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ASSESSMENT OF THE ALLERGENIC POTENTIAL OF XENOBIOTICS: *IN VIVO IN VITRO* A BACK-AND-FORTH APPROACH

Valentina GALBIATI
Matr. N. R09603

TUTOR: Chiar.mo Prof. Corrado Lodovico GALLI
CORRELATORE: Chiar.mo Prof. Alberto PANERAI

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Abbreviations

ACD	Allergic contact dermatitis
AIM2	Absent in melanoma 2
AKR1	Aldo-keto reductase family 1 member
AOP	Aderse outcome pathway
AP-1	Activator protein 1
APC	Antigen presenting cells
ASC	Apoptosis-associated speck-like protein containing a CARD domain
ATP	Adenosine triphosphate
CARD	Caspase-recruitment domain
CD[number]	Cluster of differentiation [number]
DAMPs	Damage-associated molecular patterns
DNCB	2,4-dinitrochlorobenzene
DPI	Diphenyleneiodonium
DPRA	Direct Peptide Reactivity Assay
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GPMT	Guinea Pig Maximization Test
GSH	Glutathione
GSSG	Glutathione disulfide
h-CLAT	THP-1 cell line activation assay
HClPt	Ammonium hexachloroplatinate
HMGB1	High mobility group binding protein 1
HMOX-1	Heme oxygenase (decycling) 1
Hsp	Heat shock protein
ICAM-1	Intracellular adhesion molecule 1
IFN-ϒ	Interferon ϒ
IL-[number]	Interleukine [number]
IRAK	Interleukin-1 receptor-associated kinase
KCs	Keratinocytes
Keap1	Kelch-like ECH-associated protein 1
LCs	Langerhans cells
LLNA	Local Lymph Node Assay
LPS	Lipopolysaccharides
LRR	Leucine repeated domain
MAPK	Mitogen-activated protein kinase

MDI	Diphenylmethane diisocyanates
MHC	Major Hystocapompatibility complex
MyD88	Myeloid differentiation primary response gene (88)
NADPH	Nicotinamide adenine dinucleotide phosphate
NATCH	Nucelotide-binding oligomerization domain
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells)
NK	Natural killer
NLRP3	Nod like receptor P3
NOX	NADPH oxidase
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OECD	Organisation for Economic Co-operation and Development
P2X7R	Purinergic receptor 2X7
PABA	4-aminobenzoic acid
PAMPs	Pathogen-associated molecular patterns
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PMSF	Phenylmethanesulfonylfluoride
PPD	ρ-phenylenediamine
PPRs	Pattern recognition receptors
PYD	Pyrin domain
RAGE	Receptor for advance glycation end products
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals substances
ROS	Reaactive oxigen species
SDS	Sodium lauryl sulphate
TG	Test guideline
TGF-α/β	Transforming growth factor
TIR	Toll/IL-1 receptor domain
TLR4	Toll-like receptor 4
TMA	Trimellitic anhydride
TMTD	Tetramethylthiuram disulfite
TNF-α	Tumor Necrosis Factor α
TNFR1 and 2	TNF receptor 1 and 2
TRAF	TNF receptor associated factors
TRX	Thioredoxin
TXNIP	Thioredoxin interacting protein
TXNIP	Thioredoxin-interacting protein
UVA/UVB	Ultraviolet A/B

Abstract

Allergic contact dermatitis (ACD) is an important occupational and environmental disease caused by topical exposure to low molecular weight chemical allergens. The development of ACD requires the activation of innate immune cells, such as keratinocytes (KC), necessary for the maturation and the migration of dendritic cells (DC), which in turn are required for the activation of specific T cells. Human KC constitutively express several cytokines, including pro IL-1 alpha, pro IL-1 beta and pro IL-18. *In vivo* it has been demonstrated that IL-18 plays a key proximal role in the induction of allergic contact sensitization, favoring Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF-α, IL-8 and IFN-γ (Shornick et al., 1996; Wang et al., 1999, Cumberbatch et al., 2001).

Toxicologists have the responsibility of identifying and characterizing the skin and respiratory allergenic potential of chemicals, and estimating the risk they pose to human health. Growing political and practical resistance to toxicity testing in animals has driven the development of animal-free methods for screening and prioritization of toxicants, including those causing allergic hypersensitivity.

The purpose of this thesis was to develop an alternative *in vitro* test based on the keratinocytes and IL-18 to characterize the allergenic potential of low molecular weight chemicals, and to understand the molecular mechanism(s) underlying chemical allergen-induced IL-18 production. In addition to human keratinocytes cell lines (NCTC2544, HaCaT, HPKII), commercially available reconstituted human epidermis 3D-epidermal models were also used as experimental model. Due to their anatomical location and critical role in skin inflammatory and immunological reactions, the use of the KC and skin organotypic culture as a simplified *in vitro* model to evaluate the potential toxicity of chemicals destined for

epicutaneous application is amply justified. To perform these studies 22 contact allergens, 12 photoallergens/photoirritant compounds, 3 respiratory allergens and 9 irritants chemicals were used. The choice of chemicals was dictated by the SENS-IT-IV programme as relevant and representative of the 'universe' of irritants, respiratory and contact allergens. Phototoxic chemicals were selected based on compounds used in similar published studies and reported to cause phototoxicity.

Results obtained indicate that the NCTC2544 IL-18 assay is able to discriminate contact allergens and photoallergens from irritants/photoirritants and respiratory allergens. Important factors including compound solubility, chemical reactivity and metabolic activation, which may mask the potential allergenicity of some chemicals, must be considered in the development of *in vitro* tests. Submerged cell culture may be unfavourable for many of the respiratory sensitizers, due to chemical instability; for this reason we have tested IL-18 production also in reconstituted human epidermis, which allows application in organic solvent, i.e. acetone: olive oil. The lack of metabolic activation may be a relevant problem in case of proaptens. However, NCTC 2544 cells posses both phase I and II metabolic activation capacity (Gelardi et al., 2001), and positive results were indeed obtained with the proaptens tested (eugenol and cinnamic alcohol). A sensitivity of 87%, specificity of 95% and an accuracy of 90% was obtained (Corsini et al., 2009; Galbiati et al., 2011).

In addition to being able to determine whether or not a chemical is a sensitizer (labelling) it is also equally important to determine the potency of a sensitizer (classification) in order to identify a maximum safe concentration for human exposure (risk assessment). The

combination of the epidermal equivalent potency assay with the release of IL-18 lead to the development of an *in vitro* model able to identify contact allergens and rank them according to their potency.

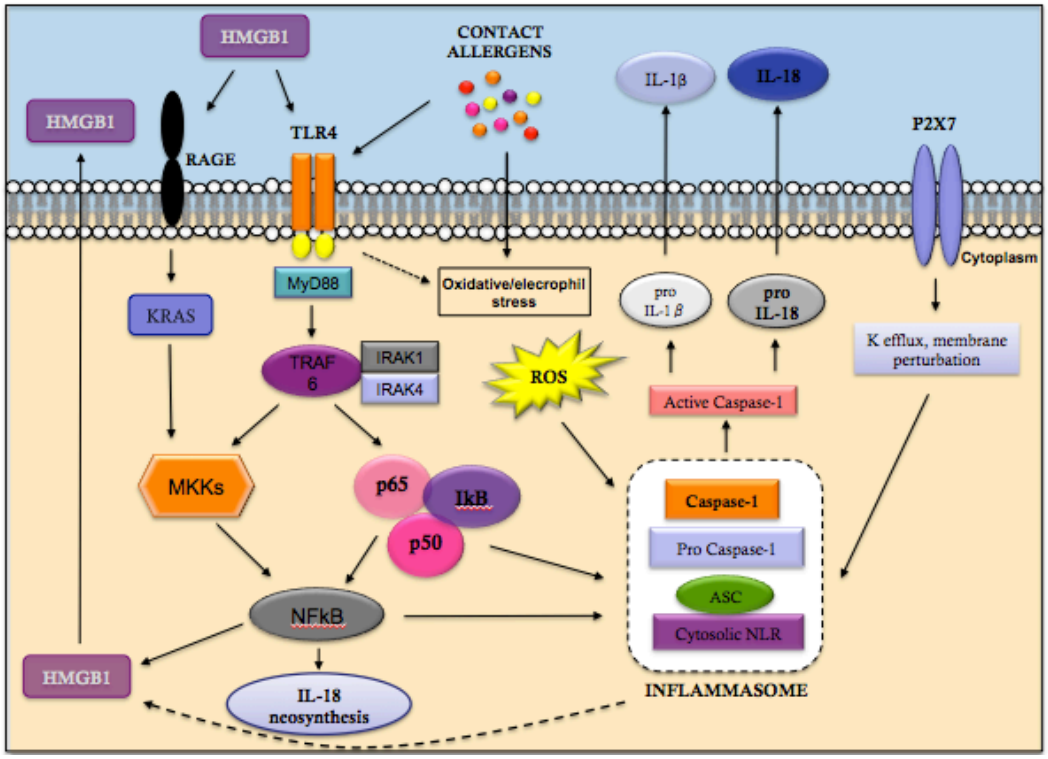
One other objective of this thesis was to study the signal transduction pathways involved in PPD, DNCB and citral-induced IL-18. For such purpose several inhibitors were used. To investigate the intracellular source of ROS, specific inhibitors of the three main cellular sources of ROS, namely *DPI*, a NADPH synthetase inhibitor; *rotenone*, a mitochondrial electron transport inhibitor; *allopurinol*, a xanthine oxidase inhibitor, were used. *Z-VAD-FMK*, a cell-permeant pan caspase inhibitor, that irreversible binds to the catalytic site of caspases, and a neutralizing *anti-TLR4* antibody were used to investigate the role of the inflammasome and TLR4. *Glycirrizic acid*, a direct inhibitor of HMGB1 protein, was used to establish the role of HMGB1 as possible DAMP associated with allergen-induced IL-18. To specifically investigate the signal transduction pathway involved in PPD-induced IL-18 production selective inhibitors were used: *GF109203X* to inhibit PKC, *PDTC* and *Bay 11-70-85* to inhibit NF-κB, and *SB203580*, as p38 MAPK inhibitor.

The results obtained during this three year of research activity have clearly shown that the *in vitro* methods based on NCTC2544 and IL-18 production are able to discriminate contact/photocontact allergens from irritants/photoirritants and respiratory allergens. Furthermore, the combined use of the epidermis *in vitro* model with the IL-18 production, beside the ability to identify sensitising compounds, is able to rank them according to their potency. With respect to the molecular mechanisms behind skin sensitization I could demonstrate that different intracellular sources of ROS are triggered by different contact allergens. Allergens-induced IL-18 production is dependent upon NF-κB and p38 MAPK

activation and TLR4 and inflammasome activation. Among the DAMPs, the evolutionarily conserved non-hystone chromatin-binding protein HMGB1 is released into the extracellular space following exposure to contact allergens, resulting in TLR4 activation and IL-18 neosynthesis.

Even if more studies are necessary to elucidate the mechanisms that are involved in chemical allergens-induced oxidative stress, the signalling pathways activated and their role in contact allergy, data clearly indicated a pivotal role of ROS in chemical allergy. Consequently, the redox state of the cell becomes imbalanced, with the activation of several pathways, including MAPK, such as SAPK/JNK, ERK1/2 and p38, NF-κB, Akt/ASK1 or Keap1/Nrf2 pathways, resulting in a inflammatory and cytotoxic response with the production of costimulatory molecules, cytokines, chemokines, and phase 1 detoxifying enzymes.

On the basis of the results obtained, the following scenario could be imagined (see Figure below): chemical sensitisers can induce oxidative stress owing to their elecrophilicity, which in turn activates the inflammasome and HMGB1 release (and possible other DAMPs), which can activate TLR4. Activation of TLR4 will results in NF-κB and p38 MAPK activation and in the neosynthesis of IL-18.



Molecular mechanism(s) underlying chemical allergen-induced IL-18 production

Chapter 1

INTRODUCTION

1.1 Allergic contact dermatitis

Chemical allergy describes adverse health effects, resulting from the stimulation of specific immune response by chemicals. The two most frequent manifestations of chemical-induced allergy are contact hypersensitivity and respiratory sensitization, both of which can have serious impact on the quality of life, and represent a common occupational health problem (Wahlberg, 1996).

Irritant and allergic contact dermatitis (ACD) is undesired side effects in the development of drugs and cosmetics as well as after contact with environmental or industrial chemicals. Whereas irritant contact dermatitis is a skin inflammation induced by primary contact with chemicals, and is though not to be mediated by lymphocytes, ACD represents a lymphocyte-mediated delayed type hypersensitivity reaction, which requires previous sensitization by the same chemicals (Basketter et al., 2008; Nosbaum et al., 2009). Because such reaction represents a true allergy, only minute quantities of chemical are necessary to elicit overt reactions. This is distinct from irritant contact dermatitis, in which the intensity of the reaction is proportional to the dose applied. An estimated 20% of all contact dermatitis is allergic (1). In industrialized countries, ACD is the most frequent manifestation of immunotoxicity, with large socio-economic consequences (Thyssen et al., 2007). Five to ten

percent of all occupational skin disease is estimated to result from skin allergy. In addition, several chemicals may also cause respiratory sensitization. The exact relation between skin and respiratory sensitization is not clear and is already for some years under scientific debate (McFadde et al., 2011). In general, low molecular-weight chemicals (haptens) are responsible for causing ACD. Most are less than 1000 Daltons and are electrophilic or hydrophilic. Some of these molecules are not inherently allergenic and must undergo metabolic transformation before participating in an allergic response. Because the skin has substantial metabolic capabilities, including many phase I and phase II enzymes, such biotransformation may occur in the skin at the site of contact with the chemical. Haptens, which are not intrinsically allergenic, must penetrate the stratum corneum and form a link with epidermal carrier proteins to form a complete allergen. They penetrate the epidermis and conjugate, most often covalently, to body proteins. The sensitizing potential of hapten cannot be reliable be predicted from its chemicals structure, although there is some correlation with the number of haptens attached to the carrier and the ability of the molecule to penetrate the skin. Also, certain contact allergens have unsatured carbon bonds and are easily oxidized. Some haptens, such as DNCB, sensitize nearly all individuals and can be used to assess cell-mediated immunity.

Contact dermatitis may occur upon exposure to any of the thousands of allergens that people are potentially exposed to in the course of a day (Table 1). Contact hypersensitivity can occur as a result of exposure to a wide variety of chemical and certain drugs (Nethercott and Holness, 1989), cosmetics (De Groot et al., 1988; Remaut, 1992; Andersen, 1993), and

various metals including nickel (Picardo et al., 1990) and chromium (Nethercott and Holness, 1989).

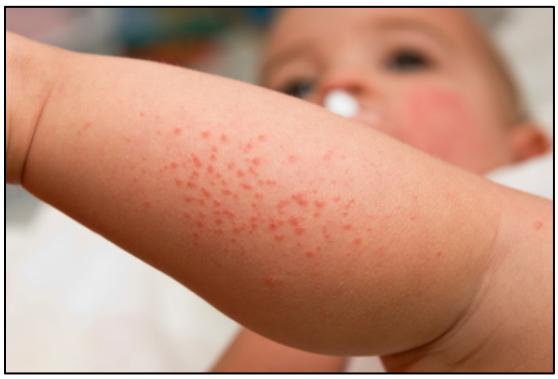


Fig.1 – Allergic Contact Dermatitis [1]

The causes of ACD are ubiquitous in the materials that come into contact with human skin. There are, however, several allergens, such as nickel, chromium, cobalt, and some food flavourings, that occur with great frequency. When an individual has a contact sensitivity to an agent, that is systematically administered (orally), a generalized skin eruption with associated symptoms such headache, malaise, and arthralgia may occur (Fig.1). Less dramatic eruptions may include flaring of a previous contact dermatitis to the same substance, vesicular hand eruptions, and an eczematous eruption in flexus areas. Systemic contact dermatitis may produce a delayed type hypersensitivity reaction or deposition of immunoglobulin's and complement components in the skin. Such deposits are potent inducers of a secondary inflammatory response and are responsible for the initial pathophysiology of many blistering and connective tissue disease of the skin.

Cross-reactions between chemicals may occur if they share functional groups critical to the formation of complete allergens (hapten plus carrier protein). These reactions may cause

difficulties in controlling contact dermatitis because avoidance of known allergens and potentially cross-reacting substances is necessary for improvement. Proper diagnosis can be hampered by concomitant sensitization to two chemicals in the same product or simultaneous sensitization to two chemicals in different products (Table 2).

A contact hypersensitivity reaction occurs in two stages: sensitization or induction and elicitation. Sensitization takes 10-14 days in humans. Once absorbed, the hapten combines with a protein and is internalized by epidermal Langerhans cells (LCs), which leave the epidermis and migrate as veiled cells, afferent lymphatic's, to the paracortical areas of regional lymph nodes. Here they present processed haptens-protein conjugates (in association with MHC class II molecules) to CD4+ lymphocytes, producing a population of memory CD4+ T cells.

The application of an allergen generally causes a modest decrease in Langerhans cell number in the epidermis within hours of application. Antigen presentation by Langerhans cell then occurs in skin and lymph nodes. Degranulation and cytokine release by mast cells and epithelial cells follows soon after contact with an allergen. TNF-α and IL-1 from many cell types, and from macrophages in particular, are potent inducers of endothelial cell adhesion molecules. These locally released cytokines produce a gradient signal for movement of mononuclear cells towards the dermo-epidermal junction and epidermis. The earliest histological change, seen after 4-8 hours, is the appearance of mononuclear cells around annex and blood vessel, with subsequent epidermal infiltration. The number of cells infiltrating the epidermis and dermis peaks at 48-72 hours. Most infiltrating lymphocytes are

CD4+, with few CD8+. Less than 1% of infiltrating cells are of a single clonal lineage. The main mechanism for T-cell recruitment is antigen independent.

Both skin resident cells, such as keratinocytes (KCs), LCs and mast cells, and immigrating leucocytes, including T lymphocytes and natural killer cells, actively participate in contact allergy (Cavani et al., 2007).

Contact hypersensitivity is primarily an epidermal reaction (Fig.2), and the dendritic Langerhans cell, located in the suprabasal epidermis, is the principle antigen-presenting cell (APC) involved. Langerhans cells are derived from bone marrow and express CD1, MHC class II antigens and surface receptor for Fc and complement.

KCs provide the structural integrity of the epidermis and have a central role in epidermal immunology. They may express MHC class II molecules and ICAM-1 in the cell membrane.

They also can release cytokines including IL-1, IL-3, IL-6, IL-8, IL-18, GM-CSF, TNF-α, TGF-α and TGF-β. Some of these cytokines can activate Langerhans cells, co-stimulate proliferative responses and recruit mast cells, and induce the secretion of immunosuppressive cytokines such as IL-10 and TGF-β, which dampen the immune response and induce clonal anergy in Th1 cells. KC can be activated by a number of stimuli, including allergens and irritants. Some antigens, such as urushiol in poison ivy, may directly induce TNF-α and IL-8.

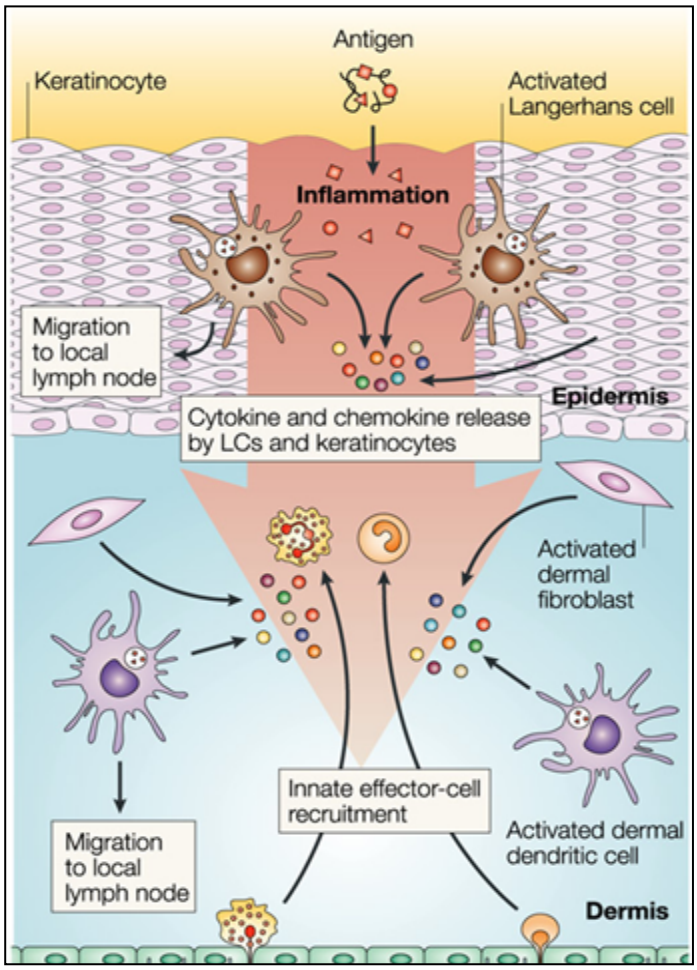


Fig.2 – Players of ACD [2]

KCs have a key role in skin sensitization, as they provide the essential danger signal, they are involved in the protein haptenation process, and supply enzymes that are necessary for the metabolic activation of prohaptens (Hennen et l., 2011; Kim and Choi, 2010). KCs play a role in all phases of ACD. They respond to allergens by producing a range of pro-inflammatory cytokines, which are important for dendritic cells maturation and migration to the draining lymph node, where the activation of the specific immune response take place (Vandebriel et al., 2005; Gober and Gaspari, 2008).

