Universidade de Aveiro Departamento de Química
2013

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Avaliação do stresse do RE induzido por DNFB em Células Dendríticas

Evaluation of ER stress induced by DNFB in Dendritic Cells

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Evaluation of ER stress induced by DNFB in Dendritic Cells

Dissertation submitted to the University of Aveiro to fulfill the requirements for the degree of Master in Biochemistry with specialization in Clinical Biochemistry, held under the scientific guidance of Dr. Bruno Neves, invited assistant professor, Department of Chemistry at University of Aveiro and Dr. Maria Teresa Cruz, assistant professor at the Faculty of Pharmacy and Investigator at the Center for Neuroscience and Cell Biology at University of Coimbra

Thanks are due to Portuguese Foundation for Science and Technology (FCT), European União, QREN, and COMPETE for funding the QOPNA research unit (PEst-C/QUI/UI0062/2013) and the research project number PTDC/SAU-OSM/099762/2008.

I dedicate this work to my family who has always been my reason to move on and never give up.

júri

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agradecimentos

Um muito obrigado à Doutora Teresa Cruz pela oportunidade e pela sua disponibilidade.
Obrigado ao Doutor Bruno Neves por todo o conhecimento transmitido, pela sua constante presença, disponibilidade e pela sua particular e incomparável orientação fundamental à realização deste trabalho.
A todos os membros da equipa do CNC do grupo *Cellular Immunology and Oncobiology* pela entreajuda, em especial ao João pela colaboração neste trabalho.
À Cláudia Dias por todas as viagens e momentos em conjunto para Coimbra. Á Elsa Maltez, à Rita Bastos, à Susana Campos, à Patrícia Soares pela amizade e suporte.
E a todos os meus amigos, pelas conversas conselhos e constante ânimo.
Ao Sérgio, pelo carinho e amparo em todos estes meses, pela exclusiva paciência e especial ajuda nesta fase.
À minha família, em especial aos meus pais e aos meus irmãos, Cláudia, Tomás e Simão pois foram sempre a razão pela qual lutei.

palavras-chave

Dermatite de contacto alérgica, células dendríticas, espécies reativas de oxigénio, stresse do RE, ATF4, IL-8, HMOX

resumo

A capacidade das células dendríticas (CD) em iniciar e modular respostas imunes, nomeadamente a dermatite de contacto alérgica (DCA), é fortemente dependente da sua ativação / estado de maturação. Os mecanismos moleculares pelos quais os sensibilizadores de pele induzem maturação das CD não são ainda completamente conhecidos. No entanto, foi demonstrado que sinais de perigo primários como a formação de espécies reativas de oxigénio (ERO) desempenham um importante papel neste processo. Nos últimos anos, inúmeras evidências têm estabelecido uma estreita ligação entre a produção de ERO, stresse do retículo endoplasmático (RE) e a patogénese de diversas doenças inflamatórias. Deste modo, pretendeu-se no presente trabalho avaliar a capacidade do sensibilizador cutâneo DNFB desencadear stresse do RE em CD e as concomitantes consequências na imunobiologia dessas células. Os resultados obtidos revelaram que o DNFB induz uma rápida e sustentada fosforilação do fator de iniciação de tradução eucariótico 2α (eIF2α), o aumento dos níveis proteicos do fator de transcrição ATF4 e uma modificação pós translacional na principal proteína chaperone do RE, GRP78. Verificou-se ainda que estes efeitos são dose-dependentes e parcialmente revertidos pelo antioxidante N-acetilcisteína, indicando que a alteração do estado redox celular está na origem da indução do stresse do RE observado. O tratamento das células com o chaperone químico, ácido 4-fenilbutírico (4-PBA) causou um aumento na apoptose induzida por DNFB, enquanto o pré-tratamento com salubrinal, um inibidor seletivo da desfosforilação de eIF2α, provocou o efeito oposto. A exacerbação pelo salubrinal da fosforilação do eIF2α induzida por DNFB causou um forte aumento da transcrição de genes de destoxificação tais como o HMOX e do gene da citoquina pró-inflamatória IL-8 tendo por sua vez o 4-PBA anulado completamente estes efeitos. Globalmente, os nossos resultados indicam que a ativação pelo DNFB do eixo eIF2α/ATF4 em CD contribui fortemente para o desenvolvimento de um microambiente pró-inflamatório e para a transcrição de genes envolvidos no restabelecimento do equilíbrio redox.

keywords

Allergic contact dermatitis, dendritic cells, reactive oxygen species, ER stress, ATF4, IL-8, HMOX

abstract

The capacity of dendritic cells (DC) to initiate and modulate immune responses, namely allergic contact dermatitis (ACD), is tightly dependent on their activation/maturation state. Molecular mechanisms driving skin sensitizers-induced DC maturation are not yet completely unrevealed, however initial danger signals such as the generation of reactive oxygen species (ROS) were shown to play an important role. In recent years innumerous evidences established a close link between ROS production, endoplasmic reticulum (ER) stress and the pathogenesis of several inflammatory diseases. Therefore, we analyzed in this work the ability of the strong sensitizer DNFB to trigger ER stress in DC and the concomitant consequences to the immunobiology of these cells. Our results revealed that DNFB induces a rapid and sustained phosphorylation of the Eukaryotic translation initiation factor 2α (eIF2α), the up regulation of ATF4 and a post translational modification at the major ER chaperone GRP78. These effects were dose dependent and partially reverted by the antioxidant N-acetylcysteine, indicating that cellular redox imbalance is in the origin of evoked ER stress. Treatment of cells with the ER chemical chaperone 4 phenylbutyric acid (4-PBA) caused an increase in DNFB-induced apoptosis while pretreatment with salubrinal, an eIF2α dephosphorylation selective inhibitor, caused the opposite effect. Exacerbation of DNFB-induced eIF2α phosphorylation by salubrinal also caused a strong increase in the transcription of detoxifying genes such as HMOX and of pro-inflammatory cytokine IL-8 while 4-PBA completely abrogated these effects. Overall, our results indicate that DNFB activation of eIF2α/ATF4 stress pathways in DC strongly contributes to generation of a pro-inflammatory microenvironment and is crucial to the transcription of genes involved in remediation of cell redox imbalance.

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Abbreviations

AARE: amino acid response element

ACD: Allergic Contact Dermatitis

ADPase: Adenosine diphosphate

AP-1: activating protein-1

APC: Antigen-presenting cells

ARE: antioxidant response element

ASK1: apoptosis signal-regulating kinase 1

ATF2: Activating transcription factor 2

ATF4: Activating transcription factor 4

ATF6: Activating transcription factor 6

ATP: Adenosine triphosphate

ATPase: Adenosine triphosphatase

BAK: Bcl-2 antagonist/killer

BAX: Bcl-2-associated X protein

Bcl-2: Apoptosis regulator B-cell leukaemia/lymphoma 2

Bcl-xL: B-cell lymphoma-extra large

BIM: Bcl2-interacting mediator of cell death

BiP: Binding immunoglobulin protein

BFA: Brefeldin A

BKC: benzalkonium chloride

bZIP: Basic leucine zipper domain

CCL2: C-C motif chemokine 2

CCL3: C-C motif chemokine 3

CCL4: C-C motif chemokine 4

CCL5: C-C motif chemokine 5

CCL8: C-C motif chemokine 8

CCL17: C-C motif chemokine 17

CCL18: C-C motif chemokine 18

CCL19: C-C motif chemokine 19

CCL22: C-C motif chemokine 22

CCR6: Chemokine receptor 6

CD1: cluster of differentiation 1

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CD11c: Complement component 3 receptor 4 subunit/ Integrin αX

CD14: Cluster of differentiation 14

CD34: Cluster of differentiation 34

CD40: Cluster of differentiation 40

CD54: Cluster of differentiation 54

CD68: Cluster of differentiation 68

CD80: Cluster of differentiation 80

CD83: Cluster of differentiation 83

CD86: Cluster of differentiation 86

CD154: Cluster of differentiation 154

CHOP: CAAT/enhancer binding protein (C/EBP) homologous protein

Cnx: calnexin

COPII: coat protein complex

CRE: cAMP-response element

CREB: cAMP response element-binding protein

Crt: calreticulin

CTLs: cytotoxic CD8+ T lymphocytes

CXCL8: C-X-C motif chemokine 8

CXCL10: C-X-C motif chemokine 10

CYP: cytochrome P450

DAMP: damage-associated molecular patterns

DAPK1: Death-associated protein kinase 1

DCs: dendritic cells

DC2: plasmacytoid dendritic cell

DC-LAMP: DC–lysosome-associated membrane protein

DMSO: Dimethyl sulfoxide

DNBS: dinitrobenzene sulfonic acid

DNCB: 2,4-dinitrochlorobenzene

DNFB: 1-Fluoro-2, 4-dinitrobenzene

ERGIC-53: ER-Golgi intermediate compartment 53 kDa protein

ERGL: Protein ERGIC-53-like

EDEM1: ER degradation enhancer, mannosidase alpha-like 1

eIF2α: eukaryotic initiation factor 2α

Elk1: ETS domain-containing protein Elk-1

ER: Endoplasmatic Reticulum

ERAD: endoplasmic-reticulum-associated protein degradation

ERAI: ER stress-activated indicator

ERK1: extracellular signal-regulated kinase 1

ERK2: extracellular signal-regulated kinase 2

ERSE: ER stress response element

FBP: fructose-1, 6-bisphosphatase

GlcI: glucosidase I

GlcII: glucosidase II

GM-CSF: granulocyte/macrophage colony-stimulating factor

GRP78: glucose regulated protein 78

GRP94: glucose-related protein 94

GSH: glutathione

GSSG: oxidized glutathione

HAC1: Homologous to ATF/CREB1

HDAC: histone deacetylase complex

HERP: homocysteine-induced ER protein

HL-60: Human promyelocytic leukemia cells

HLA-DR: human leukocyte antigen - DR

HRD1: HMG-coA reductase degradation 1

HSP: heat shock protein

ICAM 1: intercellular adhesion molecule 1

IFN-γ: Interferon gamma

IκB: inhibitor of kappa B

IL-1: interleukin 1

IL-1α: interleukin 1 alpha

IL-1β: interleukin 1 beta

IL-2: interleukin 2

IL 3Rα: Interleukin-3 receptor subunit alpha

IL-4: interleukin 4

IL-6: interleukin 6

IL-8: interleukin 8

IL-10: interleukin 10

IL-12R: interleukin 12 receptor

IL-23: interleukin 23

intDCs: interstitial Dendritic Cells

IP-10: IFN-induced protein 10

IRE1: Inositol-requiring enzyme 1

JAK: Janus kinases

JNK: c-Jun NH2-terminal kinase

Keap1: Kelch-like ECH associated protein 1

KG-1: Human myeloid cell line

LCs: Langerhans cells

LFA-3: leukocyte functional antigen-3

LHS1: Heat shock protein 70 homolog

LPS: lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MDC: myeloid dendritic cell

Mef2: myocyte enhancer factor-2

Met-$tRNA_i^{Met}$: methionyl-initiator tRNA

MHC I: major histocompatibility complex class I

MHC II: major histocompatibility complex class II

MIP-1α: Macrophage inflammatory protein-1α

MIP-1β: Macrophage inflammatory protein-1β

MKK: mitogen activated protein kinase kinase

MMP-9: Matrix metallopeptidase 9

MoDCs: monocyte-derived DCs

mRNA: messenger RNA

NAC: N-acetylcysteine

NF-E2: nuclear factor erythroid 2

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NLR: NOD-like receptors

NRF2: nuclear factor erythroid 2-related factor 2

OST: oligosaccharyltransferase

PAMP: Pathogen-associated molecular pattern

p38: 38 high osmolarity glycerol protein kinase

pDC2: pre-DC2

PCR: Polymerase chain reaction

PEPCK: phosphoenolpyruvate carboxy-kinase

PERK: protein kinase RNA-like endoplasmic reticulum kinase

PI: Propidium Iodide

PKC: protein kinase C

PMA: phorbol 12-myristate 13-acetate

PRR: Pattern Recognition Receptors

RANTES: Regulated on Activation, Normal T cell Expressed and Secreted/ CCL5

RPD3: reduced potassium dependency 3

RNA: Ribonucleic acid

RNase: ribonuclease

ROS: reactive oxygen species

RT: Reverse transcription

S1P: Site-1 protease

S2P: Site-2 protease

SAP: serum amyloid P component

SCF: stem cell factor

SCJ1: Saccharomyces cerevisiae DNAJ homolog

SDS: Sodium dodecyl sulfate

SODs: superoxide dismutase

SREBP: sterol response element binding protein

STAT: Signal transducer and activator of transcription

TAT: tyrosine amino transferase

TGF-β: transforming growth factor-β

Th: T-helper cell

TLR4: Toll-like receptor 4

TNBS: trinitrobenzene sulfonic acid

TNCB: 2,4,6-trinitrochlorobenzene

TNF-α: tumor necrosis factor-α

TRAF2: tumor necrosis factor receptor–associated factor 2

Tun: Tunicamycin

UBC7: ubiquitin conjugating enzyme 7

UGT1: UDP-glucuronosyltransferase 1

uORFs: upstream open reading frames

UPR: Unfolded protein response

VIPL: Vesicular integral-membrane protein (VIP36)-like protein

I. INTRODUCTION

1. Allergic Contact Dermatitis

Contact dermatitis defines a group of skin inflammatory disorders caused by exogenous hazardous substances. There are two types of contact dermatitis: allergic contact dermatitis (ACD) and irritant contact dermatitis. Although both diseases may have similar clinical presentations, they are physiopathologicaly distinct. ACD is a cell mediated, delayed-type hypersensitivity reaction caused by contact of the skin with low molecular weight reactive chemicals (haptens) while irritant contact dermatitis is a nonspecific localized inflammatory reaction caused by exposure to irritants (*1*, *2*). ACD is a common occupational and environmental health problem, affecting approximately 15–20% of the general population in developed countries. This pathology is the most frequent work related skin disease leading to elevated economic and social costs. Metal workers, hairdressers, health care workers, employees in the food industry, cleaners, construction workers and painters are the main affected professionals (approximately 80%).

Risk factors for allergic contact dermatitis can be subdivided into acquired and inherent. Acquired risk factors are generally inflammatory skin diseases such as irritant contact dermatitis, stasis dermatitis and possibly atopic dermatitis. In turn, inherent risk factors are associated with genetic variances that cause higher susceptibility. The most important risk factors were identified as workplace exposure, age, gender, use of consumer products and genetic predispositions. Contact dermatitis occurs twice as frequently in women as in men and often starts at a young age, with a prevalence of 15% in 12–16 year olds (*3*). Genetic predisposition involves polymorphisms in genes that regulate xenobiotic metabolism, biotransformation, cellular stress responses as well as redox balance (N-acetyltransferase, glutathione-S-transferase), inflammation, including innate and adaptive immunity (IL-1α, IL-1β, IL-6, IL-10, TNF-α) and skin barrier functions (*4*, *5*).

Polymorphisms in innate pattern recognition receptors (PRR), such as Toll-like receptors (TLR) and NOD-like receptors (NLR) or in cytokine receptors such as IL-12R, also contribute to genetic predisposition (*3*, *6*).

1.1 Physiopathology of allergic contact dermatitis

Chemical-induced inflammatory responses are initially mechanistically different from auto-inflammation and from microbe-induced inflammation, but result in the activation of similar signalling pathways (*6*). From a pathophysiological point of view, ACD can be subdivided into two temporally and spatially distinct phases: the sensitization phase and the induction phase (Figure 1). In sensitization phase, also referred to as afferent phase, haptens cross the epidermis and bind covalently to proteins converting them into immunogenic peptides (*7*). Despite the great chemical heterogeneity, a common feature of chemical sensitizers is their intrinsic electrophilicity (with the exception of metals and thiols) or their potential to be metabolized to electrophilic compounds (pre and pro-haptens) (*1*). There are also metals, such as nickel, which form noncovalent complexes with proteins and peptides. These antigenic hapten-peptide complexes are captured and processed by immature skin dendritic cells (DCs), including Langerhans cells and dermal DCs that then migrate to the paracortical region of lymph nodes. During migration, DCs suffer a complex and coordinated process of morphological, functional and phenotypic alterations designated maturation. This maturation process culminates in the acquisition of the capacity to prime naive T lymphocytes. In the lymph nodes, mature DCs present antigens to CD4 + and CD8 + T lymphocytes causing their activation and clonal expansion and leading to the formation of immunological memory against presented antigens. Specific T lymphocytes (regulatory CD4 + and effectors CD8 + T cells) leave the lymph nodes through the thoracic duct and circulate through the bloodstream to various tissues, including skin. The elicitation phase develops after the subsequent contact of the skin with the same hapten. The specific effector CD8 + T cells are mobilized to the induction site where they interact with the hapten-bearing dendritic cells, being activated. This activation leads to the production of cytokines (IFN-γ, GM-CSF, IL-2) and cytotoxins (perforin and granzyme) that cause an intense inflammatory reaction through apoptosis of keratinocytes and endothelial cells, activation of mast cells and recruitment and infiltration of neutrophils and more specific T lymphocytes. The inflammatory reaction persists for several days and progressively decreases through physiological regulatory mechanisms (*6*).

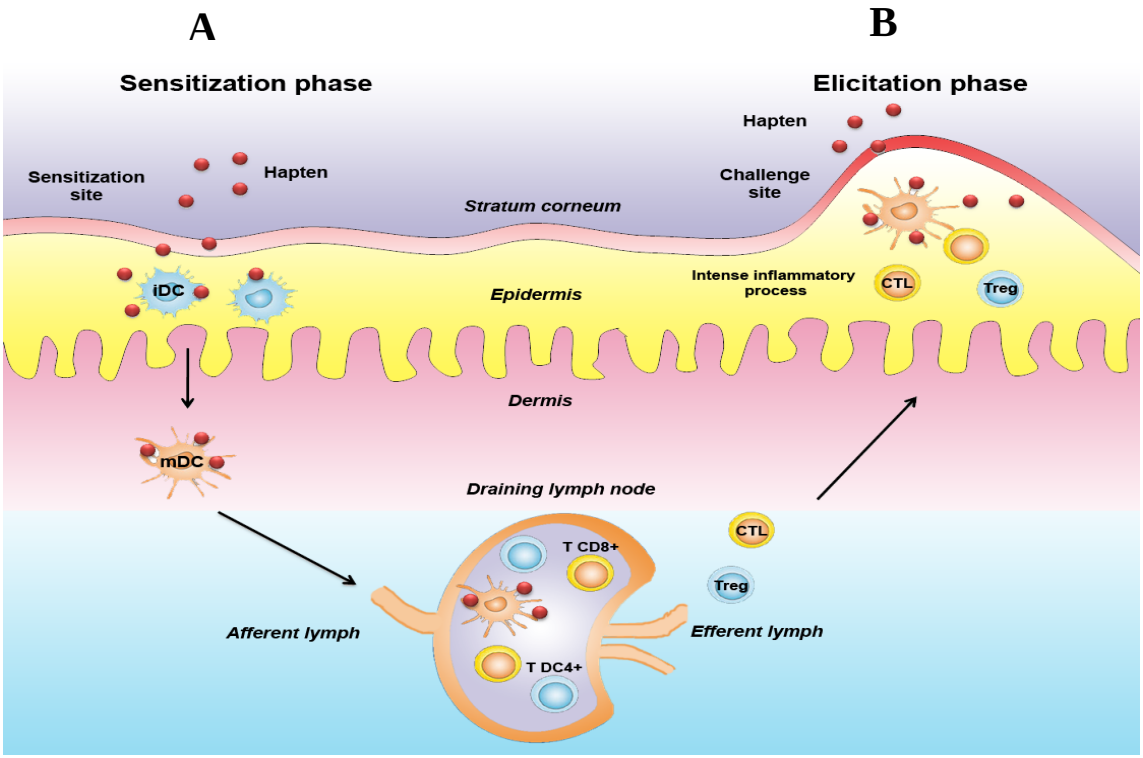


Figure 1. Physiopathology of allergic contact dermatitis. Sensitization phase (A). Following an encounter with an haptenized-peptide, dendritic cells become activated, leave the skin, migrate via the afferent lymphatics to the draining lymph nodes and differentiate into fully mature DC, able to effectively present antigens to naïveT-cells. **Elicitation phase (B).** 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. Figure taken from (*8*)

The sensitization and elicitation phases of primary ACD can be induced after a single skin contact with haptens due to the persistence of the hapten in the skin for long period of time, allowing the skin recruitment and the activation of T cells which have been primed in the lymphoid organs (*7*). Although the development of contact hypersensitivity has been described with two spatially and temporally dissociated phases, clinical evidence has demonstrated that ACD could develop after a single skin contact with a strong hapten in previously unsensitized patients, which is referred to as "primary ACD" (*9*). The pathophysiology of this primary (one step) ACD is identical to the classical (two step) ACD reaction (*7*).

2. Immunobiology and role of dendritic cells in ACD

As stated in previous section, dendritic cells play a crucial role in the sensitization phase of ACD. These cells constitute a family of highly specialized antigen-presenting cells (APC) with unique abilities to prime naïve T cell. They function as sentinels, scanning changes in their local microenvironment and transferring the information to the cells of the adaptive immune system, modulating, therefore, the outcome of immune responses.

Dendritic cells were for centuries considered part of the peripheral nervous system until the pioneer work of Ralph Steinman and Zanvil A. Cohn at the Rockefeller University, in 1973 that revealed the real function of these cells. At the time, Steinman and Cohn were studying spleen cells to understand the induction of immune responses in a mouse major lymphoid organ. They were aware from research in other laboratories that the development of immunity by mouse spleen required both lymphocytes and "accessory cells," which were of uncertain identity and function. The accessory cells were thought to be typical macrophages, but despite extensive laboratory experience with macrophages, Steinman and Cohn encountered a population of cells with unusual shapes and movements that had not been characterized before. Because the cells had unusual tree-like or "dendritic" processes, Steinman named them "dendritic cells" (*11*). By 1992, Steinman, with critical input from colleagues in Europe and Japan, developed methods to generate a large number of dendritic cells from their progenitors. At this point, dendritic cells models were available for cellular and molecular biologists, and their studies have greatly expanded dendritic cell research (*12*).

There are several subsets of DCs and their heterogeneity can be reflected by:

- Precursor Populations: in humans, different subsets of DC precursors circulate in the blood (CD14+ CD11c+ CD1-, CD14- CD11c+ CD1+ and CD14- CD11c- IL 3Rα+) (Figure 2).

- Anatomical Localization: these includes skin epidermal Langerhans cells (LCs), dermal/interstitial DCs (intDCs), splenic marginal DCs, T- zone interdigitating cells, germinal-center DCs, thymic DCs, liver DCs, and blood DCs. Even though certain

phenotypic differences have been observed among these different DC subsets, their lineage origins, maturation stages, and functional differences have not been evidently recognized.

- Function: DC subsets exert diverse functions, particularly in the regulation of B cell proliferation and polarization of T cells.

- Final result of immune response: induction of immunity or tolerance (*13*).

