007, BioLegend) according to the manufacturer's instructions.

3.2.6 Lysosomal membrane destabilization

For evaluation of lysosomal membrane destabilization, cells were stimulated with various concentrations of chemicals (EC_{30} and twice the EC_{30}) for 3 h in serum free-medium. Cells were subsequently washed, incubated for 20 min with acridine orange (5 µg/mL in culture medium) and washed again. Lysosomal destabilization was assessed by flow cytometry as a ratio between loss of emission at 675/25 nm and gain of emission at 585/40 nm (FL4 and FL2 respectively). Flow cytometry analysis was performed using a BD Accuri TM C6 flow cytometer equipped with BD Accuri C6. For the purpose of image acquisition, half of the cells were transferred to µ-slides 8-well ibidiTreat (ibidi GmbH, München, Germany) and images collected using the confocal microscope Zeiss LSM 710.

3.2.7 Cathepsin B detection

Cathepsin B activity was detected using a CV-Cathepsin B detection kit (BML-AK125, Enzo Life Sciences, Farmingdale, USA) according to the manufacturer's instructions. Briefly, cells were stimulated with various concentrations of chemicals (EC_{30} and twice the EC_{30}) for 3 h in serum free-medium. Cells were subsequently incubated with CV-$(RR)_2$ for 1 h at 37°C protected from light and gently resuspended every 20 min. Images were collected using the confocal microscope Zeiss LSM 710.

3.2.8 Analysis of gene transcription by quantitative real-time PCR

Total RNA was isolated from cells with NZYol reagent according to the manufacturer's instructions. RNA concentration was determined by OD_{260} measurement using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and samples stored in RNA Storage Solution at -80 °C until use. Briefly, 1 µg of total RNA was reverse-transcribed using the NZY First-Strand cDNA Synthesis Kit and quantitative real-time PCR (qPCR) reactions were performed, in duplicate for each sample, on a Bio-Rad CFX Connect instrument as previously described (Neves *et al.*, 2009, 2013). Gene

expression changes were analysed using Bio-Rad CFX Maestro 1:1 software. The results were normalized using *HPRT1* as reference gene. Primer sequences were designed using Beacon Designer software version 8 (Premier Biosoft International, Palo Alto, CA, USA) (Supplementary data, Table S2) and thoroughly tested. Since real-time RT-PCR results are presented as ratios of chemically treated samples vs untreated cells (control), a two-base logarithmic transformation was used to make observations symmetric and closer to a normal distribution. If x represents the fold change of a gene in one sample, then the two-base logarithmic transformation [log2(x)] is ln(x)/ln(2). Therefore, fold changes of 2 and 0.5 correspond to mean log2 values of 1 and −1, respectively.

3.2.9 Isolation of Lysosomes

Lysosomes from human cell line THP-1 were isolated with Lysosome Enrichment Kit for Tissue and Cultured Cells (Thermo Scientific, 89839) following manufacturer's instructions. The kit uses OptiPrep Cell Separation Media for the density-based separation of lysosomes from contaminating cell structures. Finally, the lysosomes were lysed in RIPA buffer, assayed by Western blotting for the levels of LAMP2 (1:1000), GRP78 (1:1000) and Actin (1:5000) and used in the following experiments. A total of 25 µg of protein in lysosome-enriched fractions was loaded into each lane according to the protein concentration measured.

3.2.10 Immunoprecipitation

Samples were immunoprecipitated with anti-DNP antibody (A150-117A) and Protein G Sepharose® 4 Fast Flow according to manufacture instructions. Briefly, 200 µg of sample were incubated in RIPA buffer with 20 µg of the polyclonal anti-DNP antibody (Bethyl laboratories, A150-117A) overnight at 4°C under rotation. Protein G Sepharose® 4 Fast Flow beads (40 µL) were then added to the DNP-labelled protein solution and incubation continued for 1 h at room temperature with agitation. The unbound proteins were removed by centrifugation and the beads with immuno-complexes washed in RIPA buffer three times. Immunoprecipitated proteins were eluted from the beads by incubation with 50 µL of sample buffer for 10 min at room temperature, with agitation. Beads were removed by centrifugation, and supernatants loaded onto 10% SDS-

PAGE. The proteins were then transferred to PVDF membrane and the immunoprecipitated DNFB-protein complexes were visualized using anti-Dinitrophenyl-KLH antibody (Fisher, A6430).

3.2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 for Mac OS X (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Unless stated otherwise, for each experimental condition, the results are presented as the mean value ± SEM of at least 3 independent experiments. Statistical significance between two groups was determined by the two-tailed unpaired Student *t*-test and between multiple groups by one-way ANOVA analysis, with a Dunnett´s or Tukey multiple comparison post-test. Significance levels are as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

3.3 RESULTS

To explore how DNFB activates the innate immune system, we used the human monocytic cell line THP1, which not only expresses multiple TLRs including TLR2 and TLR4 (Dowling *et al*., 2016) but also represents a well-established model for inflammasome activation and for skin sensitization studies (Martinon *et al*., 2002).

3.3.1 DNFB induces IL-1ß secretion through NLRP3 inflammasome activation

We started by addressing on LPS-primed THP-1 cells if DNFB treatment induced IL-1ß maturation and secretion. To ascertain the involvement of NLRP3 inflammasome components, experiments were parallelly performed on THP-1defASC (silenced for ASC) and THP-1defNLRP3 (silenced for NLRP3).

As showed in Figure 3.1a, when used alone, DNFB was unable to induce the maturation and secretion of IL-1ß. Although, using a canonical two hit model for inflammasome activation, in cells primed with LPS, exposure to DNFB induced a significative increase in the secretion of IL-1ß (Figure 3.1b). This increase is dose-dependent Figure 3.1c and almost completely abolished in THP-1 silenced for NLRP3 and

ASC. The failure in secretion of IL-1ß in THP-1 silenced for NLRP3 and ASC was not due to differences in levels of the pro IL-1ß at the moment of DNFB exposure (Figure 3.1b). Thus, the results clearly indicate that DNFB, function as a second hit, triggering the assembly of NLRP3 inflammasome components.

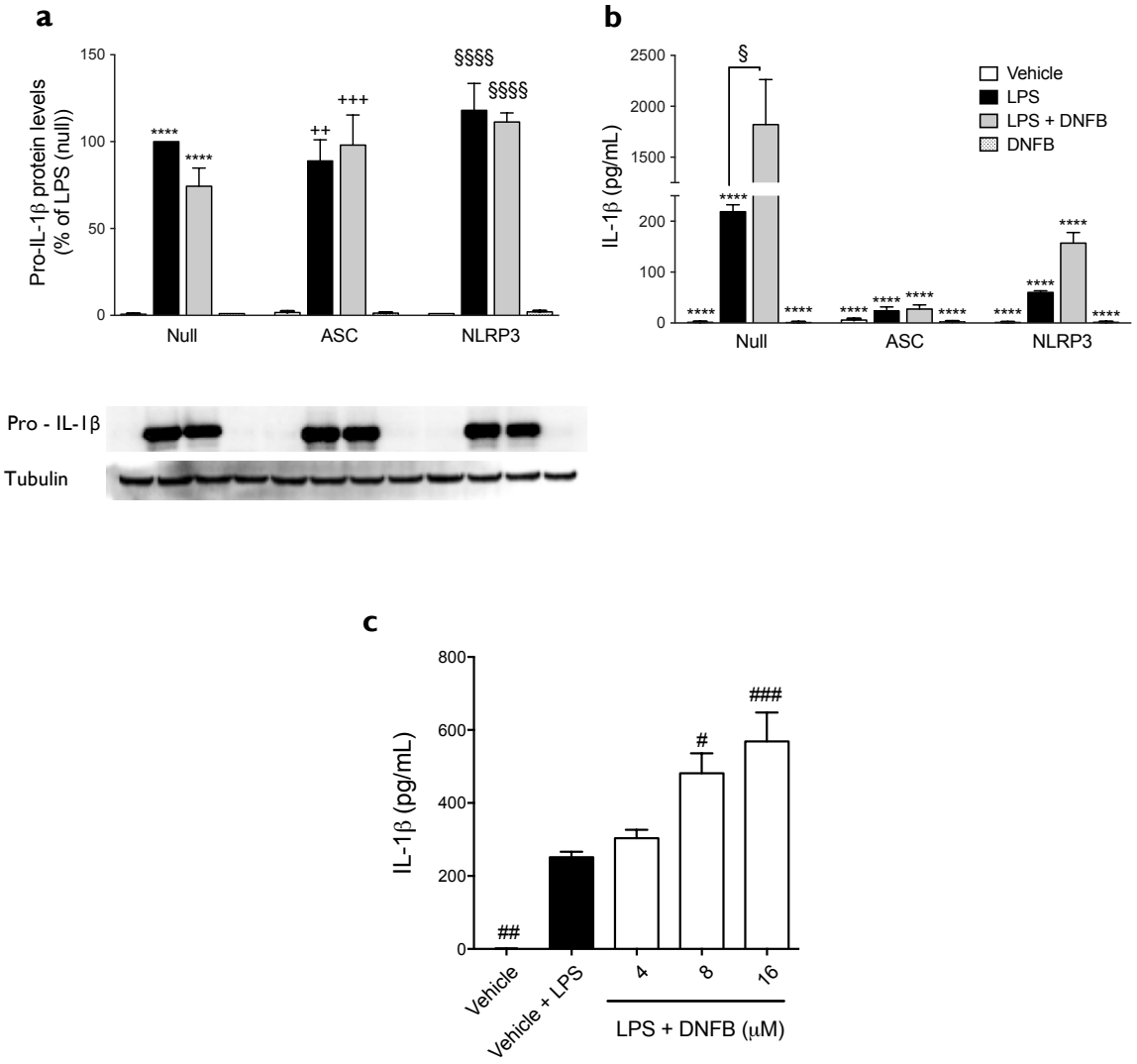


Figure 3.1 DNFB-induced secretion of IL-1ß in human THP-1 cells is mediated by the NLRP3 inflammasome. (a) Immunoblot analysis of IL-1ß production by human THP-1 cells null, NLRP3-deficient or ASC-deficient, primed for 24 h with LPS 1 µg/mL and then left unstimulated or stimulated with DNFB 16 µM for 6 h; pro-IL-1ß levels were assessed in cell lysates (a) and IL-1ß levels accessed in the respective supernatants (b). (c) ELISA of IL-1ß production in cells treated as described in a). All immunoblot data shown are representative of at least three independent experiments. Bar graph presents the mean ± SEM. Statistical analysis: (a) One-way ANOVA with Dunnett's multiple comparison test: For each cell line: ****$p < 0.0001$ compared to Null cells treated with vehicle; $^{++}p < 0.01$, $^{+++}p < 0.001$ compared to THP-1defASC cells treated with vehicle; $^{§§§§}p < 0.0001$ compared to THP-1defNLRP3 cells treated with vehicle; (b) One-way ANOVA with Dunnett's multiple comparison test: ****$p < 0.0001$ compared to Null cells treated with LPS + DNFB; t test: $^{§}p < 0.05$ (c) One-way ANOVA with Dunnett's multiple comparison test: $^{#}p < 0.05$; $^{##}p < 0.01$; $^{###}p < 0,001$ compared to null cells treated with vehicle + LPS.

3.3.2 DNFB induces lysosomal destabilization and subsequent cathepsin B leakage

To delineate the 'upstream' mechanisms involved in DNFB induced activation of NLRP3 inflammasome we first determined if DNFB could interfere with one of the several known inducers of inflammasome activation, that is lysosomal rupture and subsequent cathepsin leakage. Although lysosomal membrane permeabilization occurs mainly following crystalline particle phagocytosis (Hornung *et al*., 2008; Rajamäki *et al*., 2010; Campden *et al*., 2019), in some cases, inflammasome activators without a crystalline structure (e.g. deoxycholic acid, dextran sodium sulfate, 5-fluorouracil and gemcitabne) may still induce lysosomal damage through a yet unknown signaling pathway (Campden *et al*., 2019). Briefly, cells were treated with several concentrations of DNFB, without serum, and the lysosomal integrity was further evaluated with acridine orange. Acridine orange is a metachromatic dye that is sequestered and protonated inside the acidic lysosomal compartment, shifting its emission spectrum from green to red. Hence, loss of lysosomal integrity can be measured as a 'loss of red dots' or as a quantitative rise in green fluorescence. The results obtained showed an increase in green fluorescence and loss of red dots in THP-1 cells (both in undifferentiated and differentiated macrophages with PMA). These results were also validated in the human keratinocyte cell line HaCaT and human moDCs (Figure 3.2). As loss of red dots could both be influenced by loss of integrity or loss of the proton gradient of lysosomes, we next examined the release of lysosomal enzymes into cytosol. Interestingly, lysosomal rupture has been associated with inflammasome activation through cytosolic detection of cathepsin B. Cathepsin B is a widely expressed lysosomal cysteine cathepsin that has been implicated in processes ranging from inflammasome activation to cancer migration (Duewell *et al*., 2010; Aggarwal *et al*., 2014). Indeed, over than 100 publications address the involvement of cathepsins in inflammasome activation, the majority reporting the involvement of cathepsin B. Cathepsin B activity was evaluated by confocal microscopy with a CV-cathepsin B detection Kit that contains a cathepsin B target sequence peptide (RR)2 linked to a red fluorophore (Cresyl Violet).

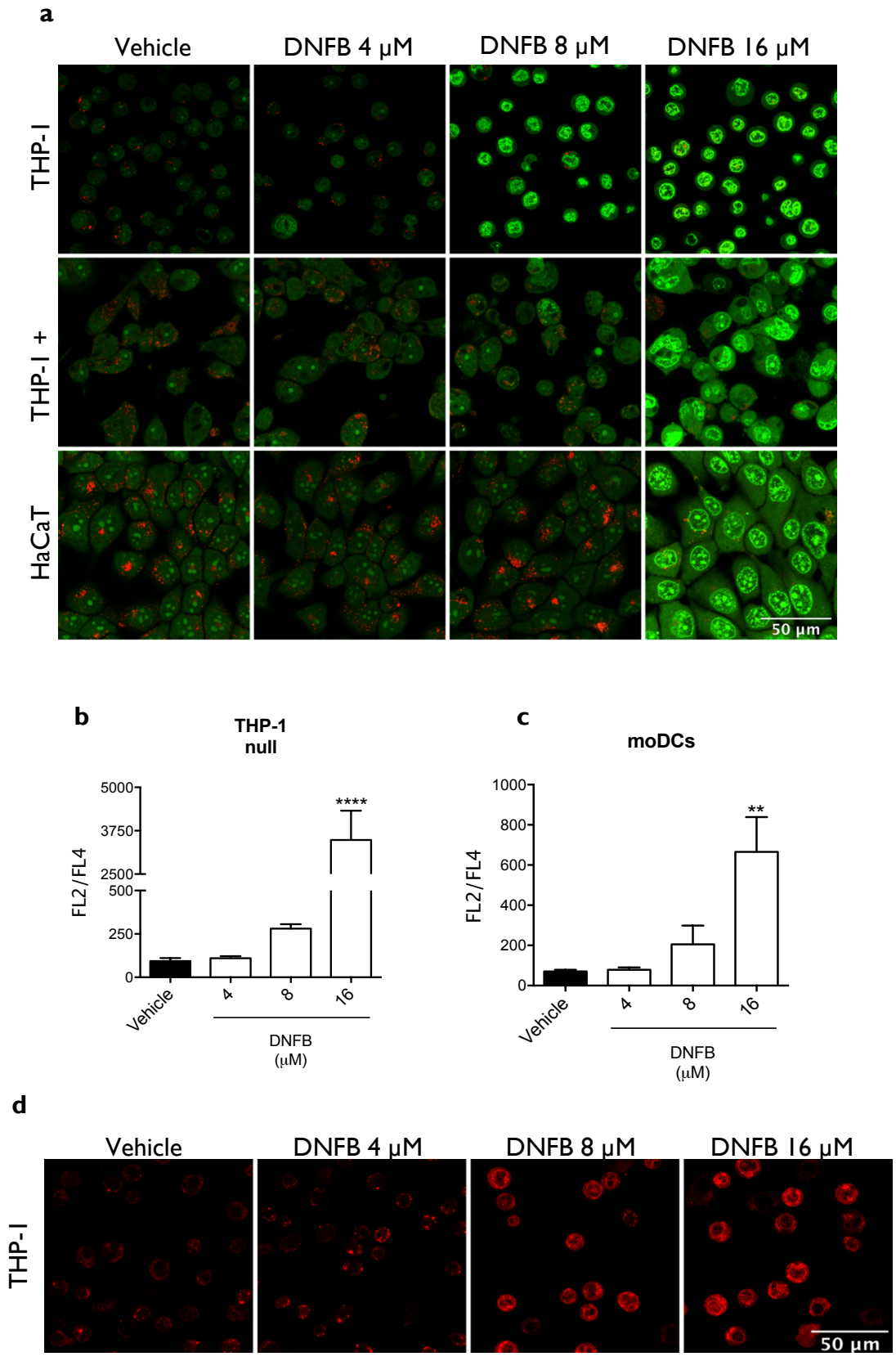


Figure 3.2 DNFB induces lysosomal destabilization and subsequent cathepsin leakage. DNFB induces lysosomal destabilization and subsequent cathepsin leakage. (a, d) Confocal microscopy of cells treated with several concentrations of DNFB in serum free medium for 3 h and further incubated with acridine orange (a) or CV-cathepsin

B (d) detection Kit to address lysosomal rupture and cathepsin B leakage respectively. Magnification: 63×. Scale bar, 50 µm. (b,c) Flow cytometry of THP-1null cells (b) and moDCs (c) treated with several concentrations of DNFB in serum free medium for 3h and further incubated with acridine orange. Lysosomal destabilization was quantified as a ratio between green (FL2) and red (FL4) fluorescence. Bar graph presents the mean ± SEM. Statistical treatment: One-way ANOVA with Dunnett's multiple comparison test: $^{**}p < 0.01$; $^{****}p < 0.0001$ compared to vehicle-treated cells.

If cathepsins are active, they will cleave the two dipeptide cathepsin targeting sequences allowing cresyl to become fluorescent upon excitation. The fluorescent product will stay inside the cell and will often aggregate inside lysosomes and other low pH areas. The low levels of background red fluorescence distributed thorough the cell could be due to constitutively synthesized serine proteases that target analogous amino acid sequences for hydrolysis. When incubating resting cells with CV-cathepsin B detection Kit, we found red fluorescence constricted to small dots, the lysosomes. Although, when cells were treated with increasing concentrations of DNFB the red fluorescence was no longer restricted to lysosomes, but brighter through the cell (Figure 3.2d). This results collectively suggest that DNFB activates NLRP3 inflammasome through lysosomal rupture and consequent cathepsin leakage.