Chemical respiratory allergy, although less common than allergic contact dermatitis, is of considerable significance, not only because asthma resulting from sensitization can be severe, but at times it can also be fatal. A variety of chemicals have been implicated as causing occupational respiratory allergy. Among these are diisocyanates, acid anhydrides, some reactive dyes, certain chlorinated platinum salts, glutaraldehyde, chloramine-T, plicatic acid and carmine (Corsini and Kimber, 2007).

Table 1 – Common contact allergens [1a]

Source	Common Allergens	
Topical medications/Hygiene products	**Antibiotics** Aminoglycosides Bacitracin Neomycin Polymyxin Sulfonamides	**Therapeutics** Benzocaine Corticosteroids Fluorouracil Idoxuridine A-Tocopherol (vit E)
	Preservatives Benzalkonium Chloride Formaldehyde	**Others** Benzophenones Cinnamic aldehyde Fragrances Lanolin PPD Propylen glycol
Plants and trees	Abietic acid Balsam of Peru Pentadecylcatechols	Rosin Sesquiterpene lactone Tuliposide A
Antiseptics	Chloramine Chlorexidine Chloroxylenol Dichlorophene	Glutaraldehyde Hexachlorophene Mercurials Thimerosal
Rubber products	Benzothiazolesulfenamides Diphenylguanidine Dithiocarbamate Hydroquinone	Mercaptobenzothiazole PPD Resorcinol Thiurams
Leather	Formaldehyde Glutaraldehyde	Potassium dichromate
Paper products	Abiet acid Dyes Formaldehyde	Nigrosine Rosin Triphenyl phosphate
Glues and bonding agents	Acrylic monomers Bisphenol A Cyanoacrylates Epichlorohydrin	Epoxy resins Formaldehyde Toluene resins Urea resins
Metals	Chromium Cobalt	Mercury Nickel

Table 2 – common cross-reacting chemicals [1a]

Chemical	Cross-reactor
Abietic acid	Pine resin
p-aminobenzoic acid	p-aminosalicylic acid, sulphonamide
Balsam of Peru	pine resin, cinnamates, benzoates
Bisphenol A	diethylstilbestrol, hydroquinone ether
Canaga oil	Benzyl salicylate
Chlorocresol	Chloroxylenol
Diazolidinyl urea	Imidazolidynil urea, formaldehyde
Ethylenediammine HCl	Aminophylline, piperazine
Formaldehyde	Arylsulfonamide resin
Hydroquinone	Resorcinol
Methyl hydroxybenzoate	Parabens
Phenol	Resorcinol, cresols, hydroquinone
Phenylenediammine	Parabens, p-aminobenzoic acid
Propyl hydroxybenzoate	Hydroquinone monobenzyl eter
Tetramethylthiuram disulfite	Tetraethylthiuram mono-and disulfide

1.1.1 Adverse outcome pathway (AOP)

An adverse outcome pathway (AOP) is the sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an in vivo outcome of interest. Each AOP represents the existing knowledge concerning the linkage between a molecular initiating event, intermediate events and an adverse outcome at the individual or population level.

Knowledge of the AOP for skin sensitization has evolved rapidly over the past decade and four events that are recognised as key ones, have been identified (Fig. 3):

1. The first key event is the interaction of the chemical with skin protein. Specifically, the target chemical or its metabolite or abiotic transformation product of the target chemical covalently binds to cysteine and/or lysine residues of skin proteins;
2. The second key event takes place in the keratinocytes. It includes inflammatory responses as well as gene expression associated with specific cell signalling pathways;
3. The third key event is the activation of dendritic cells, which is typically assessed by expression of specific cell surface markers and release of chemokines and cytokines.
4. The final key event is the specific T-cell clonal expansion, which is measured in the murine LLNA.

Following a brief description of each key steps is reported.

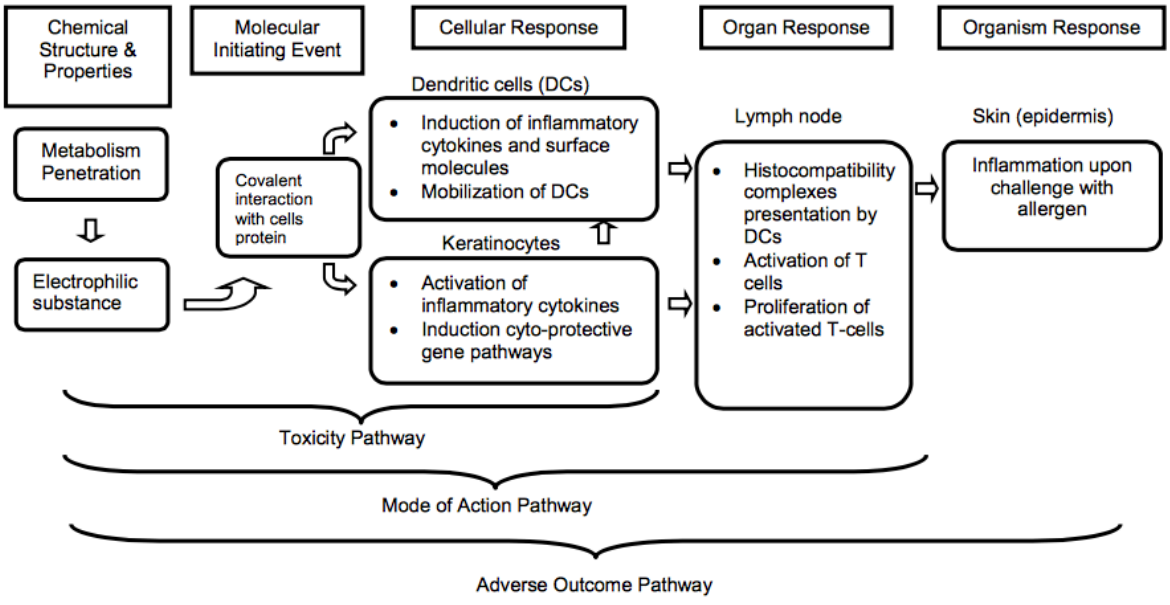


Fig. 3 – Flow diagram of the pathways associated with skin sensitization [3]

Chemical reactivity

One of the key molecular events in chemical-induced skin sensitisation is protein haptenation (the chemical modification of self-skin proteins). Chemical allergens are reactive exogenous compounds able to chemically modify skin proteins making them immunogenic and thus able to trigger a specific T-cell mediated immune response. Sensitizing chemicals have been divided according to their suspected reaction mechanisms (Divkovic et al., 2005; Aptula et al., 2005). Many compounds are not allergenic themselves but are activated before skin contact (prehaptens), i.e. via air oxidation, or in the skin, i.e. metabolically (prohaptens), to form skin sensitisers (Karlberg et al., 2008).

The skin can best be described as a nucleophilic environment, rich in water and nucleophilic functional groups present in proteins, i.e. susceptible amino acids include tyrosine, tryptophan, histidine, lysine, methionine, and cysteine. The majority of contact sensitisers are electrophiles (Divkovic et al., 2005, Karlberg et al., 2008 and Natsch et al., 2012). Five types of reactions have been recognized, including, S_N2 reactions, S_NAr reactions, Schiff base formations, Michael type additions, and acylation reactions. The possibility of a radical mechanism involved in antigen formation has also been proposed. Hydroperoxides, formed by air oxidation of many commonly used chemicals, have been shown to be strongly sensitisers. Most organic compounds, including turpentine, fragrance terpenes (i.e. limonene, linalool, geraniol), can undergo autoxidation, forming hydroperoxyde (ROOH). They are known to easily form radicals by cleavage of the labile O–O bond, the resulting radical may either bind directly with proteins or rearrange to an epoxide to form a hapten–carrier complex (electrophilic haptens). A third type of contact allergens are metal ions, i.e.

Ni, Cr, and Co, which form stable coordination complexes with proteins, then recognized as nonself.

Binding to the thiol group of cysteine is the one most often associated with sensitisers (Divkovic et al., 2005), which may lead to glutathione depletion and oxidative stress, tissue damage and increased inflammation.

On the basis of this mechanistic understanding, the relationship between reactivity of molecules toward proteins and their sensitisation potential has attracted increased interest and was used to develop experimental assay to predict the skin sensitisation potential of chemicals to replace in vivo animal tests (Gerberick et al., 2008).

A role of KC

KC form an interface between the body and the environment, they are important guardians for the detection of danger signals and the consecutive initiation of an inflammatory response. KC play a role in all phases of allergic contact dermatitis, from the early initiation phase with the elaboration of inflammatory cytokines, that plays a role in Langerhans cells (LC) migration, and T-cell trafficking, through the height of the inflammatory phase with direct interactions with epidermotrophic T-cells, through the resolution phase of allergic contact dermatitis with the production of anti-inflammatory cytokines and tolerogenic antigen presentation to effector T-cells (Gober and Gaspari, 2008). KC sense haptens, and in turn initiate a program of enhances or de novo expression of inflammatory molecules which are important for dendritic cells maturation and migration to the draining lymph node, where the activation of the specific immune response take place. Among the cytokines

produced by KC, IL-18 is of particular interest as it is a potent inducer of IFN-γ by activated T cells (Okamura et al., 1995). This cytokine has been shown to play a key proximal role in the induction of allergic contact sensitisation and to favor Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF-α, IL-18 and IFN-γ (Okamura et al., 1995, Cumberbatch et al., 2001 and Antonopoulos et al., 2008). Human keratinocytes constitutively express IL-18 mRNA and protein (Naik et al., 1999), and works published by Naik et al., 1999 and Van Och et al., 2005 showed the induction of IL-18 following exposure to contact sensitisers.

Recently, there have been several reports supporting the participation of ROS in the pathogenesis of contact dermatitis (reviewed by Corsini et al., 2013). Oxidative stress may be the starter point, as it leads to the activation of transcription factors and signalling pathways, including NF-κB and p38 MAPK, which leads to the release of cytokines and chemokines (Gloire et al., 2006; Kim and Choi, 2010). Reactive oxygen species plays also an important role in the activation of the NLRP3/NALP3 inflammasome (Martinon et al., 2010; Cumberbatch et al., 2001), which is required to direct the proteolytic maturation of inflammatory cytokines such as IL-1β and IL-18 (Martinon, 2010). The ability of contact sensitisers to induce the oxidative stress pathway in keratinocytes (Natsch and Emter, 2007) has been recently confirmed by Vandebriel et al. (2010).

The role of dendritic cells

In the induction of contact sensitisation, DC represents the most important initiators and regulators of contact allergy. Within the skin, there are at least two distinct subsets of DC,

LC, localized in the epidermis, and dermal DC (dDC). LC are the only DC subset to reside in the epidermis and, were once considered to be wholly responsible for presentation of chemical allergen to T-cells. Recently, some studies have challenged this belief, with the suggestion that also other subsets may contribute to induction and regulation of skin sensitisation (Ainscough et al., 2012).

In order to activate a specific immune response, DC must migrate through the dermis, towards the lymph nodes. The cytokines tumor necrosis factor-α, interleukin-1β and IL-18, produced by KC and DC following DAMPs generation in the skin microenvironment, are particularly important. All this is integral to the process of DC mobilization, migration and functional maturation.

In DC, Mizuashi et al. (2005) showed that all the sensitisers tested (Ni, formaldehyde, DNCB, $MnCl_2$ and thimerosal), but none of the non sensitisers reduced the GSH/GSSG ratio, which was accompanied by p38 MAPK activation. The antioxidant N-acetyl-L-cysteine, which suppressed the reduction of the GSH/GSSG ratio, abrogated p38 MAPK activation and CD86 expression, a common marker of DC maturation, induced by chemical sensitisers.

Chemical-induced oxidation of the cell surface thiols appears to be one of the triggers of DC maturation, resulting in intracellular redox imbalance (Suzuki et al., 2009 and Kagatani et al., 2010). Interestingly, in human DC, ROS were produced by both contact allergens DNCB and thimerosal and the irritant benzalkonium chloride, and were both decreased by pre-treatment with N-acetyl-L-cysteine. However, only DNCB up-regulated CD86 and HLA-DR molecules and induced protein carbonylation, suggesting the DNCB-induced ROS are somehow different from those produced by irritant (Byamba et al., 2010). The same Authors

demonstrated that DNCB induced ROS were reduced by diphenylene iodonium (DPI), while benzalkonium chloride-induced ROS were slightly decrease by N-monomethyl-L-arginine (a nitric oxide synthase inhibitor), suggesting a different cellular source of ROS. In particular, results suggest that irritant-induced ROS may consist of nitric oxides or peroxynitrite, while contact allergen-induced ROS may consist of hydrogen peroxide or superoxide, derived from the NOX2 pathway (Byamba et al., 2010). However, the involvement of mitochondria as source of ROS cannot be ruled out, as the inhibitor used DPI not only inhibits NADPH oxidase, but also potently inhibits mitochondrial ROS production (Li and Trush, 1998).

Proteins that are reversibly modulated by ROS are of high interest. In this context, protein kinases and phosphatases, which act coordinately in the regulation of signal transduction through the phosphorylation and dephosphorylation of target proteins, have been described to be key elements in ROS-mediated signaling events. The major mechanism by which these proteins may be modified by oxidation involves the presence of key redox-sensitive cysteine residues. The protein kinase C (PKC) family is composed of 10 serine/threonine protein kinases that are involved in a variety of pathways that regulate cell growth, differentiation, apoptosis, transformation and tumorigenicity. Most cells express more than one isoform, and each type of PKC mediates different cellular events. PKC isoforms are differentially distributed not only with respect to tissue but also in terms of subcellular localization, suggesting that each isoform could have specific functions. PKC isoforms differ in their structure, cofactor requirement and substrate specificity. The members of the PKC family have been divided into three major groups: the classical PKCs (cPKCs), including the α, βI, βII, and γ isoforms; the novel PKCs (nPKCs), including the θ, η, ε, δ isoforms; and the atypical PKCs (aPKCs), including the ζ and ι/λ isoforms. PKC isoforms

have been shown to contain a unique structural feature that is susceptible to oxidative modification (Cosentino-Gomes et al., 2012). In particular, two pairs of zinc fingers are found within the regulatory domain (sites of diacylglycerol and phorbol ester binding). Each zinc finger is composed of six cysteine residues and two zinc atoms. The high levels of cysteine residues render the regulatory domain susceptible to redox regulation (Giorgi et al., 2010 and Gopalakrishna and Jaken, 2000). Oxidative stress destroys the zinc finger conformation, and the autoinhibition is relieved, resulting in a PKC form that is catalytically active in the absence of Ca^{2+} or phospholipids. Currently, evidence supports the direct activation of different PKC isoforms by ROS generation (Gopalakrishna and Jaken, 2000 and DelCarlo and Loeser, 2006). In the context of skin sensitisation, however, PKC is of particular interest, as it has been demonstrated almost 20 years ago by Halliday and Lucas. (1993) to be required for LC migration, and that within the epidermis, the isoform beta is exclusively expressed in LCs, and its downregulation impaired LC function with respect to contact hypersensitivity (Goodell et al., 1996). Furthermore, not only ROS can activate PKC, but PKC also has been shown to promote the production of endogenous ROS. The β isoform of PKC induces ROS generation through mitochondrial damage (Pinton et al., 2007). PKCβ is responsible for the activation/phosphorylation of the mitochondrial p66shc protein, resulting in ROS generation (Migliaccio et al., 1999). In myeloid leukemia cells, ROS are not necessary for the translocation of PKCβII from the cytosol to the cell membrane. However, PKCβII was shown to be essential for ROS production induced by the PKC activator TPA (Datta et al., 2000). Also other PKC isoforms, including PKCα, PKCε and PKCζ, have been involved in mitochondrial ROS production in different experimental models (Wang et al., 2006 and Agudo-López et al., 2011).

Primary cells (i.e. CD34+ derived dendritic cells, monocyte derived dendritic cells) as well as dendritic cell-like cell lines ((i.e. THP-1, U-937, MUTZ-3, KG-1, HL-60, and K562) have been extensively described along with biomarkers such as cell surface markers, cytokines, chemokines and kinases and nicely reviewed by Dos Santos et al., (2009). There are currently two different sources of DC for assay development. These are DC derived from fresh blood cell precursors and DC-like cell lines. Whereas DC derived from fresh blood cell precursors are more physiologically relevant than DC-like cell lines, their implementation is limited by i) the logistics involved in supplying fresh blood to the cell culture lab, ii) ethical issues and iii) donor variation. Cell lines have the potential to provide large amounts of readily available DC-like cells without donor variation (dos Santos et al., 2009).

The following five assays use primary cells or cell lines as surrogates for dermal DC. Expression of markers which are considered prerequisites for the migration of dermal DC to the adjacent lymph nodes (key event 3 in the skin sensitisation AOP) such as cell-surface CD54 and/or CD86 or transcription of chemokine receptor (CCR)2 are used as measurements of cell activation.

1. Human cell line activation test (h-CLAT, developed by Kao and Shiseido). The h-CLAT assay uses THP-1 cells (a human monocytic leukemia cell line) as a surrogate for dermal DC. Relative fluorescence intensity (RFI) compared to vehicle-only treated control cells is used as an indicator of CD86 and CD54 induction. The h-CLAT method has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-coordinated validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) and was considered scientifically valid to be used as part of an IATA to support the

discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling (OECD GUIDELINE FOR THE TESTING OF CHEMICALS, July 2014).

2. Myeloid U937 skin sensitisation test (MUSST, L´Oreal). The MUSST assay, which uses the U937 cell line (a human histiocytic leukemia cell line), is designed to evaluate the capacity of a substance to induce dendritic cell activation. To achieve this, CD86 expression is assessed by flow cytometry. A substance inducing an increase in CD86 protein expression of P150% is considered to be a sensitiser.
3. Modified myeloid U937 skin sensitisation test (mMUSST, BASF). The mMUSST also uses the U937 cell line measuring CD86 by flow cytometry. A test substance is predicted to have a dendritic cell line activating potential when CD86 induction exceeds the threshold of 1.2 with respect to vehicle treated cells.
4. Peripheral blood monocyte-derived dendritic cell assay (PBMDC, Beiersdorf). In contrast to the above cell line-based assays, the PBMDC assay uses human peripheral blood monocyte-derived dendritic cells isolated from the fresh buffy coats of five different donors. CD1a negative/CD14 positive monocytes are selected and differentiated. CD86 expression is determined by flow cytometry. If a test substance induces on average P20% increase in CD86-positive cells compared to non-treated cells it is considered as a skin sensitiser.
5. VITOSENS (VITO). The VITOSENS assay uses differentiated CD34+ progenitor cells derived from human cord blood as surrogate for DC. The response to test substance exposure is evaluated by comparing the fold change in the expression of CCR2 (C–C

chemokine receptor type 2) and the transcription factor cAMP responsive element modulator (CREM)] compared to solvent-exposed cells (Hooyberghs et al., 2008).

It can be concluded that whilst DC cell based assays look very promising (e.g.: U-937 and THP-1 assays using CD86 as biomarker) these assays will most probably not be suitable as a stand alone assay to distinguish a potential sensitizer from a non-sensitizer to the same level of accuracy and reliability as the LLNA (dos Santos et al., 2009).

T cell activation

T-cells are typically affected by protein-hapten complexes presented by dendritic cells on MHC molecules. Molecular understanding of this process has improved in recent years (Martin et al, 2010). Briefly, MHC molecules are membrane proteins, which present the small peptide antigens placed in a "groove" of the MHC molecules during its intracellular synthesis and transport to the cell surface. In the context of the MHC molecular on the cell surface, the small peptide antigen is recognized via the T-cell receptors as a self or non-self. If this peptide antigen is recognised via the T-cell hapten complex, the T-cell will, be activated to form a memory T-cell, which subsequently proliferates. If reactivated upon hapten presentation by skin DCs cells, these memory T-cell will induce ACD (Vocanson et al., 2009).

Recognizing the importance of the process of antigen presentation (i.e. T-cell priming), in vitro T-cell priming assays have been developed (Martin et al., 2010). While first generation assays could only detect strong or extreme sensitisers, more recent development using

normal human peripheral blood depleted of regulatory cells that normally prevent the sensitisation phase, increased the probability of the detection of T- cell proliferation (Vocanson et al., 2008). A related approach is based on the hypothesis that there is a correlation between the potency of contact allergens and T-cell frequency and T-cell receptor repertoire (Kotturi et al., 2008). It is plausible that sensitisation potency may correlate with the size of the contact allergen-specific effector and regulatory T-cell pools and their diversity, and this could form the basis of a new generation of in vitro T-cell priming assays.

Perhaps the most interesting finding about lymphoid tissue, as related to the sensitisation is the selectivity of cytokine secretion. Hopkins et al. (2005), building on earlier work of Dearman and co- workers, reported that lymphoid tissue of mice exposed to classic electrophiles with conjugate proteins via nucleophilic substitution as halo-nitro-aromatic compounds (i.e. 1-chloro-2,4-dinitrobenzene and 1-fluoro- 2,4-dinitrobenzene) expresses high levels of the Th1 cytokine IFN-γ and low levels of the Th2 cytokines IL-5 and IL-10. Conversely, lymphoid tissue for mice exposed to 2,4-dinitrobenzene sulphonyl chloride, which conjugates with proteins via nucleophilic substitution as a sulphonyl halides, the acylating agent trimellitic anhydride, or fluorescein isothiocyanate, which conjugates with proteins via nucleophilic addition to the carbon atom of the isothiocyanate (-N=C=S) moiety, express high levels of the Th2 cytokines IL-5 and IL-10 and low levels of the Th1 cytokine IFN-γ (Hopkins et al., 2005). Based on differential binding to cellular and serum proteins Hopkins et al. (2005) showed that chemicals that stimulate a Th1 cytokine response bind selectively to cellular proteins, while chemicals that stimulate a Th2 cytokine response bind selectively to serum proteins. While it would be tempting to say electrophiles which

preferentially bind to cysteine express a Th1 cytokine profile and electrophiles which preferentially bind to lysine express a Th2 cytokine profile, it is most likely not that simple.