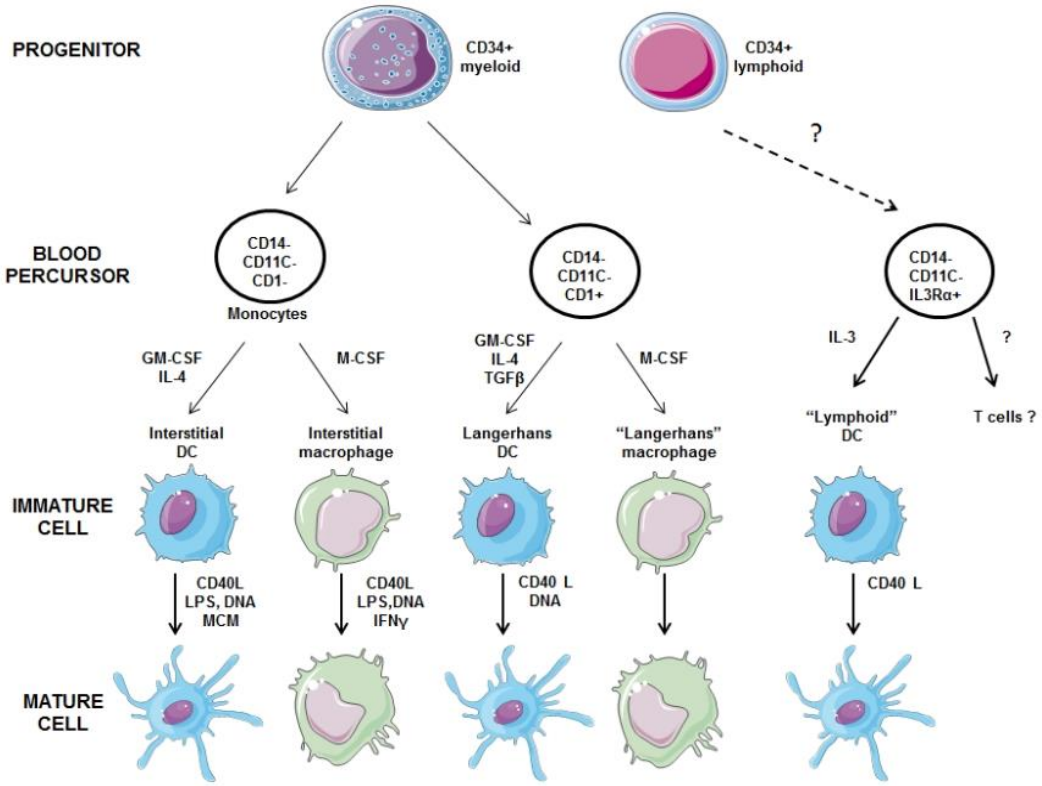


Figure 2. Origin of different human dendritic cells subtypes. Myeloid CD34+ progenitors differentiate into CD14+ and CD11c+ blood precursors that then differentiate into immature DCs in response to granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. In response to macrophage colony stimulating factor (M-CSF), blood monocytic precursors originate macrophages. CD11c+ and CD14- precursors also yield Langerhans cells when exposed to GM-CSF, IL-4 and transforming growth factor (TGF) β. The CD14- CD11c- IL-3R α + DC precursor (also called pDC2, IFN α -producing cell, or plasmacytoid T cell; a possible equivalent to the murine lymphoid DCs) may originate from the lymphoid CD34 + progenitor. The immature cells differentiate to mature cells in response to cytokines. Figure adapted from (*13*)

The plasmacytoid dendritic cell (DC2) and its immediate precursor, the plasmacytoid cell (pDC2, or pre-DC2), were recently characterized in humans. Upon stimulation, pDC2s produce high amounts of IFN-α/β and can differentiate *in vitro* into DC2s that are capable of inducing strong T-helper cell (Th) responses (*14*).

2.1 Dendritic cell maturation process

DCs are professional antigen-presenting cells originated from CD34+ bone marrow progenitor cells or CD14+ monocytes. These precursors migrate through the blood into peripheral tissues where they differentiate into immature DCs. Immature DCs scan continuously their microenvironment and are extremely efficient in the uptake and processing of antigens. After contact with danger signals such as local inflammation or microbial infection, DCs maturation process is triggered. Maturing DCs immediately migrate into secondary lymphoid organs where they present processed major histocompatibility complex (MHC)-bound peptide antigens to T-cells, thereby initiating antigen-specific T-cell responses (*15*). The maturation process is a complex and continuous course of events that results in morphological and phenotypical modifications that ultimately confers to DCs the ability to effectively stimulate T lymphocytes (*2*). Maturation of DCs can be induced by diverse danger signals such as immunoreactive molecules (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), GM-CSF, T-cell CD40L/CD154) and bacterial/ viral products such as lipopolysaccharide (LPS), lipoteichoic acid, lipoarabinomannan, CpG motifs in bacterial DNA and double-stranded viral RNA) (*13*).

Phenotypic and functional changes associated to DCs maturation consist in several coordinated events such as loss of endocytic and phagocytic receptors, changes in morphology, modification in lysosomal compartments with down regulation of CD68 and up regulation of DC–lysosome-associated membrane protein (DC-LAMP) and up regulation of cell surface expression of MHC II molecules (*16*). There is also an up regulation of co-stimulatory molecules necessary for lymphocyte activation, such as the intercellular adhesion molecule (ICAM)-1/CD54, leukocyte functional antigen-3 (LFA)-3/ CD58, B7-1/CD80, B7-2/CD86, and CD83 (exclusive marker of matured DCs). During maturation process, cytokine expression profile is significantly changed, with a general increase in the expression of TNF-α, IL-10, IL-1, IL-12, IFN-γ, IL -8, IL-6, IL-23. Regarding chemokines, after contact with the maturation stimulus, peripheral tissue DCs transiently produce the inflammatory chemokines CCL2, CCL3, CCL4, CCL5, CCL8, and CXCL8 important for the recruitment of monocytes and neutrophils to the site of infection. Subsequently, in a later maturation stage, lymphoid chemokines (CCL17, CCL18, CCL19,

CCL22 and CXCL10) are preferentially expressed which attract T and B lymphocytes, facilitating this way the interaction of these cells with DCs. The expression profile of cytokines and chemokines is essentially conditioned by the DC subtype and the stimulus that triggers the maturation process (*2*) (Figure 3).

Morphological changes that occur during DC maturation include loss of adhesive structures, cytoskeleton reorganization, and achievement of high cellular motility (*17*).

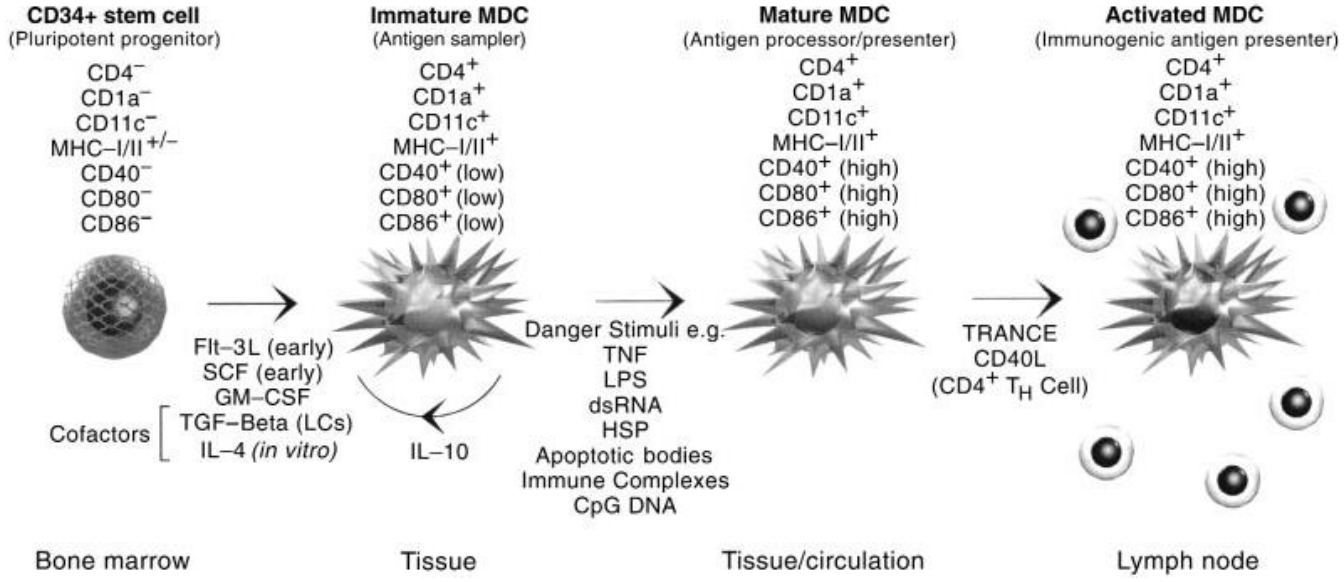


Figure 3. DCs phenotypic and functional changes triggered during maturation process. DC maturation can be triggered by multiple stimuli, including contact allergens, microorganisms, pro-inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor-α, microorganisms and microorganisms components such as lipopolysaccharide (LPS), bacterial DNA and double-stranded RNA. The maturation process is a well-coordinated succession of events characterized by changes in morphology, loss of endocytic/phagocytic receptors, shift of chemokine receptors, resulting in acquisition of migratory capacity, up-regulation of co-stimulatory molecules CD40, CD58, CD80, and CD86, alteration of secreted cytokines/chemokines and shift in lysosomal compartments. *GM-CSF, granulocyte–macrophage colony stimulating factor; HSP, heat shock protein; MDC, myeloid dendritic cell; SCF, stem cell factor; TGF, transforming growth factor.* Figure taken from (*16*).

2.2 Dendritic cell models

The study of dendritic cells remained for a long time limited by the inability to isolate and purify large numbers of their different subpopulations. This problem led the scientific community through an intensive research process in order to establish protocols for *in vitro* differentiation of DCs. The development of such protocols revealed of great importance for the study of DC immunobiology. Therefore nowadays, primary DCs are differentiated either from human cord blood CD34+ hematopoietic precursors or peripheral blood CD14+ myeloid precursors. These precursors are isolated and cultivated with several cytokine/growth factors cocktails leading to various DC subtypes such as immature interstitial DCs (using GM-CSF and IL-4) or Langerhans cells (using GM-CSF, IL-4 and transforming growth factor-β (TGF-β) (*2*). However, primary DC surrogates have certain limitations such as difficulty in access to cell sources, time-consuming and expensive cell culture procedures, and more importantly, significant donor to donor variations in cell responsiveness. To overcome these problems of primary DCs, myeloid DC-like cell lines have been extensively used in the last decades. These DCs-like cell lines, although not completely similar in phenotype, are proven to be of great value for mechanistic and functional studies. In a recent work, Santegoets and collaborators have shown that leukemia-derived cell lines are efficient in differentiation into functional DCs, expressing elevated levels of co-stimulatory and MHC class I and II molecules, and are also capable of inducing leukemia-specific cytotoxic T cells (*18*). These DC surrogates created conditions for the improvement of highly reproducible DC vaccines and provided *in vitro* model systems for deeply studies about DC physiology. Furthermore, data provided demonstrate that the human $CD34^{+}$ acute myeloid leukemic cell line MUTZ-3 shows functional properties that are essential for the *in vivo* production of CTL-mediated immunity and therefore represents the mainly helpful, sustainable model for myeloid DC differentiation and clinical DC vaccination studies (*19*). The most relevant characteristics of some common DC-like cell lines are evidenced in Table 1. In the experimental work of this thesis we used as DC-surrogate the human leukemic cell line THP1. This DC-cell model was chosen attending its easier and inexpensive maintenance in culture given that these cells have a rapid doubling time and do not require exogenous factors to growth. Furthermore THP1 cells are a good model for mechanistic studies allowing reproducible results when cultured for periods shorter than 2 months.

Table 1. Common DC-like cell lines such as THP-1, KG-1, HL-60, Monomac-6, U-937, K562, MUTZ-3, FSDC and its main characteristics.

Cell line	Characteristics
THP-1	- described for the first time in 1980; - human leukemia cell line; - individual monocytic characteristics: lysozyme production and phagocytosis ability; - distinct monocytic markers; - maintained monocytic characteristics for over 14 months; - obtain DC properties through stimulation with cytokines; - DC differentiation capabilities is very low: fewer than 5% of THP-1 cells express the classic myeloid DC marker CD1a following differentiation; - addition of calcium ionophores resulted in total differentiation and immediate maturation; - More similar to macrophages than to DCs; - express high levels of CD80, CD86, CD40, and CD83; - exhibit increased allogeneic T cell-stimulatory competence; - reduced receptor-mediated endocytosis ability (12, 16, 17).
KG-1	- $CD34^+$ myelomonocytic cell line; - derived from a patient with erythroleukemia undergoing myeloblastic degeneration (*19*); - In culture with phorbol esters produce pseudopodia and exhibit characteristics of the monocyte/macrophage lineage such as phagocytic capacities, nonspecific esterase and lysozyme-secreting activity (*20*); - Similarly to THP-1 cells present low DC differentiation capacity; - just about 10% of differentiated cells expressing CD1a (*21–24*); - Differentiated cells express the DC maturation marker CD83, intermediate-to-high levels of CD86 and HLA-DR; - present enhanced allogeneic T cell-stimulatory capacities; - maturation of the KG-1 DC was not associated with decreased antigen uptake and the acquirement of lymph node migration capacity (*23*, *25*).
HL-60	- acute promyelocytic leukemia cell line; - competence for differentiating along the granulocytic, monocyte-macrophage, or eosinophilic pathway;

	- in culture with chemicals or biological agents such as DMSO and retinoic acid, HL-60 differentiate into mature granulocytes (*4*, *26*); - when exposed to 1, 25-dihydroxy-vitamin D3 or phorbol esters, HL-60 cells differentiated in monocyte-macrophage cells (*27*, *28*); - when cultured in mild alkaline conditions HL-60 cells are able to differentiate into eosinophilic granulocytes (*29*); - limited capacity to be differentiated into DC-like cells even after cytokine exposure (*30*); - addition of calcium result in some cells differentiating into functional DCs (*31*); - unsuccessful to express MHC class II molecules; - low levels of MHC class I molecules (*31*).
Monomac-6	- human acute monocytic leukemia cell line; - marked monocyte phenotype; - present strong phagocytosis activity; - express NaF-sensitive nonspecific esterases and the mature monocyte marker CD14 (*32*); - in response to LPS, increase the expression of IL-1β, IL-6, and TNF-α; - capable of migration toward β-chemokines such as MIP-1α and -β, RANTES (*19*, *33*, *34*); - present characteristics similar human blood monocytes; - not efficient to differentiate into DC (*15*, *22*).
U-937	- histiocytic lymphoma cell line; - monocytic characteristics; - demonstrating monoblast morphology; - lysozyme production; - esterase activity; - do not show phagocytic activity (indicating that they are stopped at a relative early stage of monoblast maturity); - when stimulated with phorbol 12-myristate 13-acetate (PMA), U-937 cells mature and present monocytes morphologic phenotypic characteristics (*35*); - not efficient to differentiate into DC (*15*, *22*).
K562	- chronic myelogenous leukemia cell line; - multipotent cell line, competent to differentiating along the megakaryocytic, erythroid, and monocytic pathway; - develop DCs cytoplasmic projections when stimulated with PMA/TNF-α;

	- expression of classic DC markers such as CD86, CD40 and CD83 stays low; - without the presence of PMA/TNF-α the result is an exchange of the DC phenotype and function; - revealed to be insensitive to cytokine stimulation (*36*, *37*); - protein kinase C signaling stimulate limited DC differentiation; - not effective in developing DC differentiation (*37*).
MUTZ-3	- results from the peripheral blood of a patient with acute myelomonocytic leukemia; - morphological and phenotypical characteristics of monocytes: expression of monocyte specific esterase, myeloperoxidase enzymes, expression of monocytic marker CD14 (*19*); - requires continuous cytokine treatment for its proliferation and survival (*38*); - when treated with GM-CSF and IL-4, present characteristics of $CD34^+$ derived DC precursors and to down regulate CD14 (*22*); - LPS did not stimulate maturation of MUTZ-3-derived DC (most probable as an effect of reduced TLR4 receptor signaling and subsequent phosphorylation of p38-MAPK, ERK1, and ERK2) (*39*); - DC differentiation capacity of MUTZ-3 cells is relatively high, reaching levels between 60 and 90% (*18*, *40*); - closely resemble primary DCs with the exception of strong adhesion and podosome formation in the immature state (*21*, *22*, *41*); - express intermediate-to-high levels of co-stimulatory, adhesion, and MHC class I and II molecules; - exhibit a close DC phenotype; - revealed to be very similar to skin LC (expressing hallmarks of the LC lineage such as Langerin, membrane ATPase, and CCR6) (*42*, *43*).
FSDC	- myeloid cell line from fetal mouse skin; - obtained by infecting cell suspensions with a retroviral vector; - established by Girolomoni and co-workers in 1995; - exhibits a dendritic morphology; - its proliferation in serum-free medium was promoted by GM-CSF; - expressed strong surface-membrane ATP/ADPase activity; - intracellular staining for 2A1 antigen; - cultured in the absence of exogenous recombinant growth factors; - surface phenotype consistent with a myeloid precursor; - manifestly increased their function after treatment with GM-CSF, IL-4 or IFN-γ; - *in vitro*, pre-culture with IFN-γ was necessary for presentation of haptens to naïve T cells (*44*).

3. Molecular mechanisms of contact sensitizers-induced DC maturation

The molecular mechanisms by which sensitizers trigger and shape dendritic cell maturation is not yet completely unrevealed, however several evidences suggest that initial danger signals such generation of reactive oxygen species (ROS) and release of ATP may play an important role (*45*). ROS are significant in host defenses against bacterial infection, skin aging, cancer and various chronic diseases (*46*). ROS are molecular oxygen derivatives that have a variety of functions in cells. In addition of acting as direct bacterial killing mechanisms, ROS also stimulate the immune response through activation of signaling pathways, up-regulation of surface co-stimulatory molecules, protein carbonylation and secretion of inflammatory cytokines such as IL-12. The processes of ROS production and inactivation are strongly regulated by enzymatic and non-enzymatic antioxidant systems (*1*).

Normal skin cells generate ROS such as superoxide anion ($O_2^{\bullet-}$) and H_2O_2 as a result of normal metabolism. Both $O_2^{\bullet-}$ and H_2O_2 may be converted to the highly reactive hydroxyl radical ($OH^{\bullet-}$) by iron (Fe^{2+})-catalyzed Haber–Weiss and Fenton reactions. ROS can be formed as a result of exposure to environmental agents including reactive chemicals. Many xenobiotics are converted to toxic quinones by the family of functionally related enzymes known as cytochrome P450 (CYP). These quinones are redox-sensitive agents and are reversibly reduced to semihydroquinones/hydroquinones, which generate $O_2^{\bullet-}$. These ROS interact with lipid-rich plasma membranes and initiate a reaction known as lipid peroxidation. Numerous intracellular enzymes serve to degrade these reactive species. Some of these enzymes are specific such as superoxide dismutase (SODs), which dismuts $O_2^{\bullet-}$ to H_2O_2, whereas others have overlapping substrate affinities such as catalase and glutathione peroxidases, both of which can degrade H_2O_2 to water and O_2. Glutathione peroxidases also degrade organic peroxides to relatively non-toxic alcoholic species. These enzymes also require glutathione (GSH) during the course of peroxide degradation and convert GSH into its oxidized form, which is then recycled by the enzyme glutathione reductase (*46*). ROS generation may increase leak of Ca^{2+} from the ER lumen and in turn increases in cytosolic Ca^{2+} can stimulate mitochondrial ROS production. This results in the opening of permeability transition pore, leakage of GSH from the matrix and, as a consequence, depletion of reducing equivalents. The cellular redox homeostasis is

maintained by a dynamic interaction between reduced glutathione and protein thiols with ROS. Reduced glutathione GSH serves as a major thio-disulfide redox buffer in cells and the ratio of GSH to oxidized glutathione (GSSG) is used as an index of the redox state (*47*).

Dashlkhumbe Byamba and collaborators shown that contact allergens, in contrast to irritants, induce strong production of ROS in DC and keratinocyte cell models. In the few cases where irritants were shown to induce ROS, such as in benzalkonium chloride (BKC) treated cells, this production is not followed by DC maturation. Additionally, they shown that DNCB-induced, but not BKC-induced, ROS production increased cell surface CD86 and HLA-DR molecules on human monocyte-derived DCs (MoDCs) (*1*). The authors suggest that DNCB-induced ROS may be different from those induced by the irritant BKC and that this difference may partially explain the inability of irritants to mature DCs and trigger immune responses (*1*).

The precise intracellular signal transduction pathways activated by skin sensitizers in DCs is not fully characterized, but several works indicate that the most common observation is the increase of phosphotyrosine levels and the activation of mitogen-activated protein kinases (MAPK) in response to oxidative and electrophilic stress (*48*). Enzymes belonging to the family of MAPKs are strong candidates for activating effector proteins, since they propagate signals generated from different stimuli and have a multiplicity of signal transducing functions, converting extracellular signals into intracellular responses. Three major genetically distinct MAPK pathways are known at present in mammals: the extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal kinases (JNKs) and 38 high osmolarity glycerol protein kinase (p38 MAPKs) (*1*). Activated MAPKs can translocate into the nucleus where they phosphorylate substrates such as transcription factors. Chemical sensitizers have been shown to activate the three MAPKs, being the increase of p38 MAPK activity the most relevant event for the DC maturation process (*49*, *50*).

Haptens were also shown to trigger tyrosine phosphorylation and to activate protein kinase C (PKC), Janus kinases (JAK)/STAT pathways (Figure 4), different transcription factors such as NF-κB, NRF2/ARE, ATF-2, CREB, CHOP, Elk1, activating protein-1 (AP-1) and to promote glutathione depletion and changes in cell surface thiols (*2*, *50*).