3.3.3 DNFB induced cathepsin B leakage is responsible for NLRP3 inflammasome activation and dendritic cells maturation

To discard other danger signals as triggers for inflammasome activation we tested whether the incubation of dendritic-like cells with a known antioxidant would impair IL-1ß secretion. Briefly, cells were pre-treated with 2.5 mM of ascorbic acid before treatment with DNFB, but, as shown in Figure 3.3a, although the secretion of IL-1ß seems to have lowered compared to cells treated with DNFB, this decrease was not statistically significant. Therefore, we cannot totally discard the involvement of ROS production on DNFB induced-inflammasome activation, but its potential involvement will be of minor relevance. We also performed *in chemico* a pre-incubation of DNFB with the amount of BSA that normally exists in FBS, for 1 h at 37 °C. Cells were then treated with the mixture (haptenated BSA) in order to disclose if inflammasome activation could be happening due to frustrated phagocytosis of protein-hapten aggregates, similarly to what happens with crystalline material. As shown in Figure 3.3a, IL-1ß secretion was totally abrogated, which lead us to hypothesize that DNFB leads to lysosomal rupture due to chemical interactions,

probably by forming adducts with lysosomal proteins, rather than to a physical process, as previously reported for particulate matter, namely monosodium urate, silica, asbestos, amyloid deposits, and aluminium salts.

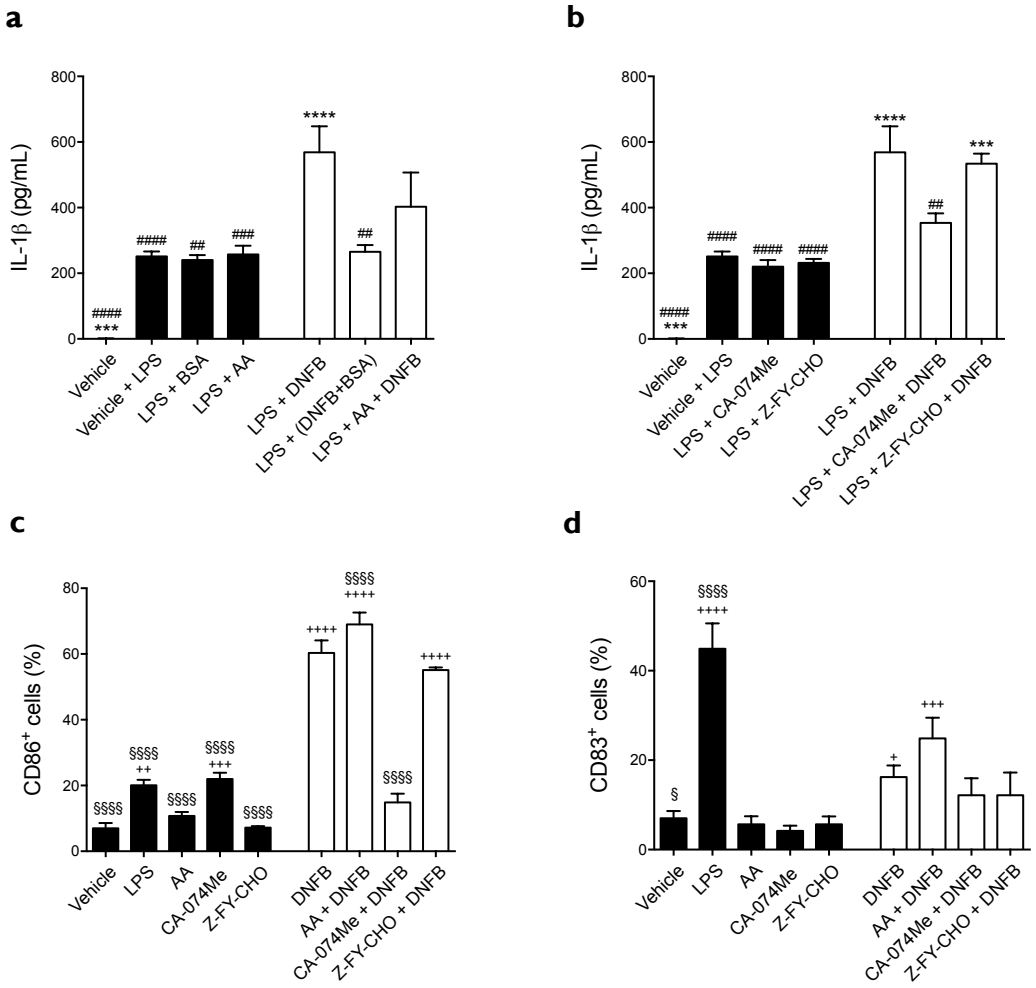


Figure 3.3 Pre-incubation with cathepsin inhibitors decreases DNFB-induced IL-1ß secretion and expression of maturation markers CD83 and CD86. (a,b) ELISA of IL-1ß production by human THP-1 cells null, primed for 24 h with LPS 1 µg/mL and left unstimulated or pre-incubated for 1 h with 2.5 mM of ascorbic acid (AA), 20 µM of cathepsin B or cathepsin L inhibitors and further treated with DNFB 16 µM for 6 h. LPS primed cells were also treated with a mix of an *in chemico* incubation of BSA with DNFB. (c,d) Flow cytometry of $DC86^+$ (c) and $CD83^+$ (d) cells that were pre-treated for 1 h with ascorbic acid, cathepsin B or cathepsin L inhibitors before stimulation with DNFB 16 µM for 6 h. Bar graph presents the mean ± SEM. Statistical treatment: One-way ANOVA with Dunnett's multiple comparison test: $^{***}p < 0.001$; $^{****}p < 0.0001$ compared to cells treated with vehicle + LPS. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ compared to cells treated with LPS + DNFB. $^{+}p < 0.05$; $^{+++}p < 0.001$; $^{++++}p < 0.0001$ compared to cells treated with vehicle. $^{\S\S}p < 0.01$; $^{\S\S\S}p < 0.001$; $^{\S\S\S\S}p < 0.0001$ compared to cells treated with DNFB.

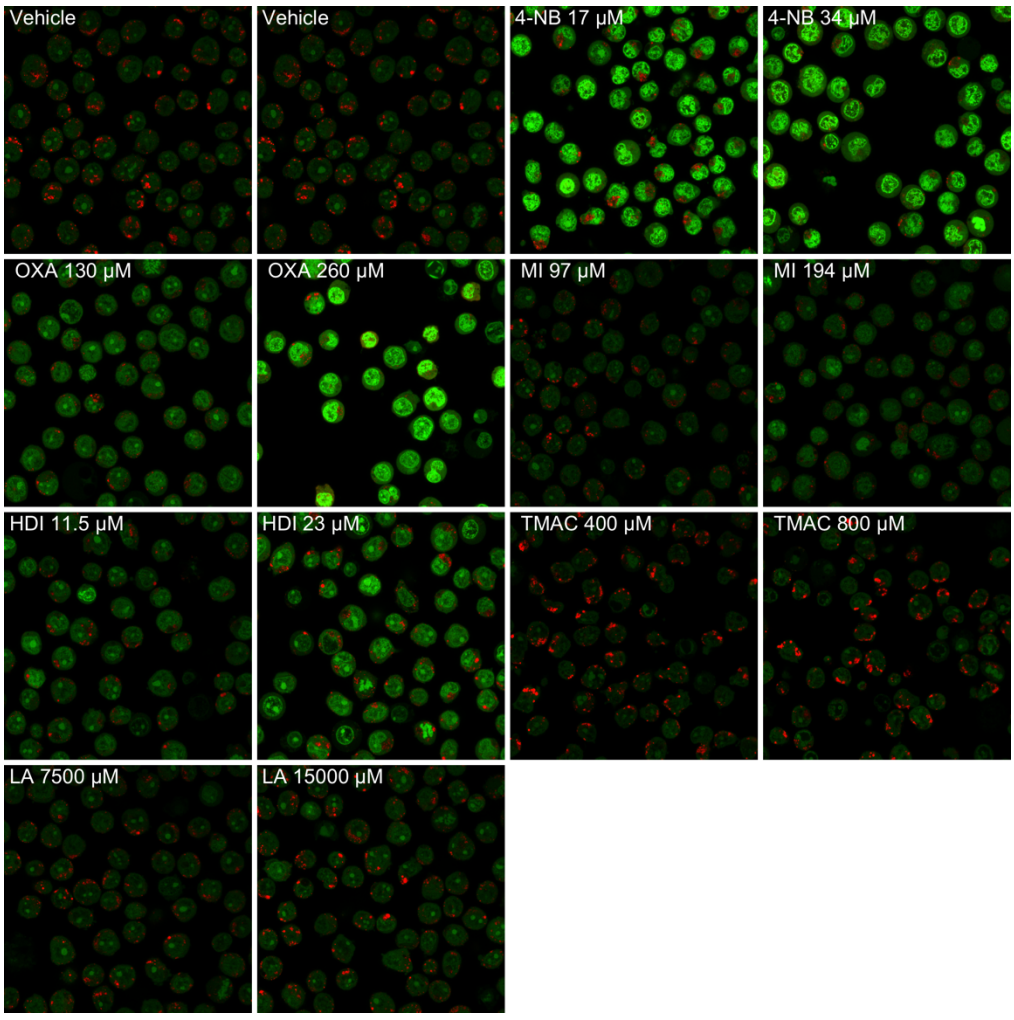
To further address the function of cathepsin leakage in DNFB-mediated inflammasome activation and DCs maturation profile, we tested two cathepsin inhibitors for their ability to inhibit DNFB-mediated IL-1ß secretion and maturation. CA-074Me was used to inhibit cathepsin B (Campden *et al.*, 2019) and Z-FY-CHO (also known as Z-Phe-

Tyr-CHO) to inhibit cathepsin L (Woo *et al.*, 1995). Among the two inhibitors tested, CA-074Me led to much less IL-1ß secretion and expression of the maturation markers CD83 and CD86 after DNFB treatment (Figure3.3,). These results support that cathepsin B leakage after lysosomal rupture, leads to NLRP3 inflammasome activation, and subsequent IL-1ß secretion, which, together with TNF, are necessary for the maturation and migration of DCs from the skin to the draining lymph node (Kondo *et al.*, 1995; Shornick *et al.*, 1996; Watanabe *et al.*, 2008; Kaplan *et al.*, 2012).

3.3.4 Thiol reactive chemical sensitizers evoke inflammasome activation through lysosomal rupture and cathepsin leakage

The most common chemicals known to cause allergic contact dermatitis are thiol reactive sensitizers.

a



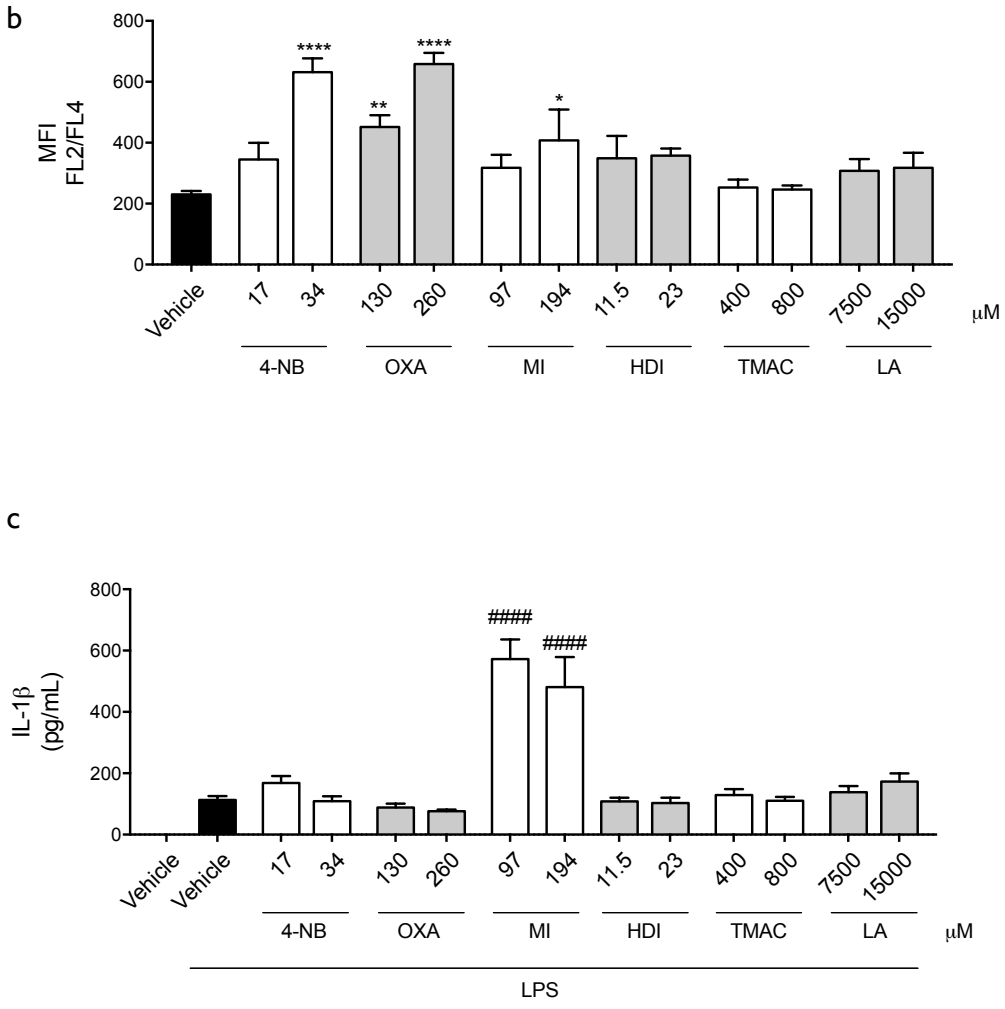


Figure 3.4 Reactivity towards Lys or Cys residues affects the ability of chemicals to induce lysosomal destabilization. (a,b) Confocal microscopy (a) and flow cytometry (b) of cells treated with several concentrations (EC30 and twice the EC30) of chemical sensitizers and non-sensitizers in serum free medium for 3 h and further incubated with acridine orange. Magnification: 63×. Scale bar, 50 μm. (c) ELISA of IL-1ß production by human THP-1 cells null, primed for 24 h with LPS 1 μg/mL and left unstimulated or treated with several concentrations of sensitizers and non-sensitizers for 6 h. Skin sensitizers: 4-nitrobenzylbromide (4-NB), methylisothiazolinone (MI) and oxazolone (OXA); Respiratory sensitizers: trimellitic anhydride chloride (TMAC) and Hexamethylene diisocyanate (HDI); Non-sensitizers: Lactic acid (LA). Statistical treatment: One-way ANOVA with Dunnett's multiple comparison test: *$p < 0.05$, **$p < 0.01$; ****$p < 0.0001$ compared to cells treated with vehicle. ####$p < 0.0001$ compared to cells treated with vehicle + LPS.

To determine whether other chemical sensitizers induced inflammation by a mechanism similar of DNFB, we repeated key experiments (IL-1ß secretion and lysosomal membrane integrity) with several skin and respiratory sensitizers presenting different reactivity profiles towards thiol and amine groups. We tested 3 groups of chemicals: 1) skin sensitizers: 4-nitrobenzylbromide (4-NB), oxazolone (OXA) and

methylisothiazolinone (MI); 2) respiratory sensitizers: hexamethylene diisocyanate (HDI) and trimellitic anhydride chloride (TMAC); 3) Non-sensitizer: lactic acid. 4-NB, OXA, MI and HDI, similarly to DNFB, have a high reactivity towards cys residues (above 75.5%) compared to TMAC (0-8.9 %, depending on the study) or LA (without reactivity towards proteins) (Supplementary data, Table 3.S3). As shown in Figure 3.5a,b, the lysosomal destabilization in cells treated with 4-NB, OXA and MI, is significantly higher and suggest to be dose dependent. As for IL-1ß, MI leads to a significant increase in IL-1ß secretion. Although not statistically significative, we can observe a slightly increase in IL-1ß levels upon 4-NB treatment (Figure 3.5c).

3.3.5 DNFB haptenizes both cytoplasmic and lysosomal proteins

Our results clearly demonstrate that DNFB and other thiol-reactive sensitizers transiently destabilize lysosomal membrane allowing the leakage of cathepsin B. Although, it remains unclear how this occurs at molecular level. We hypothesized that sensitizers would haptenize specific lysosomal proteins causing its malfunction. To further address this hypothesis, we performed immunoprecipitation of total and lysosomal extracts of DNFB treated cells. To immunoprecipitate proteins haptenated by DNFB, we incubated the samples with anti-dinitrophenol antibody overnight at 4°C and further incubated for 1 h at room temperature with Protein G Sepharose® 4 Fast Flow beads. Samples were eluted, submitted to SDS-PAGE and immunodeveloped with a different anti-dinitrophenol antibody. As shown in Figure 3.5a,b, DNP staining is specific to samples treated with DNFB. Interestingly, while DNP staining for total extracts manly occurs between 250-25 kDa, lysosomal extracts showed a noticeable staining for DNP between 250 – 11kDa (Figure 3.5c,d).

The use of anti-DNP staining for detect DNFB-haptenated proteins was validated by immunoblot of total and lysosomal extracts of vehicle and DNFB-treated cells. Indeed, as shown in Figure 3.6a, anti-DNP specifically stains DNFB-haptenated proteins. Lysosome enrichment was confirmed by a high abundance of the lysosomal marker lamp-2 and a decrease in the cytoskeleton protein actin as shown in Figure 6b. As shown in figure Figure 3.5c,d it was possible to immunoprecipitate DNFB-haptenized proteins allowing in a near future step to identify them by mass spectrometry, which is currently ongoing.