Given the complexity of ACD, a single alternative method cannot replace the LLNA, but it is necessary to combine methods through an integrated testing strategy (ITS). To ensure a mechanistic basis and cover the complexity, multiple methods should be integrated into a ITS. But the strategy most suitable for regulatory decision-making still remains to be determined (Van der Veen et al., 2014).

1.2 Photoallergy

In the course of life, the skin is exposed to radiation that spans the electromagnetic spectrum, including ultraviolet (UV), visible, and infrared radiation from the sun, artificial light sources, and heat sources. In general, the solar radiation reaching the earth that is most capable of inducing skin changes extends from 290 nm to 700 nm, the UV and visible spectra. Wavelengths on the extremes of this range are either significantly filtered by the earth's atmosphere or insufficiently energetic to cause cutaneous pathology. The absorption of light is deeper, more vital structures of the skin are dependent on a variety of optical parameters that differ from region to region on the body. Melanin is a significant chromophore in the skin because it is capable of absorbing a broad range of radiation from UVB through the visible spectrum. Other chromophores in the skin are amino acids and their breakdown products, such as tryptophan and urocanic acid, which are able to absorb light in the UVB range. Biologically, the most significant chromophore is DNA, because the resultant damage from the radiation may have lasting effects on the structure and function of the tissue.

Described as abnormal sensitivity to UV and visible light, photosensitivity may results from endogenous or exogenous factors. Illustrating the former, a variety of genetic diseases impair the cells ability to repair UV light-induced damage. The autoimmune disease lupus erithematosus also features abnormal sensitivity to UV light. In hereditary or chemically induced porphyria, enzyme abnormalities disrupt the biosynthetic pathways producing eme, leading to accumulation of porphyrin precursors or derivate throughout the body, including

the skin. These compounds in general fluoresce when exposed to UV light of 400-410 nm, and in this exited state they interact with cellular macromolecules or with molecular oxygen to generate toxic free radicals.

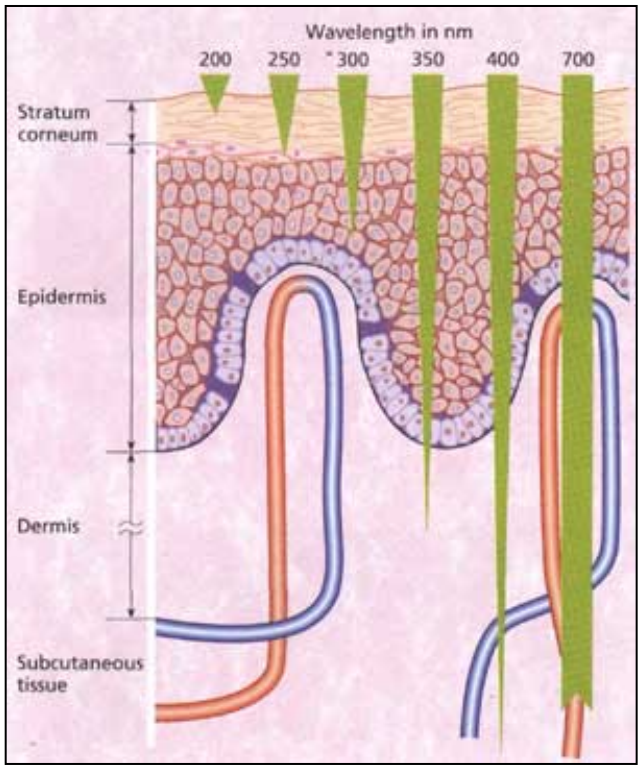


Fig.4 - Wavelength and skin [4]

Phototoxic reactions from exogenous chemicals may be produced by systemic or topical administration or exposure. In acute reactions, the skin may appear red and blister within minutes to hours after UV light exposure and resemble bad sunburn. Chronic phototoxic responses may result in hyperpigmentation and thickening of the affected areas. UVA is most commonly responsible; however, UVB may occasionally be implicated.

Not photodynamic mechanisms have been implicated in the pathogenesis of phototoxicity, psoralen being the prime examples. Upon entering the cell, psoralen intercalate with DNA in a non-photodependent manner. Subsequent excitation with UVA provokes a photochemical reaction that ultimately results in a covalently linked cycloadduct between psoralen and pyrimidine bases. This substantially inhibits DNA synthesis and repair, resulting in clinical phototoxicity reactions. Psoralens may be found in sufficiently high concentrations in plants that contact with their fruit and leaves in the presence of sunlight can cause a significant blistering eruption called phytophotodermatitis.

Photoallergy represent a true type IV delayed hypersensitivity reaction. Hence, while phototoxic reactions can occur with the first exposure to the offending chemical, photoallergy requires prior sensitization. Induction and subsequent elicitation of reaction may results from topical or systemic exposure to the agent. If topical, the reactions are termed photocontact dermatitis, while systemic exposures are termed systemic photoallergy. In most situations, systemic photoallergy is the result of the administration of medications. Generally, the mechanisms of photocontact dermatitis and of systemic photoallergy are the same as those described for the ACD, in the context of photocontact dermatitis; however, UV light is necessary to convert a potential photosensitizing chemical into a hapten that elicits an allergic response.

Safety evaluation of ingredients is an important part of the development of cosmetics and drugs. Phototoxicity, photoirritation and photoallergy are health hazard arising from exposure of skin to normally harmless levels of light in the presence of radiation-absorbing

compounds. The growing use of cosmetics in combination with relative high-UV light exposure potentiates this problem. Photoactivation and binding of photoactive compounds to proteins is a known prerequisite for the formation of immunogenic photoantigens and the induction of photoallergy. Ultraviolet A (UVA) is the action spectrum of this photoderivatization, as protein an cells are photocoulped with photohaptenic compounds by irradiation with UVA but not UVB (Tokura et al., 200; 2005). Photochemotoxic reactions may be a phototoxic or photoallergic in nature. Acute phototoxic reactions are generally characterized by erythema and oedema followed by hyperpigmentation and desquamation. Chronic repeated injury of this type may result in fragility, blistering and milia formation or even actinic keratosis and skin cancers. The photochemical mechanisms involved may differ. They include photoaddition of the chemical to biological targets such as DNA, the formation of toxic products due to the absorption of light by the phototoxic molecule, the generation of reactive oxygen species or free radicals (Epstein and Wintroub, 1985).

Phototoxic reactions are significantly more common than photoallergic reactions and mostly resemble to exaggerated sunburns. Photoallergic reactions appear only in minority of individuals and resemble ACD on sun-exposed areas, although sometimes may extend into covered areas. Generally, the physics examination and a positive patient's history of photosensitivity reactions on substances are of great importance for the diagnosis. Photocontact AD is one of the undesirable adverse effect produced by chemicals and drugs in our environment (Yamamoto and Tokura, 2003), it is a delayed type IV hypersensitivity reaction seen when exogenous agent comes into contact with the skin in the presence of UV. Various chemicals have been reported to cause photocontact dermatitis. The physical

manifestation of this increased photosensitivity will depend on the specific photosensitisers; some will reduce the threshold to sunburn while others may induce a photoallergy in susceptible individuals (Maverakis et al., 2010). Historically, the use of halogenated salicyanilide and related compounds, especially 3, 3', 4', 5-tetrachlorosalicylanilide and bithionol, resulted in a large number of patients with this skin disease. More recent causative agents include cosmetics or sunscreen products such as 6-methylcoumarine, musk ambrette, benzophenone. Likewise, there have been various culprit drugs to evoke photosensitivity, including chlorpromazine, promethazine, quinolones, such as sparfloxacin and enoxacin, piroxicum, afloqualone and non-steroidal anti-inflammatory drugs (Kurita et al., 2007).

Assessments of phototoxic properties of ingredients have generally been performed using animal test including Guinea pigs, rabbits, rats and mice (Maurer et al., 1980; Jordan, 1982; Gerberick and Ryan, 1990; Ulrich et al., 1998). For phototoxicity testing, the in vitro 3T3 NRU phototoxicity test has been adopted as an alternative to in vivo phototoxicity test (OECD no. 432). In vitro replacement for the in vivo photoallergy test have also been sought (Tokura, 2000; Lovell and Jones, 2000; Barratt et al., 2000; Neumann et al., 2005; Onoue and Tsuda, 2005; Kurita et al., 2007; Hoya et al., 2009; Karschuk et al., 2010), but to date no accepted alternative is available to identify the photoallergenic potential of new chemicals.

1.3 Keratinocytes and IL-18

KC form an interface between the body and the environment, they are important guardians for the detection of danger signals and the consecutive initiation of an inflammatory response. KC play a role in all phases of allergic contact dermatitis, from the early initiation phase with the elaboration of inflammatory cytokines, that plays a role in Langerhans cells (LC) migration, and T-cell trafficking, through the height of the inflammatory phase with direct interactions with epidermotrophic T-cells, through the resolution phase of allergic contact dermatitis with the production of anti-inflammatory cytokines and tolerogenic antigen presentation to effector T-cells (Gober and Gaspari, 2008). KC sense haptens, and in turn initiate a program of enhances or de novo expression of inflammatory molecules representing the starting point of primary inflammation. KC mount immune responses through the secretion of a variety of inflammatory cytokines, soluble proteins and ROS. KC play a role in all phase of allergic dermatitis.

Mehrotra et al. (2005) were the first to demonstrate that both contact allergens and irritants can induce ROS and nitrogen species in the human keratinocyte cell line A431. More recently, Kim et al. (2012) demonstrated in HaCaT cells that both the allergen DNCB and the irritant benzalkonium chloride can generate ROS in a time- and dose-dependent manner, however, only DNCB induced a ROS-dependent protein carbonylation, suggesting that the characteristic of ROS generated in response to chemicals might differ. Changes in ROS levels were detected after treatment for 30 min. Interestingly, not all allergens tested induced ROS production as assessed by DCFH, nickel and hydroquinone failed, suggesting that cellular oxidative stress after contact with chemicals might depend on the specific nature of the chemicals themselves. Further studies are necessary to verify oxidative stress-

dependent or oxidative stress-independent mechanisms in chemicals-induced KC activation and to establish its role in contact allergy.

As previous mentioned, human KCs constituvely express several cytokines, including pro-IL-1α, pro-IL-1β, and pro-IL-18. The IL-1 family is a group of cytokines involved in local and systemic inflammatory reactions. IL-1 induces acute-phase proteins, cytokines, and adhesion molecules. Among the cytokines produced by KC, IL-18 is of particular interest as it is a potent inducer of IFN-γ by activated T cells (Okamura et al., 1995). This cytokine has been shown to play a key proximal role in the induction of allergic contact sensitisation and to favor Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF-α, IL-18 and IFN-γ (Okamura et al., 1995, Cumberbatch et al., 2001 and Antonopoulos et al., 2008). Human keratinocytes constitutively express IL-18 mRNA and protein (Naik et al., 1999), and works published by Naik et al., 1999 and Van Och et al., 2005 showed the induction of IL-18 following exposure to contact sensitisers. IL-12 and IL-18 play important roles in the development of T helper type I (Th1) cells and are synergistic in the induction of IFN-γ by T-cells (Okamura et al., 1995). IL-18 has an important role in Th-1 type immune response enhancing the secretion of proinflammatory mediators such as TNF-α, IL-8, and IFN-γ and also in the induction of allergic contact sensitization (Shornick et al., 1996; Wang et al., 1999).

As shown in Figure 5, IL-1β and IL-18 are synthesized as preforms, which require proteolytic maturation by cysteine protease caspase-1, which must first be activated by the inflammasome (He et al., 2012; Lu et al., 2012). The inflammasome is a complex consisting

of receptor linked to inactive procaspase-1 by an intermediate apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (Menzel et al., 2011; Menu and Vince, 2011; Tschopp and Schroder, 2010). IL-1 and the inflammasome are involved in sensing contact sensitisers and triggering an innate immune response to these, which subsequently has a role in triggering the adaptive immune response to contact sensitisers in the skin (Sutterwala et al., 2006; Watanabe et al., 2007).

IL-18 plays a key proximal role in the induction of ACD (Cumberbatch et al., 2001).

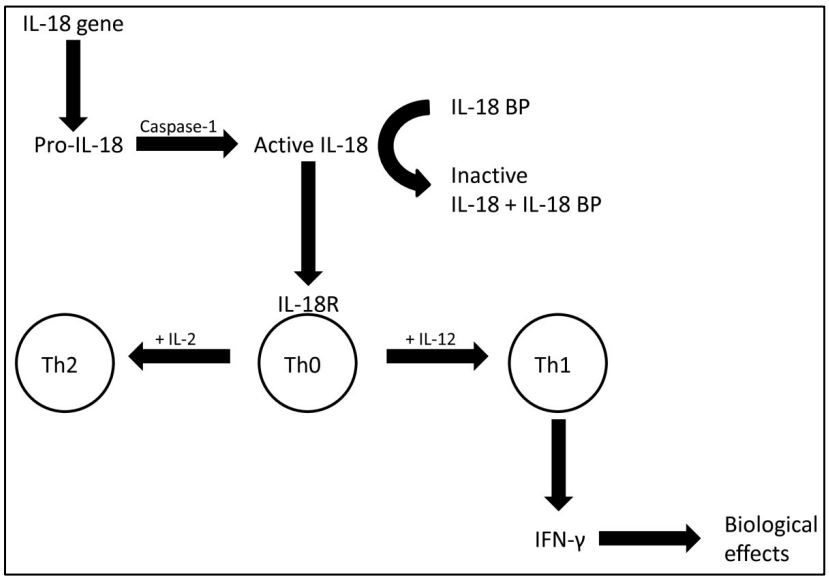


Fig.5 – IL-18 [5]

1.4 Signal transduction pathways involved in the activation of the inflammasome and IL-18 production

A wide range of proinflammatory stimuli, including pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) (Contassot et al., 2012), activate the NLRP3 inflammasome. Among DAMPs, the evolutionarily conserved non-histone chromatin-binding protein HMGB1 is released into the extracellular space during infection or injury by activated immune cells and damaged cells. HMGB1 functions as a proinflammatory mediator, it is a Toll-like receptor 4 (TLR4)-agonist, and it contributes importantly to the pathogenesis of inflammatory diseases (Lu et al., 2013). As all NLRP3 activators induced the generation of ROS, it has been reported that ROS production triggers NLRP inflammasome activation (Tshopp and Schroder, 2010). Generation of ROS induces the dissociation of thioredoxin from thioredoxin-interacting protein. Then the latter is able to bind NLRP3, which induced inflammasome activation (Zhou et al., 2010). The imbalance between the formation of ROS and the ability to detoxify these oxidizing radicals can produce a cellular state known as oxidative stress (Valko et al., 2007). It is the skin microenvironment that provides the DAMPs, including ROS, uric acid, hyaluronic acid fragments, HMGB-1, and adenosine triphosphate, that are required for the activation of pattern recognition receptors (PRRs) and full activation of DC.

1.4.1 NLRP3

The inflammasome is a cytosolic molecular complex that once activated has an enzymatic activity mediated by the recruitment and activation of caspase-1. The maintenance of

homeostasis is a primary function of the innate immune system, achieved in part through immune surveillance by PRRs, such as TLRs and NLRs, predominantly expressed by cells of the myeloid lineage (Sutterwala et al., 2014).

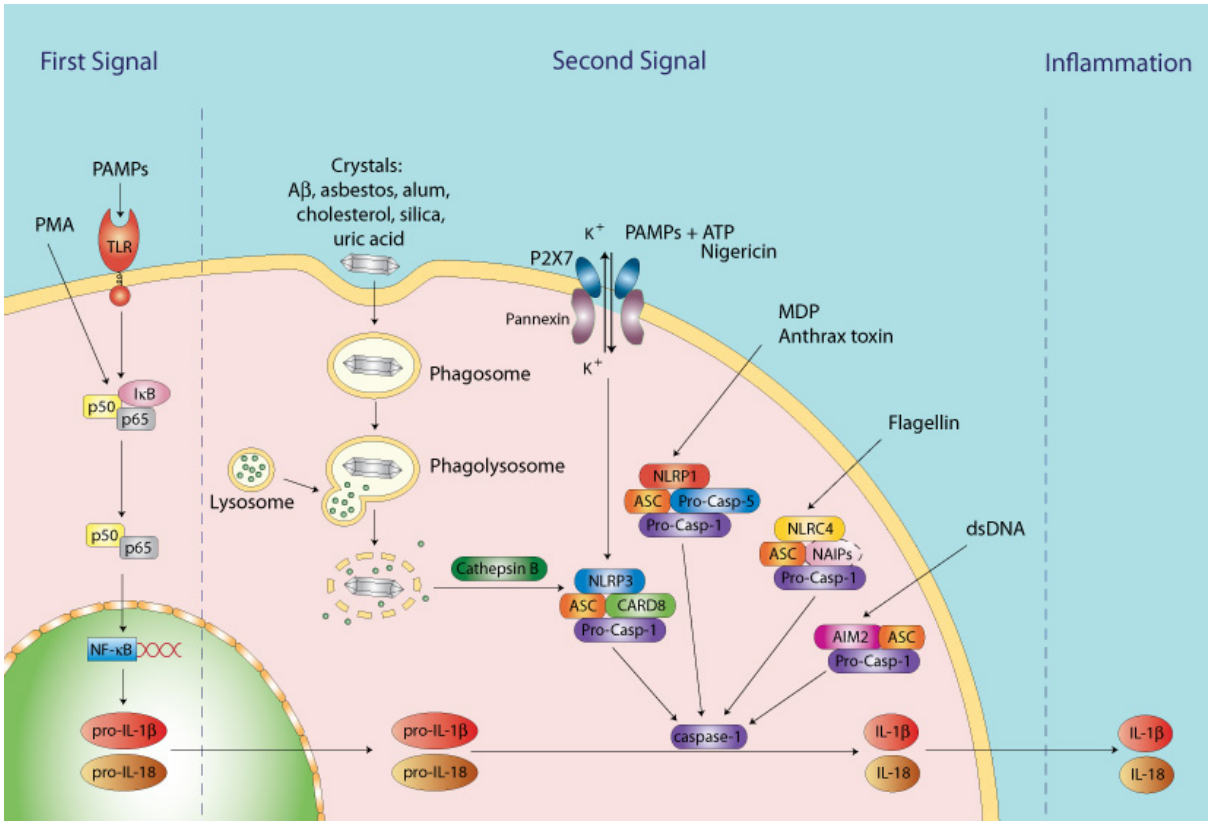


Fig 6. – Inflammasomes [5]

The cytosolic NLR family of PRRs functions to sense and respond to threats in the intracellular compartment (Fig. 6). NLR members are classified based on their shared three-domain structural homology. The N-terminal effector domain can include a pyrin domain (PYD), a caspase-recruitment domain (CARD), or a baculovirus inhibitory repeated domain, and is the basis by which NLRs may be further subcategorized. The central nucleotide-binding oligomerization domain (NATCH) is a common feature of all NLR proteins, followed by a leucine repeated domain (LRR) at the C-terminus (Ting et al., 2008).

NLRP1, NLRP3 and NLRC4, along with the pyrin and HIN domain-containing (PYHIN) family member AIM2, can be induced to associate with other proteins in large multimeric complexes termed inflammasomes.

Consisting of an NLR or PYHIN, in some cases the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD domain), and the cysteine protease procaspase-1, inflammasome formation ultimately results in the autocatalysis and activation of caspase-1 (Martinon et al., 2002; Hornung and Latz, 2010). Once enzymatically active, caspase-1 can go on to process the cytokines pro-IL-1β and pro-IL-18 into their mature secreted forms. These proinflammatory cytokines initiate an inflammatory cascade that leads to the recruitment of innate immune cells and can also determine the character of the subsequent adaptative immune response (Dinarello, 2009).

The NLRP3 inflammasome is the most extensively investigated of the inflammasome identified. The NLRP3 protein was first identified due to gain-of-function mutations in the encoding gene that are associated with the autoinflammatory cryopyrin associated periodic syndromes (Hoffman et al., 2001; Aksentijevich et al., 2002). In addition to the central NATCH and C-terminal LRR, NLRP3 is characterized by its N-terminal PYD, which allows NLRP3 to recruit the adaptor molecule ASC through PYD-PYD interactions, thus facilitating the recruitment of procaspase-1 to form the inflammasome complex (Agostini et al., 2004; Fernandes-Alnemri et al., 2007). Activation of the NLRP3 inflammasome can be achieved by a wide range of structurally dissimilar agonists, including pathogens, pore-forming toxins, environmental irritants, and endogenous DAMPs.

A two steps-model in which both priming and activating signals are required to produce a functional inflammasome is generally accepted (Sutterwala et al., 2014 - Fig.7).