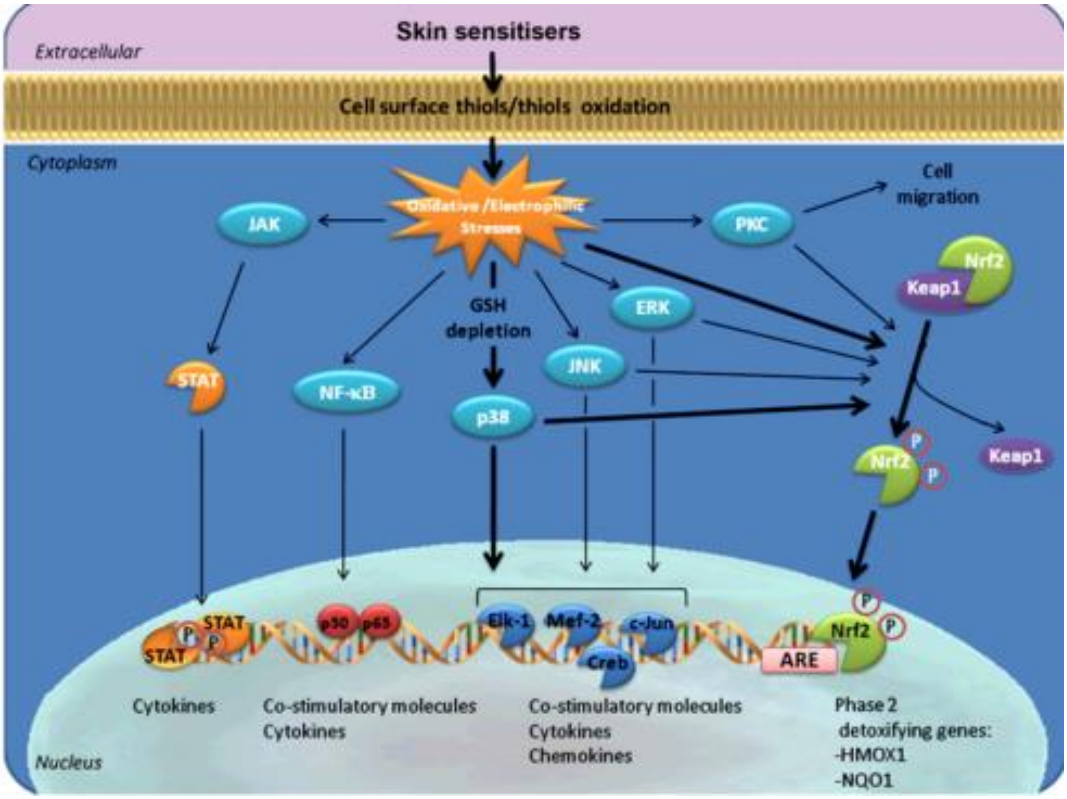


Figure 4. Signaling pathways triggered in dendritic cells by chemical sensitizer. The small chemical compound is able to covalently bind protein structures, namely lysine and cysteine residues, thus forming stable complexes with proteins. These complexes induce oxidative/electrophilic stress and consequently GSH depletion. Contact sensitizers induce maturation/activation of DCs by different signaling pathways. It would be reasonable that skin sensitisers recognized by DC as danger signals, modulate the JAK/STAT pathway. However, JAK/STAT signaling pathway in DCs activation by haptens is rather unlikely. Some sensitisers activate the NF-kB transcription factor and are probably responsible for some phenotypic modifications observed in DC. The most important class of MAPK involved in DCs activation is p38 MAPK. Activated MAPKs (p38, JNK and ERK) can translocates into the nucleus where they phosphorylate substrates such as transcription factors (Elk-1, Mef-2, Creb, c-Jun). The signalling pathway involving the repressor protein Keap1 (Kelch-like ECH associated protein 1) and the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which binds to the antioxidant response element (ARE) in the promoter region of many phase II detoxification genes is also a signalling pathway recently proposed. Finally, the involvement of PKC on DC migration is a prerequisite for skin sensitisation. Figure taken from (*50*)

In the next table were shown a variety of phenotypic changes induced by various haptens in DCs (Table 2) (*2*).

Table 2. The effects in different subpopulations of DCs exposed to contact sensitizers.

Contact sensitizer	Effects
2,4-dinitrofluorobenzene (DNFB) 1-chloro-2,4-dinitrobenzene (DNCB) Oxazolone $K_2Cr_2O_7$ 2,4,6-trinitrochlorobenzene (TNCB) Sodium dodecyl sulfate (SDS)	decrease in the Ia-antigen expression on Langerhans cells (LCs)
interferon-α (IFN-α) DNCB2-mercaptobenzothiazole p-phenylenediamine ammonium tetrachloroplatinate	internalization of MHC class II molecules on human monocyte cell line THP-1
DNCB TNCB DNFB	increased expression of class II MHC I-Aα on mouse LCs, produced of interleukin-1 (IL-1), antigen I-Aα, IL-1α, IL-1β, IFN-induced protein 10 (IP-10), and macrophage inflammatory protein 2 (MIP-2) mRNAs were up-regulated
dinitrobenzene sulfonic acid (DNBS) oxazolone	increased expression of MHC class II molecules on the XS52 mouse bone marrow-derived cell
trinitrobenzene sulfonic acid (TNBS)	up-regulating of FcεRII (CD23), FcγRII/III (CD32/CD16) and CD45
DNCB $NiCl_2$	increased the surface expression of CD54, CD86, HLA-DR antigen, and IL-1β production
TNBS fluorescein isothiocyanate or Bandrowski`s base	stimulated proliferation of autologous T lymphocytes, increased expression of CD83, CD86 and HLA-DR

$NiSO_4$ DNCB TNBS α-hexylcinnamaldehyde eugenol	up-regulation of CD54, CD86, and HLA-DR
TNCB	increase I-A/E antigen and decrease E-cadherin expression
Isoeugenol Cinnamaldehyde TNBS Bandrowski`s base p-phenylenediamine	down-regulation of E-cadherin expression on human epidermal LCs as well as a significant decrease in the percentage of E-cadherin positive cells
TNCB	produced of type IV collagenase/ Matrix metallopeptidase 9 (MMP-9) in LC-enriched epidermal cells
DNCB DNFB	expression of MMP-9
$NiCl_2$ DNCB	increase expression of mRNA for chemokine receptor 7 (CCR7)
$NiCl_2$	increase in expression of CD54, HLA-DR, a5b1- integrin, CD44 and CD44v6, and directly induced the augmentation of CD86 expression in TGF-β1+ DCs
DNCB 2-mercaptobenzothiazole Eugenol p-phenylenediamine	increased CD86 expression in THP-1 human monocyte cell line

ammonium tetrachloroplatinate	
DNCB DNFB $NiCl_2$ $MnCl_2$	down-regulation of c-fms (encoding the colony-stimulating factor CSF-1 receptor)
DPCP (diphenylcyclopropene)	Increase of cell-surface thiols in THP-1 Modified proteins such as actin and β-tubulin, molecular chaperones, and endoplasmic reticulum stress-inducible proteins (*51*)

Despite the knowledge acquired in recent years, additional studies are required to completely understand cellular stress pathways elicited by skin sensitizers in DC and the concomitant relevance for their maturation process during the early phase of ACD.

4. Oxidative stress and ER dysfunction: closely linked events

Protein folding and production of ROS are closely linked events (*47*). Uncontrolled production of ROS can directly or indirectly (or both) affect ER homeostasis and perturbations in protein folding can cause alterations in cellular redox status and increase generation of mitochondrial ROS. In recent years several studies have shown a close link between ER stress, ROS production and the pathogenesis of metabolic and degenerative diseases such as type 2 Diabetes and Alzheimer and Parkinson diseases, respectively (*47*). It was shown that in Machado-Joseph syndrome, the polyglutamine repeats present in spinocerebrocellular atrophy protein (SCA3) form cytosolic aggregates that can inhibit the proteasome. Proteasome inhibition subsequently interfere with ERAD to trigger UPR activation, caspase 12 activation, and apoptosis (*52*, *53*). In this model, the deletion of the ER stress-induced pro-apoptotic transcription factor CHOP preserved neuronal function, revealing the importance of UPR signaling.

A good example of the relation between ER stress and oxidative stress was demonstrated by the study of Hui-Juan Gao and co-workers (*54*). They observed that alterations of the ubiquitinated proteins, valosin-containing protein (VCP) and glucose regulated protein 78 (GRP 78) involved in oxidative stress, UPR and proteolysis presented a complex and dynamic interaction at the maternal-fetal interface. In this study, they developed a cell model of oxidative stress using normal decidual cells to examine cell viability and expression levels of proteins related to endoplasmic reticulum stress and UPR. They found that GRP 78 and ubiquitinated proteins were significantly up-regulated in hydrogen peroxide (H_2O_2) treated cells in a dose-dependent manner. Obtained data allow conclude that excessive oxidative stress influence proper function of UPR by decreasing VCP, thereby leading to cell damage as well as inhibition of cell growth and activation of apoptosis. In addition, when pretreated with MG132, a pharmacological proteasome inhibitor, H_2O_2-treated decidual cells became less viable and could not up-regulate the expression of GRP78 to resolve the protein folding defects. This indicates that malfunction of UPR in decidual cells might aggravate the cytotoxic effects of ROS (*54*). In addition to the intracellular responses, chronic ER stress can also, by mechanisms that remain poorly characterized, cause inflammation within affected tissues. In particular, proteins belonging to the NOD-like receptor (NLR) family have been identified as central players in innate immunity (*55*, *56*).

Some of these NLRs participate in multiprotein complexes termed inflammasomes, which mediate caspase-1-dependent maturation of the highly proinflammatory cytokine interleukin-1β (IL-1β). Of the thus far described inflammasomes, the NLRP3 inflammasome is most fully characterized (*57*). In addition to microbial and viral PAMPs (pathogen-associated molecular patterns), the NLRP3 inflammasome is unique in that it can sense the presence of endogenous damage-associated molecular patterns (DAMPs) that are danger signals associated with cellular or tissue damage or stress. In a recent work, Menu and collaborators demonstrated that three different compounds that are known to induce ER stress (BFA, Tunicamycin and thapsigargin), also activate the NLRP3 inflammasome in human and murine macrophages (*58*). They showed that ER stress causes activation of the NLRP3 inflammasome, with subsequent release of the pro-inflammatory cytokine interleukin-1β. This ER-triggered pro-inflammatory signal shares the same requirement for ROS production and potassium efflux compared with other known NLRP3 inflammasome activators, but is independent of the classical UPR. They proposed that the NLRP3 inflammasome senses and responds to ER stress downstream of a previously uncharacterized ER stress response signalling pathway distinct from the UPR, thus providing mechanistic insights to the link between ER stress and chronic inflammatory diseases (*58*).

ASK1 is an effector of the IRE1 branch of the UPR that directly connects ER stress and oxidative stress. ASK1 kinase is activated in response to a variety of cytotoxic stimuli including TNF, Fas and ROS such as H_2O_2 and it is also activated by IRE1-TRAF2 complexes in response to ER stress. Phosphorylated ASK1 subsequently activates c-Jun NH2-terminal kinase and p38 MAPK, being connoted with induction of programmed cell death. However, the roles of JNK and p38 signalling pathways during apoptosis have been controversial. Kei Tobiume and collaborators showed that TNF- and H_2O_2-induced sustained activations of JNK and p38 are lost in *ASK1*$^{-/-}$ mouse embryonic fibroblasts, and that *ASK1*$^{-/-}$ cells are resistant to TNF- and H_2O_2-induced apoptosis. TNF- but not Fas-induced apoptosis requires ROS-dependent activation of ASK1–JNK/p38 pathways. Thus, ASK1 is selectively required for TNF- and oxidative stress-induced sustained activations of JNK/p38 and apoptosis (*59*).

Although great advances were made in last decade regarding the link between oxidative and ER stresses, further research is still needed to clarify their role in the genesis and progression of inflammatory diseases.

5. Unfolded protein response

Protein folding is a vital process in all organisms and all cells have complex mechanisms to avoid protein misfolding. The effectiveness of protein-folding reactions depends on suitable environmental, genetic, and metabolic conditions and unbalances of these processes present a threat to cell viability and functionality. Every protein that enters the secretory pathway in eukaryotic cells first enters the ER, where they fold and accumulate into multisubunit complexes before pass to the Golgi complex. A sensitive surveillance mechanism exists to prevent misfolded proteins from transiting the secretory pathway and ensures that persistently misfolded proteins are directed toward a degradative pathway. The ER-associated degradation (ERAD) allows that misfolded proteins are kept in the ER lumen in complex with molecular chaperones or are directed toward degradation through the 26S proteasome (*47*).

The ER provides a unique environment that allows right protein folding as nascent polypeptide chains go into its lumen. The elevated concentrations of incompletely folded and unfolded proteins present on ER lumen tend to increase aggregation phenomena. Therefore, this organelle process high level of polypeptide-binding proteins, such as GRP78/BiP and GRP94 that works to slow protein-folding reactions and prevent abnormal interactions and aggregation. The ER lumen also needs to be an oxidizing environment in order to allow the formation of disulfide bonds. As a result, cells express many protein disulfide isomerases (PDIs) that guarantee correct disulfide-bond formation and avoid creation of wrong disulfide bonds. Additionally, ER is the primary intracellular Ca^{2+} storage/release organelle. Calcium is a crucial second messenger in numerous intracellular signalling processes and the control of its cytosolic free levels is of extreme importance for the correct functioning of the cell. Protein-folding reactions and protein chaperone functions need elevated ER intraluminal Ca^{2+} levels. The protein folding process also requires extensive quantities of energy (ATP) for chaperone function, to preserve Ca^{2+} stores, the redox homeostasis and for ERAD (*47*, *60*). Once in the ER lumen,

proteins may suffer several posttranslational modifications such as N-linked glycosylation, amino acid modifications such as proline and aspartic acid hydroxylation and γ-carboxylation of glutamic acid residues, and addition of glycosylphosphatidylinositol anchors. N-linked glycosylation is an extremely regulated process closely related to chaperone interactions which ensures that only correctly folded proteins exit the ER compartment. These processes are highly sensitive to disturbances of the ER luminal environment and are therefore affected by many environmental threats. Such insults include mechanisms that induce depletion of ER Ca^{2+}, alterations in the redox status and deprivation of energy (sugar/glucose) (*47*). Additionally, gene mutations, overload of ER compartment with newly synthesized proteins and disrupted posttranslational modification also contribute to the accumulation of unfolded proteins in the ER lumen (Figure 5).

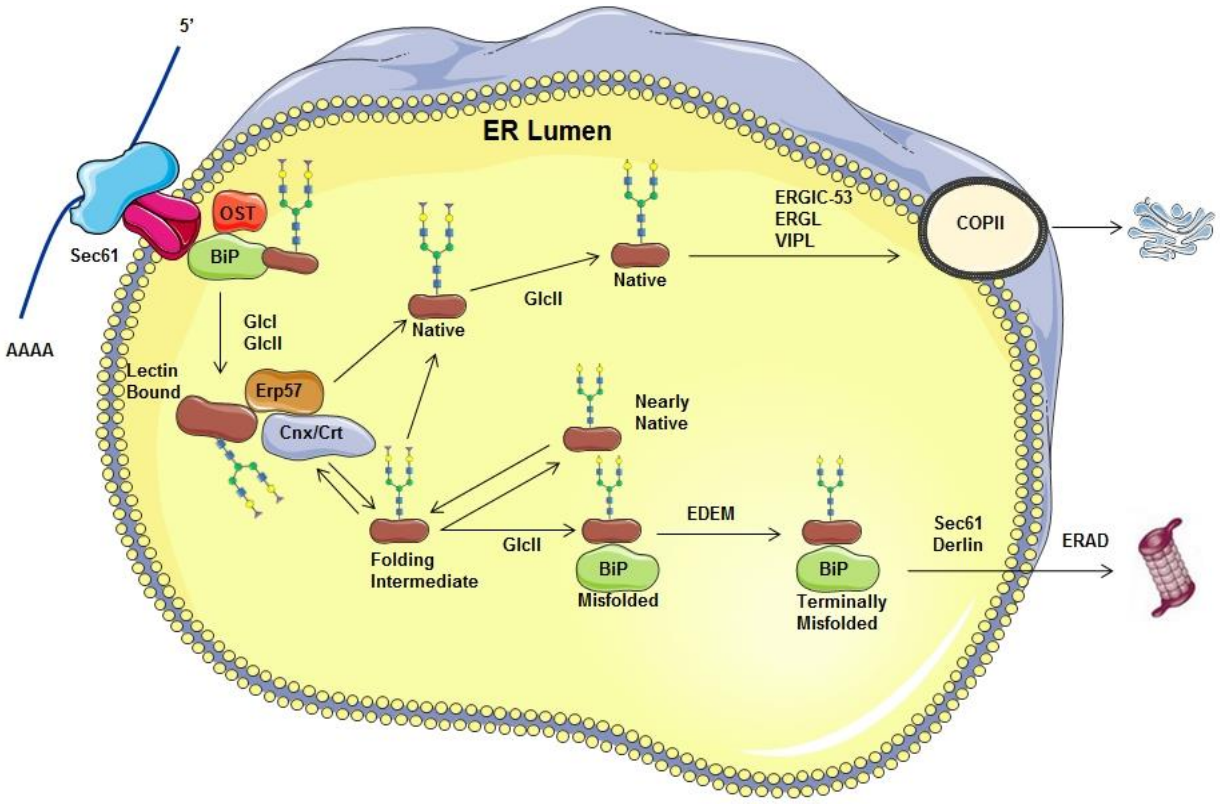


Figure 5. Overview of Protein Trafficking Mechanisms in the ER. In the transport of polypeptides through the Sec61 channel, asparagines residues are often modified by oligosaccharyltransferase (OST) through covalent addition of an oligosaccharide core. The molecular chaperone BiP interacts with nascent polypeptide chains in the ER lumen, facilitating the unidirectional transportation through the translocon. Subsequently, glucosidase I and II (GlcI and GlcII) remove the two outermost glucose residues on the oligosaccharide core structures preparing glycoproteins for association with the calnexin (Cnx) and

calreticulin (Crt). ERp57, an calnexin/calreticulin associated oxireductase facilitates protein folding by catalyzing formation of intra- and intermolecular disulfide bonds. The separation form calnexin/calreticulin, and the subsequent cleavage of the innermost glucose residue by glucosidase II, avoids additional interactions with calnexin and calreticulin. In this phase, natively folded polypeptides transit the ER to the Golgi complex. This process is assisted by mannose-binding lectins such as ERGIC-53, VIPL, and ERGL. A crucial step of protein-folding control is that nonnative polypeptides are marked for reconnection with calnexin/calreticulin by the UDP-glucose: glycoprotein glucosyltransferase (UGT1). UGT1 make possible their retention in ER and avoid the normal transport. Misfolded polypeptides are targeted for degradation; this is perhaps mediated by EDEM and Derlins. *COPII, coat protein complex; Triangles, glucose residues; squares, N acetylglucosamine residues;circles, mannose residues.* Figure adapted from (*47*)

Proper adjustment to misfolded protein accumulation requires modifications at multiple levels including transcription, translation, translocation into the ER lumen, and endoplasmic-reticulum-associated protein degradation (ERAD). The inability of cell to resolve incorrect protein folding processes leads to a sustained activation of the UPR signalling, which has been shown to induce apoptotic responses (*61–64*).

Intensive research in last decade revealed diverse mechanisms of the UPR signalling pathway being established that the response to ER stress is leaded by three transmembrane signal transducers: the kinases inositol-requiring kinase 1 (IRE1) and PERK (double-stranded RNA-activated protein kinase-like ER kinase) and the activating transcription factor 6 (ATF6) (Figure 6).

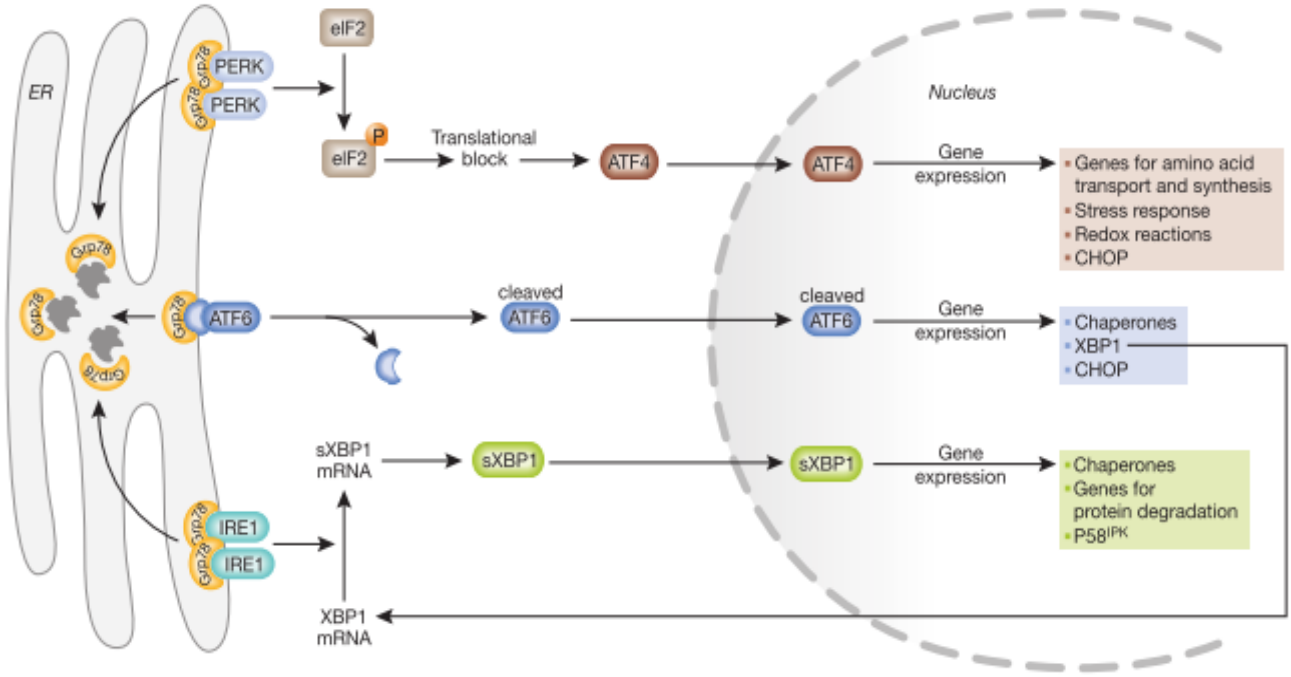


Figure 6. Signal transduction pathways of the unfolded protein response. UPR is controlled by the signal transducers IRE1, PERK, and ATF6 and their respective signaling cascades. In homeostatic conditions the chaperone protein GRP78/ BiP bind to the luminal domains of IRE1 and PERK preventing their dimerization. In ER stress conditions, the increase of unfolded proteins in the lumen of ER promotes the release of GRP78 from IRE1 allowing its dimerization. This dimerization stimulates IRE1 kinase and RNase activities leading to XBP1 mRNA splicing. Primary targets that need IRE1/XBP1 pathway for stimulation are genes encoding functions in ERAD. In the same way, ATF6 liberated from GRP78 is transported to the Golgi compartment, where it is cleaved by S1P and S2P proteases. The resultant fragments are then translocated to the nucleus where they act as potent transcriptional factors of UPR genes. Additionally, the release of, GRP78 from PERK allows its dimerization and activation which promotes the phosphorylation of eIF2α, leading to a general reduction of translational processes. Paradoxically, the eIF2α phosphorylation induces the translation of ATF4 mRNA, a transcription factor that regulates the expression of anti-oxidative stress-response genes and genes encoding proteins with pro-apoptotic functions, such as CHOP. Figure taken from (*93*)

5.1 IRE1 signalling

In the early 1990s, by a genetic screen approach, two different groups identified IRE1p/Ern1p as an ER transmembrane protein kinase that operates as a proximal sensor in the yeast UPR (*65*). IRE1p is a bi-functional protein that also presents a site-specific endoribonuclease (RNase) activity (*66*). In homeostatic conditions, IRE1p protein kinase is kept in an inactive form through interactions with the protein chaperone BiP. However, under ER stress, IRE1p is released from BiP and go through homodimerization and trans-autophosphorylation which leads to activation of its RNase activity. The RNase activity of IRE1p causes the cleavage of a 252-base intron from mRNA encoding the basic leucine zipper (bZIP)-containing transcription factor HAC1p. Subsequently, the protein encoded by spliced HAC1 (homologous to ATF/CREB 1) mRNA attach and activates the transcription of many UPR target genes (*63*, *67*).