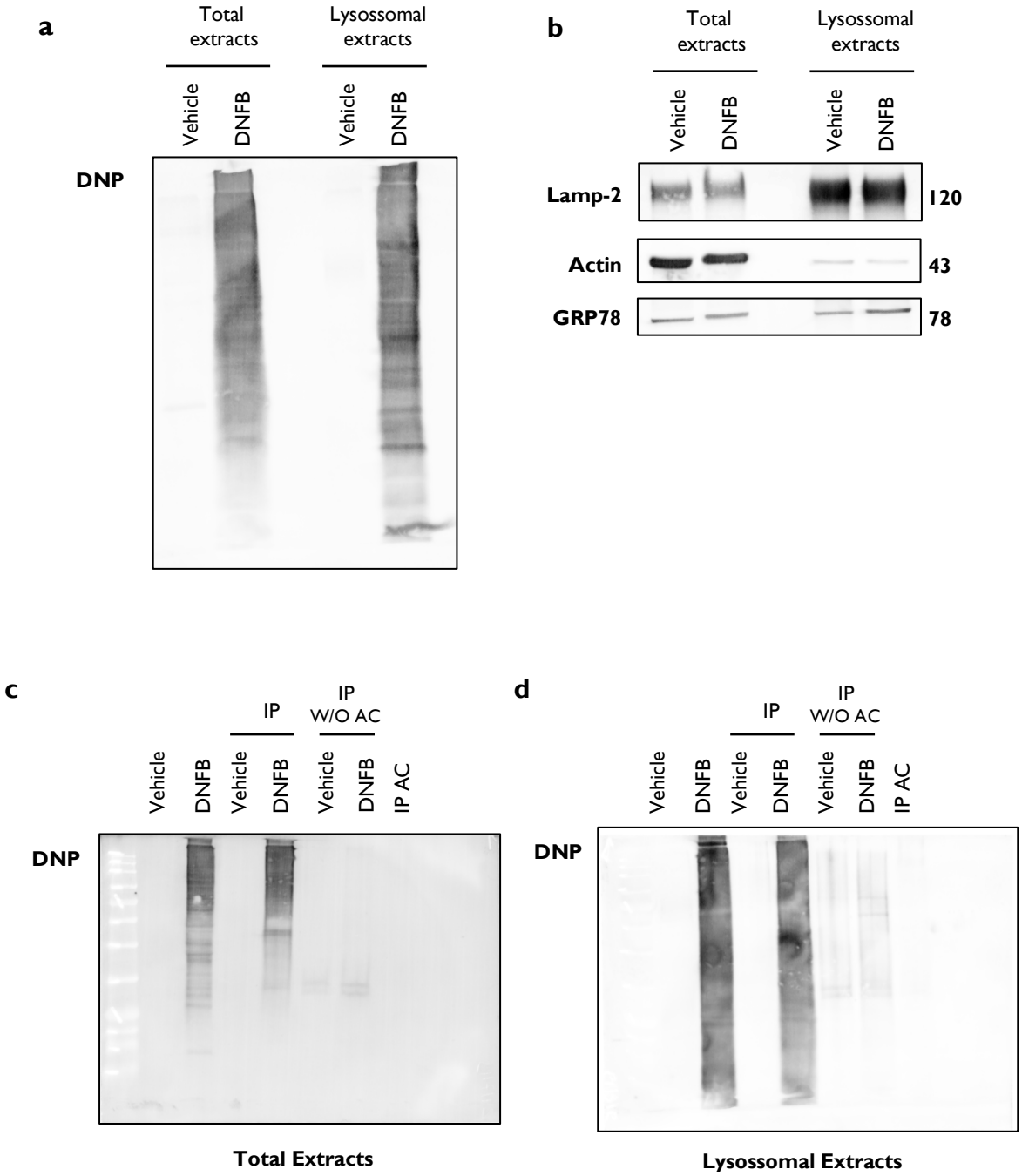


Figure 3.5. Immunoprecipitation of total extracts and lysosomal extracts of cells treated with DNFB. THP-1null cells were treated with DNFB 16 µM in serum free medium for 3 h and submitted to a lysosomal enrichment with Lysosome Enrichment Kit for Tissue and Cultured Cells according to manufacturer's instructions. Cellular contents were further extracted with RIPA buffer before (total extracts) or after lysosomal enrichment (lysosomal extracts). (a) Anti-DNP staining validation in total and lysosomal extracts of vehicle and DNFB-treated cells. (b) Lysosomal purity was assessed by immunoblot analysis of total and lysosomal extracts. (c,d) Immunoblot of the immunoprecipitation of proteins haptenated by DNFB in total extract (c) and lysosomal extracts (d). Samples were immunoprecipitated with anti-DNP (A150-117A) and further developed with anti-DNP (A6430). W/O, without.

To best of our knowledge this is the first study using anti-DNP antibodies to immunoprecipitate DNFB-haptenated proteins. This new/pioneer approach will increase the knowledge about the signaling pathways mediated by thiol-reactive sensitizers such as DNFB. Furthermore, it can be used as a starting point to further evaluate and validate specific biomarkers for sensitization.

3.4 DISCUSSION

The NLRP3 inflammasome can be activated by multiple stimuli such as extracellular ATP, alum, inhaled silica asbestos and by endogenously formed monosodium urate crystals. Typically, these substances promote potassium efflux, leakage of lysosomal cathepsin B and/or the production of ROS which are then sensed by inflammasome. These multimeric structures have been implicated in several diseases such as Alzheimer disease, cancer, diabetes or allergic contact dermatitis (Li *et al*., 2014; Mangan *et al*., 2018; Moossavi *et al*., 2018). Herein we demonstrate that the skin sensitizer DNFB activates NLRP3 inflammasome through lysosomal destabilization and subsequent cathepsin B leakage to the cytosol. Inhibition of cathepsin activity impaired NLRP3 activation and the DNFB-induced maturation of dendritic-like cells, thus disclosing a new innate immune mechanism crucial for the development of ACD. Furthermore, we demonstrate that this new mechanism of inflammasome activation may be common to other sensitizers suggesting that this pathway is shared by chemicals with high thiol reactivity.

In the context of ACD, most studies addressing inflammasome activation are related to the release of DAMPs and to the involvement of inflammasome components, such as ASC, NLRP3 and caspase 1. For instance, Watanabe and colleagues demonstrated that chemical haptens (trinitrochlorobenzene (TNCB), DNFB and dinitro-1-chlorobenzene (DNCB) activate NLRP3 inflammasome in primary human keratinocytes and murine models by an indirect pathway involving ROS and ATP. The importance of inflammasome was also demonstrated by the reduced sensitization to TNCB and DNFB in $Asc^{-/-}$, $Nlrp3^{-/-}$ and $Casp1^{-/-}$ mice (Watanabe *et al*., 2007). Other studies showed that Hexavalent chromium (Cr(VI)) induced ROS production and potassium (K^+) efflux, thereby activating the NLRP3 inflammasome (Buters *et al*., 2017; Wang *et al*., 2018). Moreover, several contact allergens induce ATP release (Weber *et al*., 2010 b; Onami *et al*., 2014) which stimulates inflammasome activation via the purinergic receptor

P2X7 (Solle *et al*., 2001; Sutterwala *et al*., 2006). Accordingly, mice lacking P2X7 fail to develop sensitization to contact allergens and do not release IL-1β in response to lipopolysaccharide (LPS) and ATP (Weber *et al*., 2010 b). Although the majority of studies report that chemical sensitizers activate inflammasome by inducing potassium efflux and/or ROS production, the actin dependent endocytic pathway has been previously shown as a prerequisite for NLRP3 inflammasome activation by many NLRP3 agonists, such as β-amyloid and alum, silica and cholesterol crystals (Eisenbarth *et al*., 2008; Halle *et al*., 2008; Hornung *et al*., 2008; Rajamäki *et al*., 2010). Curiously, Li *et al*., demonstrated that although being a insoluble crystalline, Ni^{2+}- triggered NLRP3 inflammasome assembly was independent of actin-mediated phagocytosis (Li *et al*., 2014) and that the blockade of lysosomal acidification and cathepsin B activity had no effect on NLRP3 inflammasome activation. Other authors also reported lysosomal damage with chemicals lacking a crystalline structure. Accordingly, Bruchard *et al*., demonstrated that the chemotherapeutic drugs gemcitabine and 5-fluorouracil trigger NLRP3 activation through lysosome permeabilization and cathepsin B release, without ROS activation or potassium efflux in myeloid-derived suppressor cells. They also showed that caspase-1 activation was not prevented by inhibition of phagocytosis, although inhibition of lysosomal acidification blunted caspase-1 activation (Bruchard *et al*., 2013). Indeed, insoluble crystalline structure may not be a pre-requisite for chemical-lysosome–cathepsin B induced inflammasome pathway. Supporting the above notion, our results show that the sensitizer DNFB induced NLRP3 assembly by causing a transient destabilization of lysosomal membrane with consequent leakage of the endosomal-lysosomal protease cathepsin B into the cytoplasm. This effect is not cell-specific since similar results were observed in human keratinocytes (HaCaT cell line), human macrophages differentiated from THP-1 cells and in human monocyte-derived DCs. Treatment with the cathepsin B specific inhibitor Ca-074Me dramatically decreased the amount of IL-1β elicited by DNFB and the expression of the co-stimulatory molecules CD86 and CD83. Recently, Schwenck and colleagues evaluated the effects of the cathepsin B in the effector phase of cutaneous delayed-type hypersensitivity reactions to the sensitizer 2,4,6-trinitrochlorobenzene (TNCB). To elucidate the therapeutic impact of specifically targeting cathepsin B, the authors treated mice with the selective cathepsin B inhibitor CA-074 before TNCB challenge (elicitation phase). They observed a significant decrease in the ear swelling of sensitized mice compared with sham-treated mice, accompanied by reductions in ear thickness, edema

hyperkeratosis, acanthosis and inflammatory cell infiltration (Schwenck *et al*., 2019). Besides its effect on IL-1β secretion, which has a preeminent role on ACD pathophysiology, cathepsin B was also describe to be involved in keratinocyte migration and tumor invasiveness, with poor patient prognosis in several cancer entities (Büth *et al*., 2007; Bengsch *et al*., 2014). Interestingly, Szpaderska and Frankfater were able to increase or decrease the invasiveness of several tumor cell lines, through manipulation of cathepsin B expression (either by transient expression or stable antisense cathepsin B expression) (Szpaderska *et al*., 2001). Along the same line, Büth and colleagues showed that HaCaT keratinocytes secrete cathepsin B into the extracellular space during spontaneous and induced migration. The same authors further supported these results using normal human epidermal keratinocytes (NHEK) and showed that non-cell-permeant cathepsin B-specific inhibitors delayed full regeneration of the monolayers from scratch wounding in both cell systems, HaCaT and NHEK (Büth *et al*., 2007). Although the role of cathepsin B in DCs is often associated with antigen presentation, it may also influence DCs migration capacity as described for keratinocytes and tumor cells and this is a very interesting and relevant hypothesis that should be further investigated. Indeed, cathepsin B substrates include extracellular-matrix components, type IV collagen, laminin, fibronectin (Bengsch *et al*., 2014).

Cathepsin L inhibitor slightly decreased DNFB-induced IL-1β secretion and expression of the maturation markers CD86 and CD83, although not significantly. It is important to note, however, that several studies report some uncertainties regarding CA-074Me selectivity. After phagocytosis, CA074Me is processed in the lysosomes into the highly cathepsin B-selective free acid CA-074. However this is a slow process allowing time for CA074Me to inhibit multiple cathepsins (Orlowski *et al*., 2015). Indeed, Montaser *et al*., demonstrated that in murine fibroblasts CA-074Me inhibits the activity of both cathepsin B and the very closely related cathepsin L (Montaser *et al*., 2002). Thus, we could be witnessing a much higher inhibition of IL-1β secretion and $CD86^+$ and $CD83^+$ cells with CA-074Me compared to Z-FY-CHO due to a synergistic inhibitory effect of CA-074Me on both cathepsins B and L. Therefore, we cannot rule out the involvement of cathepsin L on inflammasome activation by DNFB. Also, redundancy of cathepsins B and L has been demonstrated in a mouse model where deficiency of both cathepsins results in neonatal mortality whereas deficiency of one alone does not (Felbor *et al*., 2002). In addition, the leakage of haptenated proteins to the cytosol, upon lysosomal destabilization,

could theoretically lead to an increased DCs cross-presentation in ACD, another interesting hypothesis that should be further investigated.

To rule out that the observed mechanism was exclusive of DNFB, we tested other chemical sensitizers (skin sensitizers: OXA, 4-NB and MI; respiratory sensitizers: TMAC and HDI) and non-sensitizers (LA) for their ability to induce lysosomal destabilization. These chemicals present different reactivities towards thiol and amine residues (Supplementary data, Table S3.3). Interestingly, the chemicals with higher reactivity toward thiol groups induce lysosomal destabilization, although not all induced IL-1β secretion. Only the chemicals with a higher ratio FL2/FL4 (higher lysosomal destabilization) showed to induce IL-1β secretion. This leads us to hypothesize that inflammasome activation by these chemicals only occurs when a critical loss of lysosomal integrity is reached, but additional studies are needed to confirm this rational.

Haptenization of proteins by chemical sensitizers can lead to modifications of protein function and/or localization within the cell, similarly to what happens with post-translational modifications (Martin *et al.*, 2006; Freudenberg *et al.*, 2009) such as phosphorylation and glycosylation. One example is the regulation of Nrf2 pathway by cysteine-binding chemicals, which covalently modify the cytosolic sensor protein Keap that regulates Nrf2 (Natsch, 2009). Our results show that thiol reactive chemicals induce lysosomal destabilization and since pre-treatment with ascorbic acid had no effect on IL-1ß secretion, involvement of ROS was ruled out. On other hand, frustrated phagocytosis was also excluded because exposure of cells to DNFB-BSA pre-formed proteinaceous aggregates were unable to trigger IL-1ß secretion. Therefore, we hypothesize that thiol-reacting chemicals covalently bind lysosomal membrane proteins, impairing their function. To address this hypothesis, we optimized a protocol for the immunoprecipitation of DNFB-hapetenated proteins, which can be further identified by mass spectrometry. To further support our hypothesis, western blots of lysosomal enriched cell extracts from DNFB-treated THP-1 cells, show extensive DNP staining (Figure 3.6d), both in the immunoprecipitated or non-immunoprecipitated samples. This new method opens the possibility to perform a more narrowed proteomic study, including the proteins specifically haptenated by DNFB, which is currently ongoing in our lab.

3.5 CONCLUSIONS

Although the involvement of NLRP3 inflammasome in contact allergy is well documented, the detailed mechanisms for its assembly remain elusive. Our work provides the first evidence that thiol-reactive allergens, molecules without a crystalline structure or detergent-like effects, trigger the assembly of NLRP3 inflammasome through lysosomal membrane destabilization and consequent cathepsin leakage (Figure 3.6).

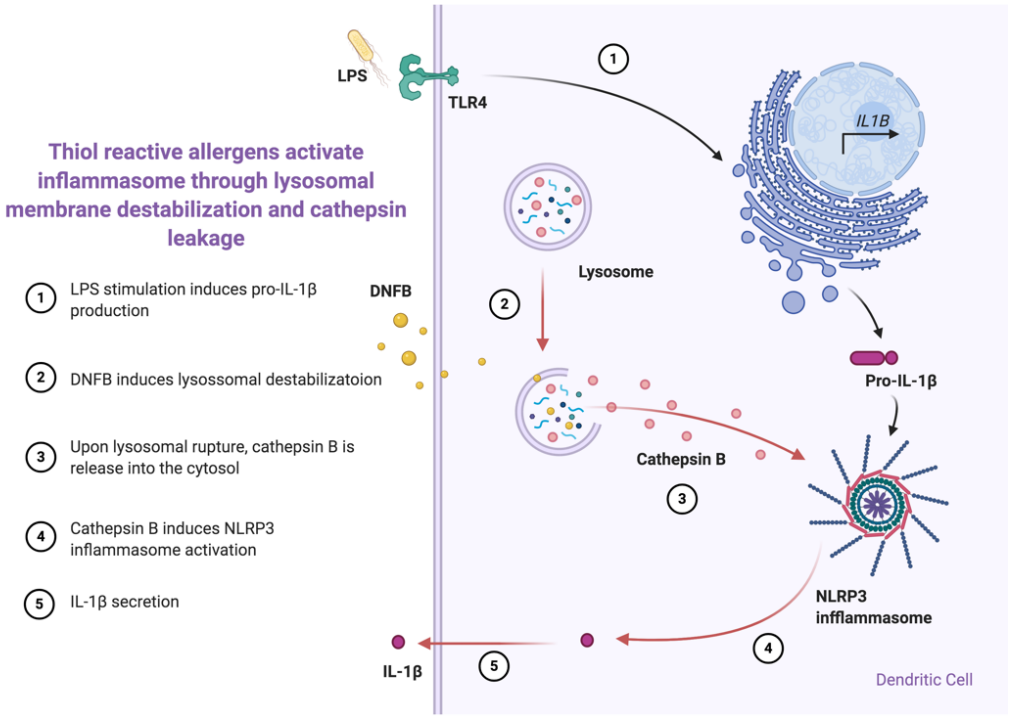


Figure 3.6. Thiol reactive sensitizers activate inflammasome through lysosomal membrane destabilization and cathepsin leakage. Our data shows that the thiol-reactive sensitizers induce lysosomal destabilization causing cathepsin B leakage, which results in inflammasome activation and subsequent IL-1ß secretion. Created with BioRender.com.

This effect is not attributable to allergen-induced ROS or frustrated phagocytosis of particulate hapten-protein complexes, relying rather in direct haptenization and functional impairment of lysosomal protein membranes. Moreover, we shown that besides the assembly of NLRP3 inflammasome and consequent maturation of IL-1ß, the presence of cathepsin B in the cytosol drastically induced CD86 and CD83 expression, to key DC co-stimulatory molecules.

Together, these results shed light on a new mechanism of inflammasome activation elicited by thiol reactive chemicals. Furthermore, the identification of the immunoprecipitated DNFB-haptenated proteins by mass spectrometry will help uncovering how chemicals induce lysosomal destabilization, which is currently ongoing in our lab. Furthermore, these results may disclose new candidate target molecules for the development of therapeutic approaches for the treatment of allergic sensitization.

ACKNOWLEDGMENTS:

This work was financed by The Johns Hopkins Center for Alternatives to Animal Testing, project nº: 2014-07, by the Portuguese national funds via FCT – Fundação para a Ciência e a Tecnologia under project UIDB/04539/2020. Isabel Ferreira was funded through FCT PhD fellowships ref SFRH/BD/110717/2015.