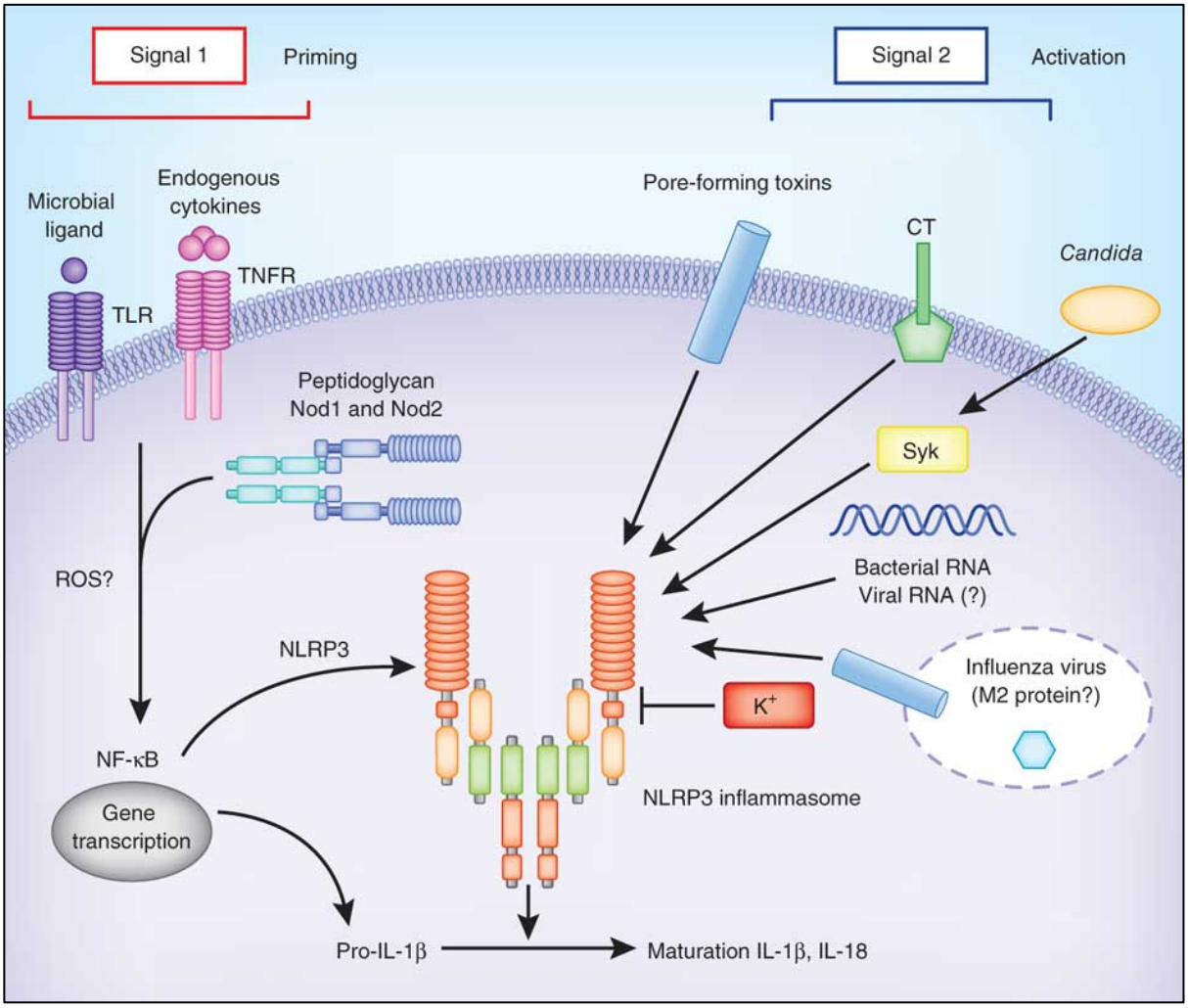


Fig. 7 – NLRP3 priming and activation [7]

Signal 1: priming

The initial inflammasome priming step upstream of activation affects NLRP3 at the transcriptional level and also serves to trigger post-translational modifications of inflammasome components that allow for oligomerization.

In general, priming stimuli can include any whose receptor signalling results in the activation of the transcription factor NF-κB, such as ligand for IL-1R, TLRs, NLRs, and the cytokine receptors TNFR1 and TNFR2 (Bauernfeind et al., 2009; Fanchi et al., 2009). The activation of NF-κB is critical for upregulating the transcription of both pro-IL-1β and NLRP3, as pro-IL-1β is not constitutively expressed and basal levels of NLRP3 are inadequate for efficient inflammasome formation. In contrast, transcriptional modulation is not required to license the inflammasome components ASC and procaspase-1 for inflammasome activation, nor the caspase-1 substrate pro IL-18, as there are found at adequate concentrations in the steady state (Schroder et al., 2012). Although NLRP3 inflammasome activation is possible if priming and activation signals are provided simultaneously, the kinetics and extent of inflammasome activation are greatly enhanced with increased availability of NLRP3 and pro-IL-1β (Latz et al., 2013; Juliana et al., 2012).

Signal 2: activation

The second step in activation of NLRP3 inflammasome is provided by one of a diverse group of agonist that triggers the specific activation of NLRP3, assembly of the inflammasome complex, and finally culminated is in the activation of caspase-1.

The activators include both exogenous and endogenous molecules such as crystalline molecules (alum, silica, asbestos, monosodium urate) that require phagocytosis for activation, ATP acting through its cell surface receptor P2X7R, and pore-forming toxins such as nigericin. As the activators are structurally dissimilar and act upon the cell in discrete ways, it has been suggested that the final activation signal, the binding of a ligand directly to NLRP3, must occur downstream from these unrelated upstream activators.

Common pathways necessary for NLRP3 inflammasome activation include cationic fluxes both to and from extracellular spaces, a well as from within the cell, and, additionally, the involvement of discrete subcellular organelles including lysosomes, mitochondria, and the endoplasmic reticulum (Sutterwala et al., 2014).

1.4.2 ROS

ROS are free reactive species that contain the oxygen atom and include hydrogen peroxide (H_2O_2), superoxide anion ($O^{\circ}_2^-$) and hydroxyl radical (OH°). These molecules are highly reactive due to the presence of unrepaired valence shell electron. ROS mainly originate as a by-product of oxygen metabolism in the electron transport chain within the mitochondria. ROS are also generated by the activity of cellular enzymes such as NADPH oxidases, xanthine oxidoreductases, lypooxigenases and cyclooxygenase (Fig. 8).

Cellular production of ROS regulates several important physiological responses, such as oxygen sensing, angiogenesis, control of vascular tone, and regulation of cell growth, differentiation and migration. While ROS is also important for cell signalling (redox signalling), sustained ROS production can cause cellular damage. To cope wit this stress, several enzyme displaying anti-oxidant activities, including thioredoxin (TRX), superoxide dismutase, glutathione peroxidase and catalase, are involved in neutralizing ROS. The imbalance between the formation of ROS and the ability to detoxify these oxiding radicals can produce a cellular state known as oxidative stress. ROS-mediated oxidative stress plays an important role in pathological processes such as aging, hypertension, atherosclerosis, cancer, ischemia, neurodegenerative disease and diabetes.

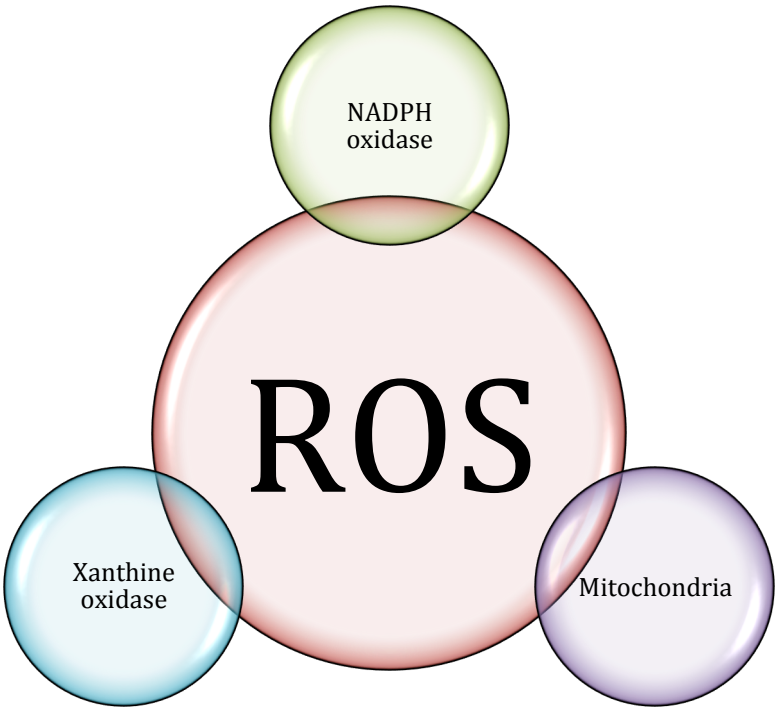


Fig.8 – different cellular font of ROS

Production of reactive ROS is crucial to the regulation of innate immune responses. Beyond its antiseptic function, release of ROS by damaged tissues can form a decreasing concentration gradient that directs leukocytes recruitment at the site of tissue injury, demonstrating that ROS can orchestrate inflammatory responses in tissues.

Redox signalling is also important in the signal pathways engaged by various inflammatory conditions. ROS production by the PRR, TLR, regulates activation of redox-regulated transcription factor (NF-kB and AP-1) and cytokines production. Recently, ROS has been proposed to play an important role in the activation of the NLRP3/NALP3 inflammasome (Martinon 2010).

Evidence indicates that allergic and inflammatory skin diseases are mediated by oxidative stress (Okayama, 2005; Byamba et al., 2010; Corsini et al., 2013). Proteomic and genomic analyses of human keratinocytes as well as of dendritic cells revealed large numbers of clearly sensitiser-specific markers, with several interesting overlaps. Particularly, markers relating to the Nrf2-mediated oxidative response and oxidative stress in general were identified repeatedly, very much in line with the prominent position that oxidative stress holds in chemical sensitisation (Ryan et al., 2004; Ade et al., 2009; Hooyberghs et al., 2008; Johansson et al., 2011; Van der Veen et al., 2013).

Electrophilicity is one of the most common features of skin contact sensitisers and it is necessary for protein haptenation. The Keap1 [Kelch-like ECH-associated protein 1]/Nrf2-signaling pathway is dedicated to the detection of electrophilic stress in cells leading to the upregulation of genes involved in protection or neutralization of chemical reactive species. Several Authors have proposed that monitoring of this pathway may provide new biomarkers (e.g., Nrf2, hmox1) for the detection of the sensitisation potential of chemicals both in KC and DC (Natsch and Emter, 2007 and Ade et al., 2009). A significant inhibition of the expression of oxidative stress associated genes and CD86 expression was observed by preincubated with N-acetyl cysteine, a glutathione precursor used to reinforce the redox potential of cells, further supporting the role of oxidative/electrophilic stress in chemical allergen-induced DC activation. Mizuashi et al. (2005) showed in human monocyte-derived dendritic cells that chemical sensitisers induced oxidative stress measuring the glutathione GSH/GSSG ratio, as a redox marker. These authors also showed that reduction of the glutathione GSH/GSSG ratio was accompanied by CD86 upregulation and p38 mitogen-activated protein kinase (p38 MAPK) activation, suggesting that the electrophilic properties

of chemical sensitisers may be perceived by DCs as a danger signal leading to DC maturation (Sasaki and Aiba, 2007).

1.4.3 NLRP3 and ROS

Most identified NLRP3 activators also trigger ROS production. Moreover, the use of antioxidants has been shown to inhibit NLRP3 inflammasome activation, suggesting that redox signalling or oxidative stress is involved in NLRP3 activation.

Extracellular ATP is an inflammatory signal that has been implicated in innate immunity in both plants and animals. In mammals, extracellular ATP binds to P2X7 receptors and activates the NLRP3 inflammasome. Treatment of macrophages with ATP results in the rapid production of ROS and the use of the broad spectrum NADPH oxidase inhibitor, diphenyleneiodonium (DPI) inhibits ATP-mediated caspase-1 activation. The NLRP3 activating particulate elements uric acid crystals, alum and particulate metals have been shown to induce ROS production. Similarly, ROS is detectable quickly upon exposure of macrophages to silica or asbestos. Other NLRP3 activators, such as the toxin nigericin, UV light and skin sensitisers activate a cellular redox imbalance required for inflammasome formation. ROS has also been implicated in NLRP3 activation by the malaria pathogenic crystal, hemozoin, the influenza virus and yeast *Candida albicans*.

Various pathways have been proposed to mediate ROS production by NLRP3 activators. Potassium efflux and decrease in cytosolic potassium concentration are the most sticking features associated with NLRP3 activators.

Some NLRP3 activators such as uric acid crystals, alum, asbestos and silica are large particulate elements that can induce the so-called frustrated phagocytosis at the cell surface. Evidence has also demonstrated that uric acid crystals can be phagocytosed. Ultrastructure studies of uric acid crystals-containing phagolysosomes show a disrupted membrane and possibly release of part of their content in the cytoplasm. In line with these observations, silica crystals and alum trigger damage and rupture of the lysosome. Importantly, the release of cathepsin B by damaged lysosomes has been proposed to mediate inflammasome activation. It is also possible that lysosomal damage and cathepsin B release act upstream of ROS production. In line with this model, cathepsin B has been shown to promote ROS production, in hepatocytes and neurons.

The observation that NOX inhibitors such as DPI inhibits inflammasome activation by virtually all NLRP3 activators identified so far suggests that NOX are involved in ROS production Indeed, DPI inhibits caspase-1 mediated IL-18 activation in mice undergoing physical stress. NOX inhibitors may have additional targets. DPI can exert inhibitory effect on mitochondrial ROS production in addition to NOX. However, in line with the possibility that NOX are involved in inflammasome activation, extracellular ATP has been shown to trigger translocation of cytosolic NOX components onto membrane-bound NOX2, forming an active macromolecular complex. Indeed, NOX2 deficiency impairs ATP-mediated ROS production by macrophages, suggesting that NOX2 may be involved in ATP-mediated NLRP3 activation. On the contrary, NOX2-deficient macrophages have no defect in inflammasome activation upon stimulation with other NLRP3 agonists including, uric acid crystals and silica while knockdown of p22phox, in the monocytic cell line THP-1 impairs inflammasome activation by hemozoin, silica, uric acid crystal and asbestos. Because p22phox deficiency

affects several NOX, it is possible that multiple NOX mediate ROS production to trigger NLRP3 inflammasome assembly (Fig. 9).

ROS production by H_2O2 activates the inflammasome; furthermore, knockdown of TRX, a cellular antioxidant protein, enhances IL-1beta activation by silica, uric acid crystals and asbestos. These findings suggest that oxidative stress could be sufficient to trigger NLRP3 activation. ROS may either directly trigger inflammasome assembly or be indirectly sensed through cytoplasmic proteins that modulate inflammasome activity. ATP-mediated ROS production has been shown to stimulate the PI3K pathway, and pharmacological inhibition of PI3K inhibits ATP-mediated caspase-1 activation suggesting that PI3K may be involved in inflammasome activation downstream of ROS.

Recently Tshopp et al. (2010) identified TXNIP/VDUP1 as an essential protein that may directly activate NLRP3 upon oxidative stress. The authors suggest, in resting cells, TXNIP/VDUP1 interacts with TRX and is therefore unable to activate NLRP3. Upon oxidative stress TXNIP/VDUP1 is released from oxidised TRX and in turn directly binds the leucine-rich region of NLRP3 leading to inflammasome assembly. Consistent with this findings, TXNIP/VDUP1-deficient macrophages treated with extracellular ATP or uric acid crystals have decreased caspase-1 and IL-1beta processing. This finding provides support for a model in which TXNIP/VDUP1 and NLRP3 set up a surveillance of cellular stress, preparing to drive inflammation in case of excessive stress or danger signals.

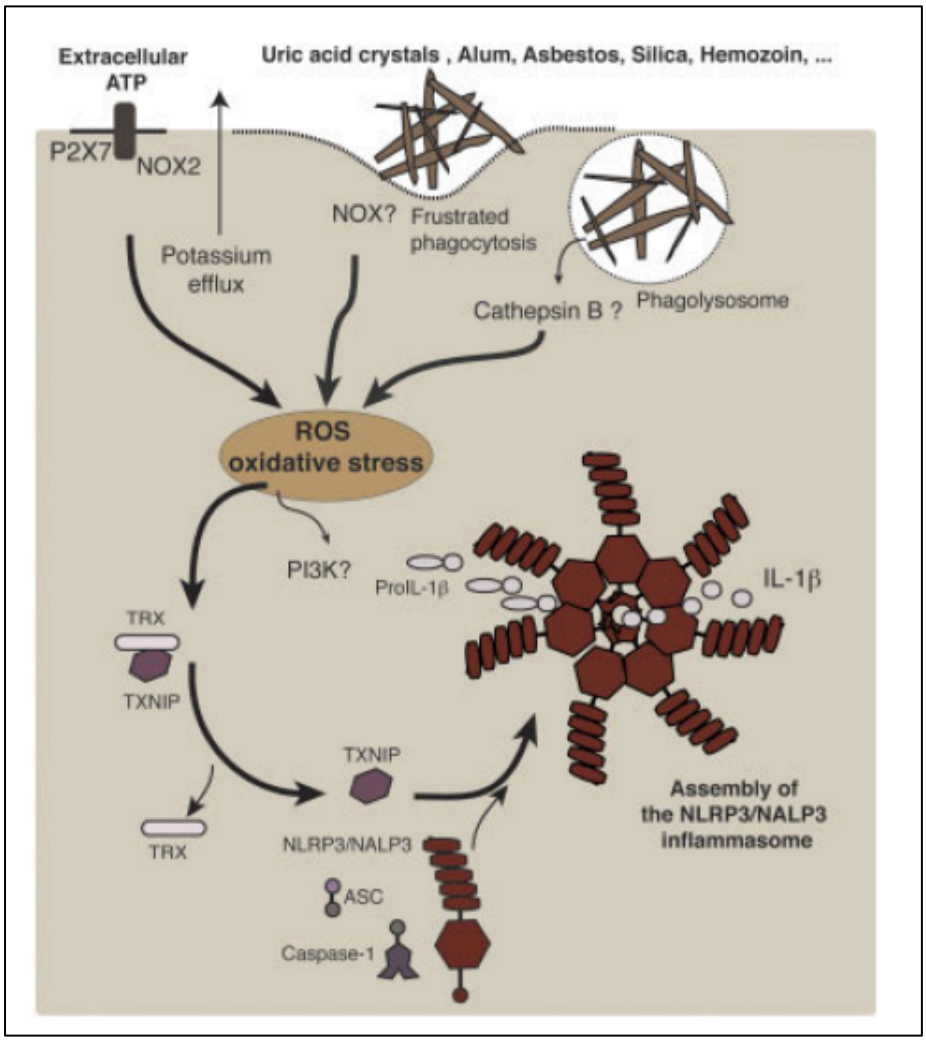


Fig. 9 – Model of NLRP3 inflammasome assembly and activation [8]

1.4.4 TLR4

In 1997, a human homologue of the Drosophila Toll protein was described, a protein later to be designated TLR4 (Fig. 10). Since that time, additional human and murine TLR proteins have been identified. Mammalian TLR proteins appear to represent a conserved family of innate immune recognition receptors. These receptors are coupled to a signalling pathway

that is conserved in mammals, insects, and plants, resulting in the activation of genes that mediate innate immune defences.

The activation of specialized receptors for pathogen-associated structures on sentinel cells is translated into the production and release of endogenous alarm mediators. IL-1β, TNF-α, IL-12 and IL-18 are rapidly released by these cells as first line defence. Interestingly, the receptors recognizing many pathogen-associated structure, the human TLRs, ands the receptors for the endogenous pro-inflammatory cytokines IL-1 and IL-18, share a cytoplasmic motif, the Toll/IL-1 receptor (TIR) domain, which is required for initiating intracellular signalling (Martin and Wesche, 2002). Thus, these receptors are not only grouped into one family because of the sharing of TIR domains and utilization of the MyD88/IRAK module but also functionally, as they include pivotal alarm receptors of the innate immune system. It needs to be stressed, however, that both IL-1 and IL-18 are not just mediators of the innate immune response. Both cytokines are centrally involved in the development, proliferation and differentiation of T helper cells and B cells. Thus, they represent key elements at the interface of innate and adaptative immune responses (Martin and Wesche, 2002).

TLR4 specifically recognizes bacterial lipopolysaccharide, along with several other components of pathogens and endogenous molecules produced during abnormal situations, such as tissue damage. Medzhitov et al. (1997) showed that activation of a human TLR leads to activation of transcription factor NF-**k**B and elements of innate immune responses. TLR2 and TLR4 signalling appear to require the adapter protein MyD88, the kinase IRAK, and TRAF6. MyD88 is a 35-kDa protein that contains three functional domains. It contains an

amino-terminal death domain, an intermediate domain, and a carboxy-terminal TIR domain, thus making MyD88 a member of the TLR family.