IRE1α and IRE1β have been identified as two mammalian homologues of yeast IRE1 (*68*). IRE1α is ubiquitously expressed in cells and tissues, being high levels found in pancreas and placenta. In turn, IRE1β is predominantly expressed in intestinal epithelial cells. These two proteins present similar cleavage specificities, suggesting that they do not recognize distinct substrates but rather have temporal and tissue specific expression (*69*, *70*). In mammals, the functional homolog of HAC1p is XBP-1 (X-box binding protein) mRNA, being the substrate for the endoribonuclease activity of IRE1α/IRE1 β (*62*, *71*). Under ER stress, IRE1 RNase activity is triggered resulting in the removal of a 26-nucleotide intron from XBP1 mRNA. This splicing process results in the translation of a larger form of XBP1 that exhibits a new transcriptional activation domain in its C-terminus. Spliced XBP1 is a potent transcriptional activator of numerous genes involved in UPR such as the ones involved in ERAD (Figure 7). This fact was demonstrated by experiments in which cells lacking IRE1 or XBP1 are unable to execute ERAD (*72*). Furthermore, IRE1 and XBP1 have shown to play crucial roles in B-cell differentiation (*73*, *74*). Upon antigenic stimulation, UPR is activated in B lymphocytes and IRE1-mediated XBP1 mRNA splicing is required to B-lymphocyte differentiation into plasma cells (*74*). These data suggest that the IRE1/XBP1 signalling pathway may be necessary for differentiation of cell types that secrete elevated levels of protein (*73*).

In addition to its endoribonuclease activity over XBP1 mRNA, IRE1 phosphorylation activates the MAP kinase signalling cascade. The IRE1 cytoplasmic domain interacts with the adaptor protein tumor necrosis factor receptor–associated factor 2 (TRAF2). IRE1 and TRAF2 interact with the mitogen activated protein kinase apoptosis signal-regulating kinase 1 (ASK1) which subsequently phosphorylates and activates JNK (*59*, *63*, *75*).

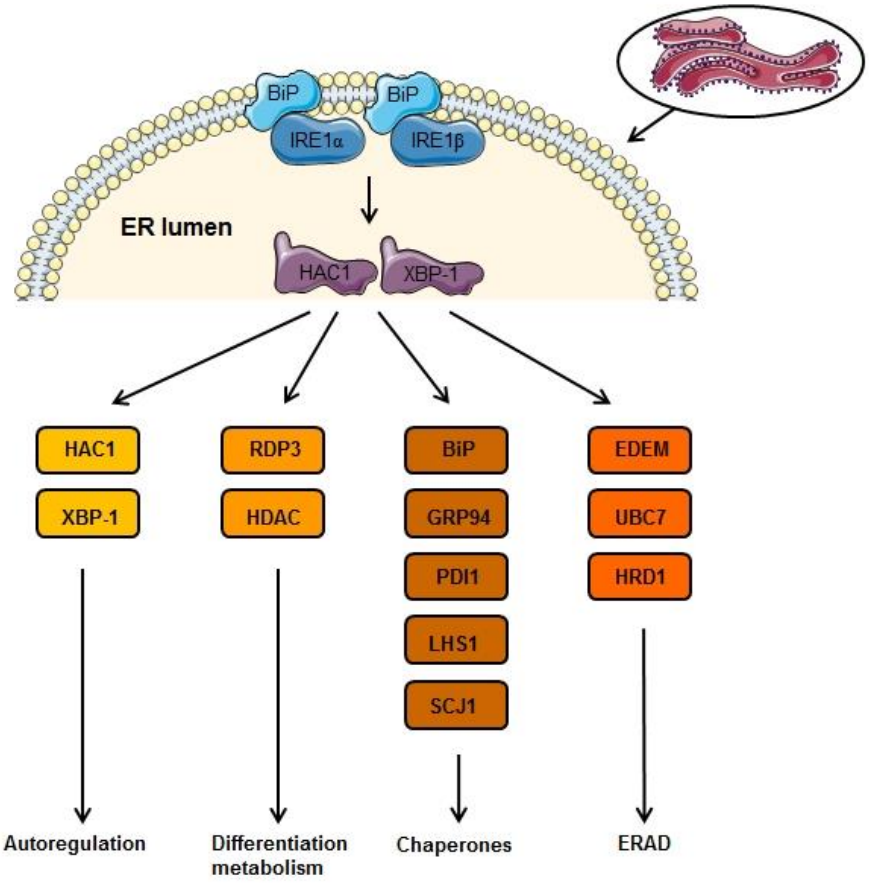


Figure 7. Signal Transduction by IRE1. HAC1 in yest and XBP-1 in mammals are potent transcriptional activators of the IRE1 signaling branch of UPR. *HAC1, homologous to ATF/CREB 1; RPD3, reduced potassium dependency 3; HDAC, histone deacetylase complex;PDI1, Protein disulfide-isomerase 1; LHS1, Heat shock protein 70 homolog; SCJ1, Saccharomyces cerevisiae DNAJ homolog; EDEM, ER degradation-enhancing alpha-mannosidase; ERAD, endoplasmic-reticulum-associated protein degradation; UBC7, ubiquitin conjugating enzyme 7; HRD1, HMG-CoA reductase degradation protein 1; XBP-1, X-box binding protein.* Figure adapted from (*62*)

5.2 PERK signalling

Activation of PERK has two major consequences: phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) and phosphorylation of the nuclear factor erythroid 2 (NF-E2) related factor (Nrf2) (Figure 8) (*62*).

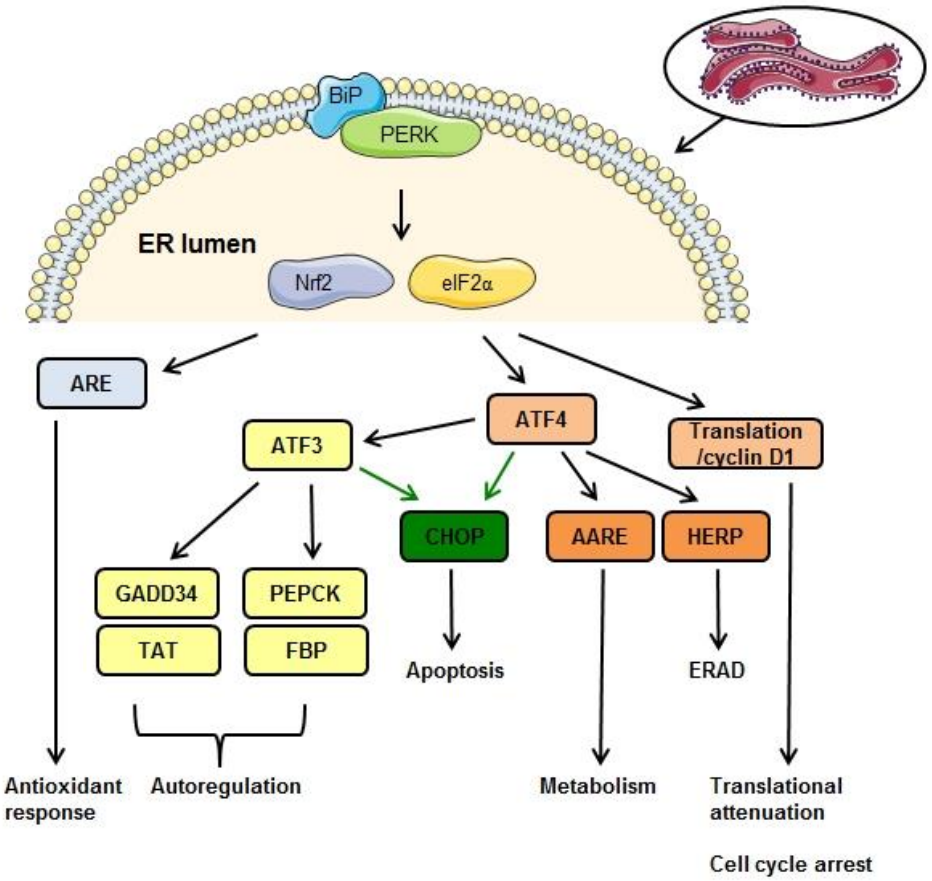


Figure 8. Signal transduction by PERK. PERK pathway has been considered as a main signal transduction pathway of ER stress. Once activated, PERK phosphorylates serine-51 of eukaryotic initiation factor 2α (eIF2α). When phosphorylated, eIF2α is unable to efficiently initiate translation, leading to inhibition of global protein synthesis. However, phosphorylated eIF2α also preferentially initiates translation of the ATF4 mRNA, which contains multiple upstream open reading frames. Transient inhibition of translation has also been suggested to contribute to the cell survival role of PERK by repressing cyclin D1 and p53 expression, which leads to cell cycle arrest. *AARE, amino acid response element; ARE, antioxidant response element; CHOP, CCAAT/enhance-binding protein (C/EBP) homologous protein; FBP, fructose-1,6-bisphosphatase; GADD34,growth arrest and DNAdamage gene 34; HERP, homocysteine-induced ER protein; NRF2, nuclear factor erythroid 2 (NF-E2) related factor; PEPCK, phosphoenolpyruvate carboxy-kinase; TAT, tyrosine amino transferase*. Figure adapted from (*62*)

In response to the accumulation of unfolded proteins, a rapid translation attenuation of mRNA occurs in order to prevent the influx of newly synthesized polypeptides into the stressed ER. This translation attenuation is mainly due to PERK-mediated phosphorylation of the eukaryotic translation initiation factor 2 into the α subunit (eIF2α). The eIF2α is part of the heterotrimeric complex eIF2, which in a GTP-bound form that binds methionyl-initiator tRNA (Met-$tRNA_i^{Met}$). The eIF2-GTP-Met-$tRNA_i^{Met}$ complex is required for AUG initiation codon recognition and association with the 60S ribosomal subunit that occurs during initiation phase of polypeptide chain synthesis. Phosphorylation of eIF2α inhibits exchange of GDP for GTP in eIF2 and decreases the concentration of the complex. Additionally, phosphorylation of eIF2α activates translation of mRNAs encoding numerous short upstream open reading frames (uORFs) (*76*). At low levels of eIF2α phosphorylation, uORFs are efficiently translated, resulting in repression of translation of the downstream ORF. When phosphorylation of eIF2α is high, the small ribosomal subunit scans through several uORFs before being loaded again with the ternary eIF2-GTPMet-$tRNA_i^{Met}$ complex, which then allows for translation of the downstream ORF (*62*). Although PERK-mediated phosphorylation of eIF2α inhibits the translational processes in general, it selectively activates the translation of one essential transcription factor of the UPR: the activating transcription factor 4 (ATF4) (*47*, *77*). ATF4 is a transcription factor that induces the expression of genes involved in amino acid metabolism, antioxidant response and apoptosis, such as the DNA damage 34 (GADD34) and the Growth arrest and DNA damage-inducible protein (GADD153)/ CHOP (*77*).

In addition to the PERK-eIF2α signaling cascade, several studies proposed that nuclear factor erythroid 2 (NF-E2) related factor (Nrf2) may also be a substrate for the PERK kinase activity (*78*). In unstressed cells, Nrf2 is found in an inactive cytoplasmic complex with the cytoskeletal anchor Kelch-like Ech-associated protein1 (Keap1). In ER stress conditions, PERK phosphorylates Nrf2, resulting in dissociation of the Nrf2-Keap1 complex, translocation of Nrf2 to the nucleus where it binds to the antioxidant response element (ARE) activating the transcription of genes encoding detoxifying enzymes (*78*). Genes regulated by ARE include the A1 and A2 subunits of glutathione S-transferase, NAD(P)H: quinone oxidoreductase, γ-glutamylcysteine synthetase, heme oxygenase 1, and UDP-glucuronosyl transferase (*79*).

A growing body of data suggests that PERK phosphorylates multiple substrates to protect cells from oxidative stress (*80*). Consistent with this observations, *perk*−/− cells accumulated ROS when exposed to ER stress (*81*). Thus, the sensitivity of *nrf2*−/− cells to ER stress results from their impaired ability to respond to an oxidative insult. The notion that an imbalance in the cell's redox status is caused by ER stress is further supported by the observation that the redox sensitive transcription factor NF-κB is activated in response to ER stress and that this activation was inhibited by antioxidants. NF-κB is one of the central effectors of innate and inflammatory responses. Although, all three UPR branches can activate NF-κB, they use different mechanisms. Generally, NF-κB is kept in an inactive form within the cytoplasm by the IκB proteins, which prevent its activation and nuclear translocation (*47*, *62*).

5.3 ATF6 signaling

The bZIP-containing activating transcription factor 6 (ATF6) was recognized as an additional regulatory protein that, like XBP1, binds to the ER stress-response element 1 (ERSE1) in the promoters of UPR-responsive genes. ATF6 has two alleles, ATF6 α (90kDa) and ATF6 β (110 kDa) that are equally found in all cell types as ER transmembrane proteins. ATF6 is located at the ER membrane and bounds to BiP in homeostatic conditions. Under ER stress conditions, BiP dissociation allows the transport of ATF6 to the Golgi complex, where it is successively cleaved by the serine protease site-1 protease (S1P) and by the metalloprotease site-2 protease (S2P). The processed forms of ATF6 α and ATF6 β translocate to the nucleus and bind to the ATF/cAMP response element (CRE) and to the ERSE1 to stimulate the transcription of target genes. The proteases S1P and S2P were first known for their important function in processing of the sterol response element binding protein (SREBP), a transcription factor that is activated on cholesterol deprivation (Figure 9) (*71*, *82*, *83*).

In addition to ATF6, CREBH an extra bZIP-containing transcription factor of the CREB/ATF family was recently identified in the ER. The transcription of CREBH is augmented by pro-inflammatory cytokines IL-6, 1L-1β, and TNFα. During ER stress,

CREBH transits to the Golgi compartment, here it is cleaved by S1P and S2P enzymes. While cleaved CREBH does not start transcription of UPR genes, it induces transcription of many acute-phase response genes, such as C-reactive protein and murine serum amyloid P component (SAP) in hepatocytes. CREBH has been shown to be a crucial transcription factor in initiation of innate immune response and in the connection of the ER stress to inflammatory responses (*47*, *62*, *84*).

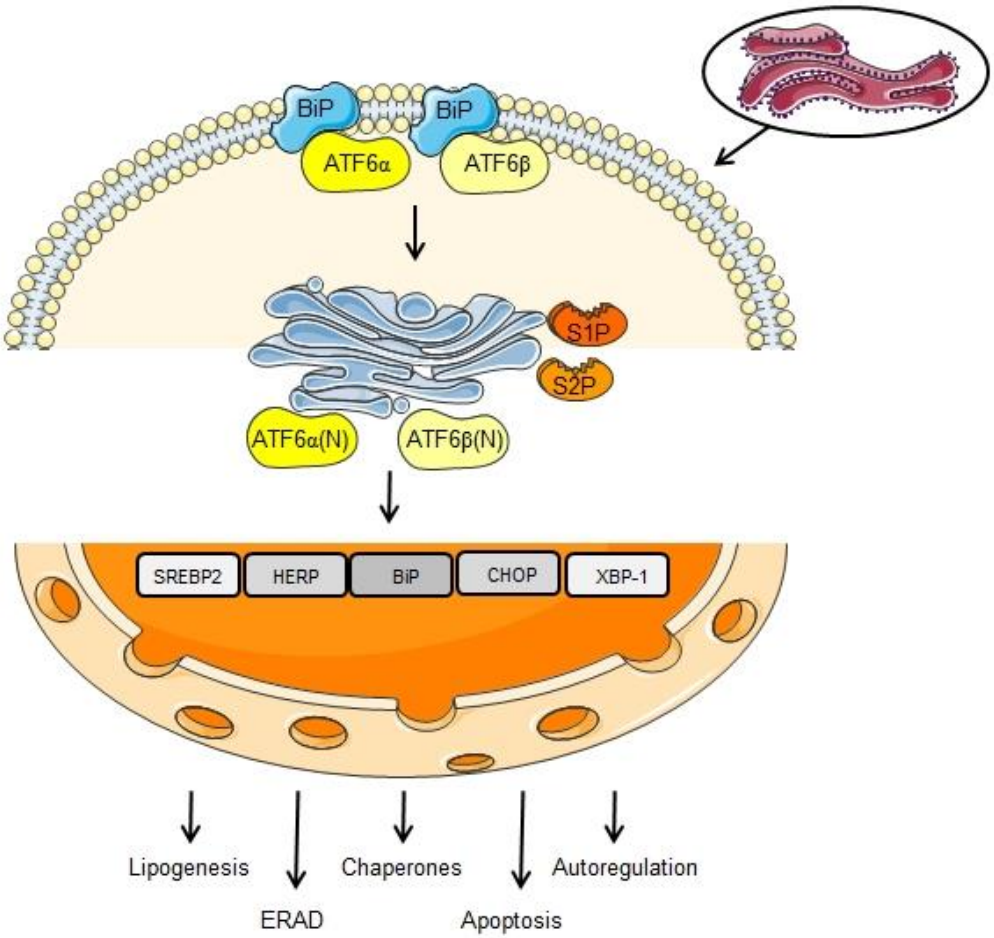


Figure 9. Signal transduction by ATF6. ATF6*α* and ATF6*β* are two homolog proteins. When protein folding in the ER is repressed, ATF6 translocates to the Golgi complex and S1P, a serine protease, cleaves ATF6 in the luminal domain. The N-terminal membrane anchored half is cleave by the S2P. The processed forms of ATF6 α and ATF6 β translocates to the nucleus where they activate the transcription of target genes. Figure adapted from (*62*)

6. ER Stress-Induced Apoptosis and Autophagy

The UPR is tailored essentially to re-establish ER homeostasis also through adaptive mechanisms involving the stimulation of autophagy (*85*). However, when persistent, ER stress can switch the cytoprotective functions of UPR and autophagy into cell death promoting mechanisms (*86*). If the UPR sensors fail to correct the accumulation of unfolded protein, both autophagy and cell death pathways are triggered (Figure 10). The ER may actually be viewed as a local where apoptotic signals are produced and integrated to trigger death responses (*64*).

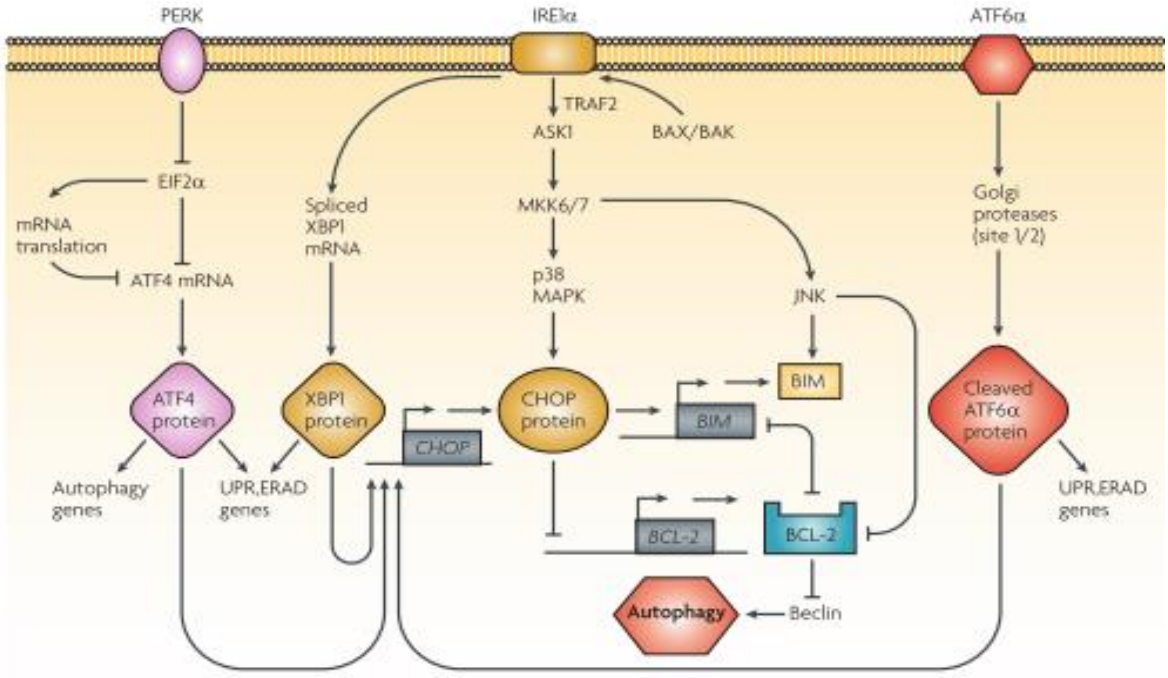


Figure 10. ER stress-induced apoptosis and autophagy. PERK activates its intrinsic kinase activity, which results in the phosphorylation of eIF2α and suppression of global mRNA translation. Under these conditions, only selected mRNAs, including ATF4, are translated. ATF4 induces expression of genes involved in restoring ER homeostasis as well as autophagy genes. ATF4, XBP1 and ATF6 all converge on the promoter of the gene encoding C/EBP homologous protein (CHOP), which transcriptionally controls expression of the genes encoding BCL2-interacting mediator of cell death (BIM) and B-cell leukaemia/lymphoma 2 (BCL-2). Activated IRE1α recruits TRAF2 (tumor necrosis factor receptor-associated factor 2) to induce JNK phosphorylation and activation. The p38 MAPK stimulates CHOP activity. JNK activates BIM, but inhibits BCL-2. Note that BCL-2-associated X protein (BAX) and BCL-2 antagonist/killer (BAK) are also reported to interact with and activate IRE1α. The autophagy protein Beclin is inhibited by direct binding to BCL-2. Release of ATF6α permits expression of UPR and ERAD genes. In a general perspective unsolved ER stress activates multiple autophagy and apoptotic pathways. MKK, mitogen activated protein kinase kinase. Figure taken from (*85*)

ER-dependent apoptosis may be triggered by several mechanisms such as PERK/eIF2α dependent induction of the pro-apoptotic transcription factor CHOP; BCL-2-associated X protein (BAX)/ BCL-2 antagonist/killer (BAK) regulated Ca^{2+} release from the ER; IRE1 mediated activation of ASK1/JNK; Cleavage and activation of pro-caspase 12 (*47*). ER stress is also able to promote ROS formation and mitochondrial ROS can as well be formatted as a result of ER stress–induced Ca^{2+} release and consequent depolarization of the internal mitochondrial membrane (*47*). Probably the most significant ER stress-induced apoptotic pathway is mediated through CHOP. CHOP/GADD153 (growth arrest and DNA damage gene 153) is a bZiP transcription factor that is induced through the PERK, IRE1 and ATF6 UPR branches (*87*, *88*). Although the precise mechanism by which CHOP mediates apoptosis is unknown, several studies demonstrated that it activates the transcription of several genes that potentiate apoptosis. These include *GADD34* (growth arrest and DNA damage gene 34), *ERO1* (Endoplasmic oxidoreductin-1), *DR5* (death receptor 5), *TRB3* (Tribbles homolog 3). *GADD34* encodes a subunit of protein phosphatase 2C that enhances dephosphorylation of eIF2α and promotes protein synthesis (*89*). *ERO1* encodes an ER oxidase that increases the oxidizing potential of the ER (*90*). *DR5* encodes a cell-surface death receptor that may activate caspase cascades (*91*). *TRB3* encodes a human orthologue of *Drosophila* tribble, and Trb3-knockdown cells are resistant to ER stress-induced apoptosis (*47*, *92*). CHOP has also been implicated in repressing the transcription of the anti-apoptotic BCL2 protein, which leads to enhanced oxidant injury and apoptosis. During ER stress, pro-apoptotic members of the BCL2 family are recruited to the ER surface and activate caspase-12. In contrast, the anti-apoptotic members prevent this recruitment, although the exact relation between these factors is still unclear. The over expression of BCL2 family members can prevent ER stress-induced apoptosis (*93*). BH3 domain (BCL2-homology domain 3)-only containing pro-apoptotic factors, such as BAX and BAK, are present at the mitochondrial and ER membranes (*93*). Under ER stress conditions, BAX and BAK oligomerize, allowing Ca^{2+} efflux into the cytoplasm where at milimolar concentrations it activates dependent and -independent caspase signaling cascades (*94*). Additionally, BH3 domain only family members, p53 up-regulated modulator of apoptosis (PUMA) and neutrophil NADPH oxidase factor (NOXA), are up-regulated by p53 during ER stress and are also involved in programmed cell death signaling cascades (*47*, *64*).