SUPPLEMENTARY DATA

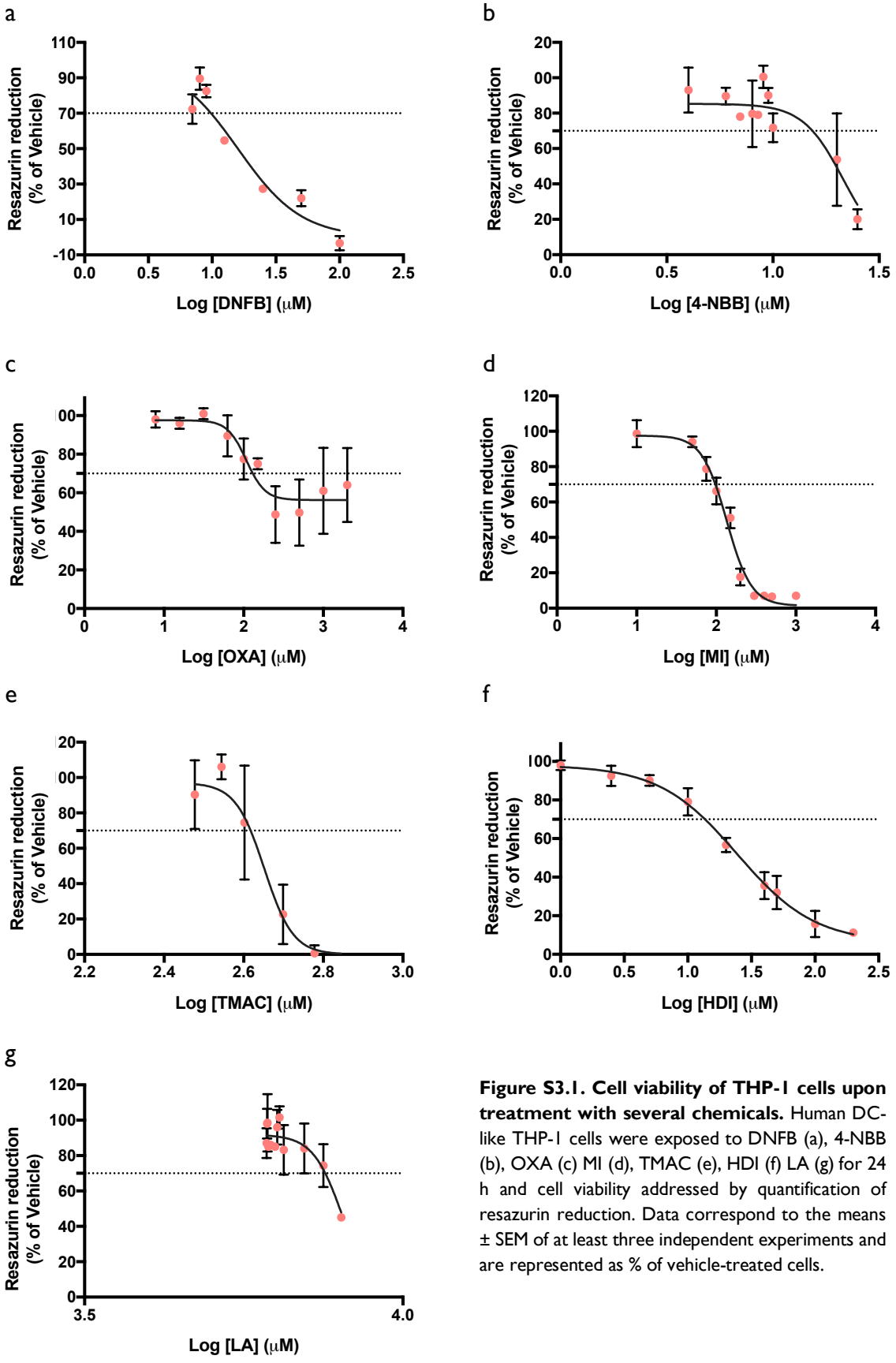


Figure S3.1. Cell viability of THP-1 cells upon treatment with several chemicals. Human DC-like THP-1 cells were exposed to DNFB (a), 4-NBB (b), OXA (c) MI (d), TMAC (e), HDI (f) LA (g) for 24 h and cell viability addressed by quantification of resazurin reduction. Data correspond to the means ± SEM of at least three independent experiments and are represented as % of vehicle-treated cells.

Table S3.1: Source, purification, supplier and reference of the primary and secondary antibodies used in Western blotting analysis.

Antibody	Host	Company	Ref	Dilution	MW
IL-1β	R	Santa Cruz	sc-7884	1:500	31,17
Tubulin	M	Sigma	T7816	1:20000	50
Lamp-2	M	Santa Cruz	sc18822	1:1000	120
Actin	M	Sigma	MAB1501	1:5000	43
GRP78	M	BD Pharmingen	610978	1:1000	78
DNP	R	Fisher	A6430	1:1000	-
DNP	G	Betyl Laboratories	A150-117A	1:1000	-
Anti-goat	R	Milipore	AP106A	1:5000	-
Anti-mouse	G	Ge Healthcare	NIF1316	1:20000	-
Anti-rabbit	G	Ge Healthcare	NIf1317	1:20000	-

Table S3.2: Primer sequences used to amplify target cDNAs.

Gene	Primer Forward	Primer reverse
IL1B	GCT TGG TGA TGT CTG GTC	GCT GTA GAG TGG GCT TAT C
IL8	CTT TCA GAG ACA GCA GAG	CTA AGT TCT TTA GCA CTC C
HPRT1	GGC TTA TAT CCA ACA CTT CG	TGA CAC TGG CAA AAC AAT G

Table S3.3: Peptide depletion values for Cysteine and Lysine residues (DPRA) for the chemicals used in the previous experiments.

Chemical	CAS	% Cys depletion	% Lys depletion	Reference
DNFB	70-34-8	87.4	78	*in house*
Methylisothiazolinone	2682-20-4	97.9	0	Natsch *et al*., 2013
Lactic acid	50-21-5	0	0.8	Natsch *et al*., 2013
4-NB	100-11-8	100	24.2	Natsch *et al*., 2013
Oxazolone	15646-46-5	75.5	49.6	Natsch *et al*., 2013
TMA	552-30-7	8.9	95.3	Lalko *et al*., 2012
TMA	552-30-7	0	43.7	Natsch *et al*., 2013
HDI	822-06-0	100	35	Lalko *et al*., 2012
HDI	822-06-0	97	34.3	Dik *et al*., 2016

CHAPTER 4

General Discussion

GENERAL DISCUSSION

Given the extent of human exposure to chemicals, it is possible to witness an arising in the development of occupational diseases such as allergic contact dermatitis and respiratory sensitization. Respiratory sensitization is a type I hypersensitivity reaction and has been associated with preferential induction of a Th2 population of T lymphocytes. Th2 cells produce high amounts of IL-4, IL-10 and IL-13, favoring humoral immune function with stimulation and differentiation of B cells to produce IgE. On the other hand, allergic contact dermatitis is a type IV hypersensitivity reaction associated with the induction of a Th1 response. Th1 cells produce high amounts of IL-2, IFN-γ and TNF-β. Interestingly, IFN-γ produced by Th1 cells also antagonizes Th2 cell responses and the production of IgE, while IL-4 produced by Th2 cells antagonizes the development of Th1 cells. As stated earlier, it seems that the immune responses induced by contact and respiratory sensitizers begin to diverge in a qualitative sense only after the initial activation of T lymphocytes (Cochrane *et al*., 2015). Indeed, the keystone in the pathogenesis of these diseases is the chemical-induced activation and maturation of DCs. DCs maturation was shown to be strongly dependent on initial danger signals such as ROS, uric acid, hyaluronic acid fragments and ATP (Corsini *et al*., 2013). Also, thiol depletion causes the activation of intracellular pathways such as p38 MAPK, ultimately leading to phenotypical and function changes that characterize DCs maturation (Kagatani *et al*., 2010) Despite the intense research about respiratory and contact sensitization, there's still a continuous uncertainty regarding the mechanism behind the different immunological responses elicited by these chemicals. Indeed, is of the uttermost importance to unveil the danger signals elicited by both kind of sensitizers, which could be related to a specific DC maturation profile and differential T cell polarization.

In order to fill this gap, the herein presented work aimed to identify and characterize the DAMPs elicited by respiratory and contact sensitizers, using THP-1 cells as a surrogate for DC. In a first approach, the nature and kinetics of ROS production elicited by contact and respiratory sensitizers were evaluated. THP-1 cells were incubated with the contact sensitizer DNFB, the respiratory sensitizer TMAC, the respiratory and contact irritant MeSA and bacterial LPS as a non-allergen immunogenic stimulus, and time course modifications of ROS production, cellular antioxidant defenses, modulation of MAPKs signaling pathways and transcription of pathophysiological relevant genes were

addressed. Similar to results obtained by Trompezinski *et al*., only sensitizers induced ROS production (Trompezinski *et al*., 2008), although with temporal and intensity differences. The contact sensitizer DNFB, which is highly electrophilic and readily reacts with thiol groups, rapidly reacted with GSH, inactivating it and subsequently leading to an increase in overall ROS. In contrast, TMAC, which mainly reacts with amine groups, induced a delayed GSH depletion at least in part due to an also later increase in mtROS and xanthine oxidase activity. Indeed, it is widely believed that strong contact sensitizers covalently bind to thiol or amino protein groups, with several studies reporting the maturation of DCs by DNCB, a structural analogue of DNFB, as a consequence of glutathione depletion (Becker *et al*., 2003; Bruchhausen *et al*., 2003; Mizuashi *et al*., 2005). Accordingly, just after 15 min exposure, DNCB depletes approximately 45% of intracellular GSH (Pickard *et al*., 2007). Interestingly, despite the different kinetics of ROS production elicited by TMAC and DNFB, p38 and JNK MAPKs showed similar activation profiles, indicating that besides inducing danger signals, chemical sensitizers directly interact with cellular proteins, modulating intracellular signaling events involved in DC maturation. Indeed, allergens can activate the immune response through direct interaction with specific receptors (e.g. TLR2 and TLR4) or signaling pathways, or indirectly, through formation/release of endogenous ligands such as DAMPs (Martin, 2017). Contrary to MAPKs activation profile, Nrf2-dependent gene expression (*HMOX* and *NQO1*) correlates with ROS kinetics. Besides inducing ROS production at early timepoints, DNFB also has a strong and direct reactivity toward the Cys residues on Keap1, a repressor protein that binds to Nrf2 and promotes its degradation by the ubiquitin proteasome pathway, which together may account for the early and marked induced expression of Nrf2-depended genes. Indeed, one of the approved test to address skin sensitization relies on Nrf2-Keap1-ARE activation (OECD, 2018 b). TMAC later induction, on the other hand, is probably solely caused by cellular oxidative stress. DNFB also showed to induce a rapidly and robust transcription of *IL1B*, *IL8* and *IL18* that was preserved overtime. Therefore, our data suggest that skin sensitizers-induced Th1 polarization results from a sustained transcription of pro-inflammatory cytokines and co-stimulatory molecules in DCs, while Th2 polarization is characterized by a modest and transitory transcription.

In a second approach, we aimed to characterize the mechanisms behind thiol-reactive-induced inflammasome activation. As stated before, danger signals regulate DC

function and are responsible for linking innate and adaptive immunity (Nace *et al*., 2011). These same danger signals are also responsible for inflammasome activation, namely NLRP3 inflammasome. Indeed, several studies document the key role of inflammasome activation in the development of ACD or contact hypersensitization (Watanabe *et al*., 2007, 2008; Chen *et al*., 2019). Furthermore, a growing body of evidence suggest a role of the inflammasome-linked cytokines IL-1β and IL-18 in asthma. Despite several studies have showing that both IL-1β, IL-18 and caspase 1 levels were increased in asthmatic patients, compared to healthy subjects, the role of inflammasomes on respiratory sensitization to low molecular weight chemicals remains incomplete. Many studies highlight the central role for caspase-1 in the pathogenesis of asthma. Indeed, mice treated with TDI show increased levels of caspase-1 as well as increased amounts of neutrophils and eosinophils and up-regulation of IL-1β (Liang *et al*., 2015). Blockage of IL-1β activity through deletion of the IL-1 receptor type 1 or administration of neutralizing antibody revokes the progression of TDI-induced asthma (Johnson *et al*., 2005). In line with these observations, IL-18 deficient mice also show decreased neutrophilic airway inflammation and remodeling in OVA-induced asthma (Yamagata *et al*., 2008). Furthermore, pre-treatment of TDI-asthmatic mice with caspase or NLRP3 inhibitors leads to a dramatic reduction in airway hyperresponsiveness, airway inflammation and remodeling as well as a decreased Th2 response and lower levels of IL-1β and IL-18 (Chen *et al*., 2019).

Although being an important inflammasome component, it is unclear whether NLRP3 is involved in other immune functions. Bruchard *et al*. showed that NLRP3 is expressed during the differentiation of $CD4^+$ T cells and is specifically involved in the polarization of Th2 cells. Although naive T cells did not express NLRP3, $CD4^+$ T cells polarized into Th0, Th1 and Th2 cells present a modest, though detectable, amounts of NLRP3 protein. The authors also demonstrated that $Nlrp3^{-/-}$ Th2 cells secreted less IL-4 than did wild-type Th2 cells, whereas $Nlrp3^{-/-}$ and WT Th1 cells secreted similar amounts of IFN-γ. Furthermore, Th2 response was impaired in Nlrp3-deficient mice, but not in Casp-1 or Asc-deficient mice, suggesting that NLRP3 was linked to Th2 response independently of inflammasome activation. Indeed, in Th2 cells, NLRP3 localizes in the nucleus, functioning as a transcription factor, where it binds to promoter regions of Th2 cell–related genes (Bruchard *et al*., 2015).

Even though inflammasome activation has an already well-established role in CHS development, the clarification of the axis skin allergens-danger signals- NLRP3

inflammasome activation remains to be elucidated. Additionally, the exact molecular mechanisms triggering sensitizer-induced inflammasome assembly remain largely unknown. To the best of our knowledge, this is the first work linking lysosomal destabilization to sensitizer-induced inflammasome activation. Indeed, inflammasome activation by contact sensitizers is often linked to extracellular ATP (Martin *et al*., 2011) while lysosomal destabilization is often associated with internalization of particulate matter by phagosomes (e.g. MSU, silica, asbestos, amyloid deposits and aluminium salts) or peroxidation of lysosome membrane lipids by ROS (Hornung *et al*., 2008; Willingham *et al*., 2008). Our results show that THP-1 cells lacking NLRP3 or ASC are unable to secrete IL-1ß in response to DNFB. Accordingly, mice lacking NLRP3 or the adaptor protein ASC show impaired CHS responses to TNCB and DNFB (Watanabe *et al*., 2007, 2008) and caspase-1 or IL-1R deficiency as well as treatment with the IL-1R antagonist anakinra, were also shown to prevent CHS (Antonopoulos *et al*., 2001; Watanabe *et al*., 2007, 2008; Weber *et al*., 2010 a). We also demonstrate that DNFB induces lysosomal destabilization and consequent cathepsin B leakage both in monocytes, macrophages, keratinocytes and human moDCs. Pre-treatment of DCs with a cathepsin B inhibitor abrogated DNFB-induced IL-1ß release and dendritic cells maturation. Interestingly, Schwenck and colleagues recently showed the importance of cathepsin B in the effector phase of cutaneous delayed-type hypersensitivity reactions to the sensitizer 2,4,6-trinitrochlorobenzene (TNCB). To elucidate the therapeutic impact of specifically targeting cathepsin B, the authors treated mice with the selective cathepsin B inhibitor CA-074 before TNCB challenge (elicitation phase). They observed a significant decrease in the ear swelling of sensitized mice compared with sham-treated mice, accompanied by reductions in ear thickness, edema hyperkeratosis, acanthosis and inflammatory cell infiltration (Schwenck *et al*., 2019). Chen and colleagues also reported that, in endothelial dysfunction during coronary arteritis by *Lactobacillus casei,* NLRP3 inflammasome is activated by cathepsin B in consequence of lysosome permeabilization. Furthermore, *Nlrp3* gene silencing or lysosome membrane stabilizing agents such as colchicine, dexamethasone, and ceramide blocked NLRP3 inflammasome formation, caspase-1 activation and IL-1ß production in mouse vascular endothelial cells (Chen *et al*., 2015).

Alongside DNFB, we also addressed the effects of the respiratory sensitizers TMAC and HDI, the non-sensitizer lactic acid (LA) and the skin sensitizers 4-nitrobenzylbromide, oxazolone and methylisothiazolinone on lysosomal integrity. While

skin sensitizers promoted lysosomal destabilization, TMAC and LA failed to do so. The chemicals with higher reactivity toward thiol groups showed to induce lysosomal destabilization, although not all induced IL-1β secretion. Only the chemicals with a higher ratio FL2/FL4 showed to induce IL-1β secretion. This leads us to hypothesize that only a critical loss of lysosomal integrity leads to inflammasome activation. Interestingly, Katnelson and colleagues show contradictory results. Using the soluble lysosomotropic agent Leu-Leu-O-methyl ester (LLME), the authors showed that, in murine BMDCs, partial increases in lysosome membrane permeabilization correlates with robust NLRP3 inflammasome activation and K^+ efflux while extremely rapid and complete collapse of lysosome integrity correlates with suppression of inflammasome signaling (Katsnelson *et al*., 2016). Although, the different type of chemicals tested, as well as the cell models used, could be responsible for the different outcomes. Therefore, it is of relevance to extend the panel of tested sensitizers to a large number of chemicals with different reactivity towards thiol and lysine residues. Furthermore, it will be interesting to further validate the obtained results using *in vivo* models through noninvasive optical imaging using protease-activatable.

CHAPTER 5

Conclusions/Future Perspectives

CONCLUSIONS/FUTURE PERSPECTIVES

The occurrence of allergic diseases has increased over the past decades and remains a burden on human health. Indeed, and although many types of allergy are caused mainly by proteins, LMW chemicals also contribute to allergic disease, namely ACD and occupational asthma. It is estimated that 20% of North America and Western Europe population is allergic to at least one chemical (Ainscough *et al*., 2013). Several studies have been published addressing the cellular and molecular events evoked by skin and respiratory sensitizers, although, the mechanism behind ACD pathophysiology remains better-defined. The full ban on animal testing in 2013 emphasized the urgency to understand the molecular mechanisms behind these pathologies and the identification of molecules that could be used to identify skin and respiratory sensitizers. In accordance to the recent toxicology paradigm defined by the United States National Research Council 'Toxicity testing in the 21st century", molecular toxicity pathways will be the key building blocks of new testing strategies. Herein, we demonstrated that skin and respiratory sensitizers induce the production of ROS, although with different kinetics and origins, which then conditions the transcription kinetics of genes involved in DC maturation. We also demonstrated that thiol-reactive sensitizers induce lysosomal destabilization, one of the canonical activators of NLRP3 inflammasome. Furthermore, to the best of our knowledge, this is the first study showing that thiol-reactive sensitizers induce NLRP3 inflammasome activation through lysosomal destabilization and cathepsin leakage.