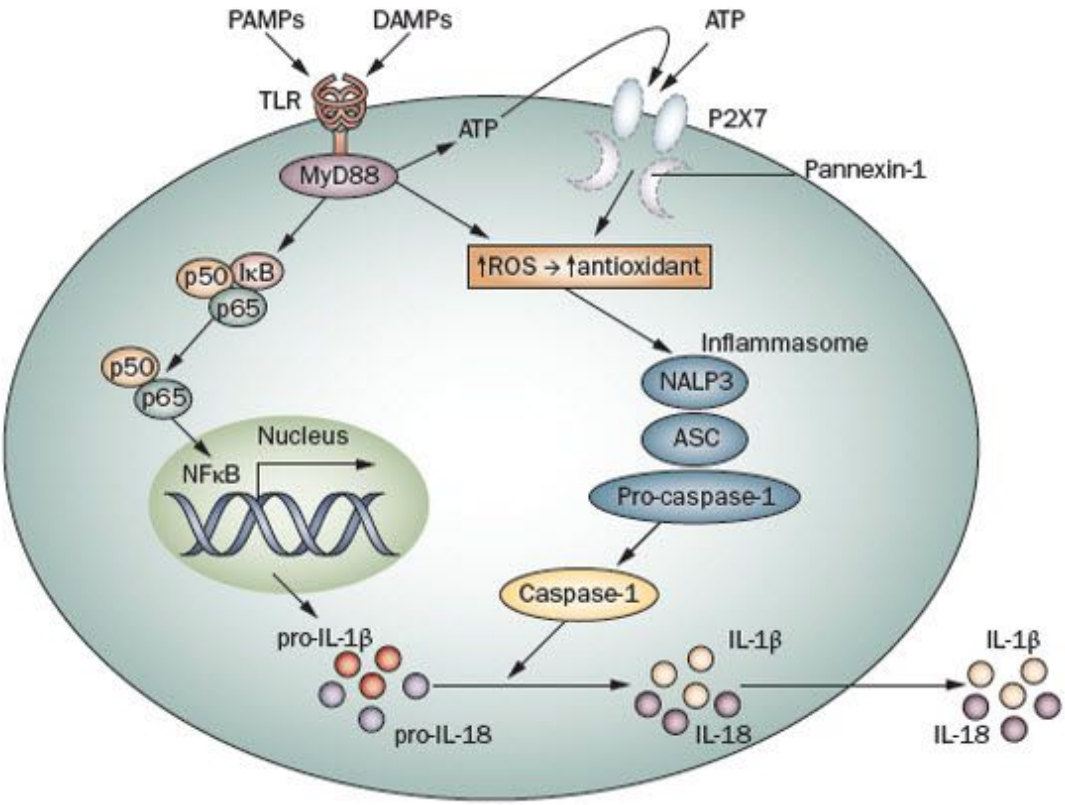


Fig. 10 – TLR4 and signal transduction pathways [9]

Using 2-hybrid analysis, MyD88 was shown to directly interact with TLR4. These data suggest that MyD88 and IRAK are part of the TLR4 signalling complex. IRAK contains an amino-terminal death domain that interacts with MyD88 (Means et al., 2000).

The observation that TLR4-deficient cells lose all the canonical responses to LPS, such as expression of proinflammatory cytokines and IFNs, has established the dogma that TLR4

accounts exclusively for all the host responses to LPS. Strikingly, recent studies have revealed the existence of host responses to LPS that do not require TLR4 (Tan and Kagan, 2014)

In addition to the NLR, other PAMPs/DAMPs receptors exist, including TLRs, c-type lectin receptors, prostanoid receptors and neuropeptide receptors (Trinchieri and Sher, 2007), that permit recognition of PAMPs that derived from bacteria, fungi and viruses. However, these TLRs are equally well equipped to recognize and respond to DAMPs that derive from the traumatic changes that may be associated with topical exposure to skin-sensitizing chemicals. Among the endogenous TLR ligands that may be elaborated in response to cell or tissue damage are fibronectin, hyaluronan, cathelicidin and heparin sulphate (McFadden et al., 2013).

Ligand activation of TLR4 and TLR2 leads to increased expression of a variety of proinflammatory cytokines, including IL-1, TNF-α, IL-6, IL-12, IL-18, IL-23 and IFN-γ. Similarly, expression of several endogenous ligands of TLR4 has been recorded following cutaneous exposure to contact allergens, including fibronectin, Hsp70, hyaluronan and β-defensin (Esser et al., 2011).

Martin et al. (2008), have shown experimentally that activation of TLR4 and TLR2 is essential for skin sensitization; thus, it would appear that stimulation of membrane-expressed TLRs via the elaboration of endogenous ligands is the key event for successful danger signalling by contact allergen.

Oxidative stress, the inflammasome and TLR4 activation are strictly cross-linked and they play a key role in the innate immune and stress responses. TLR4, after a danger signal, can

activate NF-κB and MAPK pathways, inducing the release of inflammatory cytokines such as IL-1β and IL-18. Additionally TLR4 has the ability to further induce ROS, which play a triple role: (1) they are able to directly stimulate inflammatory and cytotoxic responses; (2) they can activate the NF-κB and MAPK pathways; and (3) they can interact with the inflammasome leading to IL-1β and IL-18 release (Galbiati et al., 2014).

1.4.5 HMGB-1

HMGB1 is a ubiquitously expressed, highly conserved nuclear protein that plays important roles in chromatin organization and transcriptional regulation (Bianchi, 2009). HMGB1 was identified in 1999 as an important extracellular mediator of inflammation. When cells die in a non-programmed way (necrosis), they release HMGB1 by simple diffusion; in contrast, cells that die in a programmed way (apoptosis) avidly retain HMGB1 bound to chromatin remnants even after eventual cell lysis (Bianchi and Manfredi, 2004). This different behaviour between necrotic and apoptotic cells makes HMGB1 the primary signal of tissue damage; extracellular HMGB1 promotes local and systemic responses in the organism, including inflammation and the activation of innate and adaptative immunity (Lotze and Tracey, 2005).

Activated monocytes, macrophages, neutrophils, platelets, and dendritic and NK cells can also release HMGB1 in the extracellular space. Extracellular HMGB1 activates a large number of different physiological responses in different cell types, and can be considered a cytokine. Its beneficial roles include the promotion of tissue regeneration, by attracting stem cells and inducing them to proliferate. However, HMGB1 plays a pathogenetic role in

severe sepsis and arthritis, and may play a role in atherosclerosis and cancer (Mollica et al., 2007).

Several receptors have been implicated in HMGB1-mediated functions, including RAGE (receptor for advance glycation end products) and TLR2, TLR4 and TLR9 (Schiraldi et al., 2012). Downstream signalling is not completely understood, but involves Src, MAPK, and NF-κB activation. It is currently not known whether all receptors and all signalling pathways are required for the different responses to HMGB1; recent evidence suggest that TLR4 but not RAGE is required for cytokine release (Yang et al., 2010), and that RAGE is involved in cell migration. HMGB-1-induced cell migration requires activation of both the canonical and non-canonical NF-κB pathways, which lead to the transcription of the Cxcl12 gene (Penzo et al., 2010).

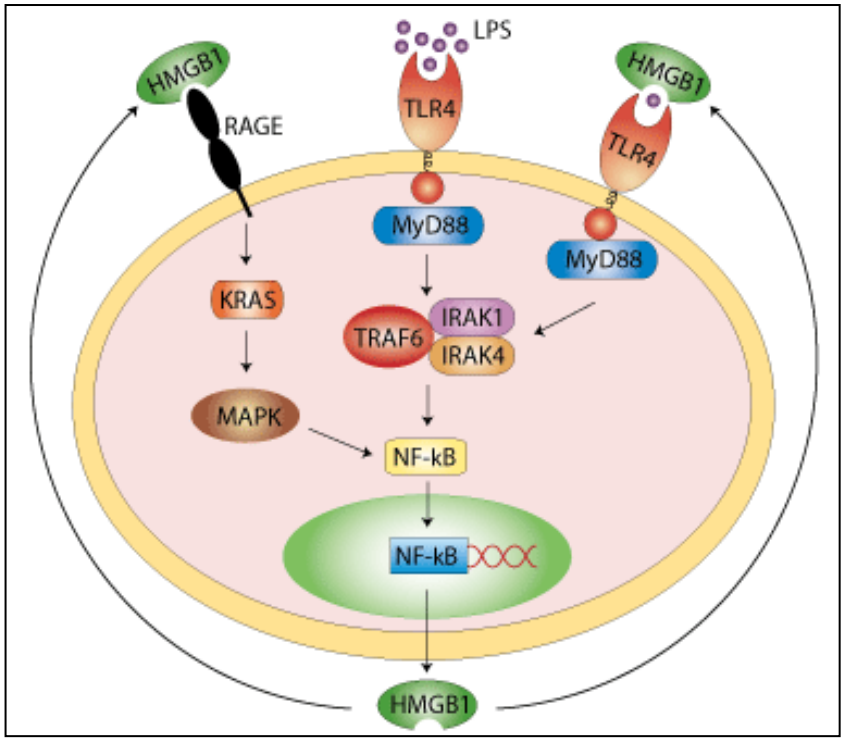


Fig. 11 – HMGB1 and signal transduction pathways [10]

1.5 Cosmetics tests and Europe

In addition to be able to determine whether or not a chemical is a sensitiser (labelling), it is also equally important to determine the potency of a sensitiser (classification) in order to identify a maximum safe concentration for human exposure (risk assessment).

The assessment of the skin sensitisation potential of chemicals represents an important component of the safety assessment, relevant in the context of several EU regulations aiming at the protection of human health. Predictive testing to identify and characterize substances causing skin sensitization historically has been based on animal tests. Officially accepted animal test methods for skin sensitization potential assessment include the Buehler occluded patch test in the Guinea pig, the Guinea Pig Maximization Test by Magnusson & Kligman (GPMT) and the Mouse Local Lymph Node Assay (LLNA) with its non-radioactive modifications (LLNA-DA and the LLNA-BrdU Elisa). The OECD Test Guideline (TG) regulates all of these *in vivo* methods: TG406 for the GPMT and Buehler test; TG429 for the LLNA; TG442A and 442B for the LLNA-DA and the LLNA-BrdU Elisa respectively. Whereas the mouse LLNA measures the response provoked during the induction of sensitization, the two Guinea pig test measure challenge induced elicitation reactions in previously sensitised animals. The LLNA is considered a reduction and refinement method compared to the traditional Guinea pig tests.

There has been a continuous effort to find alternative approaches, which avoid testing on animals wherever possible. Whenever replacement is not possible, the development of

methods that use fewer animals or cause least harm to the animals is supported. The 'Three Rs Principle' (replacement, reduction and refinement of animal use) is present in all relevant EU legislations. In early 2003, the 7th amendment to the European Union's Cosmetics Directive (76/768/EEC) was adopted, and according to it an immediate end to animal testing in the EU for cosmetic products and a complete ban of animal testing for ingredients by 11 March 2009. The animal testing ban was reinforced by a marketing ban on all cosmetic ingredients or products tested for the purposes of the Directive outside the EU after the same date. Since 11 July 2013 the new EU Regulation 1223/2009 (**Cosmetics Regulation**) is in force, which prohibits the marketing of cosmetic products, the final formulation, ingredients or combinations of ingredients, which have been tested on animals, and also prohibit each test currently, carried out using animals.

Starting from the *in vivo* observation that in mouse IL-1α expression by KC was selectively increased after *in vivo* application of contact sensitisers but not tolerogen or irritant (Enk and Katz, 1992), similar results were reproduced *in vitro* using the murine KC cell line HEL30 (Corsini et al., 1998). Same results were also obtained by Van Och et al (2005) and, furthermore, the authors observed that the ranking of potency was similar to the ranking established using the local lymph node assay. Similarly, using human KC it has been demonstrated that allergens but not irritants induced IL-12 (Muller et al., 1994; Corsini et al., 1999). Trinitrobenzene sulphonic acid induced the expression of CD40 on KC, whereas SLS did not (Coutant et al., 1999). All together these studies indicate the possibility to identify contact sensitiser using murine or human KC.

In the screening of new chemicals for human use, it should be very important both from safety and economic point of view to have biological markers to discriminate allergy and

irritation events that have different impact on human health (Corsini and Roggen, 2009; dos Santos et al., 2009; Galbiati et al., 2010).

On the other side, the EU Regulation on chemicals and their safe use – **REACH** (Registration, Evaluation, Authorization and Restriction of Chemicals substances) – which came into force in 2007, gives greater responsibility to industry to manage the risks from chemicals and to provide safety information on the substances to allow safe handling. The REACH Regulation also calls for the progressive substitution of the most dangerous chemicals where possible. Beside the ethical aspects, economic considerations also call for the timely development and validation of alternative approaches with the use of cells or tissues derived where possible from humans that are cultured under controlled conditions in the laboratory (*in vitro* methods). These can be used together with computational (*in silico*) approaches in an integrated approach to reduce the *in vivo* animal tests.

With respect to the methods which have been validated at EURL ECVAM, three of the essential steps to the induction of skin sensitization are represented: the direct peptide reactivity assay (DPRA) for covalent binding to protein; the Keratinosens™ assay for keratinocyte inflammatory responses; and the human THP-1 cell line activation test (h-CLAT) for the activation of dendritic cells. Concerning chemical reactivity, in the DPRA, generally, non-allergens and weak allergens demonstrated minimal to low peptide reactivity, whereas moderate to extremely potent allergens displayed moderate to high peptide reactivity. Classifying minimal to low reactivity as non-sensitisers and moderate to high reactivity as sensitisers, a prediction accuracy of 89% was reported. The KeratinoSens ™ assay is based

on a luciferase reporter gene under the control of an anti-oxidant response element of the human AKR1C2 gene stably inserted into HaCaT keratinocytes, while the h-CLAT address CD86 and CD54 upregulation in THP-1 cells.

As emerged from the "January 2014 Status Report for ICCR" for allergic contact dermatitis/skin sensitization validation studies for four *in vitro* test methods have been finalized. The ESAC peer review of the DPRA and the KeratinoSens™ methods were finalized and a EURL ECVAM Recommendation on the DPRA and KeratinoSens ™ was published in 2013 and in February 2014, respectively. The ESAC peer review of h-CLAT is on going.

A number of assays for the *in vitro* identification of contact and respiratory sensitization have been developed within the integrated European Framework Program 6 Project SENS-IT-IV .

The aim of the SENS-IT-IV EU Frame Programme 6 funded integrated Project (LSHB-CT-2005-018681) was to develop and optimize an integrated testing strategy consisting of *in vitro* human cell based assays, which will closely mimic sensitization mechanisms *in vivo*. These assays should provide an alternative approach to the LLNA. *In vitro* assays for risk assessment of potentially sensitizing substances can, once formally validated and implemented into European regulations, replace the LLNA (Kimber et al., 1990, 1991). In principle, a test system comprised of KC alone may not be useful in establishing allergenic potency as these cells lack antigen-presenting capacity. However, in addition to chemical processing LC activation requires the binding of cytokines produced by KC as a result of initial chemical exposure. The irritant capacity of allergens might present an additional risk factor so that irritant allergens may be stronger allergens than non-irritant ones (Grabbe et

al., 1996). In that case, the potency of chemicals to induce cutaneous sensitization may be assessed as a function of KC cytokine expression.

Given the complexity of the biological mechanisms underlying the acquisition of skin sensitization it is proposed that, in the near future, no single non-animal test method will be able to replace the currently used regulatory animal tests, instead a combination of methods addressing key mechanisms of the sensitization adverse outcome pathway (AOP) will be needed to achieve full replacement.

It is already recognised that information produced from non-animal assays can be used in regulatory decision-making, notably in term of classifying a substance as a skin sensitiser. The evolution into a full replacement for hazard identification, where the decision is not to classify, requires the generation of confidence in the *in vitro* alternative, the existence of peer reviewed publications and the knowledge that the assays are founded on key elements of the AOP for skin sensitization. At this moment it is highly unlikely that a single *in vitro* alternative method would completely replace the current animal tests. A combination of information from *in vitro* methods are likely to be necessary for confident replacement of *in vivo* assays for the identification of the presence or absence of skin sensitization hazard. Furthermore, it was noted that even the output from an Integrated Testing Strategies (ITS) would best be placed within a weight of evidence argument concerning classification.

Promising progress has been made in advancing alternative methods to animal testing over the last years, but full replacement is not yet possible. The European Commission

nevertheless believes that the most appropriate way forward is to let the marketing ban enter into force, in particular by ensuring a coherent implementation of the marketing ban and monitoring its impacts; by continuing the support for research, development and validation of new alternative methods for human safety testing; and by making alternative methods an integral part of the Union's trade agenda and international cooperation.

In conclusion, the most positive view of timing for the full replacement of animal methods is another 7–9 years (2017–2019), but alternative methods able to discriminate between sensitisers and non-sensitisers are already available. Therefore, it is clear that at the moment the more realistic way forward to fully replace animal testing seems to have as more as possible validated alternative methods (*in vitro, in silico*) covering the different biological key events of skin sensitisation, and use them in an integrated testing strategy.

Aims of the thesis

The aims of this thesis were:

1. To develop an *in vitro* test based on the use of keratinocytes and IL-18 production to identify and discriminate contact allergens from irritants and respiratory allergens;
2. To develop an in vitro test to discriminate photoallergens from phototoxic compounds;
3. To develop a single *in vitro* test for identification and ranking potency of skin sensitizing chemicals, including chemicals with low water solubility and stability in water;
4. To characterize the molecular mechanisms underlying allergen-induced IL-18 production in keratinocytes, to identify the cellular sources of reactive oxygen species and the danger signals involved.

Chapter 2

MATERIALS AND METHODS

2.1 CHEMICALS

As ***respiratory allergens*** the following chemicals were used diphenylmethane diisocyanate (MDI); trimellitic anhydride (TMA); ammonium hexachloroplatinate (HClPt). As ***skin sensitisers*** 2,4-dinitrochlorobenzene (DNCB), 4-bromobenzylbromide, 2-mercaptobenzothiazole, cinnamaldehyde, cinnamyl alcohol, eugenol, isoeugenol, resorcinol, tetramethylthiuram disulfite (TMTD), ρ-phenylenediamine (PPD), oxazolone, formaldehyde, phenyl acetaldehyde, α-hexylcinnamaldehyde, benzocaine, citral, cobalt (II) chloride, 4-nitro benzylbromide, glyoxal; as ***irritants*** sodium lauryl sulphate (SLS), salicylic acid, lactic acid, octanoic acid, methyl salicylate, tween 20, chlorobenzene, hydroxybenzoic acid, and phenol were used. Glycerol was used as negative compound.

As ***photoallergens*** 2-ethylhexyl-4-methoxicinnamate, 6-methylcoumarin, avobenzone, benzophenone, ketoprofen were used; as ***photoallergens/photoirritants*** 4-aminobenzoic acid (PABA), chlorpromazine and promethazine were used; as ***photoirritants*** 8-methoxypsoralen, acridine, ibuprofen, retinoic acid were used.

The choice of chemicals was dictated by the SENS-IT-IV program (Newsletter number 02–2007, January, 26. http://www.sens-it-iv.eu/files/newsletter/Sens-it-iv_Newsletter_08.html). Chemicals have been selected by relevant and representative of the 'universe' irritants, respiratory and contact allergens. All reagents were purchased from

Sigma (St Louis, MO, USA) at the highest purity available. Anti-TLR4 antibody, allopurinol, rotenone, and glycirrizic acid were from Sigma Aldrich, whereas Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone) and DPI were obtained from Tocris (St Louis, MO). MDI, TMA, DNCB, cinnamaldehyde, cinnamic alcohol, resorcinol, TMTD, PPD, salicylic acid, lactic acid and phenol were dissolved in DMSO (final concentration of DMSO in culture medium 0.2%), while HClPt and SLS were dissolved in PBS. Cell culture media and all supplements were from Sigma.

Maximum solubility was determined for each training chemical separately using PBS and DMSO as vehicles for the NCTC2544 IL-18 assay and 1% DMSO and acetone:olive oil (AOO, 4:1) as vehicles for the EE-potency assay. The vehicle displaying the highest solubility upon visual inspection was selected as the vehicle to be used for that particular chemical. When the solubility in PBS was equal to the solubility in DMSO, PBS was used as a vehicle for the NCTC2544 IL-18 assay. For the EE-potency assay, 1% DMSO was used when solubility was equal to AOO. Maximum solubility tested was 500 mg/ml. The highest concentration tested in the NCTC2544 IL-18 assay was 1 mg/ml and 200 mg/ml in the EE potency assay.

Table 3 – Tested chemicals and vehicles

Chemical	Category	Vehicle NCTC	Vehicle epiCS
2-mercaptobenzothiazole	Contact allergen	DMSO	AOO
4-bromo benzylbromide	Contact allergen	DMSO	
4-nitro benzylbromide	Contact allergen	DMSO	
Benzocaine	Contact allergen	DMSO	AOO
Cinnamaldehyde	Contact allergen	DMSO	AOO
Cinnamic alcohol	Contact allergen	DMSO	
Citral	Contact allergen	DMSO	AOO
Cobalt (II) chloride	Contact allergen	DMSO	DMSO
DNCB	Contact allergen	DMSO	AOO
Eugenol	Contact allergen	DMSO	AOO
Eugenol	Contact allergen	DMSO	
Formaldehyde	Contact allergen	DMSO	DMSO
Glyoxal	Contact allergen	PBS	
Glyoxal	Contact allergen	DMSO	
Isoeugenol	Contact allergen	DMSO	AOO
Isoeugenol	Contact allergen	DMSO	
Oxazolone	Contact allergen	DMSO	AOO
Phenyl acetaldehyde	Contact allergen	DMSO	AOO
PPD	Contact allergen	DMSO	AOO
Resorcinol	Contact allergen	DMSO	
TMTD	Contact allergen	DMSO	
α-hexylcinnamaldehyde	Contact allergen	DMSO	AOO
2-ethylhexyl-4-methoxicinnamate	Photoallergens		
6-methylcoumarin	Photoallergens	DMSO	
Avobenzone	Photoallergens	DMSO	
Benzophenone	Photoallergens	DMSO	
Ketoprofen	Photoallergens	DMSO	
4-aminobenzoic acid	Photoallergens/Photoirritants	DMSO	
Chlorpromazine	Photoallergens/Photoirritants	DMSO	
Promethazine	Photoallergens/Photoirritants	DMSO	
HClPt	Respiratory allergens	PBS	
MDI	Respiratory allergens	PBS	
TMA	Respiratory allergens	PBS	
Chlorobenzene	Irritants		AOO
Hydroxybenzoic acid	Irritants		AOO
Lactic acid	Irritants	PBS	AOO
Methyl salicylate	Irritants		AOO
Octanoic acid	Irritants	DMSO	AOO
Phenol	Irritants	DMSO	DMSO
Salicylic acid	Irritants	PBS	AOO
SDS	Irritants	PBS	DMSO
Tween 20	Irritants		AOO
8-methoxipsoralen	Photoirritants	DMSO	
Acridine	Photoirritants	DMSO	
Ibuprofen	Photoirritants	DMSO	
Retinoic acid	Photoirritants	DMSO	
Glycerol	Non-irritant		

2.2 CELL CULTURE

2.2.1 Human keratinocyte NCTC2544 cells

This test is used to evaluate the capacity of a compound to induce skin sensitization in vivo. The NCTC2544 IL-18 assay is based on the dose-dependent specific increase of IL-18 production by these cells upon exposure to skin-sensitizing chemicals. The production of IL-18 by the exposed cells is determined in cell lysates by sandwich ELISA, and is corrected for total protein content. IL-18 production after exposure to a potential sensitiser is compared to a positive control (PPD 60 µg/ml) and to the vehicle control. The performance of the test is good when the relative IL-18 production of the positive control is at least 1.5 times higher than that of the vehicle control.