IRE1 branch of the UPR also directly relates with cell death and survival. IRE 1 phosphorylation activates the MAP kinase signalling cascade. The IRE1 cytoplasmic domain interacts with the adaptor protein tumor necrosis factor receptor–associated factor 2 (TRAF2) that in turn interacts with the mitogen activated protein kinase, ASK1 which subsequently phosphorylates and activates JNK leading to apoptosis (*59, 63, 75*). TRAF2 is involved in other mechanisms that control programmed cell death such as the association with and activation of caspase-12 and the activation of the transcriptional repressor ATF3 leading to apoptosis. UPR activation of IRE1 may also initiate the extrinsic apoptotic pathway. IRE1 interacts with tumor necrosis factor receptor 1 (TNFR1) to form a complex with TRAF2 and ASK1 to mediate JNK activation. Furthermore, the expression of TNF-α is up-regulated by the IRE1 pathway during ER stress. ROS can directly activate ASK1 by disrupting an ASK1-thioredoxin (TDX) complex through oxidation of TDX, and thereby lead to activation of JNK, p38 MAP kinase, and cell death. The Jun activation domain-binding protein (JAB1) may be a feedback regulator because it can interact with IRE1 and inhibit XBP1 mRNA splicing and BiP transcription. Thus, oxidative stress and ER stress may induce cell death using the same molecular complex consisting of IRE1/TRAF2/ASK1/TDX (*64, 95*)**.** TNF-α was also shown to activate the UPR in a ROS-dependent manner indicating that an intricate relation exists between death-receptor signalling, oxidative stress, and activation of the UPR (*45, 51*).

Recent studies showed that the Selenoprotein S (SEPS1), an ER resident protein related to production of inflammatory cytokines, may play an important role in the control of ER stress-mediated apoptotic processes (*96*). The over expression of SEPS1 in macrophages showed to have a protective effect against ER stress-induced cytotoxicity and apoptosis (*96*). The protective action of SEPS1 is largely dependent on ER stress-mediated cell death signal with less effect on non-ER stress component cell death signals. Conversely, suppression of SEPS1 resulted in sensitization of macrophages to ER stress-induced cell death (*96*).

In order to clear the ER from the accumulation of terminally misfolded protein aggregates that cannot be degraded by the proteasome, the UPR may activate also the autophagy machinery. Proteasomal degradation and autophagy are the two main mechanisms that are in charge of protein clearance in the cell (*97*). In spite of its role as a self-digestion mechanism, autophagy is mainly activated to protect against cell death. Through the autophagic process, complete cytoplasmic portions, including organelles and other cytoplasmic components are "swallow up" by double membrane vesicle designated autophagosomes (*98*). The maturation of these vesicles involves their fusion with lysosomes, which leads in turn to the degradation of the autophagosome components by the lysosomal degradative enzymes (*99*). A variety of stress signals such as nutrient starvation or treatment with different agents that induce ER stress (DTT, Tunicamycin, Thapsigargin, or proteasome inhibitors) stimulate the autophagy process (*100–105*). Also, autophagy can facilitate cells to survive with ER stress or participate in the mechanism of ER stress- induced cell death (*86*).

The molecular pathway that is responsible for the initiation of autophagy after ER stress is not completely established Maiko Ogata and co-workers suggested an involvement of IRE1 signalling via JNK-1 but not XBP1, and independent of PERK1 and ATF6 (*103*). Moreover, the phosphorylation of Beclin 1 by JNK-1 is essential for the induction of autophagy under starvation conditions (*106*). IRE1 interact also with TRAF-2 and activates JNK (*103*). JNK has been proposed to regulate autophagy through BCL-2 phosphorylation, which prevents this protein of interacting (and inhibiting) the essential autophagy regulator Beclin-1. Consequently, it is plausible that activation of the IRE1/TRAF2/JNK branch of ER stress may regulate autophagy through modulation of Beclin-1 function and expression. One the other hand, Claudio Hetz and collaborators shown that XBP-1 ablation increases autophagy and protects from the toxicity induced by the aggregates of the enzyme superoxide dismutase 1 in a model of Amyotrophic Lateral Sclerosis. These data suggest that the XBP-1 may play a different role than TRAF2/JNK on the regulation of autophagy by the IRE1 branch of the UPR (*107*). A different mechanism may involve Death-associated protein kinase 1 (DAPK1) activation by ER stress leading to Beclin 1 phosphorylation, which induces autophagy by reducing Beclin 1 binding to B-cell lymphoma-extra large (BCL-xL) (*108*, *109*). From another standpoint *Kouroku et al.* verified that ER stress caused activation of autophagy via PERK1 and eIF2

(*101*). Indication for a link between UPR-PERK branch and autophagy was obtained from ectopic expression of polyglutamine (polyQ) proteins. A dominant-negative form of PERK or genetic substitution of Serine 51 of eIF2α by Ala prevented polyQ protein-induced autophagy, strongly suggesting that PERK-dependent eIF2α phosphorylation plays an important role in the activation of autophagy in response to the accumulation of unfolded proteins (*101*). Calcium release due to ER stress has also been implicated in the pathway leading to autophagy induction (*102*).

7. ER stress in human diseases

In recent years, numerous works have revealed a close relation between the ER stress activated pathways and the genesis and progression of several inflammatory processes (*80*). The sensors of the UPR PERK, IRE1, and ATF6 contribute to up-regulating inflammatory processes in several pathologies, including obesity, type 2 diabetes, cancer, intestinal bowel and airway diseases and neurodegenerative diseases (*61*, *84*). ER stress does not assume always a deleterious effect and in cancer represents a paradoxal case. In one hand, current studies have revealed that various types of tumors might need an inflammatory microenvironment mostly because inflammation can be protumorigenic (*110–112*). On the other hand it was recently shown that ER stress accompanied by ROS production can promote the 'regeneration' of antitumor immunity by inducing immunogenic apoptosis in cancer cells (*113*). Recently, Abhishek and co-workers proposed that ER stress can help or delay disease evolution through the inflammatory pathways, depending on the cell type, disease stage, and type of ER stressor (Figure 11) (*84*).

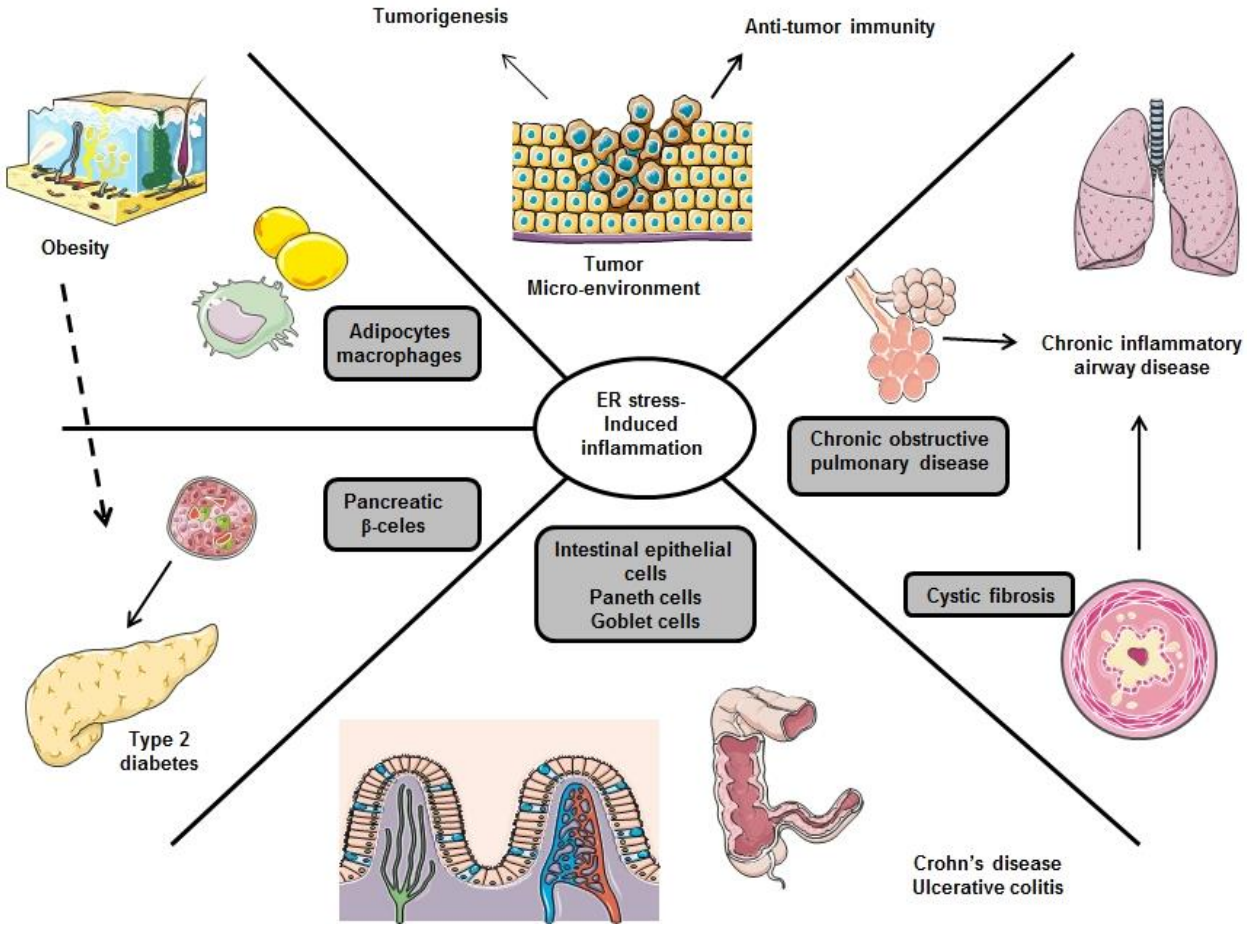


Figure 11. Paradoxal effects of ER stress in health and disease. The crosstalk between inflammation and ER stress in the pathogenesis of several diseases. ER stress-induced inflammation has deleterious effects on pancreatic β cells, contributing to the progression of type 2 diabetes. As well, ER stress causes dysfunctions in adipocytes promoting the progression of obesity-associated inflammation that in turn is associated with suppression of insulin receptor signaling observed in type 2 diabetes. Similarly, ER stress-induced inflammation can change intestinal epithelial cells, Paneth cells, and goblet cells, probably assisting the progression of inflammatory bowel diseases (IBDs) such as Crohn's disease and ulcerative colitis. The progression of cystic fibrosis and cigarette smoke-induced chronic obstructive pulmonary disease has been also associated with ER stress-induced inflammation. The connection between ER stress-induced inflammation and cancer has dichotomous effects. Even though ER stress-induced inflammation has been publicized to support tumorigenesis, it has also been shown that ER stress prevents tumorigenesis by inducing immunogenic cell death-based antitumor immunity. Figure adapted from (*84*)

8. Effects of ER stress in dendritic cell immunobiology

As stated in the initial topics of the present thesis, dendritic cells are heterogeneous cell populations with unique immunomodulatory abilities. Upon activation by a danger signal, DC become mature, enter draining lymphatic vessels and migrate to the T-cell zones in the draining lymph nodes where they present processed antigens to T lymphocytes. The maturation process is an extremely complex and well-coordinated succession of events characterized by changes in morphology, loss of endocytic/ phagocytic receptors, shift of chemokine receptors, resulting in acquisition of migratory capacity, up-regulation of co-stimulatory molecules, alteration of secreted cytokines/chemokines and shift in lysosomal compartments (*16*). Although molecular events associated to this process have not yet been completely defined, it is known that activating mitogen-activated protein kinases, and activation of transcription factors such as nuclear factor κ-B (NF-κB), activating transcription factor-2 (ATF-2) or cAMP response element-binding protein (CREB) are intimately involved (*2*). However, despite some evidences, data regarding the role of ER stress and UPR signalling effectors on the immunobiology of DCs is very scarce.

In a recent study, Goodall and co-workers investigated the effect of ER stress on the inflammatory cytokine production by monocyte-derived DC (*114*). They demonstrated that activation of ER stress, in combination with Toll-like receptor (TLR) agonists, markedly enhanced mRNA expression of the unique p19 subunit of IL-23, and also significantly augmented secretion of IL-23 protein. These effects were not seen for IL-12 secretion. The IL-23 gene was found to be a target of the ER stress- induced transcription factor C/EBP homologous protein (CHOP), which exhibited enhanced binding in the context of both ER stress and TLR stimulation. Knockdown of CHOP in U937 cells significantly reduced the synergistic effects of TLR and ER stress on IL-23p19 expression, but did not affect expression of other LPS-responsive genes (*114*).

In another work, the endogenous changes and mechanisms underlying the maturation and activation of DC triggered by high mobility group box-1 protein (HMGB1) were explored (*115*). Since ER stress activates an adaptive UPR that facilitates cellular survival and repair, Xiao-mei Zhu and collaborators hypothesized that HMGB1 may regulate the function of DC by modulating ER stress (*115*). In this study, HMGB1 stimulation induced,

in a time- and dose-dependent manner, a significant ER stress response in DCs. This was demonstrated by the up-regulation of a number of ER stress markers such as GRP78. XBP-1 gene silencing in splenic DCs, when compared with untransfected or nontargeting-transfected DCs, induces decreased levels of co-stimulatory molecules CD80, CD86 as well as major histocompatibility complex (MHC)-II and cytokines after HMGB1 treatment. Moreover, XBP-1 silenced DCs after treatment with HMGB1 failed to stimulate notable proliferation and differentiation of T cells, unlike normal DCs or nontargeting-transfected DCs. Gene silencing of XBP-1 also resulted in down-regulation of the receptor for advanced glycation end products (RAGE) in splenic DCs treated with HMGB1. These findings demonstrate an important role for ER stress and its regulator XBP-1 in HMGB1-induced maturation and activation of DCs (*115*).

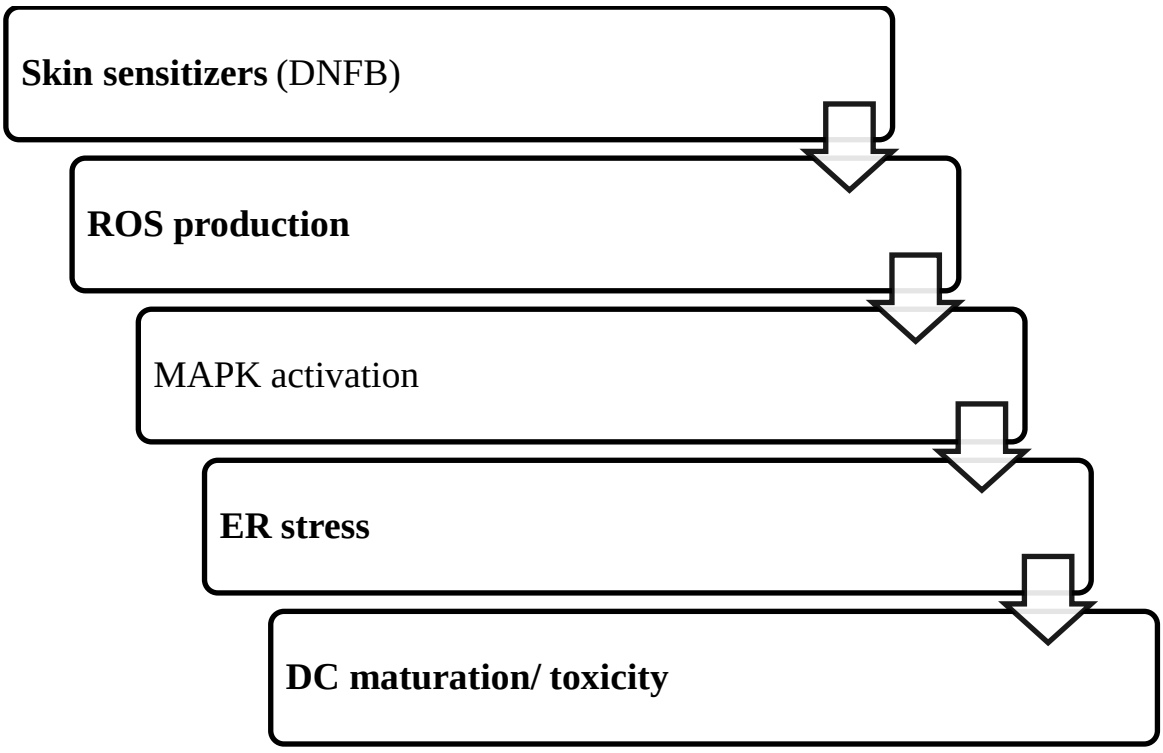
In another study where murine DCs were stimulated by polyIC, a synthetic mimic of virus dsRNA, the obtained data suggested that induced ER stress greatly potentiates the expression of inflammatory cytokines and IFN-β (*95*). Both toll-like receptor 3 and melanoma differentiation-associated gene-5 were involved in the enhanced IFN-β production, which was associated with increased activation of NF-kB and IRF3 signaling, as well as the observed XBP-1splicing. Surprisingly, silencing of XBP-1 reduced polyIC-stimulated IFN-β expression in the presence or absence of ER stress, indicating that XBP-1 may be essential for polyIC signaling and ER stress-amplified IFN-β production. Overexpression of spliced XBP-1 (XBP-1s) synergistically augmented polyIC-induced inflammatory response. This data suggest that evolutionarily conserved ER stress response and XBP-1 may function collaboratively with innate immunity to maintain DC cellular homeostasis (*95*).

Therefore, much remains unknown regarding the impact of ER stress on DC response to danger signals such as ROS, pathogens, pathogen molecules, inflammatory cytokines, and allergens.

II. HYPOTHESIS AND OBJECTIVES

DCs maturation process triggered by skin sensitizers is at least in part due to the formation of ROS and alteration of cell redox status. In this process, ROS and depletion of thiol groups activate intracellular signalling pathways such as p38 MAPK and JNK. It is also known that ER and oxidative stress are interrelated. Oxidative stress can affect ER homeostasis and perturbations in protein folding can cause alterations in cellular redox status increasing the generation of ROS.

Therefore, the work hypothesis for this dissertation is that: **ER stress may be involved in skin sensitizers-induced DC maturation through a ROS dependent mechanism.**



The general objectives of the present work are:

1. To study the ability of skin sensitizers to induce ER stress in dendritic cells;
2. To elucidate possible mechanisms for the plausible ER stress induction;
3. To evaluate the relevance of induced ER stress in apoptotic/ autophagic processes in sensitizer-treated cells;
4. To evaluate the relevance of induced ER stress in the maturation/activation status of sensitizer-treated cells;

III. MATERIALS

Materials

Resazurin, penicillin streptomycin, Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal Bovine Serum and TRIzol reagent were from Invitrogen (Paisley, UK). The protease and phosphatase inhibitor cocktails were obtained from Roche (Mannheim, Germany). Pharmacological inhibitors SB203580, U0126, SP600125 and Salubrinal were from Calbiochem (San Diego, California, USA) and 4-PBA form Merck (Darmstadt, Germany). The anti-tubulin antibody was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Antibodies against phospho-p44/p42 MAPK (ERK1/ERK2), phospho-p38 MAPK, phospho-SAPK/JNK, phospho-eIF2α, CHOP, and total p38 MAPK were from Cell Signaling Technologies (Danvers, MA, USA). The anti-JNK1 and anti-ERK antibodies were from Millipore (Bedford, MA) and XBP1-s and GRP78 antibodies were purchased from Biolegend (San Diego, CA, USA). The alkaline phosphatase-linked secondary antibodies and the enhanced chemifluorescence (ECF) reagent were obtained from GE Healthcare (Chalfont St. Giles, UK), and the polyvinylidene difluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA, USA). iScript kit and SYBR green were obtained from BioRad (Hercules, CA, USA). Primers were from MWG Biotech (Ebersberg, Germany). All other reagents were from Sigma Chemical Co. (St. Louis, Mo, USA) or from Merck (Darmstadt, Germany).

IV. METHODS

Methods

1. Cell culture

THP-1 human monocytic cell line (ATCC TIB-202, American Tissue Culture Collection, Manassas, VA) was cultured and maintained at a cell density between $0{,}2x10^6$ and $1x10^6$ cells/mL in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% of inactivated FBS, 25 mM glucose, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.05 mM of 2-mercaptoethanol. Cells were subcultured every 3–4 days and kept in culture for a maximum of 2 months.

2. Resazurin Cell viability assay

Cell viability was assessed by resazurin assay (*116*). Briefly, $0.2x10^6$ cells/well in a 96 well plate were exposed to different DNFB concentrations (2 µM, 4 µM, 8 µM, 10 µM and 15 µM), for 24 h. Three hours before the end of exposure, resazurin solution was added to each well to a final concentration of 50 µM. Absorbance was then read at 570 and 600 nm in a standard spectrophotometer MultiSkan Go (Thermo Fisher Scientific, Waltham, MA, USA). DNFB concentration that causes a loss of 20% viability (EC_{20} values) was determined by linear regression and used hereinafter in all experiment unless stated otherwise.

3. Cell lysate preparation and Western blot analysis

To obtain the lysates for Western blot analysis, THP-1 cells were plated at $2.4x10^6$ cells/well in 6-well microplates in a final volume of 3 ml. The cells were then incubated with 8 µM DNFB for the indicated time periods (1 h, 2 h, 4 h, 8 h and 24 h) . As a positive control, parallel assays were performed with 10 µg/ml Tunicamycin, a known inducer of ER stress. In some experiments cells were exposed to different concentrations of DNFB (4 µM, 8 µM, 16 µM and 24 µM) for 1 or 8 hours. To assess the possible crosstalk between the UPR response and the DNFB activation of MAPK kinases, cells were pre-treated for

1h with the pharmacological inhibitors 4-PBA (2.5 mM), NAC (5 mM), SB203580 (15 µM), U0126 (15 µM), SP600125 (25 µM) and Salubrinal (25 µM) and then stimulated with 8 µM DNFB for the indicated periods. At the end of exposure, cells were washed in 1 ml ice-cold PBS and harvested 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 and 1 mM DTT) freshly supplemented with protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany). The nuclei and the insoluble cell debris were removed by centrifugation at 4ºC, at 12,000*g* for 10 min. 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 95ºC, 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 posterior use. Western blot analysis was performed to evaluate the effect of DNFB on several ER stress related proteins and on the activation of MAPK kinases and autophagy processes. Briefly, 30 µg of protein were separated by electrophoresis on a 12% (v/v) SDS-polyacrylamide gel, transferred to polyvinylidene fluoride (PVDF) membranes and 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 4ºC with the primary antibodies. Antibodies and respective dilutions are indicated in Table 3. The membranes were then washed with TBS-T and incubated, for 1 h at room temperature, with alkaline phosphatase-conjugated anti-rabbit (1:20,000) or anti-mouse (1:20,000) antibodies. The immune complexes were detected using the Enhanced Chemifluorescence reagent and the membranes were scanned for blue excited fluorescence on the Thyphon (GE Heatlcare, Chalfont St. Giles, UK) and analyzed using the software Total Lab 2009 (TotalLab Ltd, Durham, USA). To demonstrate equivalent protein loading, membranes were stripped and reprobed with antibodies to total ERK1/2, SAPK/JNK, p38 MAPK, or with an anti-tubulin antibody. All the antibodies were prepared in 1% (w/v) fat-free dry milk in TBS-T. Blots were developed with alkaline phosphatase-conjugated secondary antibodies and visualized by enhanced chemifluorescence.