Overall, the present study brought new insights about the DAMPs and molecular pathways differentially elicited by skin and respiratory sensitizers, which preferentially react with thiol and amine groups, respectively. Furthermore, in order to obtain a direct correlation between inflammasome activation by lysosomal disruption with specific classes of sensitizers or sensitizer's reactivity, it would be of interest to expand the panel of allergens tested. Importantly, the identification of the immunoprecipitated DNFB-haptenated proteins by mass spectrometry would help to uncover the mechanism behind chemical-induced lysosomal destabilization. Additionally, we intend to validate our *in vitro* results using a cathepsin B inhibitor on a mouse model of hypersensitivity, thus opening new avenues for the development of novel drugs for the treatment of ACD. Last, but not least, the identification of new molecular targets for skin and respiratory sensitization could be included in the development/optimization of alternative tests to identify hazard

and potency of possible chemicals sensitizers, as demanded by the new European legislation.

CHAPTER 6

References

REFERENCES

Adam, C.; Wohlfarth, J.; Haußmann, M.; Sennefelder, H.; Rodin, A.; Maler, M.; Martin, S. F.; Goebeler, M.; Schmidt, M., 2017: Allergy-Inducing Chromium Compounds Trigger Potent Innate Immune Stimulation Via ROS-Dependent Inflammasome Activation. *Journal of Investigative Dermatology*., **137**, 367–376.

Adams, E. J., 2014: Lipid presentation by human CD1 molecules and the diverse T cell populations that respond to them. *Current Opinion in Immunology*., **26**, 1–6.

Ade, N.; Antonios, D.; Kerdine-romer, S.; Boisleve, F.; Rousset, F.; Pallardy, M., 2007: NF-κB plays a major role in the maturation of human dendritic cells induced by NiSO4 but not by DNCB. *Toxicological Sciences*., **99**, 488–501.

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. *Toxicological Sciences*., **107**, 451–460.

Aggarwal, N.; Sloane, B. F., 2014: Cathepsin B: Multiple roles in cancer. *Proteomics - Clinical Applications*., **8**, 427–437.

Agner, L. E.; Martins, M.; Manoel, V.; Dos Reis, S., 2011: Immunopathology of allergic contact dermatitis. *Anais Brasileiros de Dermatologia*., **86**, 419–452.

Aiba, S.; Manome, H.; Nakagawa, S.; Mollah, Z. U. A.; Mizuashi, M.; Ohtani, T.; Yoshino, Y.; 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. *The Journal of Investigative Dermatology*., **120**, 390–399.

Aiba, S., 2007: Dendritic cells - Importance in allergy. *Allergology International*., **56**, 201–208.

Ainscough, J. S.; Frank Gerberick, G.; Dearman, R. J.; Kimber, I., 2013: Danger, intracellular signaling, and the orchestration of dendritic cell function in skin sensitization. *Journal of Immunotoxicology*., **10**, 223–234.

Allam, R.; Lawlor, K. E.; Yu, E. C. W.; Mildenhall, A. L.; Moujalled, D. M.; Lewis, R. S.; Ke, F.; Mason, K. D.; White, M. J.; Stacey, K. J.; Strasser, A.; O'Reilly, L. A.; Alexander, W.; Kile, B. T.; Vaux, D. L.; Vince, J. E., 2014: Mitochondrial apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming. *EMBO reports*., **15**, 982–990.

Alvarez, D.; Vollmann, E. H.; von Andrian, U. H., 2008: Mechanisms and Consequences of Dendritic Cell Migration. *Immunity*., **29**, 325–342.

Amigorena, S.; Savina, A., 2010: Intracellular mechanisms of antigen cross presentation in dendritic cells. *Current Opinion in Immunology*., **22**, 109–117.

Antonios, D.; Ade, N.; Kerdine-Römer, S.; Assaf-Vandecasteele, H.; Larangé, A.; Azouri, H.; Pallardy, M., 2009: Metallic haptens induce differential phenotype of human dendritic cells through activation of mitogen-activated protein kinase and NF-κB pathways. *Toxicology in Vitro*., **23**, 227–234.

Antonios, D.; Rousseau, P.; Larangé, A.; Kerdine-Römer, S.; Pallardy, M., 2010: Mechanisms of IL-12 Synthesis by Human Dendritic Cells Treated with the Chemical Sensitizer NiSO 4 . *The Journal of Immunology*., **185**, 89–98.

Antonopoulos, C.; Cumberbatch, M.; Dearman, R. J.; Daniel, R. J.; Kimber, I.; Groves, R. W., 2001: Functional Caspase-1 Is Required for Langerhans Cell Migration and Optimal Contact Sensitization in Mice. *The Journal*

of Immunology., **166**, 3672–3677.

Aptula, A. O.; Roberts, D. W.; Pease, C. K., 2007: Haptens, prohaptens and prehaptens, or electrophiles and proelectrophiles. *Contact Dermatitis*., **56**, 54–56.

Arias, M.; Zabaleta, J.; Rodríguez, J. I.; Rojas, M.; París, S. C.; García, L. F., 1997: Failure to induce nitric oxide production by human monocyte-derived macrophages. Manipulation of biochemical pathways. *Allergologia et Immunopathologia*., **25**, 280–288.

Arkusz, J.; StEogonekpnik, M.; Sobala, W.; Dastych, J., 2010: Prediction of the contact sensitizing potential of chemicals using analysis of gene expression changes in human THP-1 monocytes. *Toxicology Letters*., **199**, 51–59.

Arrighi, J. F.; Rebsamen, M.; Rousset, F.; Kindler, V.; Hauser, C., 2001: A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. *The Journal of Immunology*., **166**, 3837–3845.

Arthur, J. C.; Lich, J. D.; Ye, Z.; Allen, I. C.; Gris, D.; Wilson, J. E.; Schneider, M.; Roney, K. E.; O'Connor, B. P.; Moore, C. B.; Morrison, A.; Sutterwala, F. S.; Bertin, J.; Koller, B. H.; Liu, Z.; Ting, J. P. Y., 2010: Cutting edge: NLRP12 controls dendritic and myeloid cell migration to affect contact hypersensitivity. *Journal of Immunology*., **185**, 4515–4519.

Ashikaga, T.; Hoya, M.; Itagaki, H.; Katsumura, Y.; Aiba, S., 2002: Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive endpoints for contact sensitizers. *Toxicology in Vitro*., **16**, 711–716.

Banchereau, J.; Briere, F.; Caux, C.; Davoust, J.; Lebecque, S.; Liu, Y. J.; Pulendran, B.; Palucka, K., 2000: Immunobiology of dendritic cells. *Annual Review of Immunology*., **18**, 767–811.

Baur, X.; Aasen, T. B.; Sherwood Burge, P.; Heederik, D.; Henneberger, P. K.; Maestrellie, P.; Schlünssen, V.; Vandenplas, O.; Wilken, D., 2012: The management of work-related asthma guidelines: A broader perspective. *European Respiratory Review*., **21**, 125–139.

Becker, D.; Valk, E.; Zahn, S.; Brand, P.; Knop, J., 2003: Coupling of contact sensitizers to thiol groups is a key event for the activation of monocytes and monocyte-derived dendritic cells. *Journal of Investigative Dermatology*., **120**, 233–238.

Bengsch, F.; Buck, A.; Günther, S. C.; Seiz, J. R.; Tacke, M.; Pfeifer, D.; Von Elverfeldt, D.; Sevenich, L.; Hillebrand, L. E.; Kern, U.; Sameni, M.; Peters, C.; Sloane, B. F.; Reinheckel, T., 2014: Cell type-dependent pathogenic functions of overexpressed human cathepsin B in murine breast cancer progression. *Oncogene*., **33**, 4474–4484.

Bhatia, J.; Sarin, A.; Wollina, U.; Lotti, T.; Navarini, A. A.; Mueller, S. M.; Grabbe, S.; Saloga, J.; Rokni, G. R.; Goldust, M., 2020: Review of biologics in allergic contact dermatitis. *Contact Dermatitis*., **83**, 179–181.

Birben, E.; Sahiner, U. M.; Sackesen, C.; Erzurum, S.; Kalayci, O., 2012: Oxidative stress and antioxidant defense. *The World Allergy Organization Journal*., **5**, 9–19.

Blanco, P.; Palucka, A. K.; Pascual, V.; Banchereau, J., 2008: Dendritic cells and cytokines in human inflammatory and autoimmune diseases. *Cytokine & Growth Factor Reviews*., **19**, 41–52.

Boislève, F.; Kerdine-Römer, S.; Pallardy, M., 2005: Implication of the MAPK pathways in the maturation of human dendritic cells induced by nickel and TNF-alpha. *Toxicology*., **206**, 233–244.

Boukamp, P.; Petrussevska, R. T.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig, N. E., 1988: Normal

keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *Journal of Cell Biology*., **106**, 761–771.

Brand, P.; Plochmann, S.; Valk, E.; Zahn, S.; Saloga, J.; Knop, J.; Becker, D., 2002: Activation and translocation of p38 mitogen-activated protein kinase after stimulation of monocytes with contact sensitizers. *The Journal of Investigative Dermatology*., **119**, 99–106.

Bruchard, M.; Mignot, G.; Derangère, V.; Chalmin, F.; Chevriaux, A.; Végran, F.; Boireau, W.; Simon, B.; Ryffel, B.; Connat, J. L.; Kanellopoulos, J.; Martin, F.; Rébé, C.; Apetoh, L.; Ghiringhelli, F., 2013: Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nature Medicine*., **19**, 57–64.

Bruchard, M.; Rebé, C.; Derangère, V.; Togbé, D.; Ryffel, B.; Boidot, R.; Humblin, E.; Hamman, A.; Chalmin, F.; Berger, H.; Chevriaux, A.; Limagne, E.; Apetoh, L.; Végran, F.; Ghiringhelli, F., 2015: The receptor NLRP3 is a transcriptional regulator of TH2 differentiation. *Nature Immunology*., **16**, 859–870.

Bruchhausen, S.; Zahn, S.; Valk, E.; Knop, J.; Becker, D., 2003: Thiol antioxidants block the activation of antigen-presenting cells by contact sensitizers. *Journal of Investigative Dermatology*., **121**, 1039–1044.

Buters, J.; Biedermann, T., 2017: Chromium(VI) Contact Dermatitis: Getting Closer to Understanding the Underlying Mechanisms of Toxicity and Sensitization! *Journal of Investigative Dermatology*., **137**, 274–277.

Büth, H.; Luigi Buttigieg, P.; Ostafe, R.; Rehders, M.; Dannenmann, S. R.; Schaschke, N.; Stark, H. J.; Boukamp, P.; Brix, K., 2007: Cathepsin B is essential for regeneration of scratch-wounded normal human epidermal keratinocytes. *European Journal of Cell Biology*., **86**, 747–761.

Byamba, D.; Kim, T. G.; Kim, D. H.; Je, J. H.; Lee, M. G. G.; Byamba, D.; Gyun Kim, T.; Hyun Kim, D.; Hwan Je, J., 2010: The roles of reactive oxygen species produced by contact allergens and irritants in monocyte-derived dendritic cells. *Annals of Dermatology*., **22**, 269–278.

Calmeiro, J.; Carrascal, M.; Gomes, C.; Falcão, A.; Cruz, M. T.; Neves, B. M., 2018: Highlighting the Role of DC-NK Cell Interplay in Immunobiology and Immunotherapy. In: Chapoval, S. P. (ed.), *Dendritic Cells*. IntechOpen.

Campden, R. I.; Zhang, Y., 2019: The role of lysosomal cysteine cathepsins in NLRP3 inflammasome activation. *Archives of Biochemistry and Biophysics*., **670**, 32–42.

Cartier, A.; Bernstein, D. I., 2019: Occupational asthma: Definitions, epidemiology, causes, and risk factors. In: Post, T. W. (ed.), *UpToDate*. UpToDate, Waltham, MA.

Chary, A.; Hennen, J.; Klein, S. G.; Serchi, T.; Gutleb, A. C.; Blömeke, B., 2018: Respiratory sensitization: toxicological point of view on the available assays. *Archives of Toxicology*., **92**, 803–822.

Chen, S.; Yao, L.; Huang, P.; He, Q.; Guan, H.; Luo, Y.; Zou, Z.; Wei, S.; Peng, G.; Yan, J.; Chen, R.; Zhang, Q.; Tao, A., 2019: Blockade of the NLRP3/Caspase-1 Axis Ameliorates Airway Neutrophilic Inflammation in a Toluene Diisocyanate-Induced Murine Asthma Model. *Toxicological Sciences*., **170**, 462–475.

Chen, Y.; Li, X.; Boini, K. M.; Pitzer, A. L.; Gulbins, E.; Zhang, Y.; Li, P. L., 2015: Endothelial Nlrp3 inflammasome activation associated with lysosomal destabilization during coronary arteritis. *Biochimica et Biophysica Acta - Molecular Cell Research*., **1853**, 396–408.

Chipinda, I.; Hettick, J. M.; Siegel, P. D., 2011: Haptenation: Chemical Reactivity and Protein Binding. *Journal of Allergy*., **2011**, 1–11.

Choi, G. S.; Trinh, H. K. T.; Yang, E. M.; Ye, Y. M.; Shin, Y. S.; Kim, S. H.; Park, H. S., 2018: Role of

clusterin/progranulin in toluene diisocyanate-inducedoccupational asthma. *Experimental & Molecular Medicine*., **50**, 1–10.

Cochrane, S. A.; Arts, J. H. E.; Ehnes, C.; Hindle, S.; Hollnagel, H. M.; Poole, A.; Suto, H.; Kimber, I., 2015: Thresholds in chemical respiratory sensitisation. *Toxicology*., **333**, 179–194.

Collin, M.; Bigley, V., 2018: Human dendritic cell subsets: an update. *Immunology*., **154**, 3–20.

Corsini, E.; Mitjans, M.; Galbiati, V.; Lucchi, L.; Galli, C. L.; Marinovich, M., 2009: Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicology in Vitro*., **23**, 789–796.

Corsini, E.; Galbiati, V.; Nikitovic, D.; Tsatsakis, A. M., 2013: Role of oxidative stress in chemical allergens induced skin cells activation. *Food and Chemical Toxicology*., **61**, 74–81.

Cosentino-Gomes, D.; Rocco-Machado, N.; Meyer-Fernandes, J. R., 2012: Cell signaling through protein kinase C oxidation and activation. *International Journal of Molecular Sciences*., **13**, 10697–10721.

Cox, D. P.; Cardozo-Pelaez, F., 2007: High throughput method for assessment of cellular reduced glutathione in mammalian cells. *Journal of Environmental Protection Science*., **1**, 23–28.

Cumberbatch, M.; Dearman, R. J.; Antonopoulos, C.; Groves, R. W.; Kimber, I., 2001: Interleukin (IL)-18 induces Langerhans cell migration by a tumour necrosis factor-alpha- and IL-1beta-dependent mechanism. *Immunology*., **102**, 323–330.

Curtsinger, J. M.; Johnson, C. M.; Mescher, M. F., 2003: CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *Journal of Immunology*., **171**, 5165–5171.

Das, J.; Chen, C. H.; Yang, L.; Cohn, L.; Ray, P.; Ray, A., 2001: A critical role for NF-κB in Gata3 expression and TH2 differentiation in allergic airway inflammation. *Nature Immunology*., **2**, 45–50.

Davis, B. K.; Wen, H.; Ting, J. P. Y., 2011: The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annual Review of Immunology*., **29**, 707–735.

De Smedt, A. C.; Van Den Heuvel, R. L.; Zwi Berneman, N.; Schoeters, G. E., 2001: Modulation of phenotype, cytokine production and stimulatory function of CD34+-derived DC by NiCl(2) and SDS. *Toxicology in Vitro*., **15**, 319–325.

Dearman, R. J.; Betts, C. J.; Humphreys, N.; Flanagan, B. F.; Gilmour, N. J.; Basketter, D. A.; Kimber, I., 2003: Chemical allergy: considerations for the practical application of cytokine profiling. *Toxicological Sciences*., **71**, 137–145.

Dhingra, N.; Gulati, N.; Guttman-Yassky, E., 2013: Mechanisms of contact sensitization offer insights into the role of barrier defects vs. intrinsic immune abnormalities as drivers of atopic dermatitis. *Journal of Investigative Dermatology*., **133**, 2311–2314.

Di Virgilio, F., 2007: Liaisons dangereuses: P2X7 and the inflammasome. *Trends in Pharmacological Sciences*., **28**, 465–472.

Diepgen, T. L.; Ofenloch, R. F.; Bruze, M.; Bertuccio, P.; Cazzaniga, S.; Coenraads, P. J.; Elsner, P.; Goncalo, M.; Svensson, Å.; Naldi, L., 2016: Prevalence of contact allergy in the general population in different European regions. *British Journal of Dermatology*., **174**, 319–329.

Dinkova-Kostova, A. T.; Holtzclaw, W. D.; Kensler, T. W., 2005: The role of Keap1 in cellular protective

responses. *Chemical Research in Toxicology*., **18**, 1779–1791.

dos Santos, G. G.; Reinders, J.; Ouwehand, K.; Rustemeyer, T.; Scheper, R. J.; Gibbs, S., 2009: Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound. *Toxicology and Applied Pharmacology*., **236**, 372–382.

Dowling, J. K.; Dellacasagrande, J., 2016: Toll-like receptors: Ligands, cell-based models, and readouts for receptor action. *Methods in Molecular Biology*. Humana Press Inc., Vol. 1390pp. 3–27.

Duewell, P.; Kono, H.; Rayner, K. J.; Sirois, C. M.; Vladimer, G.; Bauernfeind, F. G.; Abela, G. S.; Franchi, L.; Nũez, G.; Schnurr, M.; Espevik, T.; Lien, E.; Fitzgerald, K. A.; Rock, K. L.; Moore, K. J.; Wright, S. D.; Hornung, V.; Latz, E., 2010: NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*., **464**, 1357–1361.

Dumitriu, I. E.; Baruah, P.; Valentinis, B.; Voll, R. E.; Herrmann, M.; Nawroth, P. P.; Arnold, B.; Bianchi, M. E.; Manfredi, A. A.; Rovere-Querini, P., 2005: Release of High Mobility Group Box 1 by Dendritic Cells Controls T Cell Activation via the Receptor for Advanced Glycation End Products. *The Journal of Immunology*., **174**, 7506–7515.