NCTC2544 cells (obtained from Institute Zooprofilattico di Brescia, Brescia, Italy) were cultured in 96- and 24-well plates at a cell density of 1.5–2.5 × 10^5 cells/ml, to a confluency of 80%. Cells were cultured in 0.1 or 0.5 ml, respectively of RPMI-1640 containing 2 mM l-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, supplemented with 10% heat-inactivated fetal calf serum (culture medium) and cultured at 37 °C in 5% CO_2.

Primary human KC were obtained from Invitrogen (Carlsbad, CA, USA). Cells were seeded at 2.5 × 10^4/ml in 24-well plate. Cells were grown in serum free supplemented keratinocyte growth medium (Sigma) to 80–90% density prior to treatment. Cells were grown at 37 °C in 5% CO_2. Second and third passages post-cryopreserved seedings were used for experiments.

To study the impact of cell density on the selective production of IL-18 by contact allergens, NTCT 2544 cells were seeded in 24-well plate at different density (1–10 × 10^5 cells/ml). After overnight adherence, cells were treated for 24 h with PPD (60 µg/ml) or DMSO as vehicle

control or after 2 h of adherence, cells were treated for 24 h with PPD (60 µg/ml), cinnamaldehyde (30 µg/ml), salicylic acid (500 µg/ml) or DMSO as vehicle control.

To develop a high throughput assay, NCTC 2544 were seeded at the cell density of 1.5–2.5 × 10^5 cells/ml in 96-well plate and treated after overnight adherence for 24 h with increasing concentration of contact sensitisers (DNCB, PPD, isoeugenol, cinnamaldehyde, TMTD, resorcinol, cinnamic alcohol and eugenol), respiratory allergens (hexachloroplatinate, MDI and TMA) and irritants (phenol, SDS, lactic acid and salicylic acid).

For experiments using HPK II kindly provided by Prof Fusening (Heidelberg University, Germany), and HaCaT cells lines (ATCC, LCG Standards, Sesto San Giovanni, Italy), cells were cultured in 24-well plate at a cell density of 1.5 × 10^5 cells/ml. NCTC 2544, HPK II and HaCaT cell lines were treated for 24 h with PPD (60 µg/ml) or DMSO as vehicle control.

To investigate the signal transduction pathway involved in PPD-induced IL-18 production, NCTC 2544 were treated with several inhibitors, namely, the selective inhibitor of protein kinase C α and β1 isoforms GF109203X (5 µM), the inhibitor of NF-κB and anti-oxidant pyrrolidine dithiocarbamate (PDTC 100 µM), the irreversible inhibitor of I-κB Bay 11-70-85 (1 µM), and the inhibitor of p38 MAPK SB203580 (100 nM), for 1 h, and then PPD (60 µg/ml) or DMSO as vehicle control were added for 24 h. p38 MAPK and NF-κB activation were assessed by Western blot analysis as described below.

NCTC2544 cells were treated with several inhibitors, namely Z-VAD-FMK (10 µM), anti-TLR4 antibody (1 µg/well), glycirrizic acid (100 µM), DPI (0.5 µM), rotenone (20 µM), and allopurinol (10 µM) for different durations (30 minutes for anti-TLR4 antibody and 1 hour for all the other inhibitors), and then PPD (30 µg/ml), DNCB (2 µg/ml) and citral (30 µg/ml) or

DMSO as vehicle control were added for 24 hours.

The chemical concentration that resulted in a CV80 was the start concentration during the IL-18 experiments. When no CV80 could be determined, the maximum solubility is used as the starting concentration.

2.2.2 Human epidermal equivalent

epiCS® previously known as EST1000 ™ (Epidermal Skin Test-1000) (CellSystems, Biotechnology GmbH, Troisdorf, Germany); SkinEthic ™ RHE (Reconstructed Human Epidermis) (SkinEthic Laboratories, Lyon, France); EpiDerm™ (MatTek Corp., Ashland, USA and Bratislava, Slovakia) and VU University in house EE (VUMC-EE) were used in this study. EE were maintained according to the suppliers' instructions at 37 °C, 5% CO2 and 95% relative humidity. After 24 h (1–24 h for EpiDerm™) of equilibration time, EE were exposed to chemicals as described below and as described in detail previously (dos Santos et al., 2011 and Teunis et al., 2013). All reconstructed epidermal models were derived from normal human keratinocytes. For VUMC-EE, human foreskin was obtained from healthy donors with informed consent. The VU University Medical Centre approved the experiments described in this paper. The study was conducted according to Declaration of Helsinki Principles.

A total of 17 contact sensitisers and 10 non-sensitisers were studied (Table 1). At DiSFeB and VUMC, the 13 sensitisers, marked with an asterisk in Table 1, were tested coded in order to further challenge the EE potency assay using the epiCS® model.

At DiSFeB and VUMC where epiCS® was used (and VUMC-EE for limited experiments): (1) EE were allowed to equilibrate for 24 h at 37 °C, 5% CO2 and then; (2) chemicals were dissolved in either acetone olive oil (AOO 4:1) or 1% DMSO in culture medium. At VUMC and DiSFeB the vehicles and chemicals were purchased from the same supplier: acetone (Cat. No. 20066.321) from VWR (Radnor, Pennsylvania (VS); olive oil (cat no. 01514) and DMSO (Cat. No. 154938) from Sigma-Aldrich (St. Louis, MO, USA). With the exception of 2-mercaptobenzothiazol which was purchased from Fisher-Scientific (ACROS Organics; Loughborough, UK), all chemicals were purchased from Sigma-Aldrich (Sigma, Aldrich, SAFC; St Louis, Missouri, USA). Chemicals were > 95% pure with the exception of formaldehyde which was 36.5%–38% in H2O. Isoeugenol was a 98% mixture of the cis and trans form and oxazolone was purified by recrystallization. For experiments, Finn Chamber filter paper discs 7.5 (for epiCS®) or 11 mm (for VUMC-EE) (Epitest LTD Oy, Finland) were impregnated with chemicals or vehicle controls and applied topically to the EE stratum corneum; (3) cultures were incubated with chemicals for 24 h; (4) after chemical exposure, filter paper discs were removed and; (5) metabolic activity was determined immediately by the MTT assay and culture supernatants were harvested and stored at − 20 °C for IL-18 analysis by ELISA. Chemical exposure and dose finding was performed as described in detail previously (dos Santos et al., 2011 and Teunis et al., 2013).

In parallel, at MatTek and MB Research Labs where EpiDerm ™ was used, a protocol was developed in which 12 chemicals were tested: (1) Epiderm tissues were allowed to equilibrate for 1–24 h at 37 °C, 5% CO2 and then; (2) test chemicals were directly pipetted to the EE surface, dissolved in either ethanol (25 µl) for lipid soluble compounds or distilled

water (100 µl) for water-soluble compounds (Deng et al., 2011). The only exception was ethanol: DMSO 4:1 (25 µl) which was used as vehicle for 10% MBT exposure of EpiDerm™ (Table 1). For MB Research Labs and MatTek Corp., absolute ethanol (200 proof), sterile tissue culture water (ultrapure) and all other chemicals were at least 98% pure ACS grade, and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sterile DMEM-based tissue culture medium was supplied by MatTek (Ashland, MA, USA); (3) cultures were incubated with chemicals for 24 h; (4) after chemical exposure, the epidermal surface was washed with PBS to remove chemicals and; (5) metabolic activity was determined immediately by the MTT assay and culture supernatants were harvested and stored at − 20 °C for IL-18 analysis by ELISA.

In addition, a comparison of the effects of different vehicles (AOO, ethanol, water) on 0.15% DNCB, 0.3% citral and 1.0% eugenol induced IL-18 release and cell viability was conducted. This experiment was performed at MB Research in SkinEthic™ RHE EE model, n = 2 replicates from a single experiment.

2.3 CELL VIABILITY

The MTT assay measures mitochondrial activity, which is representative for cell viability, by quantifying dehydrogenase activity in the mitochondria (Gerlier and Thomasset, 1986). To make sure no, or very little, cytotoxic effects occur during the NCTC2544 IL-18 experiments, cytotoxicity was assessed by MTT assay. Hundred microliter of cells ($1.5–2.5 \times 10^5$/ml) were seeded in a 96-well plate, up to a confluency of 80%, and exposed to decreasing concentrations (using CV80 as highest concentrations) of test chemicals, vehicle control

(0.2% DMSO or culture medium) and positive control (PPD, 60 µg/ml in 0.2% DMSO).

After an incubation period of 24 h, medium was aspirated and 100 µl/well of MTT solution (0.75 mg/ml in culture medium) was added. Cells were incubated for 3 h at 37 °C, medium was aspirated and cells were lysed in 100 µl/well of a mixture of HCl 1N:isopropanol (1:24). The absorbance of the resulting solutions was read at a wavelength of 595 nm in a micro-plate reader (molecular devices or equivalent). Cell viability was calculated for each chemical by linear regression analysis. Concentrations resulting in a cell viability lower than 80% were discarded from further analysis. The CV_{80} was the highest concentration tested for IL18 production.

Five decreasing concentrations (2 step dilutions), starting with the EC_{60} concentration, of the training chemicals, unexposed, vehicle control (1% DMSO or AOO) and the positive control were tested and 2 independent experiments were performed by each laboratory.

After a 24 h exposure, test chemicals or filter paper discs impregnated with chemicals were removed from EE or the epidermal surface and then washed with PBS to remove chemicals (the MatTek protocol). The MTT analysis was performed in 24-well plates. Thiazolyl blue tetrazolium bromide (MTT) (Sigma) solution in PBS (0.5 mL of a 5 mg/mL solution) was added to the 24-well plate, EE cultures were placed on top and further incubated for 2–3 h under standard culture conditions. Cultures were then transferred to a new 24 well plate containing 0.3–0.9 mL isopropanol to dissolve the formazine crystals. Plates were incubated overnight, covered with parafilm and protected from the light at room temperature. The absorbance of the formazan dissolved in isopropanol was measured at either 540 or 570 nm and expressed as a percentage relative to the absorbance value of control vehicle.

2.4 CYTOKINE PRODUCTION

For IL-18 production, cells were incubated (24 well plates) for 24 h with four decreasing concentrations of the training chemicals (starting with the CV80 concentration), vehicle control (0.2% DMSO or culture medium) and the positive control PPD. Each condition (chemical concentration, positive control or vehicle) was tested in quadruplicate per independent experiment. Each lab performed three independent experiments.

After incubation with chemicals, culture medium was aspirated, monolayers gently washed once with 1 ml PBS and cells were lysed in 0.25 ml 0.5% Triton X-100 in PBS. Plates were stored at −80 °C until measurement. Intracellular IL-18 content was assessed by specific sandwich ELISA commercially available (MBL, Nagoya, Japan). IL-18 coating antibody was used at a working concentration of 1 µg/ml; IL-18 biotinylated antibody was used at a working concentration of 250 pg/ml; IL-18 standard was used at a starting concentration of 1000 pg/ml. All ELISA components were dissolved in PBS, containing 1% BSA. Results are expressed in pg/mg of total intracellular protein content. The protein content of the cell lysates was determined by the BCA method (for details see supplement B: 'NCTC2544 IL-18 SOP'). All IL-18 samples were analysed using dilutions in PBS supplemented with 1% BSA. The OD of the samples was only accepted when the OD was well within the linear range of the standard curve.

Stimulation index (SI) was calculated as follow:

$$\mathrm{SI} = \frac{\text{IL-18 in chemical-treated cells}}{\text{IL-18 in vehicle-treated cells}}$$

The following preliminary prediction model was used: if the fold increase in intracellular IL-18 is over 1.2 compared to vehicle the chemical is classified as contact sensitiser (labeled as R43). The positive and negative controls are included in each plate. The positive control meets the acceptance criteria if the fold increase in intracellular IL-18 is over 1.5 compared to vehicle treated cells. For a given chemical, the same classification must be obtained in at least two out of three independent experiments. The 1.2 fold increase should be at least at one of the concentrations tested.

In the 96-well plate format, total IL-18 production was assessed (both intracellular and released). In this case after incubation, 20 µl of 3% Triton X-100 (0.5% final concentration) in PBS was added to each well. Plates were then frozen at −80 °C, and 100 µl directly transferred from the 96-well plate to the ELISA plate to asses IL-18 content. In this case, results are expressed as pg/ml.

For IL-1α production, EE cultures were incubated for 24 h with 5 decreasing (2 step dilutions) concentrations of the training chemicals (starting with the EC_{60} concentration), unexposed, vehicle control (1% DMSO or AOO) and the positive control resorcinol. Two independent experiments were performed by each lab.

The amount of IL-1alpha present in culture supernatants was determined by ELISA (R&D Systems). IL-1alpha coating antibody was used at a working concentration of 2 µg/ml; IL-1alpha biotinylated antibody was used at a working concentration of 12.5 ng/ml; IL-1alpha standard was used at a starting concentration of 500 pg/ml. All were dissolved in 0.5% BSA/0.005% Tween. Results were expressed in pg/ml and the concentration resulting in IL-$1\alpha_{2\times}$ value can be measured by correlating to the vehicle control.

2.5 WESTERN BLOTTING

For Western blot analysis of p38 MAPK activation and I-κB degradation, as measure of NF-κB activation, NCTC 2544 cells were seeded in 6-well plate at a cell density of 2.5×10^5 cells/ml (2 ml/well). After 24 h of adherence, cells were treated with PPD (60 μg/ml) or DMSO as vehicle control for different times (5–60 min). After incubation, culture medium was discarded, 1 ml of cold PBS was added, cells were collected by scraping, transferred in Eppendorf tubes and centrifuge for 5 min at 1200 rpm at 5 °C. The supernatant was discarded and cells lysed in 100 μl of homogenization buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA pH 7.5, 0.5% Triton X-100, 50 μM PMSF, 2 μg/mL aprotinin, 1 μg/mL pepstatin and 1 μg/mL leupeptin) and denatured for 10 min at 100 °C. The protein content of the cell lysate was measured using a commercial kit (Bio-Rad, Segrate, Italy). For the Western blot analysis, 15 μg of extracted proteins were electrophoresed into a 15% SDS–polyacrylamide gel under reducing conditions. The proteins were then transferred to PVDF membrane (Amersham, Little Chalfont, UK). The different proteins were visualized using activated p38 (1:1000), non-activated p38 (1:500) I-κB (1:1000) as primary antibodies (Sigma), and developed using enhanced chemioluminescence (Sigma). The image of the blot was acquired with the Molecular Imager Gel Doc XR (Bio-Rad). The optical density of the bands was calculated and analyzed by means of the Image 1.47 program for digital image processing (Wayne Rasband, Research Service Branch, NIMH, NIH, Bethesda, MD, USA).

2.6 PERFORMANCE CRITERIA – EE POTENCY ASSAY

For the positive control: Exposure to 60 mg/ml (475 mM) resorcinol should result in 25–75%

(preferably 50%) decrease in cell viability compared to vehicle control (1% DMSO or 4:1 AOO). If the value obtained is outside this range the EE batch did not fulfill the Quality Criteria required for the assay and the experiment is considered as invalid. For the negative control: Vehicle exposure should not result in more than 30% decrease in cell viability compared to unexposed cultures. If the vehicle value obtained is higher than 30% the EE batch did not fulfill the Quality Criteria required for the assay. If a chemical then fails to result in >60% cytotoxicity, this chemical is excluded from the assay. Chemical concentrations found to result in an EC_{50} or IL-1$\alpha_{2\times}$ can be used to rank according to potency.

2.7 DATA ANALYSIS

For NCTC2544 IL-18 assays, all experiments were repeated at least three times, with representative results shown. Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using InStat software version 3.0a (GraphPad Software, La Jolla, CA, USA). Statistical differences were determined using ANOVA followed by a multiple comparison test (Dunnett's multiple comparison test or Tukey's t-test) as indicated in the legends. Effects were designated significant if $p < 0.05$.

For EE, experiments were performed in duplicate in two of the laboratories (VUMC, DiSFeB) and at least once in each lab by MB Research Labs and MatTek Corp. Different EE batches were used in each experiment. Data are presented as an average of the 2 independent experiments ± difference (VUMC, DiSFeB), or as average ± difference of duplicate tissues within a single experiment (EpiDerm™).

Sensitiser labelling by means of assessing IL-18 release: different prediction models were developed for the epiCS® and EpiDerm™ models. Based on data obtained testing sensitisers and non-sensitisers, for the epiCS® model a cut-off of 5-fold increase of IL-18 release above vehicle control and ≤ 40% cell viability was found to be the best for the discrimination of contact allergens, while for EpiDerm™ a cut-off of 1.6-fold increase of IL-18 above vehicle control and between 5 and 50% cell viability (Prediction Model 1.6 ×) was found to be optimal to identify allergens.

EE potency assay: the EE-EC50 value is the effective chemical concentration required to reduce metabolic activity (corresponding to cell viability) to 50% of the maximum value. The 100% value for cell viability corresponds to the vehicle control (1% DMSO culture medium or acetone/olive oil 4: 1). EE-EC50 values were obtained by linear regression analysis based on changes in metabolic activity (MTT). Statistical significances were determined by Kruskal–Wallis (nonparametric, one way ANOVA) using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant for $p < 0.05$.

IL-18 potency assessment: IL-18 SI-2 and SI-5 were obtained by linear regression analysis based on the chemical concentration resulting in a 2 fold or 5 fold release in IL-18. Statistical significances were determined by Kruskal–Wallis (nonparametric, one way anova) using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant for $p < 0.05$.

Correlations were determined by nonparametric two-tailed correlation Spearman Analyses using GraphPad Software, San Diego, CA, USA.

Chapter 3

RESULTS

3.1 USE OF IL-18 PRODUCTION IN A HUMAN KERATINOCYTE CELL LINE TO DISCRIMINATE CONTACT SENSITISERS FROM IRRITANTS AND LOW MOLECULAR WEIGHT RESPIRATORY ALLERGENS

Abstract

Assessment of allergenic potential of chemicals is performed using animal models, such as the murine local lymph node assay, which does not distinguish between respiratory and contact allergens. Progress in understanding the mechanisms of skin sensitization, provides us with the opportunity to develop in vitro tests as an alternative to in vivo sensitization testing.

The aim of the present study was to evaluate the possibility to use intracellular interleukin-18 (IL-18) production to assess in vitro the contact sensitization potential of low molecular weight chemicals. The human keratinocyte cell line NCTC2455 was used. Cells were exposed to contact allergens (cinnamaldehyde, dinitrochlorobenzene, glyoxal, isoeugenol, p-phenylediamine, resorcinol, tetramethylthiuram disulfide, 2-mercaptobenzothiazole, 4-nitrobenzylbromide), to proaptens (cinnamyl alcohol, eugenol), to respiratory allergens (diphenylmethane diisocyanate, trimellitic anhydride, ammonium hexachloroplatinate) and to irritants (sodium lauryl sulphate, salicylic acid, phenol). Cell associated IL-18 were

evaluated 24 later. At not cytotoxic concentrations (cell viability higher of 75%, as assessed by MTT reduction assay), all contact sensitisers, including proaptens, induced a dose-related increase in IL-18, whereas both irritants and respiratory failed. Similar results were also obtained using primary human keratinocytes. Results were reproducible, and the method could be transferred to another laboratory, suggesting the potential use of the test in immunotoxicity testing strategies. Overall, results obtained indicated that cell-associated IL-18 may provide an in vitro tool for identification and discrimination of contact versus respiratory allergens and/or irritants.

Determination of 75% cell viability

The concentration of chemical resulting in 75% of viability or CV_{75} respect to vehicle treated cells 24 h after treatment was calculated for all chemicals. About 500–1000 μg/ml were the highest concentration tested. Results are presented in Table 1. For the majority of chemicals CV_{75} represented the highest concentration tested. In Table 1 is also reported the category and classification according to the LLNA EC3 values (Basketter et al., 1999 and Gerberick et al., 2005). Both weak and extreme sensitisers were used.

Table 1 - Confluent cells were treated for 24 h with different chemicals or DMSO (0.2% final concentration) as vehicle control. Results are expressed as the concentration of chemical resulting in 75% cell viability, as assessed by MTT reduction. CV_{75} was calculated by linear regression analysis of data.

In the last column, the LLNA EC3 values expressed as% and its relative potency classification in parenthesis are reported. According to Basketter et al. (1999), potency classification is based on the mathematical estimation of the concentration of chemical necessary to obtain a threshold positive response (SI = 3); this is termed the EC3 value. Chemicals with an EC3 value (%) ⩾ 10 to <100 are classified as weak, ⩾1 to <10 moderate, ⩾0.1 to <1 strong, <0.1 extreme.