Table 3. Dilutions of antibodies used for Western Blot.

Antibody	Dilution in TBS-T 1% milk
phospho-eIF2α (Cell Signaling)	1/1000
XBP1-s (BioLegend)	1/250
GRP78 (Biolegend)	1/1000
CHOP (Cell Signaling)	1/1000
ATF4 (Cell Signaling)	1/1000
phospho-ERK (Cell Signaling)	1/1000
phospho- p38 (Cell Signaling)	1/1000
phospho-JNK (Cell Signaling)	1/1000
LC 3 B I/II (Cell Signaling)	1/1000
p68 (Cell Signaling)	1/1000
Tubulin (Sigma Aldich)	1/5000
ERK (Millipore)	1/2000
p38 (Cell Signaling)	1/1000
JNK1 (Millipore)	1/1000

4. RNA extraction

For RNA extraction, THP-1 cells were plated at $2.4x10^6$ cells/well in 6-well microplates in a final volume of 3 ml and treated with 8 µM DNFB or 10 µg/mL Tunicamycin during 3, 6 or 24h. When pharmacological inhibitors of MAPK kinases or ER stress inducers were used, drugs were added 1 hour before DNFB stimulation. Total RNA was isolated from cells with the TRIzol® reagent according to the manufacturer's

instructions. Briefly, cells were washed with ice-cold PBS harvested and homogenized in 1 ml of Trizol by pipetting vigorously. After addition of 200 µL of chloroform the samples were vortexed, incubated for 2 min at room temperature and centrifuged at 12,000×g, for 15 min, at 4ºC. The aqueous phase containing RNA was transferred to a new tube and RNA precipitated with 500 µL of isopropanol for at least 10 minutes at room temperature. Following a 10 minutes centrifugation at 12,000g, the pellet was washed with 1 ml 75% ethanol and resuspended in 30 µl 60ºC heated RNase free water. The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA). Quality was inspected for absence or genomic DNA, protein or guanidine isotiocianate contamination trough assessment of 260/280 and 260/230 ratios. RNA was stored in RNA Storage Solution (Ambion, Foster City, CA, USA) at -80ºC until use.

5. Real-time Reverse Transcription Polymerase Chain Reaction

For the synthesis of cDNA the iScript™ Select cDNA Synthesis Kit was used according to manufacturer instructions. Briefly, 2 µL of random primers and the necessary volume of RNase-free water to complete 15µL were added to 1 µg of total RNA. After this, 5µL of a Master Mix containing 1 µL of iScript reverse transcriptase and 4 µL of 5x Reaction Buffer were added to each sample. A protocol for cDNA synthesis was run on all samples (5 min at 25ºC, 30 min at 42ºC, 5 min at 85ºC and then put on hold at 4ºC). After the cDNA synthesis, the samples were diluted with RNase-free water up to a volume of 100 µL. Real-time PCR was performed in a 20 µL volume containing 2,5 µL cDNA (25 ng), 10 µL 2x Syber Green Supermix, 2 µL of each primer (250 nM) and 3,5 µL H_2O PCR grade. Samples were denatured at 95ºC during 3 min. Subsequently, 40 cycles were run for 10 sec at 95ºC for denaturation, 30 sec at the appropriate annealing temperature and 30 sec at 72ºC for elongation. Real-time RT-PCR reactions were run in duplicate for each sample on a Bio-Rad My Cycler iQ5. Primers were designed using Beacon Designer® Software v8.0, from Premier Biosoft International and thoroughly tested. Primer sequences used are given in Table 4.

On each real-time PCR plate there was a non-template control present for each pair of primers analyzed. For determination of primer-pair specific efficiencies, a 4 points

dilution series of control sample for each pair of primers was run on each experiment (*117*). Amplification reactions were monitored using SYBR-Green reagent. After amplification, a threshold was set for each gene and Ct-values were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software v2.1. The software enables analyzing the results with the Pfaffl method (*118*), a variation of ΔΔCT method corrected for gene-specific efficiencies, and to report gene expression changes as relative fold changes compared to control samples. The results were normalized using HPRT-1 and GAPDH as reference genes. These genes were determined with Genex® software (MultiD Analyses AB) as the most stables for the treatment conditions used.

Table 4. Primer sequences used for qRT-PCR assays

Gene Name	Forward primer	Reverse primer	RefSeq ID
GAPDH	ACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATCC	NM_002046.4
HPRT1	TGACACTGGCAAAACAATG	GGCTTATATCCAACACTTCG	NM_000194.2
IL1β	GCTTGGTGATGTCTGGTC	GCTGTAGAGTGGGCTTATC	NM_000576.2
IL8	CTTTCAGAGACAGCAGAG	CTAAGTTCTTTAGCACTCC	NM_000584.3
CD86	GAACCTAAGAAGATGAGT	TCCAGAATACAGAAGATG	NM_175862.4
CD40	TGATAGTGAACAACTGGAA	CCATAGGCAATATACATACATAA	NM_001250.4
DDIT3	TAAAGATGAGCGGGTGGCAG	CTGCCATCTCTGCAGTTGGA	NM_004083.5
HSPA5	TCTTGTTGGTGGCTCGACTC	ATCTGGGTTTATGCCACGGG	NM_005347.4
VIMP	AACCCGTTGTCTGGTGAAGG	GAAAAGCGTGCGTAAGGCAA	NM_203472.1
HERPUD1	GTCTCAGGGACTTGCTTCCA	CCTTGGCGTTGATTTCTGGC	NM_014685.3

6. ROS Detection by Immunocytochemistry

To measure reactive oxygen species (ROS) formation after DNFB exposure, the fluorogenic probe Cell ROX® Green Reagent was used. This probe, upon oxidation, binds to DNA with a strong increase in fluorescence. Therefore its signal is primarily localized in the nucleus and mitochondria. The fluorescence resulting from CellROX® Oxidative Stress Reagent was captured by conventional fluorescence microscopy. For this procedure THP-1 cells ($1x10^5$) were plated in Poly-L-Lysine coated wells of µ-Chamber slides (IBIDI GmbH, Germany). After an overnight stabilizing period the cells were loaded with 5 µM Cell ROX® Green Reagent for 30 min and then washed three times with culture medium. Following this, cells were treated with 8 µM DNFB during 30 min and then washed with PBS before fixation during 15 min with 4% paraformaldehyde. The cells were then permeabilized with 0.2% (v/v) Triton-X-100, 200 mM Glycine in PBS for 10 min at room temperature and F-actin was stained by adding Alexa Fluor 555 Phalloidin (Molecular Probes) 1:100 in PBS during 30 min. After three washing steps cells were finally exposed to nuclear label DAPI (100 nM) for 2 min and the slides analyzed with a fluorescent microscope (Nikon Corporation, Japan) at 630X magnification. Images were captured with a DS-Fi2 High-definition digital camera and analyzed in NIS-Elements Imaging Software (Nikon Corporation, Japan).

7. Cell Cycle analysis by flow cytometry

The effect of DNFB exposure on THP-1 cell cycle was analyzed by flow cytometry with Propidium Iodide (PI). PI binds to DNA by intercalating into the double stranded macromolecule being the quantity of blinded dye proportional to the quantity of DNA. The quantification of the content DNA allowed us to know the distribution of a cell population along the different phases of the cell cycle. For this procedure $1x10^6$ cells were plated in 12 multiwell plates in a final volume medium of 1.2 mL and pre-treated with 2.5 mM PBA, 25 µM SP600125 or 25 µM Salubrinal and then stimulated with 8 µM DNFB during 24 hours. After the incubation time, cells were centrifuged for 5 min at 300 g, washed with PBS and the pellet resuspended in the residual liquid. The cells were then fixed by adding drop wise 200 µL of ice cold 70% ethanol while slowly vortexing. The cells were left in

ethanol at – 4ºC until analysis. For PI staining, the cells were washed in 1 ml of 2% BSA in PBS and centrifuged during 5 min at 500g. The supernatant was removed and the pellet was resuspended in the residual liquid. Next, 0.5 mL of propidium iodide solution (PI/RNase) was added to the pellet and mixed well. The samples were incubated for 15 minutes at room temperature before to analysis.

8. Statistical analysis

The results are presented as mean ± S.D., and the statistic difference between two groups was determined by the two-sided unpaired Student's t test. For multiple group comparisons, the One-Way ANOVA test, with a Bonferroni´s Multiple Comparison post-test was used. The tests were performed using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA). Statistically significant values are as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$

V. RESULTS

1. Determination of EC20 DNFB concentration

Resazurin was used to assess the metabolic capacity of cells and by extension their viability. This method provides a rapid and easy way to estimate the number of viable cells as they reduce resazurin into resorufin leading to an increase in emitted fluorescence and a change in medium color. Since a certain level of cytotoxicity is required for effective DC activation (*119*) we determined the concentration of DNFB that induced a maximum of 20% cytotoxicity (EC_{20}), and used this concentrations along the subsequent experiments (Figure 12).

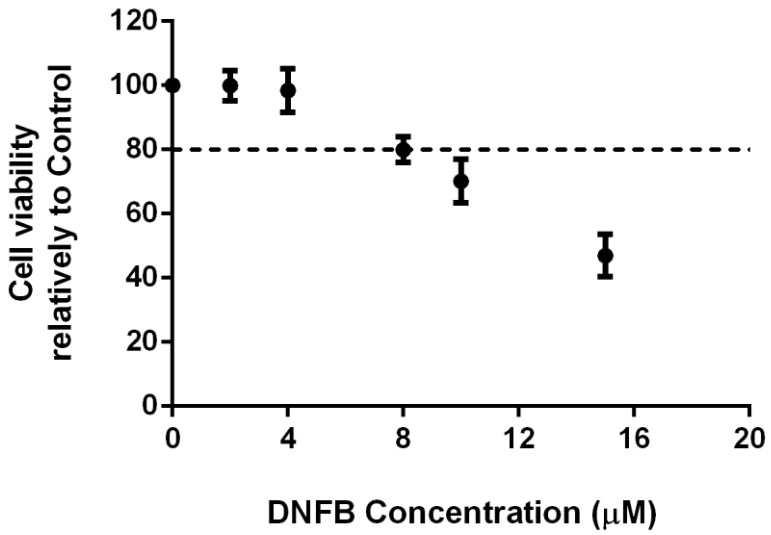


Figure 12. Effect of DNFB on THP-1 cell viability. $0.2x10^6$ cells/well in a 96 well plate were exposed during 24 hours at the indicated concentrations (2 µM, 4 µM, 8 µM, 10 µM and 15 µM). The concentration that causes a 20% loss in cell viability (EC_{20}) was determined by linear regression.

According to the established criterion, the determined EC_{20} DNFB concentration was 8 µM. This concentration was used hereinafter in all experiment unless stated otherwise. From our results we also observe that for concentrations superior to 4 µM, cell viability decreases linearly with the increase of DNFB.

2. DNFB treatment induces a rapid increase in ROS production

The production of ROS following exposure of DCs to skin sensitizers is well documented and viewed as a primary danger signal that subsequently triggers intracellular signaling cascades that ultimately culminates in cell maturation (*120*). As we hypothesize that DNFB may trigger ER stress through a ROS dependent mechanism we initially analyze its ability to effectively induce oxidative stress in THP-1 cells.

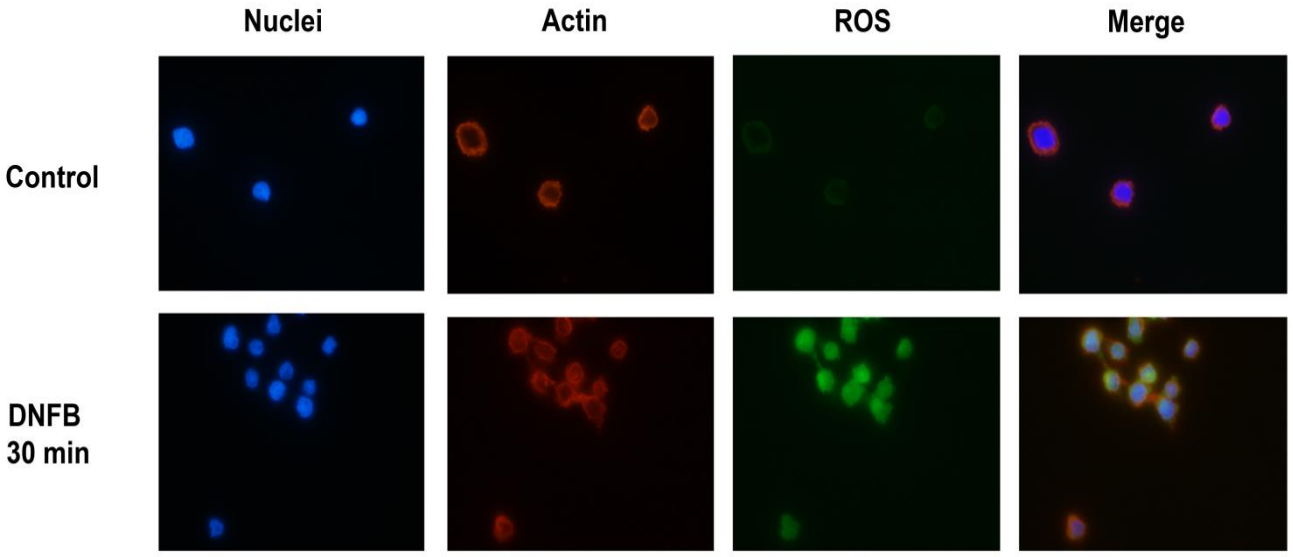


Figure 13. DNFB treatment induces a rapid increase in ROS levels. THP1 cells were loaded with the fluorogenic probe Cell ROX® Green Reagent (green) for 30 min and then treated with 8 µM DNFB during additional 30 min. Alexa Flúor 555 Phalloidin was used for visualization of F actin cytoskeleton fibers (red) and DAPI to highlight the nuclei (blue). Images representative of different fields were acquired with a DS-Fi2 High-definition digital camera coupled to a Nikon fluorescent microscope (magnification 630x) and analyzed in NIS-Elements Imaging Software (Nikon Corporation, Japan).

For this propose we measured, by immunofluroscence microscopy, the levels of oxidative stress with the ROS sensitive probe Cell ROX® Green Reagent (Figure 13). This probe is weakly fluorescent while in a reduced state but is converted in a highly fluorescent form upon oxidation by peroxides, peroxynitrite, or hydroxyl radical and subsequent binding to DNA. The signal is therefore primarily localized in the nucleus and mitochondria. We observed that DNFB causes a strong and rapid ROS generation in THP-

1 exposed cells, confirming that this event may represent an early danger signal during the sensitization phase of allergic contact dermatitis.

3. DNFB induces a rapid and sustained phosphorylation of eIF2α, an up regulation of ATF4 and a posttranslational modification of the ER major chaperone GRP78

To test our hypothesis that skin sensitizers such as DNFB may induce ER stress in DC, affecting their immunobiology, we started to test its effects on the levels of key proteins from the three branches of the UPR. THP-1 cells were incubated with 8 µM DNFB for the indicated time periods (1h, 2h, 4h, 8h and 24h) and the protein levels of GRP78, CHOP, p-eIF2α, ATF4 and XBP-1s (Figure 14) were assessed by Western Blot. Parallel experiments were performed with 10 µg/ml Tunicamycin a known inducer of ER stress.

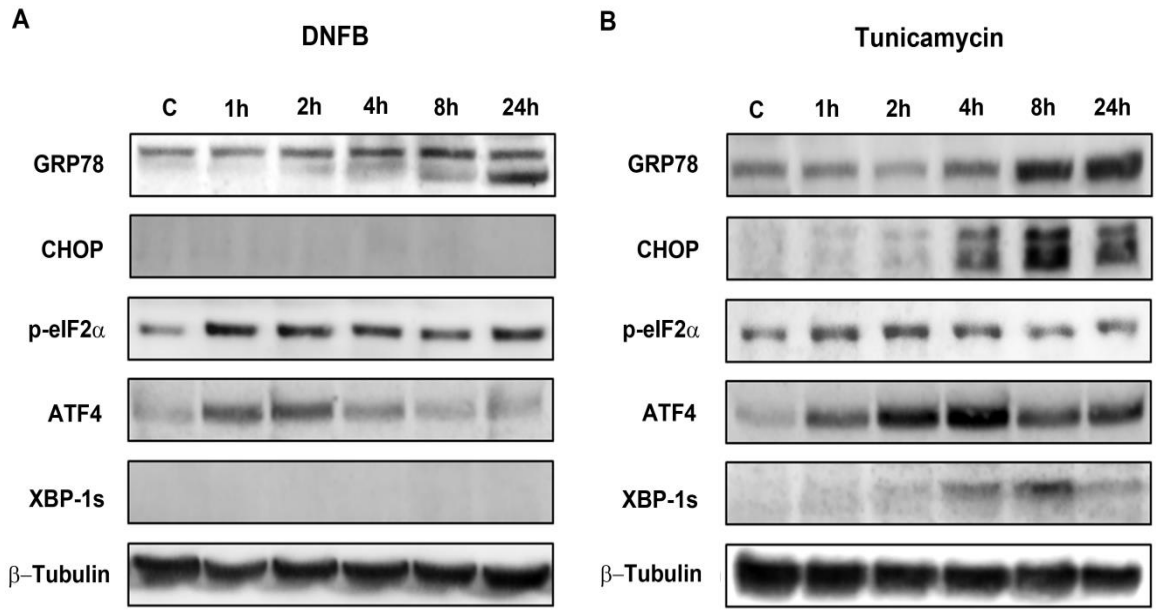
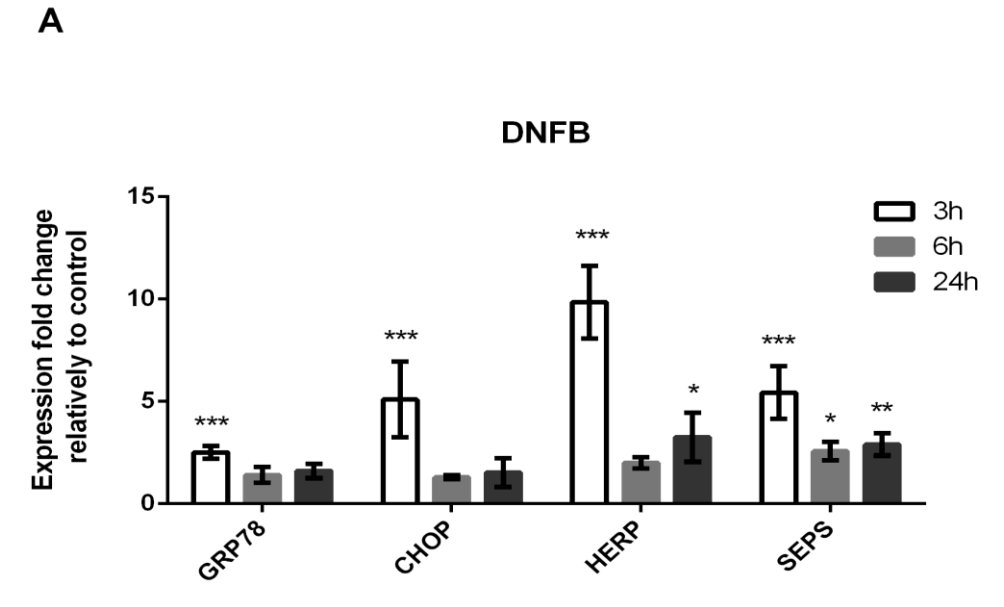


Figure 14. Effects of DNFB (A) and Tunicamycin (B) on the levels of several ER stress related proteins. Proteins were extracted after 1h, 2h, 4h, 8h and 24h of stimulation and 30 µg of protein were loaded on a 12% SDS-polyacrylamide gel. Protein levels of GRP78, CHOP, p-eIF2α, ATF4 and XBP-1s were assessed by Western Blot. The results are representative of at least three independent experiments. To ensure that equal protein loading between samples, the membranes were stripped, and reprobed with a β-Tubulin antibody. C, control

We observed that, in contrast to Tunicamycin, DNFB does not trigger a canonical ER stress response. As expected, Tunicamycin induced the expression of proteins from the three UPR branches, namely CHOP, GRP78, ATF4, phosphorylated eIF2α and the protein resultant from the spliced form of XBP1 mRNA (Figure 14B). From the studied ER stress markers, DNFB appears to mainly affect the PERK-eIF2α-ATF4 branch, causing a strong and time sustained phosphorylation of eIF2α and an early increase of ATF4 levels (Figure 14A). Of note is also the fact that DNFB treatment although just slightly up regulates the levels of chaperone GRP78, causes the appearance of a low molecular weight immunoreactive protein form in a time dependent way (Figure 14A). This suggests that DNFB may induce a posttranslational modification in GRP78 protein with possible consequences to its functions.

4. DNFB treatment modestly affects the transcription of ER stress related genes

Additionally, we performed quantitative real-time RT-PCR experiments to evaluate the effect of DNFB on the expression of several genes under control of the unfolded protein response. THP-1 cells were treated with 8 µM DNFB and 10 µg/mL Tunicamycin during 3, 6 or 24 h and the mRNA levels of CHOP, GRP78, SEPS1 and HERP were analyzed (Figure 15).



B

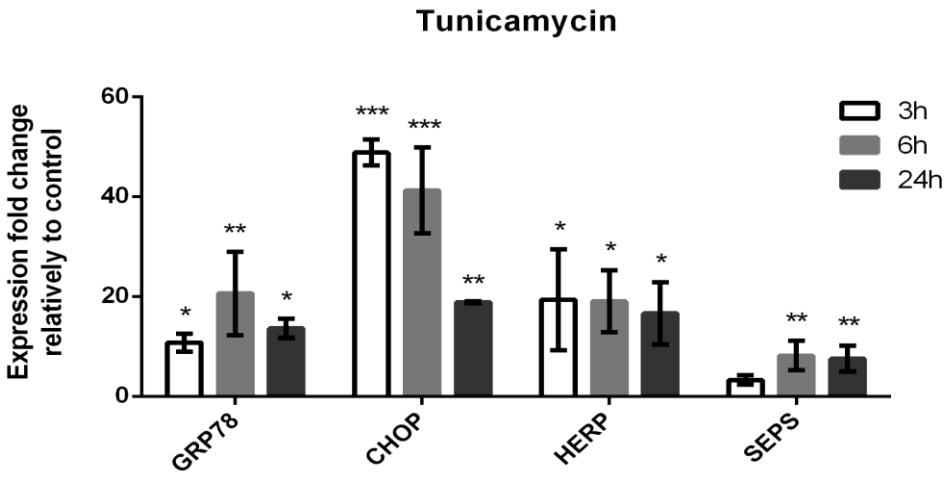


Figure 15. Effect of DNFB (A) and Tunicamycin (B) in the mRNA levels of ER stress related genes. RNA was extracted after the 3h, 6h and 24h of stimulation. The relative expression of indicated genes was assessed by Quantitative Real Time-PCR and normalized using HPRT-1 and GAPDH as reference genes. Each value represents the mean ± S.D. from at least three independent experiments. . (*p<0.05; **p<0.01; ***p<0,001 DNFB or Tunicamycin vs control;

Obtained results revealed that THP-1 exposure to DNFB modestly induce common ER stress controlled transcripts. Among studied genes, the most robustly up regulated were the detoxifying proteins SEPS1 and HERP, with maximal increases observed at 3h post treatment (3.9 and 6.7 fold change relatively to control for SEPS1 and HERP, respectively). Regarding CHOP and GRP78, despite statistically significant increases observed at 3h post treatment, they are very modest and the levels rapidly decrease to values similar to untreated cells (Figure 15A). In contrast, treatment of THP-1 cells with Tunicamycin caused a significant and sustained increase of all the genes analyzed, being CHOP and HERP the most robustly up regulated (Figure 15B).