Eisenbarth, S. C.; Colegio, O. R.; O'Connor, W.; Sutterwala, F. S.; Flavell, R. A., 2008: Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*., **453**, 1122–1126.

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. *Toxicological Sciences*., **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. *Toxicology and Applied Pharmacology*., **245**, 281–290.

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

Esser, P. R.; Martin, S. F., 2017: Pathomechanisms of Contact Sensitization. *Current Allergy and Asthma Reports*., **17**, 1–10.

Felbor, U.; Kessler, B.; Mothes, W.; Goebel, H. H.; Ploegh, H. L.; Bronson, R. T.; Olsen, B. R., 2002: Neuronal loss and brain atrophy in mice lacking cathepsins B and L. *Proceedings of the National Academy of Sciences of the United States of America*., **99**, 7883–7888.

Ferrari, D.; Pizzirani, C.; Adinolfi, E.; Lemoli, R. M.; Curti, A.; Idzko, M.; Panther, E.; Di Virgilio, F., 2006: The P2X 7 Receptor: A Key Player in IL-1 Processing and Release. *The Journal of Immunology*., **176**, 3877–3883.

Ferreira, I.; Silva, A.; Martins, J. D.; Neves, B. M.; 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 Biology*., **16**, 75–86.

Franchi, L.; Eigenbrod, T.; Muñoz-Planillo, R.; Nuñez, G., 2009: The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nature immunology*., **10**, 241–247.

Frank, S.; Zacharowski, K.; Wray, G. M.; Thiemermann, C.; Pfeilschifter, J., 1999: Identification of copper/zinc superoxide dismutase as a novel nitric oxide-regulated gene in rat glomerular mesangial cells and kidneys of endotoxemic rats. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*., **13**, 869–882.

Freudenberg, M. A.; Esser, P. R.; Jakob, T.; Galanos, C.; Martin, S. F., 2009: Innate and adaptive immune responses in contact dermatitis: Analogy with infections. *Giornale Italiano di Dermatologia e Venereologia*., **144**, 173–185.

Fyhrquist, N.; Lehto, E.; Lauerma, A., 2014: New findings in allergic contact dermatitis. *Current Opinion in Allergy and Clinical Immunology*., **14**, 430–435.

Gagliardo, R.; Chanez, P.; Mathieu, M.; Bruno, A.; Costanzo, G.; Gougat, C.; Vachier, I.; Bousquet, J.; Bonsignore, G.; Vignola, A. M., 2003: Persistent Activation of Nuclear Factor-κB Signaling Pathway in Severe Uncontrolled Asthma. *American Journal of Respiratory and Critical Care Medicine*., **168**, 1190–1198.

Galbiati, V.; Papale, A.; Galli, C. L.; Marinovich, M.; Corsini, E., 2014: Role of ROS and HMGB1 in contact allergen-induced IL-18 production in human keratinocytes. *Journal of Investigative Dermatology*., **134**, 2719–2727.

Galbiati, V.; Cornaghi, L.; Papale, A.; Donetti, E.; Marinovich, M.; Corsini, E., 2019: Study on the inflammasome nlrp3 and blimp-1/nlrp12 after keratinocyte exposure to contact allergens. *Toxicology Letters*., **313**, 130–136.

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. *Toxicological Sciences*., **97**, 417–427.

Gober, M.; Gaspari, A., 2008: Allergic contact dermatitis. *Current Directions in Autoimmunity*., **10**, 1–26.

Goebeler, M.; Trautmann, A.; Voss, A.; Bröcker, E. B.; Toksoy, A.; Gillitzer, R., 2001: Differential and sequential expression of multiple chemokines during elicitation of allergic contact hypersensitivity. *American Journal of Pathology*., **158**, 431–440.

Gonçalo, M.; Pinho, A.; Agner, T.; Andersen, K. E.; Bruze, M.; Diepgen, T.; Foti, C.; Giménez-Arnau, A.; Goossens, A.; Johanssen, J. D.; Paulsen, E.; Svedman, C.; Wilkinson, M.; Aalto-Korte, K., 2018: Allergic contact dermatitis caused by nail acrylates in Europe. An EECDRG study. *Contact Dermatitis*., **78**, 254–260.

Groves, E.; Dart, A. E.; Covarelli, V.; Caron, E., 2008: Molecular mechanisms of phagocytic uptake in mammalian cells. *Cellular and Molecular Life Sciences*., **65**, 1957–1976.

Guedes, S.; Neves, B.; Vitorino, R.; Domingues, R.; Cruz, M. T.; Domingues, P., 2017: Contact dermatitis: in pursuit of sensitizer's molecular targets through proteomics. *Archives of Toxicology*., **91**, 811–825.

Guilliams, M.; Ginhoux, F.; Jakubzick, C.; Naik, S. H.; Onai, N.; Schraml, B. U.; Segura, E.; Tussiwand, R.; Yona, S., 2014: Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nature Reviews Immunology*., **14**, 571–578.

Guo, H.; Callaway, J. B.; Ting, J. P. Y., 2015: Inflammasomes: Mechanism of action, role in disease, and therapeutics. *Nature Medicine*., **21**, 677–687.

Guo, H.; Liu, H.; Jian, Z.; Cui, H.; Fang, J.; Zuo, Z.; Deng, J.; Li, Y.; Wang, X.; Zhao, L.; Geng, Y.; Ouyang, P.; Lai, W.; Chen, Z.; Huang, C., 2019: Nickel induces inflammatory activation via NF-kB, MAPKs, IRF3 and NLRP3 inflammasome signaling pathways in macrophages. *Aging*., **11**, 11659–11672.

Halle, A.; Hornung, V.; Petzold, G. C.; Stewart, C. R.; Monks, B. G.; Reinheckel, T.; Fitzgerald, K. A.; Latz, E.; Moore, K. J.; Golenbock, D. T., 2008: The NALP3 inflammasome is involved in the innate immune response to amyloid-β. *Nature Immunology*., **9**, 857–865.

Halliday, G. M.; Lucas, A. D., 1993: Protein kinase C transduces the signal for Langerhans' cell migration

from the epidermis. *Immunology*., **79**, 621–626.

Harijith, A.; Ebenezer, D. L.; Natarajan, V., 2014: Reactive oxygen species at the crossroads of inflammasome and inflammation. *Frontiers in Physiology*., **5**, 352.

Hedley, D. W.; Chow, S., 1994: Evaluation of methods for measuring cellular glutathione content using flow cytometry. *Cytometry*., **15**, 349–358.

Helou, D. G.; Martin, S. F.; Pallardy, M.; Chollet-Martin, S.; Kerdine-Römer, S., 2019a: Nrf2 involvement in chemical-induced skin innate immunity. *Frontiers in Immunology*., **10**, 1004.

Helou, D. G.; Noël, B.; Gaudin, F.; Groux, H.; El Ali, Z.; Pallardy, M.; Chollet-Martin, S.; Kerdine-Römer, S., 2019b: Cutting Edge: Nrf2 Regulates Neutrophil Recruitment and Accumulation in Skin during Contact Hypersensitivity. *The Journal of Immunology*., **202**, 2189–2194.

Heo, J. H.; Heo, Y.; Lee, H. J.; Kim, M.; Shin, H. Y., 2018: Topical anti-inflammatory and anti-oxidative effects of porcine placenta extracts on 2,4-dinitrochlorobenzene-induced contact dermatitis. *BMC Complementary and Alternative Medicine*., **18**, 331.

Hoeffel, G.; Ripoche, A. C.; Matheoud, D.; Nascimbeni, M.; Escriou, N.; Lebon, P.; Heshmati, F.; Guillet, J. G.; Gannagé, M.; Caillat-Zucman, S.; Casartelli, N.; Schwartz, O.; De la Salle, H.; Hanau, D.; Hosmalin, A.; Marañón, C., 2007: Antigen crosspresentation by human plasmacytoid dendritic cells. *Immunity*., **27**, 481–492.

Holgate, S. T., 2012: Innate and adaptive immune responses in asthma. *Nature Medicine*., **18**, 673–683.

Honda, T.; Egawa, G.; Grabbe, S.; 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.

Hornung, V.; Bauernfeind, F.; Halle, A.; Samstad, E. O.; Kono, H.; Rock, K. L.; Fitzgerald, K. A.; Latz, E., 2008: Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature Immunology*., **9**, 847–856.

Hossenbaccus, L.; Linton, S.; Garvey, S.; Ellis, A. K., 2020: Towards definitive management of allergic rhinitis: Best use of new and established therapies. *Allergy, Asthma and Clinical Immunology*., **16**, 1–17.

Hulette, B. C.; Ryan, C. A.; Gildea, L. A.; Gerberick, G. F., 2005: Relationship of CD86 surface marker expression and cytotoxicity on dendritic cells exposed to chemical allergen. *Toxicology and Applied Pharmacology*., **209**, 159–166.

Humeniuk, P.; Dubiela, P.; Hoffmann-Sommergruber, K., 2017: Dendritic cells and their role in allergy: Uptake, proteolytic processing and presentation of allergens. *International Journal of Molecular Sciences*., **18**, 1491.

Idzko, M.; Hammad, H.; Van Nimwegen, M.; Kool, M.; Willart, M. A. M.; Muskens, F.; Hoogsteden, H. C.; Luttmann, W.; Ferrari, D.; Di Virgilio, F.; Virchow, J. C.; Lambrecht, B. N., 2007: Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nature Medicine*., **13**, 913–919.

Imbalzano, E.; Quartuccio, S.; Di Salvo, E.; Crea, T.; Casciaro, M.; Gangemi, S., 2017: Association between HMGB1 and asthma: A literature review. *Clinical and Molecular Allergy*., **15**, 1–9.

Ito, T.; Yang, M.; Wang, Y. H.; Lande, R.; Gregorio, J.; Perng, O. A.; Qin, X. F.; Liu, Y. J.; Gilliet, M., 2007: Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *The Journal of Experimental Medicine*., **204**, 105–115.

Jacquoilleot, S.; Sheffield, D.; Olayanju, A.; Sison-Young, R.; Kitteringham, N. R.; Naisbitt, D. J.; Aleksic, M., 2015: Glutathione metabolism in the HaCaT cell line as a model for the detoxification of the model sensitisers 2,4-dinitrohalobenzenes in human skin. *Toxicology Letters*., **237**, 11–20.

Jaworska, J.; Harol, A.; Kern, P. S.; Gerberick, G. F., 2011: Integrating non-animal test information into an adaptive testing strategy - skin sensitization proof of concept case. *ALTEX*., **28**, 211–225.

Jo, E. K.; Kim, J. K.; Shin, D. M.; Sasakawa, C., 2016: Molecular mechanisms regulating NLRP3 inflammasome activation. *Cellular and Molecular Immunology*., **13**, 148–159.

Johnson, V. J.; Yucesoy, B.; Luster, M. I., 2005: Prevention of IL-1 signaling attenuates airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *Journal of Allergy and Clinical Immunology*., **116**, 851–858.

Jongbloed, S. L.; Kassianos, A. J.; McDonald, K. J.; Clark, G. J.; Ju, X.; Angel, C. E.; Chen, C. J. J.; Dunbar, P. R.; Wadley, R. B.; Jeet, V.; Vulink, A. J. E.; Hart, D. N. J.; Radford, K. J., 2010: Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *Journal of Experimental Medicine*., **207**, 1247–1260.

Kadowaki, N.; Ho, S.; Antonenko, S.; De Waal Malefyt, R.; Kastelein, R. A.; Bazan, F.; Liu, Y. J., 2001: Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *Journal of Experimental Medicine*., **194**, 863–869.

Kagatani, S.; Sasaki, Y.; Hirota, M.; Mizuashi, M.; Suzuki, M.; Ohtani, T.; Itagaki, H.; Aiba, S., 2010: Oxidation of cell surface thiol groups by contact sensitizers triggers the maturation of dendritic cells. *Journal of Investigative Dermatology*., **130**, 175–183.

Kaiko, G. E.; Horvat, J. C.; Beagley, K. W.; Hansbro, P. M., 2008: Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology*., **123**, 326–338.

Kanitakis, J.; Morelon, E.; Petruzzo, P.; Badet, L.; Dubernard, J. M., 2011: Self-renewal capacity of human epidermal Langerhans cells: observations made on a composite tissue allograft. *Experimental Dermatology*., **20**, 145–146.

Kaplan, D. H.; Igyártó, B. Z.; Gaspari, A. A., 2012: Early immune events in the induction of allergic contact dermatitis. *Nature Reviews Immunology*., **12**, 114–124.

Kapsenberg, M. L., 2003: Dendritic-cell control of pathogen-driven T-cell polarization. *Nature Reviews Immunology*., **3**, 984–993.

Kashem, S. W.; Igyártó, B. Z.; Gerami-Nejad, M.; Kumamoto, Y.; Mohammed, J.; Jarrett, E.; Drummond, R. A.; Zurawski, S. M.; Zurawski, G.; Berman, J.; Iwasaki, A.; Brown, G. D.; Kaplan, D. H., 2015: Candida albicans morphology and dendritic cell subsets determine T helper cell differentiation. *Immunity*., **42**, 356–366.

Kastenmuller, W.; Gasteiger, G.; Subramanian, N.; Sparwasser, T.; Busch, D. H.; Belkaid, Y.; Drexler, I.; Germain, R. N., 2011: Regulatory T cells selectively control CD8+ T cell effector pool size via IL-2 restriction. *Journal of Immunology*., **187**, 3186–3197.

Katoh, S.; Matsumoto, N.; Kawakita, K.; Tominaga, A.; Kincade, P. W.; Matsukura, S., 2003: A role for CD44 in an antigen-induced murine model of pulmonary eosinophilia. *Journal of Clinical Investigation*., **111**, 1563–1570.

Katsnelson, M. A.; Lozada-Soto, K. M.; Russo, H. M.; Miller, B. A.; Dubyak, G. R., 2016: NLRP3 inflammasome signaling is activated by low-level lysosome disruption but inhibited by extensive lysosome disruption: Roles for K+ efflux and Ca2+ influx. *American Journal of Physiology-Cell Physiology*., **311**, C83–C100.

Kavasi, R. M. M.; Berdiaki, A.; Spyridaki, I.; Papoutsidakis, A.; Corsini, E.; Tsatsakis, A.; Tzanakakis, G. N.; Nikitovic, D., 2019: Contact allergen (PPD and DNCB)-induced keratinocyte sensitization is partly mediated through a low molecular weight hyaluronan (LMWHA)/TLR4/NF-κB signaling axis. *Toxicology and Applied Pharmacology*., **377**, 114632.

Kimber, I.; Dearman, R. J., 1997: Chemical respiratory hypersensitivity: an introduction. *Toxicology of Chemical Respiratory Hypersensitivity*. Taylor & Francis London, pp. 1–6.

Kimber, I.; Dearman, R. J., 2005: What makes a chemical a respiratory sensitizer? *Current Opinion in Allergy and Clinical Immunology*., **5**, 119–124.

Kimber, I.; Basketter, D. A.; Gerberick, G. F.; Ryan, C. A.; Dearman, R. J., 2011: Chemical allergy: Translating biology into hazard characterization. *Toxicological Sciences*., **120**, S238–S268.

Kimber, I.; Basketter, D. A.; Thyssen, J. P.; Dearman, R. J.; McFadden, J. P., 2014: Chemical allergy in humans: Fresh perspectives. *Journal of Immunotoxicology*., **11**, 203–204.

Kimber, I.; Poole, A.; Basketter, D. A., 2018: Skin and respiratory chemical allergy: confluence and divergence in a hybrid adverse outcome pathway. *Toxicology Research*., **7**, 586–605.

Kissenpfennig, A.; Henri, S.; Dubois, B.; Laplace-Builhé, C.; Perrin, P.; Romani, N.; Tripp, C. H.; Douillard, P.; Leserman, L.; Kaiserlian, D.; Saeland, S.; Davoust, J.; Malissen, B., 2005: Dynamics and function of langerhans cells in vivo: Dermal dendritic cells colonize lymph node areasdistinct from slower migrating langerhans cells. *Immunity*., **22**, 643–654.

Kobayashi, A.; Kang, M. I.; Watai, Y.; Tong, K. I.; Shibata, T.; Uchida, K.; Yamamoto, M., 2006: Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Molecular and Cellular Biology*., **26**, 221–229.

Koeper, L. M.; Schulz, A.; Ahr, H. J.; Vohr, H. W., 2007: In vitro differentiation of skin sensitizers by cell signaling pathways. *Toxicology*., **242**, 144–152.

Kondo, S.; Pastore, S.; Fujisawa, H.; Shivji, G. M.; McKenzie, R. C.; Dinarello, C. A.; Sauder, D. N., 1995: Interleukin-1 receptor antagonist suppresses contact hypersensitivity. *Journal of Investigative Dermatology*., **105**, 334–338.

Kong, B. Y.; Bolton, H.; Kim, J. W.; Silveira, P. A.; Fromm, P. D.; Clark, G. J., 2019: On the other side: Manipulating the immune checkpoint landscape of dendritic cells to enhance cancer immunotherapy. *Frontiers in Oncology*., **9**, 50.

Kool, M.; Willart, M. A. M.; van Nimwegen, M.; Bergen, I.; Pouliot, P.; Virchow, J. C.; Rogers, N.; Osorio, F.; Reis e Sousa, C.; Hammad, H.; Lambrecht, B. N., 2011: An Unexpected Role for Uric Acid as an Inducer of T Helper 2 Cell Immunity to Inhaled Antigens and Inflammatory Mediator of Allergic Asthma. *Immunity*., **34**, 527–540.

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

Kostner, L.; Anzengruber, F.; Guillod, C.; Recher, M.; Schmid-Grendelmeier, P.; Navarini, A. A., 2017: Allergic Contact Dermatitis. *Immunology and Allergy Clinics of North America*.

Kühn, U.; Brand, P.; Willemsen, J.; Jonuleit, H.; Enk, A. H.; van Brandwijk-Petershans, R.; Saloga, J.; Knop, J.; Becker, D., 1998: Induction of Tyrosine Phosphorylation in Human MHC Class II-Positive Antigen-Presenting Cells by Stimulation with Contact Sensitizers. *The Journal of Immunology*., **160**, 667–673.

la Sala, A.; Ferrari, D.; Corinti, S.; Cavani, A.; Di Virgilio, F.; Girolomoni, G., 2001: Extracellular ATP Induces a Distorted Maturation of Dendritic Cells and Inhibits Their Capacity to Initiate Th1 Responses. *The Journal of Immunology*., **166**, 1611–1617.