Table 1. Concentration of the chemicals that induced 75% viability (CV_{75}) and their classification.

Chemical	CV_{75} (µg/ml)	Category	LLNA EC3 (%)
HClPt	15	Respiratory allergen	Not available
MDI	>500	Respiratory allergen	0.3 (strong)
TMA	>500	Respiratory allergen	9.2 (moderate)
Cinnamaldehyde	40	Contact allergen	2.8 (moderate)
Cinnamyl alcohol	300	Contact allergen	21 (weak)
DNCB	5	Contact allergen	0.05 (extreme)
Eugenol	225	Contact allergen	12 (weak)
Glyoxal	50	Contact allergen	1.3 (moderate)
Isoeugenol	225	Contact allergen	1.2 (moderate)
PPD	108	Contact allergen	0.22 (strong)
Resorcinol	>1000	Contact allergen	6 (moderate)
TMTD	32	Contact allergen	5.6 (moderate)
2-Mercaptobenzothiazole	125	Contact allergen	5.8 (moderate)
4-Nitrobenzylbromide	2.5	Contact allergen	0.05 (extreme)
Phenol	70	Irritant	Non sensitiser
Lactic acid	>500	Irritant	Non sensitiser
Salicylic acid	250	Irritant	Non sensitiser
SLS	30	Irritant	14.0 (weak, false positive)
Glycerol	>1000	Negative	Non sensitiser

Effect of the selected chemicals on cell-associated IL-18

Preliminary experiments were carried out to determine the best time, cell density and serum concentrations to measure cell-associated IL-18. In time course experiments, cells, at 80–90% density, were treated for different times (5, 24, 30 and 48 h) with salicylic acid (250 μg/ml), and PPD (60 μg/ml). As shown in Fig. 1 A, statistically significant increase in intracellular IL-18 was detected in cells treated with PPD already after 5 h of treatment, reaching a plateau after 24–30 h, while no changes were observed following treatment with the irritant salicylic acid. Therefore, the incubation time of 24 h was chosen for all other investigations.

The effect of cell density on the response to the contact allergen PPD was investigated seeding cells at different density (1 to 10×10^5 cells/ml in 24-well plate). After over night adherence, cells were treated for 24 h with PPD (60 μg/ml) or DMSO as vehicle control. As shown in Fig. 1 B, 1 to 2.5×10^5 cells/ml represents the optimal cell density, higher cell density ($>5 \times 10^5$ cells/ml) resulted in a loss of effect. Experiments were therefore conducted using 1.5 to 2.5×10^5 cells/ml.

To investigate the effect of serum concentrations on contact allergen-induced increase in intracellular IL-18, cells were treated with cinnamaldehyde (60 μg/ml), DNCB (0.5 μg/ml) and PPD (60 μg/ml) or DMSO as vehicle control in the presence of 1 or 10% of FCS. As shown in Table 2, similar results were obtained under the two experimental conditions, with the exemption of cinnamaldehyde, which showed a higher response if cells were cultured in 10% FCS. Therefore, all experiments were conducted using 10% FCS.

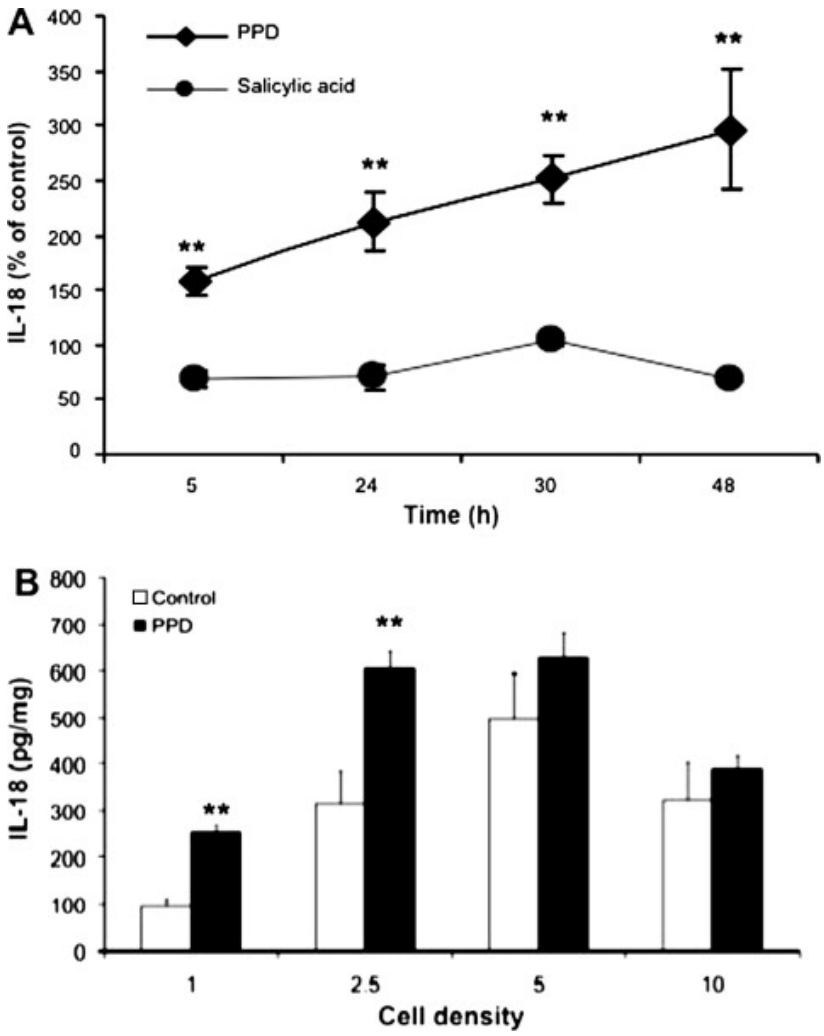


Fig. 1. Time course and effect of cell density on intracellular IL-18. Panel A, time course. NCTC 2544 cells were treated for different times (5, 24, 30, 48 h) with PPD (60 μg/ml), and salicylic acid (250 μg/ml) or vehicle control. Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{**}p < 0.01$ versus vehicle treated cells. Panel B, effect of cell densitiy: NTCT 2544 cells were seeded in 24-well plate at different density (1–10×10^5 cells/ml) and treated for 24 with PPD (60 μg/ml) or DMSO as vehicle control. Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Tukey's multiple comparison test, with $^{**}p < 0.01$ versus vehicle treated cells (control).

Treatment	1% FCS (IL-18% of control)	10% FCS (IL-18% of control)
Cinnamaldehyde 60 µg/ml	584 ± 87	839 ± 148[§]
DNCB 0.5 µg/ml	146 ± 5	176 ± 29
PPD 60 µg/ml	414 ± 56	358 ± 54

Table 2. Effect of FCS on contact allergen-induced intracellular IL-18 production. NTCT 2544 cells were treated with three different contact allergens or DMSO (0.2% final concentration) as vehicle control. Twenty-four hours later intracellular IL-18 was evaluated as described in Section 2. Results are expressed as% of control. Each value represents the mean ± SD, n = 4. Statistical analysis was performed by Tukey's multiple comparison test, with [§]$p < 0.05$ versus cells treated in 1% FCS.

Effect of contact allergens on cell-associated IL-18

NCTC 2544 cells were then treated for 24 h with increasing concentrations of the contact allergens DNCB, TMTD, cinnamaldehyde, cinnamic alcohol, eugenol, isoeugenol, resorcinol, PPD. As shown in Fig. 2, all contact allergens, including the proaptens cinnamic alcohol and eugenol, induced a dose-related increase in intracellular IL-18. Additional contact allergens tested, not reported in Fig. 2, included glyoxal, 4-nitro benzylbromide, 2-mercaptobenzothiazole. Also for these chemicals a statistically significant increase was observed. In particular, the treatment with glyoxal 25 µg/ml the intracellular level of IL-18 was 283 ± 52[*] pg/mg, with 50 µg/ml 371 ± 107[**] (control 132 ± 49); with 4-nitrobenzylbromide 1.25 µg/ml was 368 ± 15[*], with 2.5 µg/ml was 442 ± 14[**] (control 293 ± 13); with 2-mercaptobenzothiazole 62 µg/ml was 817 ± 57[**] and with 125 µg/ml was 960 ± 111[**] (control 594 ± 29).

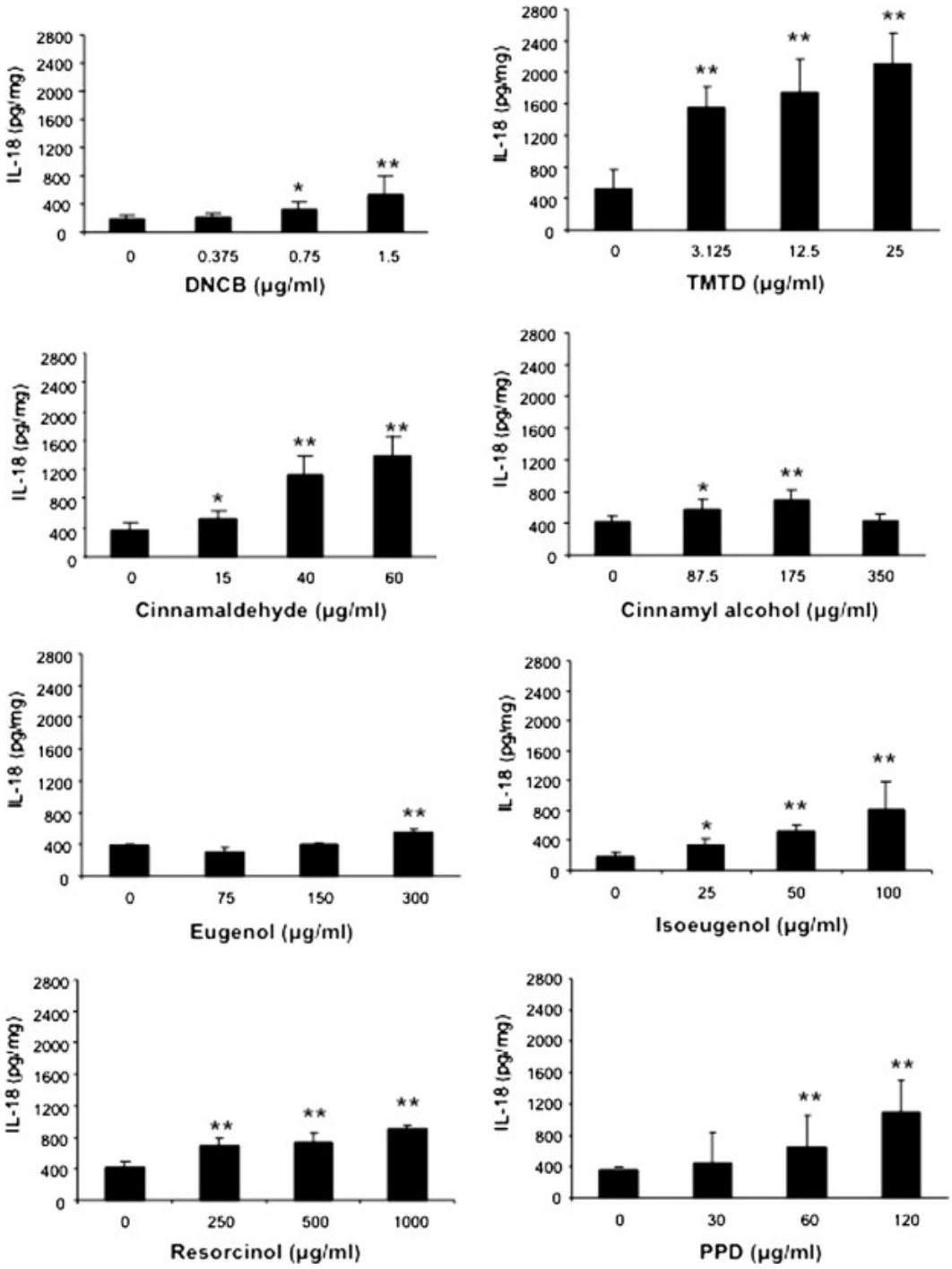


Fig. 2. Dose response effects of contact sensitisers on cell-associated IL-18. NCTC 2544 cells were treated for 24 h with increasing concentrations of DNCB, TNTD, cinnamaldehyde, cinnamyl alcohol, eugenol, isoeugenol, resorcinol and PPD. Cells incubated with vehicle were included as a control (0). Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle treated cells (0).

Effect of respiratory allergens and irritants on cell-associated IL-18

We then tested the effect of the respiratory allergens MDI, TMA, HClPt and the irritants SDS, salicylic acid, lactic acid, and phenol. None of them was able to increase intracellular IL-18 content (Fig. 3), suggesting a selective up-regulation by contact allergens only. We additionally tested glycerol, which also failed to induce IL-18.

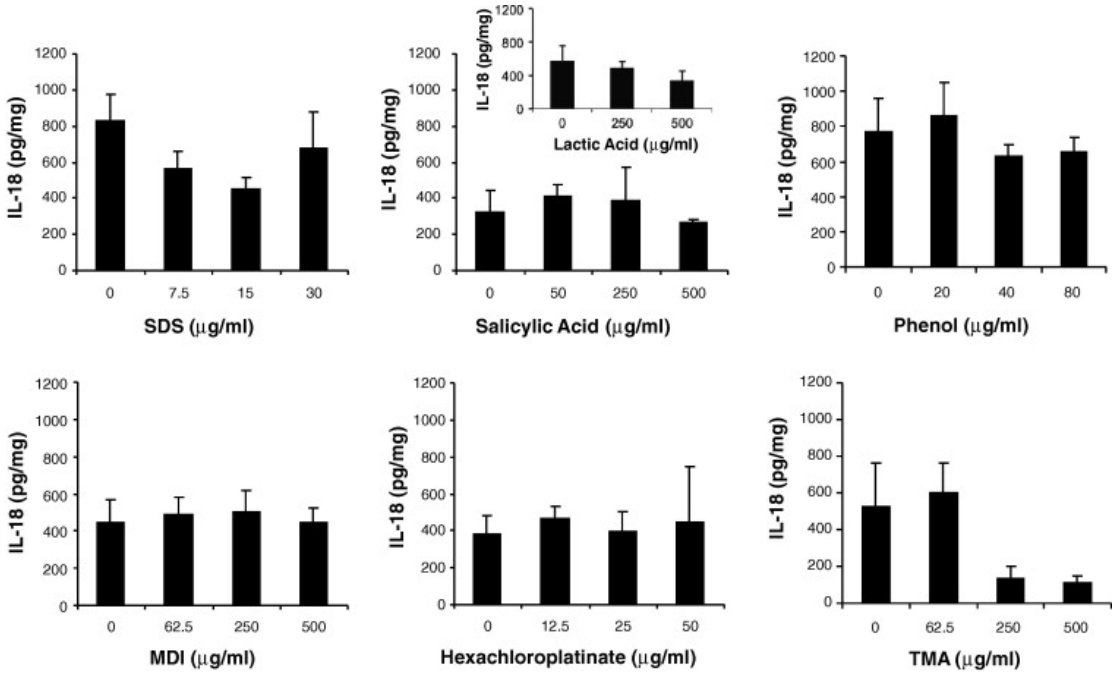


Fig. 3. Dose response effects of irritants and respiratory sensitisers on cell-associated IL-18. NCTC 2544 cells were treated for 24 h with increasing concentrations of SDS, salicylic acid, lactic acid, phenol, MDI, hexachloroplatinate, TMA. Cells incubated with vehicle were included as a control (0). Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test.

Data reproducibility

We investigated the reproducibility of data by comparing the results obtained in seven independent experiments conducted over a three months period treating NCTC 2544 cells

with PPD 60 µg/ml. As shown in Fig. 4, in all experiments PPD induced a statistically significant increase in intracellular IL-18, with a stimulation index ranging from 1.8 to 3.6.

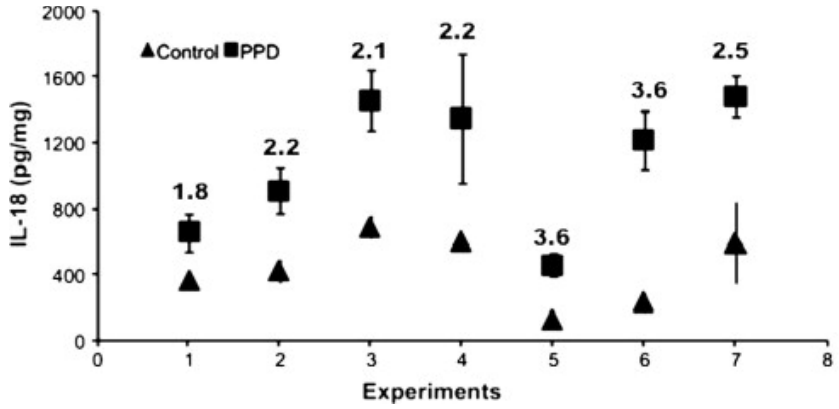


Fig. 4. Reproducibility of data. Results obtained in seven independent experiments performed over a three months period are reported. NCTC 2544 cells were treated for 24 h with PPD (60 µg/ml) or with DMSO as vehicle control. Intracellular IL-18 was evaluated as described in Section 2. Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Student's t-test, in all experiments PPD induced a statistical significant increase in cell-associated IL-18. The numbers reported in figure represent the stimulation index of the individual experiment.

Effects of selected chemicals on cell-associated IL-18 in primary human KC

We then asked the question if these results were peculiar of this cell line or if similar results could be obtained using other keratinocyte models. We could demonstrate that similar results were obtained also using primary human KC. As shown in Table 3, while cinnamaldehyde induced a dose-related increase in intracellular IL-18 content, HClPt, SDS and phenol failed, confirming the results obtained with NCTC 2544 cell line, and indicating the possibility to use other keratinocyte models.

Chemical (μg/ml)	Intracellular IL-18 (pg/mg)
Control	507 ± 73
HClPt 25	617 ± 110
HClPt 50	482 ± 224
HClPt 100	582 ± 162
Cinnamaldehyde 12.5	1455 ± 367*
Cinnamaldehyde 25	1256 ± 218*
Cinnamaldehyde 50	889 ± 76*
SDS 15	450 ± 13
SDS 30	198 ± 50
Phenol 40	527 ± 134
Phenol 80	438 ± 24

Table 3. Effects of selected chemicals on IL-18 production in primary human keratinocytes. Primary keratinocytes were treated with different concentrations of selected chemicals or DMSO (0.2% final concentration) as vehicle control. Twenty-four hours later intracellular IL-18 content was evaluated as described in Section 2. Results are expressed as pg/mg of total protein content. Each value represents the mean ± SD, n = 3. Statistical analysis was performed by Dunnett's multiple comparison test, with $^{*}p < 0.05$ versus vehicle-treated control cells.

Discussion

In the present work, intracellular IL-18 was investigated after exposure of NCTC 2544 cells to contact allergens, respiratory allergens, and irritants. We showed that exposure of NCTC 2544 cells to contact allergens results in a dose-related induction of intracellular IL-18, whereas exposure to respiratory allergens and irritants fails to induce IL-18 production, indicating the possibility to use IL-18 it to identify contact allergens.

We had focused our attention on IL-18 since this cytokine has been shown to play a key proximal role in the induction of allergic contact sensitization and to favor Th-1 type

immune response by enhancing the secretion of pro-inflammatory mediators such as TNF-α, IL-8 and IFN-γ, (Okamura et al., 1995, Cumberbatch et al., 2001 and Antonopoulos et al., 2008). IL-18 has not apparent role in irritant contact dermatitis (Antonopoulos et al., 2008), indicating that the role of IL-18 in contact hypersensitivity is not simply part of a general requirement for IL-18 in skin inflammation. Furthermore, Hartwig et al. (2008) demonstrated using IL-18-deficient mice that the absence of IL-18 does not affect any of the asthma-specific parameters, including allergen-specific IgE, histological changes of the lungs, infiltrating leukocytes, serum cytokine levels and airway hyperresponsiveness.

We therefore investigated if skin sensitisers could selectively up-regulate this cytokine in human KC. We could indeed demonstrate that contact sensitisers, including proaptens, increase in a dose-related manner intracellular IL-18. In contrast, irritants and respiratory allergens failed to up-regulate IL-18. The measure of the release of IL-18 did not offer any advantage, since a passive release of this constitutively expressed cytokine could be observed at high concentrations with both allergens and irritants, likely to be due to a cell damage effect (data not shown). Also the evaluation of IL-18 mRNA expression did not provide additional advantage. KC constitutively express high IL-18 mRNA levels, which could not be modulated further following treatment with contact allergens as assessed by real time PCR (data not shown). The latest finding is consistent with data obtained by Naik et al. (1999) in primary human KC treated with DNCB.

Cultures of adult human KC are readily available, even commercially, but differences in metabolism and response to stimuli can exist between cultures derived from different donors (Berghard et al., 1990); therefore, established lines of human KC represent a good

alternative. We obtained similar results using both the NCTC 2544 cell line and primary human KC, confirming the relevance of the proposed model and the possibility to use different source of KC.

Important factors including compound solubility, chemical reactivity and metabolic activation, which may mask the potential allergenicity of some chemicals must be considered in the development of in vitro tests. This may be the case of MDI and TMA, highly instable in water. TMA is readily hydrolyzed in water to trimellitic acid and MDI produces inert, solid, insoluble polyurea. The hydrolysis of isocyanate in aqueous solution is rapid, with a half-life of less of 20 s. If submerged cell culture may be unfavourable for many of the respiratory sensitisers, due to chemical instability, we have tested IL-18 production in reconstituted human epidermis, which allows application in organic solvent, i.e. acetone: olive oil. Preliminary results obtained testing DNCB, phenol, salicylic acid, TMA and MDI showed an increase in IL-18 only following treatment with DNCB, which may support results obtained with submerged keratinocytes.