5. Effect of DNFB over p-eIF2α, GRP78 and ATF4 is concentration dependent and related to induced redox-imbalance

In order to evaluate the relationship between DNFB-induced redox imbalance and the observed effects on p-eIF2α, GRP78 and ATF4, cells were pre-treated with the antioxidant N-acetylcysteine (NAC) and the levels of these proteins subsequently analyzed by Western Blot (Figure 16).

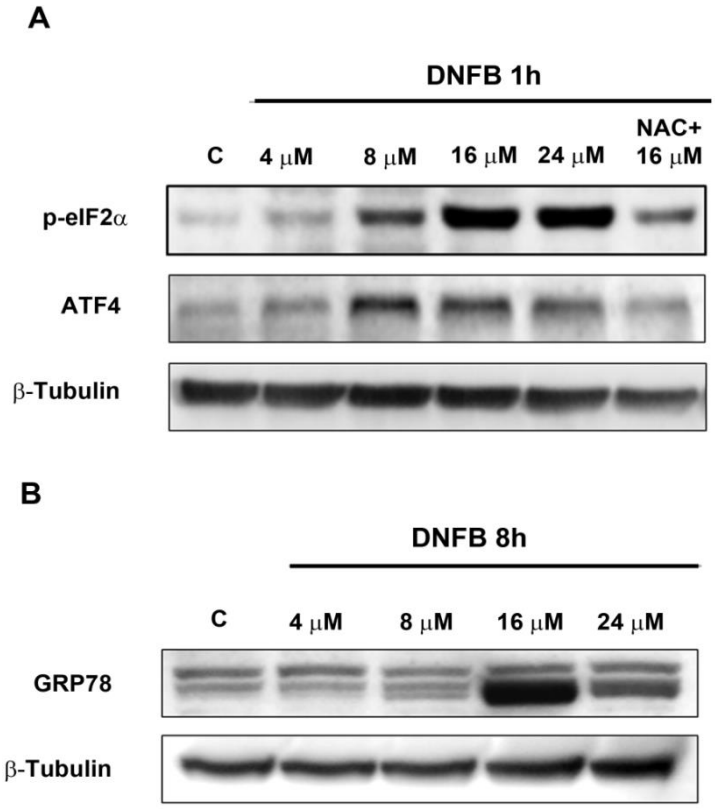


Figure 16. Effect of different concentrations of DNFB and NAC on p-eIF2α, GRP78 and ATF4 protein levels. Cells were exposed to 4μM, 8μM, 16μM and 24μM of DNFB. It was used 16μM of DNFB simultaneously with NAC. Proteins were extracted after 8 hours to analyse the production of GRP78, ATF 4 and 1hour to analyse the production of p-eIF2α. After extraction, 30μg of the protein lysates was loaded on the gel. Protein expression of p-eIF2α, GRP78 and ATF4 was assessed with Western Blot. Equal protein loading between samples was controlled with β-Tubulin. Data are representative of three independent experiments. C, Control.

We observed that the effects of DNFB over the analyzed proteins were dose-dependent. A substantial increase of p-eIF2α, ATF4 and posttranslational modified form of GRP78 was observed when cells were exposed to growing concentrations of DNFB (Figure 16 A and B). However, in cells exposed to DNFB during 8h and to higher concentrations (24 µM) the effects were significantly lower. This suggests that cells are only able to trigger recovery mechanisms until the insult reaches a given threshold and for stress levels beyond this limit predominate signals conducting to cell death. Pre-treatment with N-acetyl-L-cysteine (NAC) markedly suppressed the DNFB-induced phosphorylation of eIF2α and the expression of ATF4. This indicates that the observed activation of eIF2α/ATF4 UPR branch is, at least in part, a consequence of redox imbalance caused by DNFB.

6. Crosstalk between the DNFB-activated MAPK kinases and the UPR response

The activation of mitogen-activated protein kinases (MAPK), particularly p38 MAPK, is in DC a common observation after skin sensitizers exposure (*49*, *119*). Therefore, we assessed in a first step the effects of DNFB on the levels of phosphorylated p-38, ERK1/2 and JNK1/2 in a 24 hours' time-course experiment. As can be seen in Figure 17A, exposure to DNFB rapidly and intensively activates p38 MAPK and JNK signaling pathways in THP-1 cells. These two kinases presented however a distinct behaviour over time: while JNK1/2 remained highly phosphorylated during the 24h of the assay, p38 presented maximal activation at 1h post cell treatment with phosphorylation progressively decaying over time (Figure 17A). From the obtained results, ERK1/2 is in THP-1 cells constitutively activated, with high phosphorylation levels detected in untreated cells. Moreover, DNFB treatment does not induced substantial ERK1/2 activation over the detected basal levels.

Next we analyzed the existence of a possible crosstalk between the DNFB-activated MAPKs and the observed eIF2α phosphorylation. For this propose cells were pre-treated with the pharmacological inhibitors NAC (5 mM), SB203580 (15 µM), U0126 (15 µM), SP600125 (25 µM) and Salubrinal (25 µM) for 1h and exposed to 8 µM DNFB during an additional hour (Figure 17B).

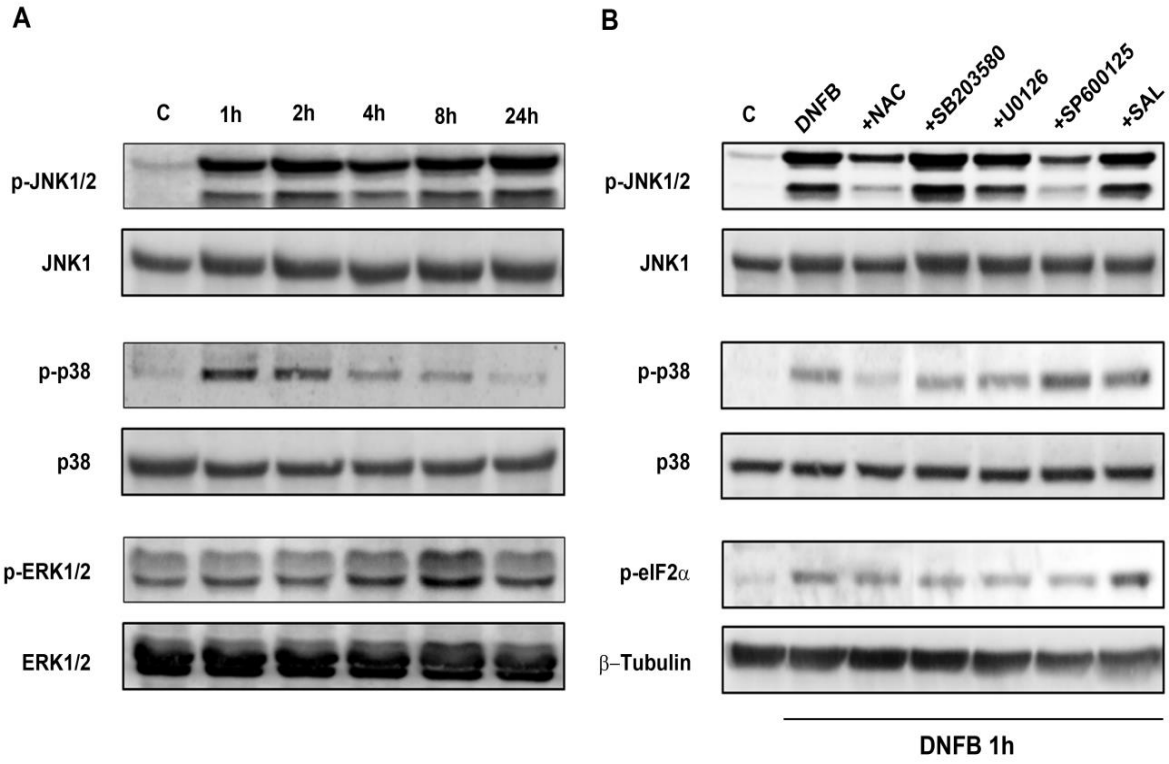


Figure 17. (A) Effect of DNFB treatment on the activation of MAPK kinases. Proteins were extracted after 1h, 2h, 4h, 8h and 24h of stimulation and 30 µg of protein were loaded on a 12% SDS-polyacrylamide gel. Phosphorylated levels of JNK1/2, p38 and ERK1/2 were assessed by Western Blot. To ensure equal protein loading between samples, the membranes were stripped and reprobed with total JNK1, total p38, total ERK1/2 antibodies. The results are representative of three independent experiments. **(B) Evaluation of the possible crosstalk between DNFB-induced MAPK activation and eIF2α phosphorylation**. THP1 cells were pre-treated for 1h with the NAC (5 mM), SB203580 (15 µM), U0126 (15 µM), SP600125 (25 µM) or Salubrinal (25 µM), and then stimulated with 8 µM DNFB. Proteins were extracted after 1h of stimulation and 30 µg of protein were loaded on a 12% SDS-polyacrylamide gel. Phosphorylated levels of JNK1/2, p38 and eIF2α were assessed by Western Blot. The results are representative of two independent experiments. Equal protein loading between samples was controlled by stripping and reprobing membranes with total JNK1, total p38 and β-Tubulin antibodies. C, control; NAC, N-acetylcysteine; SAL, Salubrinal.

We observed that pre-treatment of cells with N-acetylcysteine (NAC) efficiently reduces the DNFB-induced phosphorylation of JNK, p38 and eIF2α. These results suggest that in fact ROS production and resultant redox imbalance is a key early danger signal that is central to the activation of several signalling pathways in skin sensitizers-exposed DC. Regarding the possible crosstalk between DNFB-induced MAPKs activation and eIF2α

phosphorylation we observed that the inhibition of p38 kinase activity by SB203580 and the inhibition of phosphorylation of JNK1/2 by SP600125 are accompanied by a slight decreased in the levels of p-eIF2α. This observation points to a possible link between the activity of these MAPK kinases and the regulation of eIF2α phosphorylation in THP-1 cells triggered by the allergen DNFB. In contrast, eIF2*α* phosphorylation status seems to not have influence in the activation of MAPKs, as shown in Figure 17B. Treatment of cells with the eIF2α specific phosphatase inhibitor Salubrinal resulted in increased phosphorylation of eIF2α without relevant effects on the levels of p-JNK1/2 and p-38.

7. Evaluation of DNFB effects in Autophagy

Given that activation of PERK-eIF2α-ATF4 was shown to regulate important aspects of autophagic process (*121*) we assessed the possible induction of autophagy in DNFB-treated cells. For this propose we analyzed by Western blot the levels of microtubule-associated protein light chain 3 (LC3) and p62 in cells exposed to 8 µM DNFB for the indicated time periods (1h, 2h, 4h, 8h and 24h) (Figure 18A) or to different DNFB concentrations (4 µM, 8 µM, 16 µM and 24 µM) during 8 hours (Figure 18B). LC3 and p62 were widely used to monitor autophagy: the amount of LC3‑II is directly correlated with the number of autophagosomes (*122*) and p62 protein levels allow to distinguish between induction of autophagy or blockade of autophagic process (*99*).

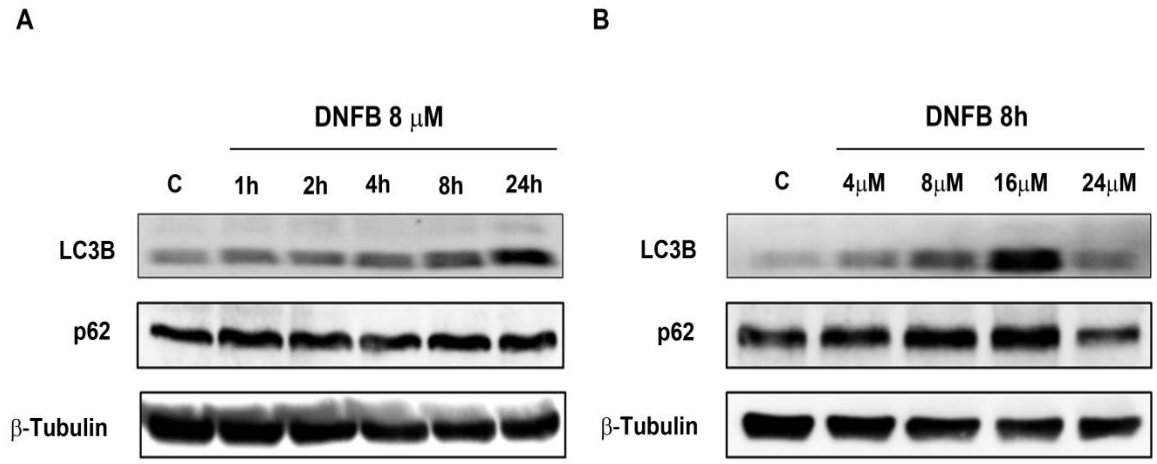


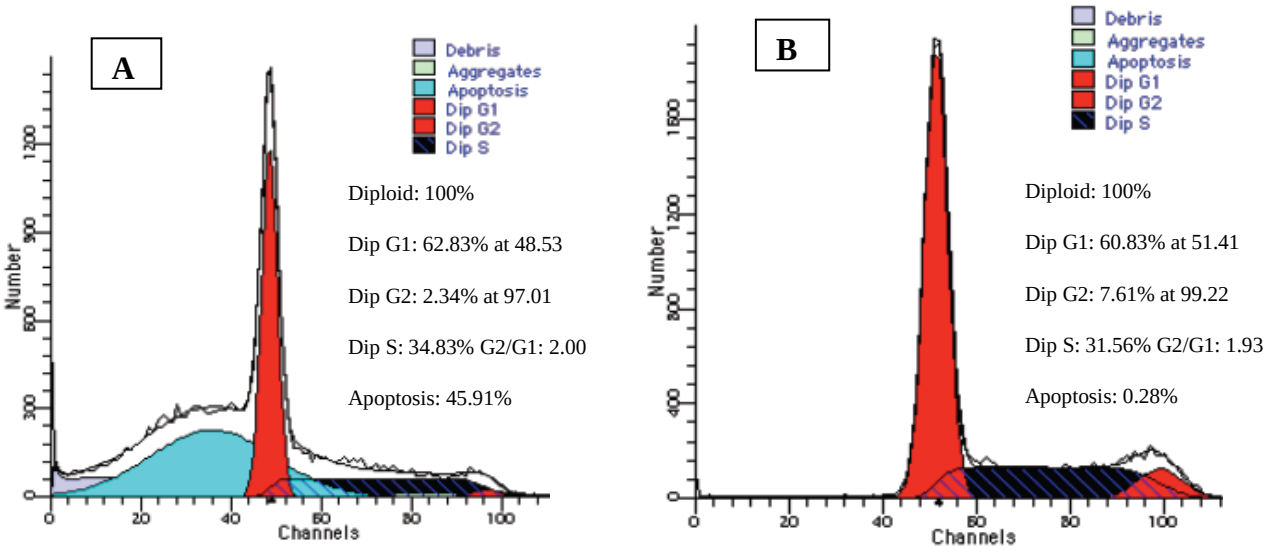
Figure 18. Time course and effect of different DNFB concentrations on the expression of LC 3 I/II and p62. Cells were exposed to DNFB for the indicated time periods (A) or to the indicated concentrations (B). After extraction, 30 µg of protein were loaded on a 12% SDS-polyacrylamide gel and Western blot was performed to evaluate the levels of LC3 and p62. To ensure equal protein loading between samples, the membranes were stripped, and reprobed with a β-Tubulin antibody. The results are representative of two independent experiments. C, control.

DNFB treatment induced a time and concentration dependent increase in the levels of LC3BII. As the amount of LC3B at a certain time point does not specifically indicates activation of autophagy, and may evenly be caused by a blockade of autophagic flux, we checked the levels of p62. p62 protein (sequestosome 1) binds to LC3, serving as a

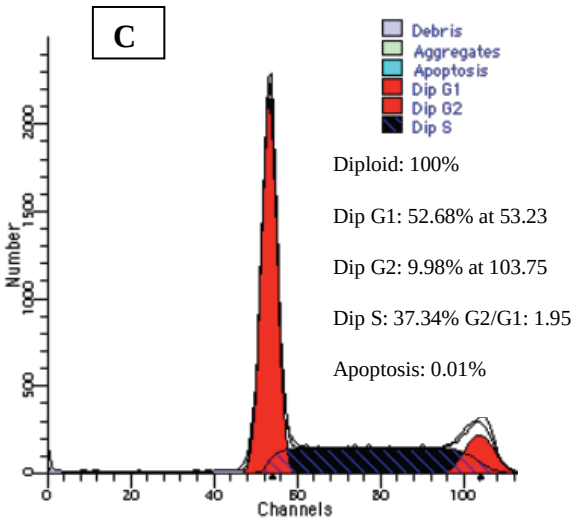
selective substrate of autophagy (*122*). Therefore, in situations where autophagy is activated, the levels of p62 are expected to decrease. We verified along the time course experiment that DNFB-treated cells do not present decreased levels of 62. Rather, we observed a slight concentration-dependent increase in cells treated during 8 hours. Taken together these results suggest that DNFB treatment does not activate autophagy in DC and but rather block the autophagic flux.

8. Apoptosis and cell cycle analysis by Flow Cytometry

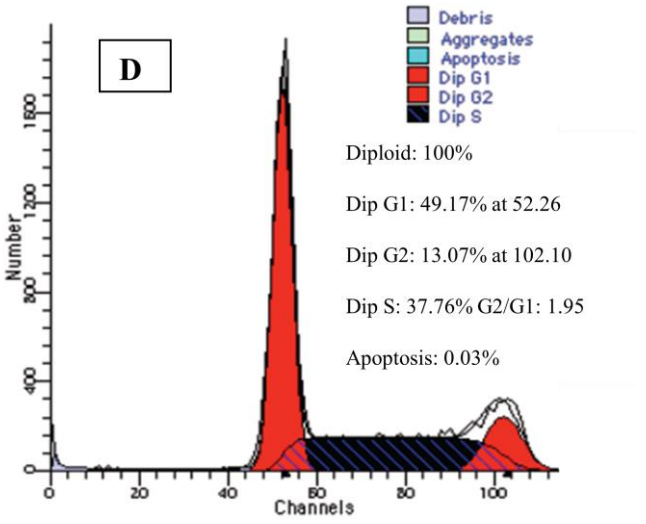
A major consequence of eIF2α phosphorylation is the blockade of mRNA translation leading to inhibition of global protein synthesis. Transient inhibition of translation has been suggested to contribute to the cell survival by repressing cyclin D1 and p53 expression, which lead to cell cycle arrest (*123*). As we detected a sustained eIF2α phosphorylation in DNFB-treated DC we tried to address if this event modulates the normal cell cycle. The effect of DNFB on THP-1 cell cycle was analyzed by flow cytometry with Propidium Iodide (PI). This procedure allows the distribution of a cell population along the different phases of the cell cycle according to its DNA content. Additionally it also enables the estimation of the apoptotic cells (Figure 19A).



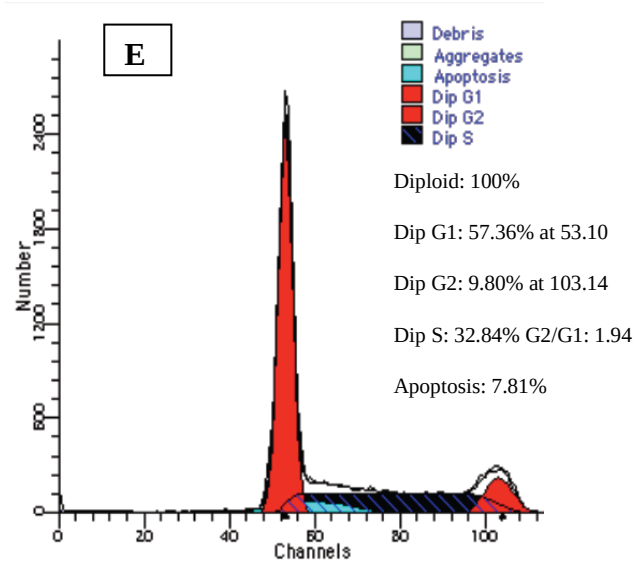
Cells stimulated with Staurosporine **Control 8 h**