La Sala, A.; Sebastiani, S.; Ferrari, D.; Di Virgilio, F.; Idzko, M.; Norgauer, J.; Girolomoni, G., 2002: Dendritic cells exposed to extracellular adenosine triphosphate acquire the migratory properties of mature cells and show a reduced capacity to attract type 1 T lymphocytes. *Blood*., **99**, 1715–1722.

Lalko, J. F.; Kimber, I.; Dearman, R. J.; Gerberick, G. F.; Sarlo, K.; Api, A. M., 2011: Chemical reactivity measurements: potential for characterization of respiratory chemical allergens. *Toxicology in Vitro*., **25**, 433–445.

Lalko, J. F.; Kimber, I.; Gerberick, G. F.; Foertsch, L. M.; Api, A. M.; Dearman, R. J., 2012: The direct peptide reactivity assay: selectivity of chemical respiratory allergens. *Toxicological Sciences*., **129**, 421–431.

Lalko, J. F.; Kimber, I.; Dearman, R. J.; Api, A. M.; Gerberick, G. F., 2013: The selective peptide reactivity of chemical respiratory allergens under competitive and non-competitive conditions. *Journal of Immunotoxicology*., **10**, 292–301.

Lambrechts, N.; Verstraelen, S.; Lodewyckx, H.; Felicio, A.; Hooyberghs, J.; Witters, H.; Van Tendeloo, V.; Van Cauwenberge, P.; Nelissen, I.; Van Den Heuvel, R.; Schoeters, G., 2009: THP-1 monocytes but not macrophages as a potential alternative for CD34+ dendritic cells to identify chemical skin sensitizers. *Toxicology and Applied Pharmacology*., **236**, 221–230.

Landsteiner, K.; Jacobs, J., 1936: Studies on the sensitization of animals with simple chemical compounds. *Journal of Experimental Medicine*., **64**, 643–657.

Langerhans, P., 1868: Ueber die Nerven der menschlichen Haut. *Archiv für Pathologische Anatomie und Physiologie und für Klinische Medicin*., **44**, 325–337.

Latz, E.; Xiao, T. S.; Stutz, A., 2013: Activation and regulation of the inflammasomes. *Nature Reviews Immunology*., **13**, 397–411.

Lauterbach, H.; Bathke, B.; Gilles, S.; Traidl-Hoffmann, C.; Luber, C. A.; Fejer, G.; Freudenberg, M. A.; Davey, G. M.; Vremec, D.; Kallies, A.; Wu, L.; Shortman, K.; Chaplin, P.; Suter, M.; O'Keeffe, M.; Hochrein, H., 2010: Mouse CD8α+ DCs and human BDCA3+ DCs are major producers of IFN-λ in response to poly IC. *Journal of Experimental Medicine*., **207**, 2703–2717.

Lee-Sayer, S. S. M.; Dong, Y.; Arif, A. A.; Olsson, M.; Brown, K. L.; Johnson, P., 2015: The where, when, how and why of hyaluronan binding by immune cells. *Frontiers in Immunology*., **6**, 150.

Lepoittevin, J. P. P., 2006, February 1: Metabolism versus chemical transformation or pro- versus prehaptens? *Contact Dermatitis*., **54**, 73–74.

Li, R. R.; Wang, J.; Li, R. R.; Zhu, F.; Xu, W.; Zha, G.; He, G.; Cao, H.; Wang, Y.; Yang, J., 2018: ATP/P2X7-NLRP3 axis of dendritic cells participates in the regulation of airway inflammation and hyper-responsiveness in asthma by mediating HMGB1 expression and secretion. *Experimental Cell Research*., **366**, 1–15.

Li, X.; Zhong, F., 2014: Nickel induces interleukin-1β secretion via the NLRP3-ASC-caspase-1 pathway. *Inflammation*., **37**, 457–466.

Liang, J.; Zhao, H.; Yao, L.; Tang, H.; Dong, H.; Wu, Y.; Liu, L.; Zou, F.; Cai, S., 2015: Phosphatidylinositol 3-kinases pathway mediates lung caspase-1 activation and high mobility group box 1 production in a toluene-diisocyanate induced murine asthma model. *Toxicology Letters*., **236**, 25–33.

Liu, D.; Rhebergen, A. M.; Eisenbarth, S. C., 2013: Licensing Adaptive Immunity by NOD-Like Receptors. *Frontiers in Immunology*., **4**, 486.

Liu, L.; Inoue, H.; Nakayama, H.; Kanno, R.; Kanno, M., 2007: The Endogenous Danger Signal Uric Acid Augments Contact Hypersensitivity Responses in Mice. *Pathobiology*., **74**, 177–185.

Liu, Z.; Roche, P. A., 2015: Macropinocytosis in phagocytes: regulation of MHC class-II-restricted antigen presentation in dendritic cells. *Frontiers in physiology*., **6**, 1.

Lui, G.; Manches, O.; Angel, J.; Molens, J. P.; Chaperot, L.; Plumas, J., 2009: Plasmacytoid dendritic cells capture and cross-present viral antigens from influenza-virus exposed cells. *PloS one*., **4**, e7111.

Luís, A.; Martins, J. D.; Silva, A.; Ferreira, I.; Cruz, M. T.; Neves, B. M., 2014: Oxidative stress-dependent activation of the eIF2α–ATF4 unfolded protein response branch by skin sensitizer 1-fluoro-2, 4-dinitrobenzene modulates dendritic-like cell maturation and inflammatory status in a biphasic manner. *Free Radical Biology and Medicine*., **77**, 217–229.

Lukacs-Kornek, V.; Engel, D.; Tacke, F.; Kurts, C., 2008: The role of chemokines and their receptors in dendritic cell biology. *Frontiers in Bioscience*., **13**, 2238–2252.

Mangan, M. S. J. J.; Olhava, E. J.; Roush, W. R.; Seidel, H. M.; Glick, G. D.; Latz, E., 2018: Targeting the NLRP3 inflammasome in inflammatory diseases. *Nature Reviews Drug Discovery*., **17**, 588–606.

Martin, S.; Merfort, I.; Thierse, H. J., 2006: Interactions of Chemicals and Metal Ions with Proteins and Role for Immune Responses. *Mini-Reviews in Medicinal Chemistry*., **6**, 247–255.

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.; Freudenberg, M. A., 2008a: Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. *Journal of Experimental Medicine*., **205.**, 2151–2162.

Martin, S. F.; Jakob, T., 2008b: From innate to adaptive immune responses in contact hypersensitivity. *Current Opinion in Allergy and Clinical Immunology*., **8**, 289–293.

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: European Journal of Allergy and Clinical Immunology*., **66**, 1152–1163.

Martin, S. F., 2012: Allergic contact dermatitis: Xenoinflammation of the skin. *Current Opinion in Immunology*., **24**, 720–729.

Martin, S. F., 2017: The role of the innate immune system in allergic contact dermatitis. *Allergologie Select*., **1**, 39–43.

Martin, S. F.; Rustemeyer, T.; Thyssen, J. P., 2018: Recent advances in understanding and managing contact dermatitis. *F1000Research*.

Martinon, F.; Burns, K.; Tschopp, J., 2002: The Inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Molecular Cell*., **10**, 417–426.

Martinon, F.; Pétrilli, V.; Mayor, A.; Tardivel, A.; Tschopp, J., 2006: Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*., **440**, 237–241.

Martins, J. D.; Silva, A.; Ferreira, I.; Gonçalo, M.; Custódio, J. B. A.; Lopes, M. C.; Domingues, M. R. M.; Neves, B. M.; Cruz, M. T., 2016: Adenosine diphosphate involvement in THP-1 maturation triggered by the contact

allergen 1-fluoro-2,4-dinitrobenzene. *Toxicology Research*., **5**, 1512–1521.

Matos, T. J.; Duarte, C. B.; Gonçalo, M.; Lopes, M. C., 2005: Role of oxidative stress in ERK and p38 MAPK activation induced by the chemical sensitizer DNFB in a fetal skin dendritic cell line. *Immunology and Cell Biology*., **83**, 607–614.

Matsue, H.; Edelbaum, D.; Shalhevet, D.; Mizumoto, N.; Yang, C.; Mummert, M. E.; Oeda, J.; Masayasu, H.; Takashima, A., 2003: Generation and function of reactive oxygen species in dendritic cells during antigen presentation. *Journal of Immunology*., **171**, 3010–3018.

Megherbi, R.; Kiorpelidou, E.; Foster, B.; Rowe, C.; Naisbitt, D. J.; Goldring, C. E.; 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.

Mitjans, M.; Galbiati, V.; Lucchi, L.; Viviani, B.; Marinovich, M.; Galli, C. L.; Corsini, E., 2010: Use of IL-8 release and p38 MAPK activation in THP-1 cells to identify allergens and to assess their potency in vitro. *Toxicology in Vitro*., **24**, 1803–1809.

Miyazawa, M.; Ito, Y.; Yoshida, Y.; Sakaguchi, H.; Suzuki, H., 2007: Phenotypic alterations and cytokine production in THP-1 cells in response to allergens. *Toxicology in vitro : an international journal published in association with BIBRA*., **21**, 428–437.

Mizuashi, M.; Ohtani, T.; Nakagawa, S.; Aiba, S., 2005: Redox imbalance induced by contact sensitizers triggers the maturation of dendritic cells. *Journal of Investigative Dermatology*., **124**, 579–586.

Mizumoto, N.; Mummert, M. E.; Shalhevet, D.; Takashima, A., 2003: Keratinocyte ATP Release Assay for Testing Skin-Irritating Potentials of Structurally Diverse Chemicals. *Journal of Investigative Dermatology*., **121**, 1066–1072.

Mokra, D.; Drgova, A.; Pullmann, R.; Mikolka, P.; Antosova, M.; Mokry, J., 2012: Changes in several inflammatory and oxidation markers after ovalbumin-sensitization in a guinea pig model of allergic asthma - a pilot study. *Acta Medica Martiniana*., **12**, 5–11.

Molinari, G.; Colombo, G.; Celenza, C., 2014: Respiratory Allergies: A General Overview of Remedies, Delivery Systems, and the Need to Progress. *ISRN Allergy*., **2014**, 1–15.

Montaser, M.; Lalmanach, G.; Mach, L., 2002: CA-074, but not its methyl ester CA-074Me, is a selective inhibitor of cathepsin B within living cells. *Biological Chemistry*., **383**, 1305–1308.

Monzon, M. E.; Fregien, N.; Schmid, N.; Falcon, N. S.; Campos, M.; Casalino-Matsuda, S. M.; Forteza, R. M., 2010: Reactive oxygen species and hyaluronidase 2 regulate airway epithelial hyaluronan fragmentation. *Journal of Biological Chemistry*., **285**, 26126–26134.

Moossavi, M.; Parsamanesh, N.; Bahrami, A.; Atkin, S. L.; Sahebkar, A., 2018: Role of the NLRP3 inflammasome in cancer. *Molecular cancer*., **17**, 158.

Musumeci, D.; Roviello, G. N.; Montesarchio, D., 2014: An overview on HMGB1 inhibitors as potential therapeutic agents in HMGB1-related pathologies. *Pharmacology and Therapeutics*., **141**, 347–357.

Muti, A. D.; Pârvu, A. E.; Muti, L. A.; Moldovan, R.; Mureşan, A., 2016: Vitamin E effect in a rat model of toluene diisocyanate-induced asthma. *Clujul Medical*., **89**, 499–505.

Muto, J.; Morioka, Y.; Yamasaki, K.; Kim, M.; Garcia, A.; Carlin, A. F.; Varki, A.; Gallo, R. L., 2014: Hyaluronan digestion controls DC migration from the skin. *Journal of Clinical Investigation*., **124**, 1309–1319.

Muttardi, K.; White, I. R.; Banerjee, P., 2016: The burden of allergic contact dermatitis caused by acrylates. *Contact Dermatitis*., **75**, 180–184.

Nace, G.; Evankovich, J.; Eid, R.; Tsung, A., 2011: Dendritic cells and damage-associated molecular patterns: Endogenous danger signals linking innate and adaptive immunity. *Journal of Innate Immunity*., **4**, 6–15.

Nair, S.; Archer, G. E.; Tedder, T. F., 2012: Isolation and generation of human dendritic cells. *Current Protocols in Immunology*., **99**, 7.32.1-7.32.23.

Nakahara, T.; Moroi, Y.; Uchi, H.; Furue, M., 2006: Differential role of MAPK signaling in human dendritic cell maturation and Th1/Th2 engagement. *Journal of Dermatological Science*., **42**, 1–11.

Nakanishi, K.; Yoshimoto, T.; Tsutsui, H.; Okamura, H., 2001: Interleukin-18 regulates both Th1 and Th2 responses. *Annual Review of Immunology*., **19**, 423–474.

Nassau, S.; Fonacier, L., 2020: Allergic Contact Dermatitis. *Medical Clinics of North America*., **104**, 61–76.

Natsch, A.; Emter, R., 2008: Skin sensitizers induce antioxidant response element dependent genes: Application to the in vitro testing of the sensitization potential of chemicals. *Toxicological Sciences*., **102**, 110–119.

Natsch, A., 2009: The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers-Functional relevance and a hypothesis on innate reactions to skin sensitizers. *Toxicological Sciences*., **113**, 284–292.

Neisius, U.; Brand, P.; Plochmann, S.; Saloga, J.; Knop, J.; Becker, D., 1999: Detection of increased tyrosine phosphorylation in murine Langerhans cells after stimulation with contact sensitizers. *Archives of Dermatological Research*., **291**, 22–27.

Neuberger, A.; Ring, S.; Silva-Vilches, C.; Schrader, J.; Enk, A.; Mahnke, K., 2017: Expression of CD73 slows down migration of skin dendritic cells, affecting the sensitization phase of contact hypersensitivity reactions in mice. *Journal of Dermatological Science*., **87**, 292–299.

Neves, B. M.; Cruz, M. T.; Francisco, V.; Garcia-Rodriguez, C.; Silvestre, R.; Cordeiro-da-Silva, A.; Dinis, A. M.; Batista, M. T.; Duarte, C. B.; Lopes, M. C., 2009: Differential roles of PI3-Kinase, MAPKs and NF-κB on the manipulation of dendritic cell Th1/Th2 cytokine/chemokine polarizing profile. *Molecular Immunology*., **46**, 2481–2492.

Neves, B. M.; Gonçalo, M.; Figueiredo, A.; Duarte, C. B.; Lopes, M. C.; Cruz, M. T., 2011: Signal transduction profile of chemical sensitisers in dendritic cells: an endpoint to be included in a cell-based in vitro alternative approach to hazard identification? *Toxicology and Applied Pharmacology*., **250**, 87–95.

Neves, B. M.; Rosa, S. C.; Martins, J. D.; Silva, A.; Gonçalo, M.; Lopes, M. C.; Cruz, M. T., 2013: Development of an in vitro dendritic cell-based test for skin sensitizer identification. *Chemical Research in Toxicology*., **26**, 368–378.

Nikitovic, D.; Berdiaki, A.; Galbiati, V.; Kavasi, R. M. M.; Papale, A.; Tsatsakis, A.; Tzanakakis, G. N.; Corsini, E., 2015: Hyaluronan regulates chemical allergen-induced IL-18 production in human keratinocytes. *Toxicology Letters*., **232**, 89–97.

Nizzoli, G.; Krietsch, J.; Weick, A.; Steinfelder, S.; Facciotti, F.; Gruarin, P.; Bianco, A.; Steckel, B.; Moro, M.; Crosti, M.; Romagnani, C.; Stölzel, K.; Torretta, S.; Pignataro, L.; Scheibenbogen, C.; Neddermann, P.; De Francesco, R.; Abrignani, S.; Geginat, J., 2013: Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. *Blood*., **122**, 932–942.

Novak-Bilić, G.; Vučić, M.; Japundžić, I.; Meštrović-štefekov, J.; Stanić-Duktaj, S.; Lugović-Mihić, L., 2018:

Irritant and allergic contact dermatitis – skin lesion characteristics. *Acta Clinica Croatica*., **57**, 713–720.

Nukada, Y.; Miyazawa, M.; Kosaka, N.; Ito, Y.; Sakaguchi, H.; 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. *The Journal of Toxicological Sciences*., **33**, 175–185.

OECD, 2012a: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. *OECD Environment, Health and Safety Publications Series on Testing and Assessment 168*., 1–46.

OECD, 2012b: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 2: Use of the AOP to Develop Chemical Categories and Integrated Assessment and Testing Approaches. *OECD Environment, Health and Safety Publications Series on Testing and Assessment 168*., 1–59.

OECD, 2018a: Test No. 442E: In Vitro Skin Sensitisation: In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation. OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris, France.

OECD, 2018b: Test No. 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method. OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris, France.

Okayama, Y., 2005: Oxidative stress in allergic and inflammatory skin diseases. *Current Drug Targets - Inflammation & Allergy*., **4**, 517–519.

Onami, K.; Kimura, Y.; Ito, Y.; Yamauchi, T.; Yamasaki, K.; Aiba, S., 2014: Nonmetal haptens induce ATP release from keratinocytes through opening of pannexin hemichannels by reactive oxygen species. *Journal of Investigative Dermatology*., **134**, 1951–1960.

Orlowski, G. M.; Colbert, J. D.; Sharma, S.; Bogyo, M.; Robertson, S. A.; Rock, K. L., 2015: Multiple Cathepsins Promote Pro-IL-1β Synthesis and NLRP3-Mediated IL-1β Activation. *Journal of Immunology*., **195**, 1685–1697.

Owen, J. L.; Vakharia, P. P.; Silverberg, J. I., 2018: The Role and Diagnosis of Allergic Contact Dermatitis in Patients with Atopic Dermatitis. *American Journal of Clinical Dermatology*., **19**, 293–302.

Palm NW; Medzhitov R, 2009: Pattern recognition receptors and control of adaptive immunity. *Immunology Reviews*., **227**, 221–233.

Paudel, Y. N.; Shaikh, M. F.; Chakraborti, A.; Kumari, Y.; Aledo-Serrano, Á.; Aleksovska, K.; Alvim, M. K. M. H.; Othman, I., 2018: HMGB1: A common biomarker and potential target for TBI, neuroinflammation, epilepsy, and cognitive dysfunction. *Frontiers in Neuroscience*., **12**, 628.

Peiser, M.; Tralau, T.; Heidler, J.; Api, A. M.; Arts, J. H. E.; Basketter, D. A.; English, J.; Diepgen, T. L.; Fuhlbrigge, R. C.; Gaspari, A. A.; Johansen, J. D.; Karlberg, A. T.; Kimber, I.; Lepoittevin, J. P.; Liebsch, M.; Maibach, H. I.; Martin, S. F.; Merk, H. F.; Platzek, T. et al., 2012: Allergic contact dermatitis: Epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. *Cellular and Molecular Life Sciences*., **69**, 763–781.

Pendse, S. S.; Behjati, S.; Schatton, T.; Izawa, A.; Sayegh, M. H.; Frank, M. H., 2006: P-glycoprotein functions as a differentiation switch in antigen presenting cell maturation. *American Journal of Transplantation*., **6**, 2884–2893.

Pennino, D.; Eyerich, K.; Scarponi, C.; Carbone, T.; Eyerich, S.; Nasorri, F.; Garcovich, S.; Traidl-Hoffmann, C.; Albanesi, C.; Cavani, A., 2010: IL-17 Amplifies Human Contact Hypersensitivity by Licensing Hapten Nonspecific Th1 Cells to Kill Autologous Keratinocytes. *The Journal of Immunology*., **184**, 4880–4888.

Pickard, C.; Smith, A. M.; Cooper, H.; Strickland, I.; Jackson, J.; Healy, E.; Friedmann, P. S., 2007: Investigation of mechanisms underlying the T-cell response to the hapten 2,4-dinitrochlorobenzene. *The Journal of Investigative Dermatology*., **127**, 630–637.

Platt, C. D.; Ma, J. K.; Chalouni, C.; Ebersold, M.; Bou-Reslan, H.; Carano, R. A. D.; Mellman, I.; Delamarre, L., 2010: Mature dendritic cells use endocytic receptors to capture and present antigens. *Proceedings of the National Academy of Sciences of the United States of America*., **107**, 4287–4292.

Qin, R.; Lampel, H. P., 2015: Review of Occupational Contact Dermatitis—Top Allergens, Best Avoidance Measures. *Current Treatment Options in Allergy*., **2**, 349–364.

Rajamäki, K.; Lappalainen, J.; Öörni, K.; Välimäki, E.; Matikainen, S.; Kovanen, P. T.; Kari, E. K., 2010: Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: A novel link between cholesterol metabolism and inflammation. *PLoS ONE*., **5**, e11765.

Randolph, G. J.; Beaulieu, S.; Pope, M.; Sugawara, I.; Hoffman, L.; Steinman, R. M.; Muller, W. A., 1998: A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. *Proceedings of the National Academy of Sciences of the United States of America*., **95**, 6924–6929.

Reis e Sousa, C., 2006: Dendritic cells in a mature age. *Nature Reviews Immunology*., **6**, 476–483.

Remy, S.; Verstraelen, S.; Van Den Heuvel, R.; Nelissen, I.; Lambrechts, N.; Hooyberghs, J.; Schoeters, G., 2014: Gene expressions changes in bronchial epithelial cells: Markers for respiratory sensitizers and exploration of the NRF2 pathway. *Toxicology in Vitro*., **28**, 209–217.

Ring, S.; Oliver, S. J.; Cronstein, B. N.; Enk, A. H.; Mahnke, K., 2009: CD4+CD25+ regulatory T cells suppress contact hypersensitivity reactions through a CD39, adenosine-dependent mechanism. *Journal of Allergy and Clinical Immunology*., **123**, 1287–1296.

Rustemeyer, T.; van Hoogstraten, I. M. W.; von Blomberg, B. M. E.; Scheper, R. J., 2020: Mechanisms of Allergic Contact Dermatitis. *Kanerva's Occupational Dermatology*. 3rd edn. Springer International Publishing, Cham, pp. 1–41.

Sallusto, F.; Cella, M.; Danieli, C.; Lanzavecchia, A., 1995: Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *The Journal of Experimental Medicine*., **182**, 389–400.

Santegoets, S. J. A. M.; van den Eertwegh, A. J. M.; van de Loosdrecht, A. A.; Scheper, R. J.; de Gruijl, T. D., 2008: Human dendritic cell line models for DC differentiation and clinical DC vaccination studies. *Journal of Leukocyte Biology*., **84**, 1364–1373.

Schnurr, M.; Then, F.; Galambos, P.; Scholz, C.; Siegmund, B.; Endres, S.; Eigler, A., 2000: Extracellular ATP and TNF-α Synergize in the Activation and Maturation of Human Dendritic Cells. *The Journal of Immunology*., **165**, 4704–4709.

Schwenck, J.; Maurer, A.; Fehrenbacher, B.; Mehling, R.; Knopf, P.; Mucha, N.; Haupt, D.; Fuchs, K.; Griessinger, C. M.; Bukala, D.; Holstein, J.; Schaller, M.; Menendez, I. G.; Ghoreschi, K.; Quintanilla-Martinez, L.; Gütschow, M.; Laufer, S.; Reinheckel, T.; Röcken, M. et al., 2019: Cysteine-type cathepsins promote the effector phase of acute cutaneous delayed-type hypersensitivity reactions. *Theranostics*., **9**, 3903–3917.

Shi, Y.; Evans, J. E.; Rock, K. L., 2003: Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*., **425**, 516–521.

Shih, Y. H.; Sun, C. C.; Tseng, Y. H.; Chu, C. Y., 2015: Contact dermatitis to topical medicaments: A

retrospective study from a medical center in Taiwan. *Dermatologica Sinica*., **33**, 181–186.

Shornick, L. P.; De Togni, P.; Mariathasan, S.; Goellner, J.; Strauss-Schoenberger, J.; Karr, R. W.; Ferguson, T. A.; Chaplin, D. D., 1996: Mice deficient in IL-1β manifest impaired contact hypersensitivity to trinitrochlorobenzene. *Journal of Experimental Medicine*., **183**, 1427–1436.

Silva, A.; Nunes, C.; Martins, J.; Dinis, T. C. P.; Lopes, C.; Neves, B.; Cruz, T., 2014: Respiratory sensitizer hexamethylene diisocyanate inhibits SOD 1 and induces ERK-dependent detoxifying and maturation pathways in dendritic-like cells. *Free Radical Biology and Medicine*., **72**, 238–246.

So, A.; Thorens, B., 2010: Uric acid transport and disease. *Journal of Clinical Investigation*., **120**, 1791–1799.

Solle, M.; Labasi, J.; Perregaux, D. G.; Stam, E.; Petrushova, N.; Koller, B. H.; Griffiths, R. J.; Gabel, C. A., 2001: Altered cytokine production in mice lacking P2X7 receptors. *Journal of Biological Chemistry*., **276**, 125–132.

Soumelis, V.; Liu, Y. J., 2006: From plasmacytoid to dendritic cell: Morphological and functional switches during plasmacytoid pre-dendritic cell differentiation. *European Journal of Immunology*., **36**, 2286–2292.

Steinman, R. M.; Cohn, Z. A., 1973: Identification of a novel cell type in peripheral lymphoid organs of mice: I. Morphology, quantitation, tissue distribution. *Journal of Experimental Medicine*., **137**, 1142–1162.

Steinman, R. M.; Cohn, Z. A., 1974: Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *The Journal of Experimental Medicine*., **139**, 380–397.

Steinman, R. M.; Banchereau, J., 2007: Taking dendritic cells into medicine. *Nature*., **449**, 419–426.

Stępnik, M.; Arkusz, J., 2003: Molecular events associated with dendritic cells activation by contact sensitizers. *International Journal of Occupational Medicine and Environmental Health*., **16**, 191–199.

Stern, R.; Asari, A. A.; Sugahara, K. N., 2006: Hyaluronan fragments: An information-rich system. *European Journal of Cell Biology*., **85**, 699–715.

Sung, C. T.; McGowan, M. A.; Machler, B. C.; Jacob, S. E., 2019: Systemic Treatments for Allergic Contact Dermatitis. *Dermatitis*., **30**, 46–53.

Sutterwala, F. S.; Ogura, Y.; Szczepanik, M.; Lara-Tejero, M.; Lichtenberger, G. S.; Grant, E. P.; Bertin, J.; Coyle, A. J.; Galán, J. E.; Askenase, P. W.; Flavell, R. A., 2006: Critical role for NALP3/CIAS1/cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity*., **24**, 317–327.

Swanson, K. V.; Deng, M.; Ting, J. P. Y., 2019: The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nature Reviews Immunology*., **19**, 477–489.

Szpaderska, A. M.; Frankfater, A., 2001: An Intracellular Form of Cathepsin B Contributes to Invasiveness in Cancer. *Cancer Research*., **61**, 3493–3500.

Takahashi, T.; Kimura, Y.; Saito, R.; Nakajima, Y.; Ohmiya, Y.; Yamasaki, K.; 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.

Thyssen, J. P.; Linneberg, A.; Menné, T.; Johansen, J. D., 2007: The epidemiology of contact allergy in the general population - Prevalence and main findings. *Contact Dermatitis*., **57**, 287–299.

Tietze, C.; Blömeke, B., 2008: Sensitization assays: Monocyte-derived dendritic cells versus a monocytic cell line (THP-1). *Journal of Toxicology and Environmental Health - Part A: Current Issues*., **71**, 965–968.

Tominaga, K.; Yoshimoto, T.; Torigoe, K.; Kurimoto, M.; Matsui, K.; Hada, T.; Okamura, H.; Nakanishi, K., 2000: IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. *International Immunology*., **12**, 151–160.

Tonc̈ić, R. J.; Lipozenc̈ić, J.; Martinac, I.; Gregurić, S., 2011: Immunology of allergic contact dermatitis. *Acta Dermatovenerologica Croatica*., **19**, 51–68.

Trompezinski, S.; Migdal, C.; Tailhardat, M.; Le Varlet, B.; Courtellemont, P.; Haftek, M.; Serres, M., 2008: Characterization of early events involved in human dendritic cell maturation induced by sensitizers: cross talk between MAPK signalling pathways. *Toxicology and Applied Pharmacology*., **230**, 397–406.

Tsuchiya, K.; Hara, H., 2014: The inflammasome and its regulation. *Critical reviews in immunology*., **34**, 41–80.

Ullah, M. A.; Loh, Z.; Gan, W. J.; Zhang, V.; Yang, H.; Li, J. H.; Yamamoto, Y.; Schmidt, A. M.; Armour, C. L.; Hughes, J. M.; Phipps, S.; Sukkar, M. B., 2014: Receptor for advanced glycation end products and its ligand high-mobility group box-1 mediate allergic airway sensitization and airway inflammation. *Journal of Allergy and Clinical Immunology*., **134**, 440–450.

Usatine, R. P.; Riojas, M., 2010: Diagnosis and management of contact dermatitis. *American Family Physician*., **82**, 249–255.

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

van Amsterdam, J.; van Loveren, H.; Ezendam, J., 2011: Sensitizing potency of chemical respiratory allergens in humans : Can animal data be used to estimate human potency? *RIVM letter report*., 340300004.

van der Veen, J. W.; Gremmer, E. R.; Vermeulen, J. P.; van Loveren, H.; Ezendam, J., 2013: Induction of skin sensitization is augmented in Nrf2-deficient mice. *Archives of Toxicology*., **87**, 763–766.

van Triel, J. J.; van Bree, B. W. J.; Roberts, D. W.; Muijser, H.; Duistermaat, E.; Woutersen, R. A.; Kuper, C. F., 2011: The respiratory allergen glutaraldehyde in the local lymph node assay: Sensitization by skin exposure, but not by inhalation. *Toxicology*., **179**, 115–122.

Venereau, E.; Casalgrandi, M.; Schiraldi, M.; Antoine, D. J.; Cattaneo, A.; De Marchis, F.; Liu, J.; Antonelli, A.; Preti, A.; Raeli, L.; Shams, S. S.; Yang, H.; Varani, L.; Andersson, U.; Tracey, K. J.; Bachi, A.; Uguccioni, M.; Bianchi, M. E., 2012: Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. *Journal of Experimental Medicine*., **209**, 1519–1528.

Vénéreau, E.; Ceriotti, C.; Bianchi, M. E., 2015: DAMPs from cell death to new life. *Frontiers in Immunology*., **6**, 442.

Vocanson, M.; Hennino, A.; Rozières, A.; Poyet, G.; Nicolas, J. F., 2009: Effector and regulatory mechanisms in allergic contact dermatitis. *Allergy: European Journal of Allergy and Clinical Immunology*., **64**, 1699–1714.

Vos, T.; Abajobir, A. A.; Abbafati, C.; Abbas, K. M.; Abate, K. H.; Abd-Allah, F.; Abdulle, A. M.; Abebo, T. A.; Abera, S. F.; Aboyans, V.; Abu-Raddad, L. J.; Ackerman, I. N.; Adamu, A. A.; Adetokunboh, O.; Afarideh, M.; Afshin, A.; Agarwal, S. K.; Aggarwal, R.; Agrawal, A. et al., 2017: Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*., **390**, 1211–1259.

Vyas, J. M.; Van der Veen, A. G.; Ploegh, H. L., 2008: The known unknowns of antigen processing and presentation. *Nature reviews. Immunology*., **8**, 607–618.

Wakabayashi, N.; Dinkova-Kostova, A. T.; Holtzclaw, W. D.; Kang, M. I.; Kobayashi, A.; Yamamoto, M.; Kensler, T. W.; Talalay, P., 2004: Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proceedings of the National Academy of Sciences of the United States of America*., **101**, 2040–2045.

Wang, B. J.; Sheu, H. M.; Guo, Y. L.; Lee, Y. H.; Lai, C. S.; Pan, M. H.; Wang, Y. J., 2010: Hexavalent chromium induced ROS formation, Akt, NF-κB, and MAPK activation, and TNF-α and IL-1α production in keratinocytes. *Toxicology Letters*., **198**, 216–224.

Wang, B. J.; Chiu, H. W.; Lee, Y. L. Y. H.; Li, C. Y.; Wang, Y. J.; Lee, Y. L. Y. H., 2018: Pterostilbene Attenuates Hexavalent Chromium-Induced Allergic Contact Dermatitis by Preventing Cell Apoptosis and Inhibiting IL-1β-Related NLRP3 Inflammasome Activation. *Journal of Clinical Medicine*., **7**, 489.

Watanabe, H.; Gaide, O.; Pétrilli, V.; Martinon, F.; Contassot, E.; Roques, S.; Kummer, J. A.; Tschopp, J.; French, L. E., 2007: Activation of the IL-1β-processing inflammasome is involved in contact hypersensitivity. *Journal of Investigative Dermatology*., **127**, 1956–1963.

Watanabe, H.; Gehrke, S.; Contassot, E.; Roques, S.; Tschopp, J.; Friedmann, P. S.; French, L. E.; Gaide, O., 2008: Danger Signaling through the Inflammasome Acts as a Master Switch between Tolerance and Sensitization. *The Journal of Immunology*., **180**, 5826–5832.

Weber, F. C.; Esser, P. R.; Müller, T.; Ganesan, J.; Pellegatti, P.; Simon, M. M.; Zeiser, R.; Idzko, M.; Jakob, T.; Martin, S. F., 2010a: Lack of the purinergic receptor P2X7 results in resistance to contact hypersensitivity. *Journal of Experimental Medicine*., **207**, 2609–2619.

Weber, F. C.; Esser, P. R.; Müller, T.; Ganesan, J.; Pellegatti, P.; Simon, M. M.; Zeiser, R.; Idzko, M.; Jakob, T.; Martin, S. F., 2010b: Lack of the purinergic receptor $P2X_7$ results in resistance to contact hypersensitivity. *The Journal of Experimental Medicine*., **207**, 2609–2619.

Willart, M. A. M.; Lambrecht, B. N., 2009: The danger within: Endogenous danger signals, atopy and asthma. *Clinical and Experimental Allergy*., **39**, 12–19.

Willart, M. A. M.; Hammad, H., 2010: Alarming dendritic cells for allergic sensitization. *Allergology International*., **59**, 95–103.

Willingham, S. B.; Ting, J. P. Y., 2008: NLRs and the dangers of pollution and aging. *Nature Immunology*., **9**, 831–833.

Wolff, K.; Johnson, R. A.; Saavedra, A. P.; Roh, E. K., 2017: ECZEMA/DERMATITIS. *Fitzpatrick's Color Atlas and Synopsis of Clinical Dermatology, 8e*. McGraw-Hill Education, New York, NY.

Woo, J. T.; Sigeizumi, S.; Yamaguchi, K.; Sugimoto, K.; Kobori, T.; Tsuji, T.; Kondo, K., 1995: Peptidyl aldehyde derivatives as potent and selective inhibitors of cathepsin L. *Bioorganic and Medicinal Chemistry Letters*., **5**, 1501–1504.

Xiao, Y.; Xu, W.; Su, W., 2018: NLRP3 inflammasome: A likely target for the treatment of allergic diseases. *Clinical and Experimental Allergy*., **48**, 1080–1091.

Yakubu, S. I.; Yakasai, I. A.; Musa, A., 2011: Spectrofluorimetric assay method for glutathione and glutathione transferase using monobromobimane. *Journal of Basic and Clinical Pharmacy*., **2**, 151–158.

Yamagata, S.; Tomita, K.; Sato, R.; Niwa, A.; Higashino, H.; Tohda, Y., 2008: Interleukin-18-deficient mice exhibit diminished chronic inflammation and airway remodelling in ovalbumin-induced asthma model. *Clinical and Experimental Immunology*., **154**, 295–304.

Yeang, H. X. A.; Hamdam, J. M.; Al-Huseini, L. M. A.; Sethu, S.; Djouhri, L.; Walsh, J.; Kitteringham, N.; Park, B. K.; Goldring, C. E.; Sathish, J. G., 2012: Loss of transcription factor nuclear factor-erythroid 2 (NF-E2) p45-related factor-2 (Nrf2) leads to dysregulation of immune functions, redox homeostasis,and intracellular signaling in dendritic cells. *Journal of Biological Chemistry*., **287**, 10556–10564.

Yuk, J. M.; Jo, E. K., 2013: Crosstalk between autophagy and inflammasomes. *Molecules and Cells*., **36**, 393–399.

Zembala, M.; Siedlar, M.; Pryjma, J.; Marcinkiewicz, J., 1994: Human monocytes are stimulated for nitric oxide releasein vitro by some tumor cells but not by cytokines and lipopolysaccharide. *European Journal of Immunology*., **24**, 435–439.

Zhang, X.; Chen, X.; Song, H.; Chen, H. Z.; Rovin, B. H., 2005: Activation of the Nrf2/antioxidant response pathway increases IL-8 expression. *European Journal of Immunology*., **35**, 3258–3267.