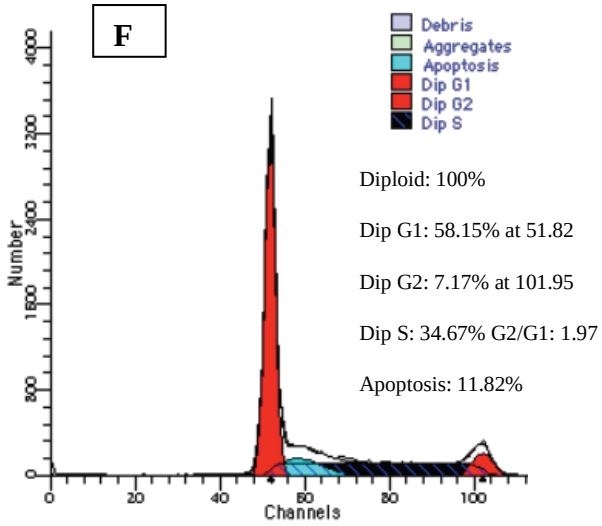
DNFB 4 µM-8 h



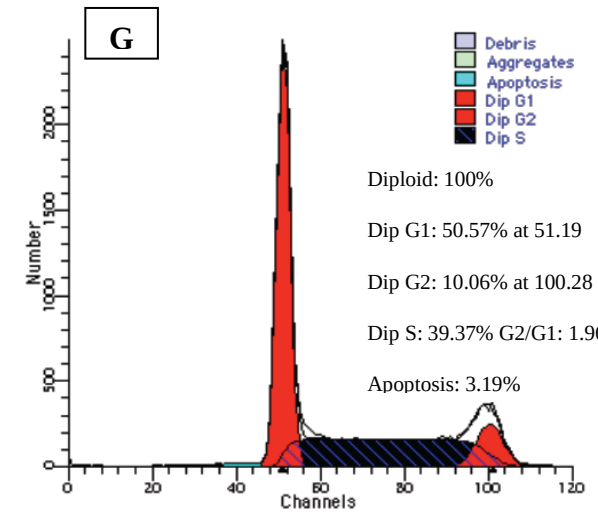
DNFB 8 µM-8 h



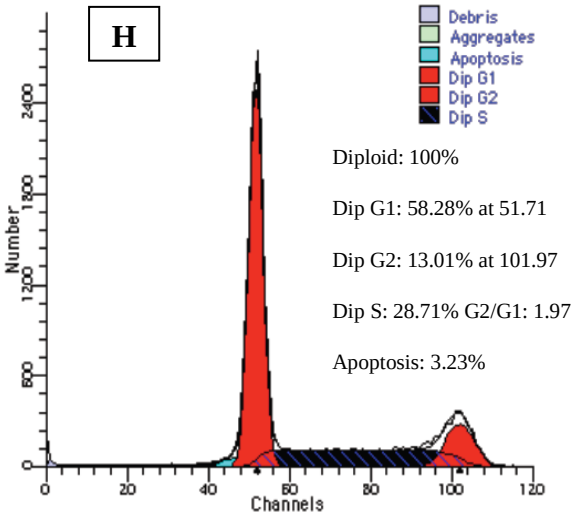
DNFB 16 µM-8 h



DNFB 24 µM-8 h

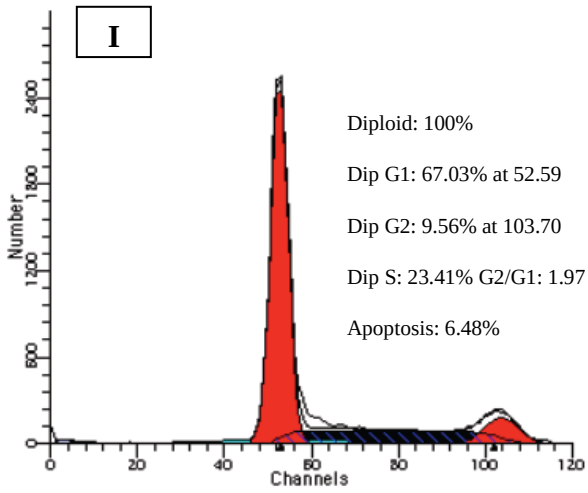


Control 24 h



DNFB 8 µM-24 h

DNFB 8 µM + 4-PBA 2,5Mm-24 h



DNFB 8 µM + Salubrinal 25µM-24 h

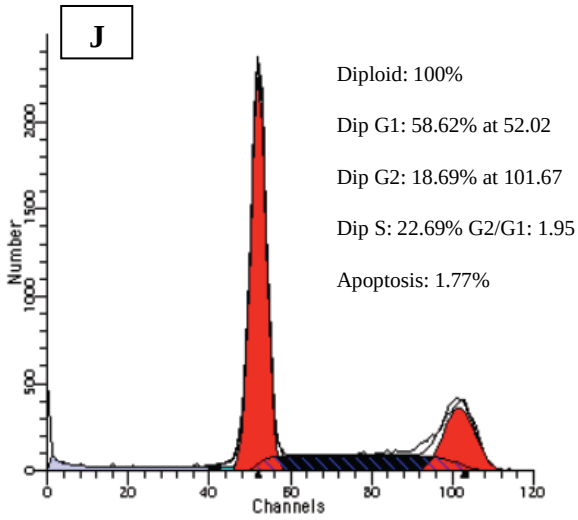
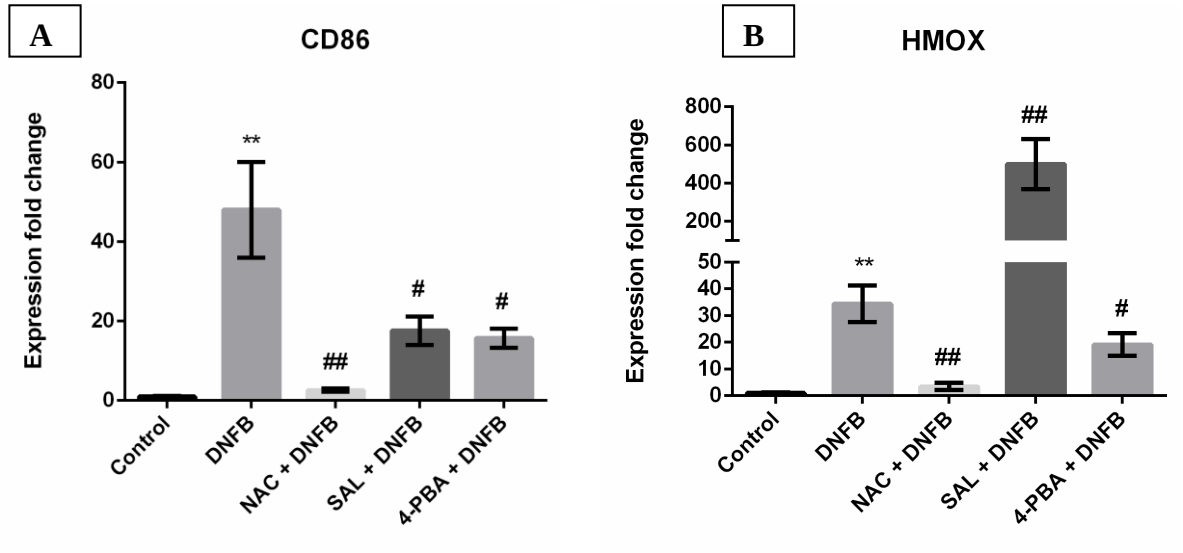


Figure 19. Effect of DNFB exposure on THP-1 cell cycle. Flow cytometry was done after 8 and 24 hours of stimulation with DNFB. Cells were stained with PI for the different conditions. (A) Cells stimulated with Staurosporine, (B) control 8 h (C) DNFB 4 µM-8 h, (D) DNFB 8 µM-8 h, (E) DNFB 16 µM-8 h, (F) DNFB 24 µM-8 h, (G) control 24 h (H) DNFB 8 µM-24 h (I) DNFB 8 µM + 4-PBA 2,5Mm-24 h and (J) DNFB 8 µM + Salubrinal 25µM-24 h. Cells were distributed along the different phases of the cell cycle: cells in G2 and M phases that have double DNA content of those in G0 and G1 phases, and a region correspondent to cells in phase S. The percentage of apoptotic cells on the different conditions is indicated in each histogram. As a positive control of apoptosis THP-1 cells were treated for 8 h with Staurosporine. The results are representative of one independent experiment.

Concerning the effects of DNFB on THP-1 cell cycle, we do not detect evidences of cell cycle arrest. Regarding the effects of DNFB on programmed cell death, obtained results revealed that apoptosis is barely detectable in cells treated with up to 8 µM DNFB for 8 hours (0.03%). However, populations with apoptotic characteristics increased significantly after exposure to 16 µM (7.81%) and 24 µM DNFB (11.82%). In 24h exposure experiments we observed that 8 µM DNFB (EC20 concentration) barely increased the percentage of apoptotic cells: 3.19% for control vs 3.23% for DNFB treated cells. Prevention of DNFB-induced ER stress by 4-PBA resulted in a 2 fold increase in apoptosis (6.48%) while cell exposure to eIF2α dephosphorylating inhibitor salubrinal caused the opposite effect (1.77% apoptotic cells). These results suggest that DNFB-activation of eIF2α-ATF4 UPR branch may play an important role in cell recovery from redox imbalance.

9. Activation of p-eIF2*α* – ATF4 axis is required for DNFB induced *IL8* and *HMOX* gene transcription

Finally, we addressed the relevance of eIF2α-ATF4 activation to DNFB-induced DCs maturation/inflammatory status. For this propose THP-1 cells were pre-treated with 5 mM NAC, 2.5 mM 4-PBA or 25 µM Salubrinal and stimulated with 8 µM DNFB for additional 24h. Then, quantitative real-time RT-PCR was performed to evaluate the mRNA levels of pro-inflammatory cytokines IL-1β and IL-8, co-stimulatory molecule CD86 and of the detoxifying protein HMOX. We observed that DNFB causes a significant increase in the mRNA levels of all the genes analyzed and that this induced transcription was almost completely blocked by cell pre-treatment with NAC (Figure 20 A,B,C and D) . Once again this result evidences the key role of ROS in skin sensitizers-induced DC activation/maturation. DNFB-induced transcription of CD86 and IL-1β was shown to be significantly decreased by both Salubrinal and 4-PBA (Figure 20 A and C). Interestingly, HMOX and IL-8 mRNA levels where significantly increased by pre-treatment with Salubrinal (Figure 20 B and D) indicating that these genes are under transcriptional control of eIF2α-ATF4 axis. In fact, the marked increase in the expression of the detoxifying protein HMOX in cells where eIF2α is maintained in a phosphorylated state by Salubrinal may partially explain the observed decrease apoptosis in this condition. This corroborates our hypothesis that eIF2α-ATF4 pathway activation is a key event in cell response to DNFB-induced oxidative insult.



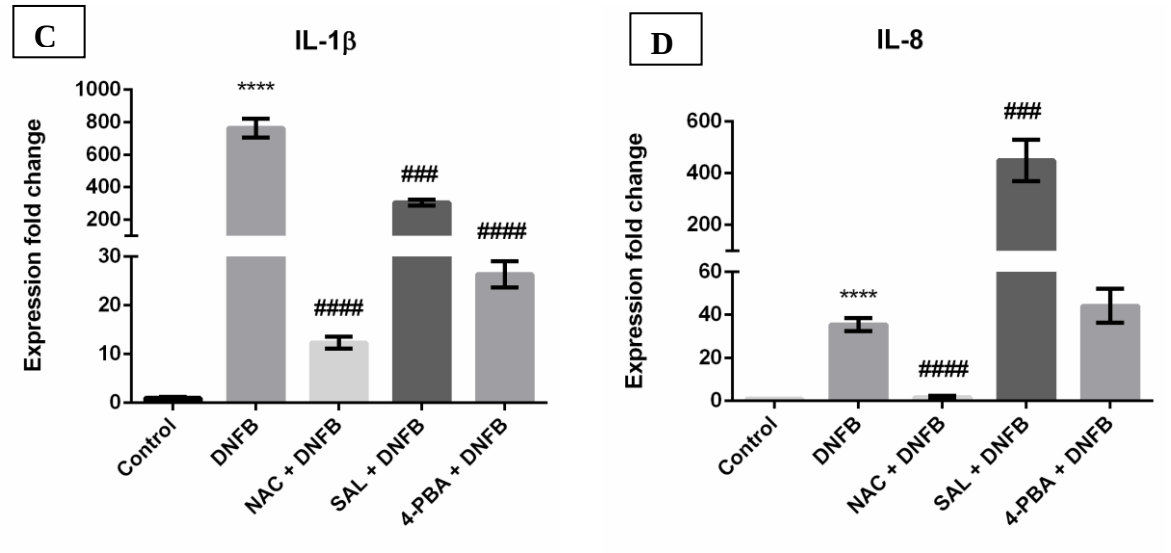


Figure 20. Effect of DNFB, NAC, Salubrinal and 4-PBA in the mRNA levels of CD86 (A), HMOX (B), IL-1β (C) and IL-8 (D). THP-1 cells were pre-treated with NAC (5 mM), 4-PBA (2.5 mM) and Salubrinal (25 µM) for 1h and then stimulated with 8 µM DNFB during 24h. Quantitative Real Time RT-PCR was then performed to evaluate CD86, HMOX, IL-1β and IL-8 mRNA levels. Each value represents the mean ± S.D. from two independent experiments (****$p<0.0001$ control vs DNFB and; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; $^{\#\#\#\#}p<0.0001$ DNFB vs DNFB+inhibitors).Gene expression changes were analyzed using the built-in iQ5 Optical system software v2.1. NAC, N-acetylcysteine; SAL, Salubrinal.

VI. DISCUSSION

Discussion

Allergic contact dermatitis, a delayed-type hypersensitivity reaction caused by skin exposure to low molecular weight reactive chemicals, is one of the most ordinary occupational diseases in developed countries. A keystone in the pathogenesis of this disease is the chemical-induced activation/maturation of DCs. Mature DCs migrate to lymph nodes and effectively present processed antigens to naïve T cells, polarizing that way effector and memory T cell populations. The DC maturation process was shown to be strongly dependent on initial danger signals such as imbalance of cell redox status and release of cellular ATP (*45*, *46*, *120*). ROS deplete thiol groups and cause the activation of intracellular signaling pathways such as p38 MAPK and JNK that ultimately drive phenotypical, and functional changes that characterize DC maturation (*49*, *50*). In recent years numerous studies have shown a close link between ER stress, ROS production and the pathogenesis of several chronic inflammatory, neurodegenerative, cardiovascular and autoimmune diseases such as type 1 diabetes (*47*, *93*, *124*, *125*). Oxidative stress affects ER functions through perturbation of protein processing/transport and by disturbing calcium ER homeostasis and perturbations in protein folding cause alterations in cellular redox status by increasing mitochondrial ROS production (*45*, *47*, *54*). Despite recent intense research activity regarding ER stress and its consequences, the information regarding its effects in DC immunobiology remains scarce. Therefore, in this work we assessed the effects of the extreme sensitizer DNFB on the induction of ER stress in THP-1 cells, as well as the relevance of mounted UPR for cell maturation/toxicity.

As in our hypothesis sensitizers-induced redox imbalance may result in ER stress we started to evaluate the ability of DNFB to effectively induce oxidative stress in THP-1 cells. In accordance to recent works we observed a rapid and strong increase of ROS in DNFB treated cells (*120*, *126*, *127*). Electrophilic chemicals such as DNFB react with cysteine residues of proteins and cause a rapid depletion of intracellular GSH levels leading to an accumulation of ROS. Next we directly assessed the ability of DNFB to trigger ER stress in THP-1 cells through evaluation of the levels of ER stress sensory proteins and transcription of related genes. We found that in contrast to Tunicamycin, a well establish ER stressor (*62*, *70*, *92*, *95*), DNFB does not canonically activates the three UPR branches, whit minimal effects on XBP1 splicing, CHOP/GADD153 and GRP78

expression. Moreover, DNFB treatment modestly affected the transcription of these genes being the genes coding for the detoxifying proteins SEPS1 and HERP the most robustly up regulated. SEPS1 has been reported as a new ER stress-dependent survival factor that protects macrophage against ER stress-induced cellular dysfunction (*96*) and HERP, a mammalian homolog of Usa1p, was shown to be up-regulated in response to ER stress in a variety of cell lines being crucial for ERAD (*62*, *73*, *107*, *125*). Therefore, DNFB induced effects over ER stress related genes point to the activation of an UPR branch mainly involved in cell recovery from insult.

Although DNFB slightly affected the transcription and expression of intact GRP78, cell treatment caused a time and concentration-dependent appearance of a low molecular weight immunoreactive form of the protein, suggesting that a posttranslational modification may be occurring. Several studies report GRP78 post-translational modifications such as phosphorylation, ADP ribosylation and oxidative cleavage, being the existence of these modified forms correlated with the physiological activity of the ER (*128–130*). Attending that the protein form detected in DNFB-treated cells presents a low molecular weight it is plausible to speculate that DNFB-induced ROS formation may be cause the oxidative cleavage of GRP78.

Among other ER stress sensory proteins analyzed we observed that DNFB causes a rapid and sustained phosphorylation of eIF2α and an increase in the expression of the bZIP transcription regulator ATF4. These effects were shown to be concentration-dependent and markedly abrogated by cells pretreatment with the antioxidant NAC. It is therefore shown a direct relation between DNFB-induced redox imbalance and the activation of eIF2α – ATF4 axis, leading us to hypothesize that DNFB may be selectively activating the PERK-eIF2α – ATF4 branch of UPR. This result must however be interpreted with caution because in mammals eIF2α may also be phosphorylated by PKR, a sensor of viral RNA, by general control non depressible 2 (GCN2), a sensor of aminoacid starvation, and by heme-regulated inhibitor (HRI), in heme depletion conditions (*86*).

Direct evaluation of PERK phosphorylation was shown to be difficult, and in fact we were not able to detect phosphorylated-PERK, neither in DNFB or Tunicamycin-treated cells. However, our hypothesis that DNFB is activating eIF2α – ATF4 axis through ER stress effector kinase PERK is strengthen by the fact that besides the phosphorylation of eIF2α in serine 51, PERK was recently shown to directly phosphorylates and activates

NRF2 (*78*), a transcription factor intimately related to cell response to skin sensitizer-induced electrophilic stress (*131*).

Like IRE1 and ATF6, PERK is believed to bind the chaperone protein GRP78 under normal conditions. As unfolded proteins accumulate during ER stress, GRP78 dissociates, allowing PERK to autophosphorylate and dimerize, which induces transphosphorylation and consequent eIF2α phosphorylation in serine-51 (*123*). The main consequence of eIF2α phosphorylation is a rapid mRNA translation attenuation in order to prevent the influx of newly synthesized polypeptides into the stressed ER (*62*). Besides this general inhibition of protein synthesis, specific proteins such as ATF4 are up-regulated. The transcription factor ATF4 controls the expression of ER stress target genes including amino acid transporters, detoxifying proteins and cell death related proteins, among others (*47*). CHOP is one of the most studied targets of ATF4, playing a crucial role in ER stress-induced apoptosis. Although increased levels of ATF4 were detected in DNFB-treated cells, we do not observe a concomitant expression of CHOP. This could be explained by earlier reports suggesting that ATF4 alone is not sufficient for CHOP expression (*132*) (*133*). Our findings reinforce the idea that additional UPR signals other than those transmitted by the PERK- eIF2α – ATF4 branch are required for CHOP induction in response to ER stress.

The exact molecular mechanisms driving sensitizer-induced DC maturation were not completely known, however innumerous studies evidence the activation of mitogen-activated protein kinases (MAPKs) as a crucial event (*49, 75*). MAPKs participate in a diversity of cell processes by controlling transcriptional or translational regulation. ERKs have a central role in survival and mitogenic signaling, while JNKs and p38 MAP kinases are preferentially activated by environmental stresses and are actively involved in various stress responses including cell death, survival and differentiation (*75*). Given that ER stress effector proteins such as IRE1α were shown to crosstalk with MAPKs, particularly JNK (*51*), we analyzed the existence of a possible link between DNFB-induced phosphorylation of eIF2α and MAPKs. In accordance to previous studies we observed that DNFB rapidly triggers p38 and JNK signaling pathways and that this activation is dependent on sensitizer-induced oxidative stress (*120, 134*).

The inhibition of p38 kinase activity and of JNK 1/2 phosphorylation with pharmacological inhibitors was accompanied by a slight decrease in the levels of p-eIF2*α*. This result suggests the possible existence of a link between DNFB-induced MAPK activation and the phosphorylation of eIF2*α*. Supporting this notion of crosstalk, Liang SH and collaborators reported that PERK (the eIF2alpha kinase) is required to activate JNK and p38 MAPK and induce the expression of immediate-early genes upon disruption of ER calcium homoeostasis (*135*).

The ER stress response may be viewed as a mechanism to protect the cells from different alterations affecting this organelle. However, when the intensity and/or duration of the damage do not allows an efficient recovery, ER stress results into cell death. Similarly, autophagy can help cells to cope with ER stress, for instance contributing to the elimination of unfolded or aggregated proteins or participate in the mechanism of ER stress-induced cell death (*104*). Therefore, we addressed the relevance of the observed DNFB-induced eIF2α – ATF4 axis activation in cell cycle, apoptotic and autophagic processes. Previous studies showed a connection between ROS production, activation of PERK- eIF2α-ATF4 pathway and autophagy (*136*). In these studies, extracellular matrix detachment of mammary epithelial cells resulted in ROS production that activates canonical PERK- eIF2α-ATF4 pathway that in turn coordinately induces the autophagy regulators ATG6 and ATG8, sustains ATP levels, and reduces redox imbalance.

In our experiments we found that DNFB treatment resulted in a time and concentration dependent increase in LC3BII indicating a possible increase in autophagic process. However, as autophagy could not be estimated by the singular analysis of LC3B we also checked the levels of p62. We found that rather than decrease as expected in induced autophagy, p62 levels remained unaltered and seem even to increase in concentration-dependent experiments. Although an induction in autophagy could not be completely discarded, our results indicate that DNFB mainly causes the blockade of autophagic flux. The impairment of autophagy was recently correlated with the induction of a pro-inflammatory response; therefore it is reasonable to speculate that DNFB-induced ER-stress could trigger an impairment of the autophagy process and concomitantly activate the inflammatory response driven by contact sensitizers.

Regarding the effects in apoptosis we verified that exposure during 24h to 8 µM DNFB caused minimal cell death. However, the prevention of DNFB-induced ER stress by pre-treatment with the chemical chaperone 4-PBA resulted in a duplication of apoptotic cell population. In contrast, a 2 fold reduction was obtained when cells were pre-treated with the eIF2α dephosphorylation inhibitor Salubrinal. This indicates that DNFB-activation of eIF2α-ATF4 UPR branch is intimately related to cell ability to recovery from redox imbalance. Supporting this hypothesis we observed that while 4-PBA caused a significant decrease in DNFB-induced transcription of the detoxifying gene *HMOX*, Salubrinal drastically potentiates the increase evoked by the sensitizer.

Finally we assessed the eventual relevance of eIF2α-ATF4 axis to DNFB-induced DC activation/ maturation status. In accordance to previous studies performed in other DC models we observed that DNFB causes a significant increase in the transcription of the co-stimulatory molecule CD86 and of the pro-inflammatory cytokines IL-1β and IL-8 (*3, 137*). As expected, pretreatment of cells with NAC almost completely abrogates these effects. The simultaneous exposure of cells to 4-PBA resulted in a slight decrease in CD86 and in a drastic decrease of IL-1b transcription, indicating that DNFB-evoked ER stress is part of the intricate signaling cascade that drives DC maturation. Surprisingly, treatment with Salubrinal and the concomitant sustained activation of the eIF2α-ATF4 axis resulted in a dramatic exacerbation of DNFB-induced IL-8 transcription. As 4-PBA does not significantly alter IL-8 mRNA levels evoked by DNFB, we hypothesize that although not essential to IL-8 transcription the eIF2α-ATF4 axis may synergize with other transcription factor, leading to the observed effect. Overall, our results indicate that DNFB activation of eIF2α-ATF4 stress pathway in DC is crucial to the remediation of cell redox imbalance through transcriptional control of detoxifying genes and strongly contributes to the generation of a pro-inflammatory microenvironment.

VII. General conclusions

General conclusions

Overall, we concluded:

- DNFB induced intense and early ROS production;
- DNFB does not trigger a canonical ER stress response in contrast to Tunicamycin, inducing only p-eIF2a-ATF4 UPR branch;
- MAPK activation by DNFB probably crosstalk with p-eIF2a;Activation of p-eIF2α – ATF4 axis exerts strong cytoprotective effects through exacerbation of detoxifying gene transcription;
- DNFB-evoked ER stress is part of the intricate signaling cascade that drives DC maturation;
- Activation of p-eIF2α – ATF4 axis contributes to the generation of a proinflammatory microenvironment.

Future work

- Evaluation of other kinases that also activate the p-eIF2α such as double-stranded RNA-activated protein kinase (PKR; activated in viral responses), general control non derepressible 2 (GCN2; activated upon aminoacid starvation) and heme-regulated inhibitor (HRI; activated in heme depletion);
- Evaluation of the effects of p-eIF2α – ATF4 axis activation in DNFB-induced apoptosis by a more direct technique;
- -evaluation whether the impairment of autophagy is dependent on p-eIF2α – ATF4 axis activation and contributes to the inflammatory response triggered by DNFB;
- Assessment of the effects of p-eIF2α – ATF4 axis activation in DNFB-induced gene transcription of other detoxifying genes: NQO1, TRX, TRXR;
- Evaluation of the possible synergizing effects with keap-NRF2 pathway.

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