The lack of metabolic activation may be a relevant problem in case of proaptens. However, NCTC 2544 cells posses both phase I and II metabolic activation capacity (Gelardi et al., 2001), and positive results were indeed obtained with the proaptens eugenol and cinnamic alcohol (Fig. 2). A comparison between the phase I enzyme activities expressed in normal human KC and in several human keratinocyte cell lines, namely HaCaT, SVK14 and NCTC 2544, was established by Cotovio et al. (1997). From that study it appeared that in NCTC cell line both basal and induced levels of 7-ethoxycoumarin O-deethylase (ECOD) activity (mainly CYP 1A- and CYP 2B-related), are higher than those found in normal. The NCTC 2544

cell line not only compares well with other in vitro models such as reconstructed epidermis and primary cultured KC, but have even better metabolic properties. The characterization of phase I and phase II metabolism expressed in these cells gives sustained evidence for their suitability to in vitro toxicology studies (Gelardi et al., 2001).

In terms of potency, the calculation of concentration of allergen that induced a 1.4 increase in cell-associated IL-18 by linear regression analysis of data gave the following values: DNCB (0.5 μg/ml), 4-bromobenzylbromide (2.0 μg/ml), 2-mercaptobenzothiazole (92 μg/ml), cinnamaldehyde (11.0 μg/ml), cinnamyl alcohol (103 μg/ml), eugenol (344 μg/ml), isoeugenol (12.8 μg/ml), glyoxal (9.0 μg/ml), resorcinol (206.0 μg/ml), PPD (31.7 μg/ml), TMTD (1.04 μg/ml). If compared with the available in vivo LLNA EC3 values a significant correlation ($R = 0.754$, $p = 0.0098$) was obtained. Therefore, IL-18 production by KC may also be seen as a possible tool for the prediction of sensitizing strength of a particular chemical. However, investigations are needed to better address the question of potency.

In conclusion, IL-18 production by NCTC 2544 cells, or primary KC, may represent a promising in vitro model for the screening of potential contact allergens and may be useful for ranking chemicals also for their sensitizing potency resulting in a reduction of animal testing. The test may also be complementary to the LLNA, allowing for a rapid and easy discrimination of contact sensitisers from respiratory allergens. Our results warrant further analysis of panels of potential allergens and irritants. It is, however, important to stress that due to the complexity of hypersensitivity reactions and the chemical diversity of allergens, a more realistic approach should consider several markers of activation and different cell types, including dendritic like cells.

3.2 FURTHER DEVELOPMENT OF THE NCTC 2544 IL-18 ASSAY TO IDENTIFY IN VITRO CONTACT ALLERGENS

Abstract

Several European Union legislations request the use of in vitro methods for toxicological evaluations, including sensitization, in order to increase consumer safety but also to reduce the use of animals. The EU project SENS-IT-IV addresses the need of developing predictive in vitro tests to assess contact and respiratory hypersensitivity reactions. In this context, we have recently reported the possibility to use IL-18 production in the human keratinocyte cell line NCTC 2544 to discriminate contact sensitiser from irritants and low molecular weight respiratory allergens. The aims of the present study were to further develop this assay in order to optimize experimental conditions; to develop a 96-well plate format to establish a high throughput assay; to test the performance of other available keratinocyte cell lines, and to understand the signal transduction pathway involved in p-phenylenediamine (PPD)-induced IL-18 production.

If cells reach confluence at the moment of treatment, the ability to identify contact allergens is lost; therefore a careful check for the optimal cell density using PPD as reference contact allergen is critical. In our hands, a cell density of 1–2.5 × 10^5 cells/ml gave optimal stimulation. In order to develop a high throughput test, cells seeded in 96-well plate were exposed to contact allergens (2,4-dinitrochlorobenzene, p-phenylenediamine, isoeugenol, cinnamaldehyde, tetramethylthiuram disulfite, resorcinol, cinnamic alcohol and eugenol), irritants (phenol, sodium laurel sulphate, lactic acid and salicylic acid) and respiratory

allergens (hexachloroplatinate, diphenylmethane diisocyanate, trimellitic anhydride). A selective increase in total (intracellular plus released) IL-18 was observed 24 h later in cells treated with contact allergens, whereas no changes were observed following treatment with respiratory allergens and irritants, confirming previous results obtained in a 24-well format assay. A selective induction of IL-18 was also obtained testing with PPD other keratinocyte cell lines, namely HPKII and HaCaT, with the HPKII showing the highest stimulation index. Regarding the signal transduction pathway, we could demonstrate using selective inhibitors a role for oxidative stress, NF-κB and p38 MAPK activation in PPD-induced IL-18 production.

In conclusion, results obtained suggest that the production of IL-18 represents a promising endpoint for the screening of potential contact allergens. The assay can be performed in a 96-well plate format, different keratinocyte cell lines can be used, and a role for oxidative stress in contact allergen-induced IL-18 was demonstrated.

Role of cell density and adhesion time on contact allergen-induced intracellular IL-18

The effect of cell density and adhesion time on the response to the contact allergen PPD was investigated seeding cells at different density (1–10 × 10^5 cells/ml in 24-well plate) and treating them after o.n. or 2 h seeding. As shown in Fig. 1A, 1–2.5 × 10^5 cells/ml represents the optimal cell density, higher cell density (>5 × 10^5 cells/ml) resulted in a loss of effect. This is also evident comparing the stimulation index: the higher SI was observed at cell density of 1–2.5 × 10^5 cell/ml. A critical point in the performance of the assay is, therefore, the cell density: if cells reach confluence the discriminatory capacity is lost. This problem

can be overcome treating cells 2 h after seeding, time sufficient to allow cells to adhere. As shown in Fig. 1C, a statistical significant upregulation of intracellular IL-18 was observed at all cell densities tested, including $5–10 \times 10^5$ cells/ml. Furthermore, as shown in Fig. 1B, seeding cells at two different density ($1.5–2.5 \times 10^5$ cells/ml), and treating them after 2 h with PPD (60 μg/ml), cinnamaldehyde (30 μg/ml), salicylic acid (250 μg/ml) or DMSO as vehicle control for 24 h, only the contact allergens induced an increase in intracellular IL-18, whereas salicylic acid fails, indicating that the discriminatory capacities are preserved.

Selective induction of total IL-18 by contact allergens

In order to develop a high throughput assay, a 96-well plate format assay was developed. NCTC 2544 cells were seeded in 96-well plate and treated for 24 h with representative contact allergens, respiratory allergens or skin irritant. The highest concentration tested for each chemical was the CV_{80}. Following treatment the total IL-18 content (intracellular and released) was assessed. NCTC 2544 cells were treated with increasing concentrations of the contact allergens DNCB, PPD, isoeugenol, cinnamaldehyde, TMTD, resorcinol, cinnamic alcohol and eugenol. As shown in Fig. 2, all contact allergens, including the pro-haptens cinnamic alcohol and eugenol, induced a statistical significant increase in total IL-18, confirming results previously obtained in 24-well format assay (Corsini et al., 2009).

We then tested the effect of the respiratory allergens HClPt, MDI, TMA and the irritants phenol, SDS, lactic acid and salicylic acid on total IL-18. None of them was able to increase total IL-18 (Fig. 3 and Fig. 4), confirming the selective up-regulation of IL-18 by contact allergens.

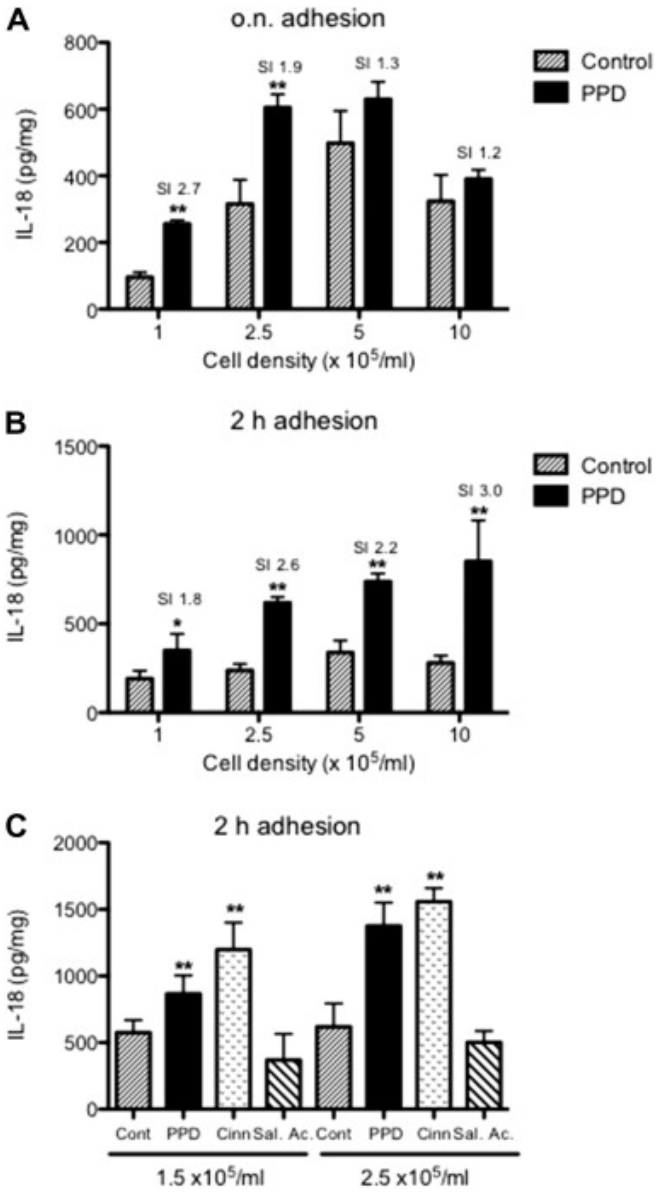


Fig. 1. Effect of cell density and adhesion time on intracellular IL-18. Panel A, over night adhesion. NCTC 2544 were seeded in 24-well plate at different density (1–10 × 10^5 cells/ml). After overnight adherence, cells were treated for 24 h with PPD (60 μg/ml) or DMSO (0.2% final concentration) as vehicle control. Results are expressed as mean ± SD, n = 4. Panel B, 2 h adhesion. NCTC 2544 were seeded in 24-well plate at different density (1–10 × 10^5 cells/ml). After 2 h adherence, cells were treated for 24 h with PPD (60 μg/ml) or DMSO (0.2% final concentration) as vehicle control. Results are expressed as mean ± SD, n = 4. Panel C, 2 h adhesion. NCTC 2544 were seeded in 24-well plate at two different density: 1.5–2.5 × 10^5 cells/ml. After 2 h of adherence, cells were treated for 24 h with PPD (60 μg/ml), cinnamaldehyde (30 μg/ml), salicylic acid (200 μg/ml) or DMSO (0.2% final concentration) as vehicle control. Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Student's multiple comparison test for panel A, B and Dunnet's multiple comparison test for panel B, with $^{**}p < 0.01$ versus vehicle treated cells (control). SI, stimulation index.

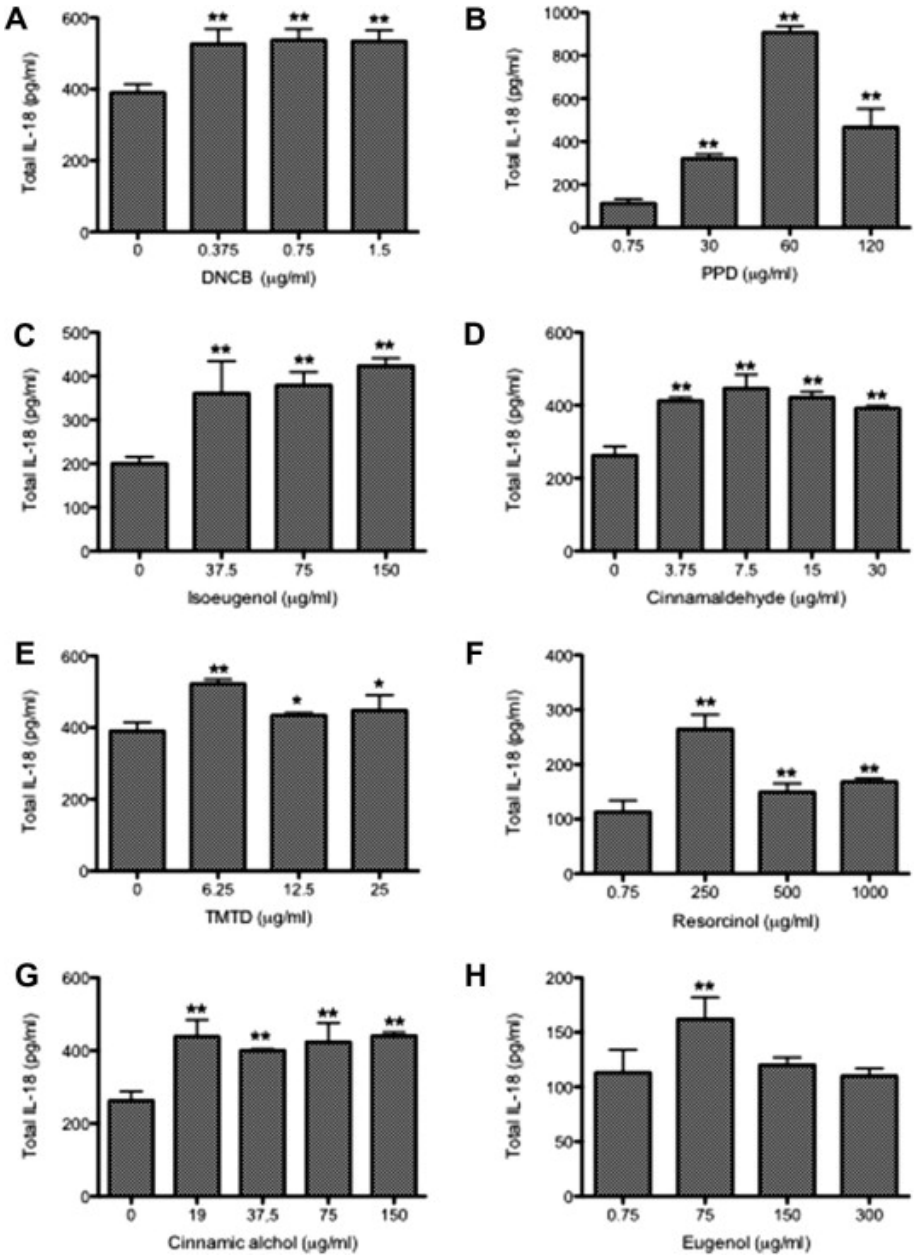


Fig. 2. Effects of contact sensitisers on total IL-18 production. NCTC 2544 seeded in 96-well plate were treated for 24 h with increasing concentration of DNCB (A), PPD (B), isoeugenol (C), cinnamaldehyde (D), TMTD (E), resorcinol (F), cinnamic alcohol (G) and eugenol (H). Cells incubated with vehicle were included as control (0). Results are expressed as mean ± SD. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle treated cells (0).

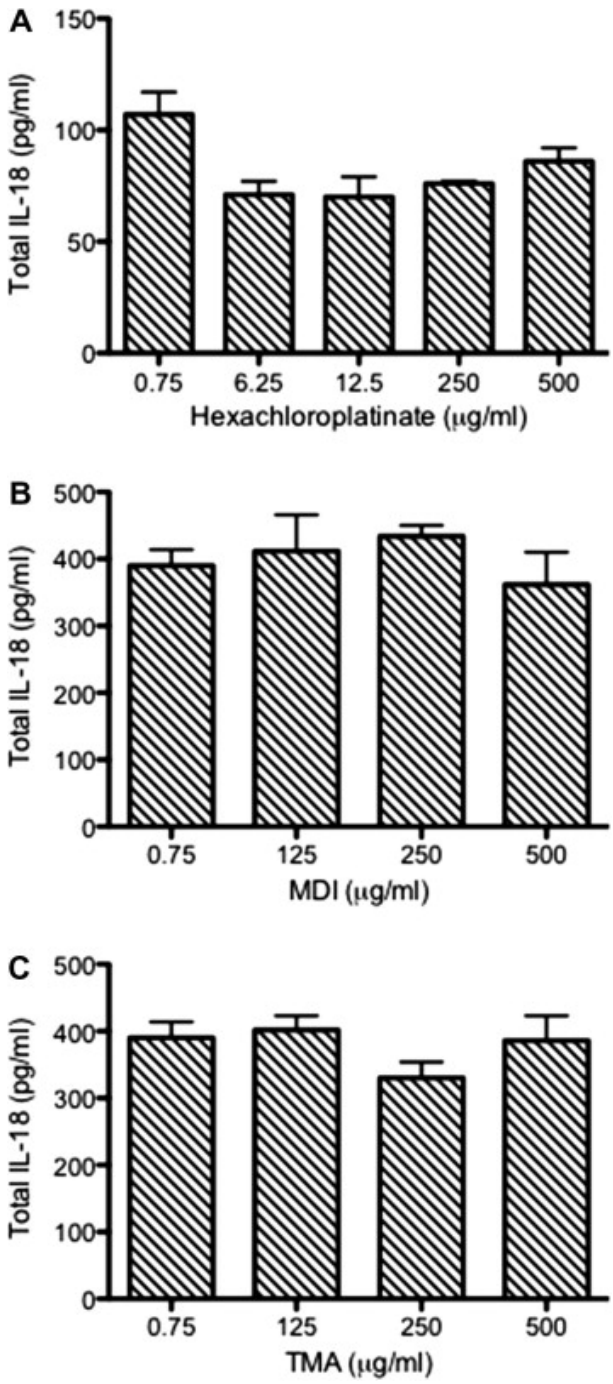


Fig. 3. Effects of respiratory allergens on total IL-18 production. NCTC 2544 seeded in 96-well plate were treated for 24 h with increasing concentration of hexachloroplatinate (A), MDI (B) and TMA (C). Cells incubated with vehicle were included as a control (0). Results are expressed as mean ± SD. Statistical analysis was performed with Dunnett's multiple comparison test with $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle treated cells (0).

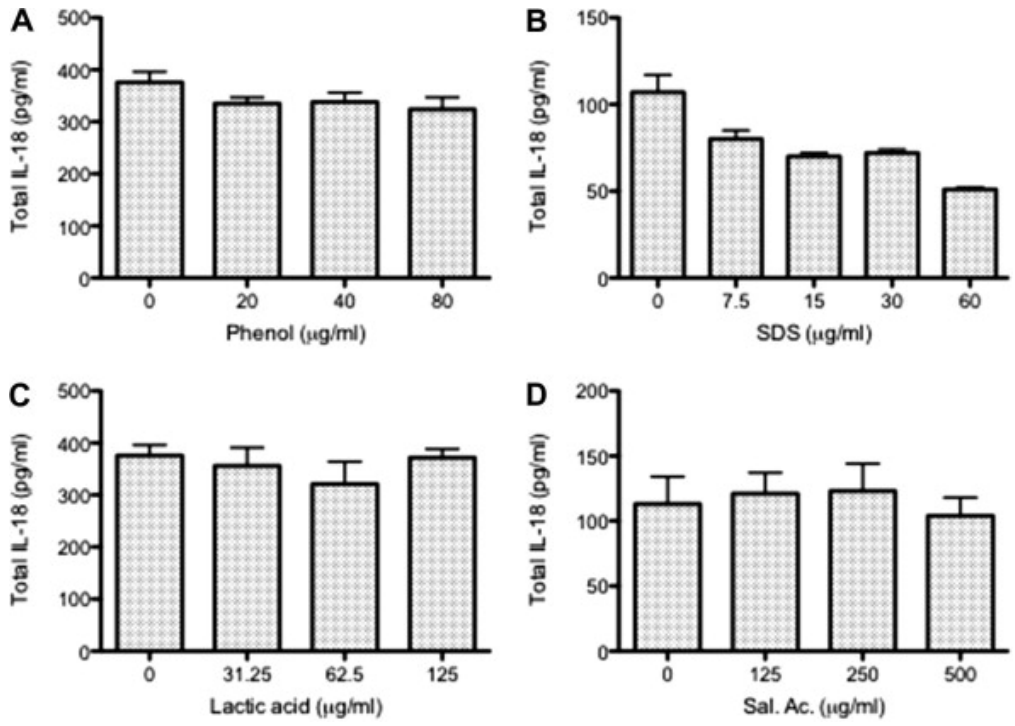


Fig. 4. Effects of irritants on total IL-18 production. NCTC 2544 seeded in 96-well plate were treated for 24 h with increasing concentration of phenol (A), SDS (B), lactic acid (C) and salicylic acid (D, Sal. Ac.). Cells incubated with vehicle were included as a control (0). Results are expressed as mean ± SD. Statistical analysis was performed with Dunnett's multiple comparison test with $^{*}p < 0.05$ and $^{**}p < 0.01$ versus vehicle treated cells (0).

Effects of PPD on cell-associated IL-18 in different human KC

We then investigated if these results were peculiar of this cell line or if similar results could be obtained using other keratinocyte cell lines. At this purpose, two other KC cell lines were used, namely HaCaT and HPK II. As shown in Fig. 5, the treatment with PPD induced an increase in intracellular IL-18 content in all three cell lines tested, with the HPKII showing the highest stimulation index: 3.4 ± 0.7 vs 1.6 ± 0.3 in NCTC 2544 and 2.1 ± 0.3 in HaCaT cells ($p < 0.05$). These results confirm the possibility to use different keratinocyte models.

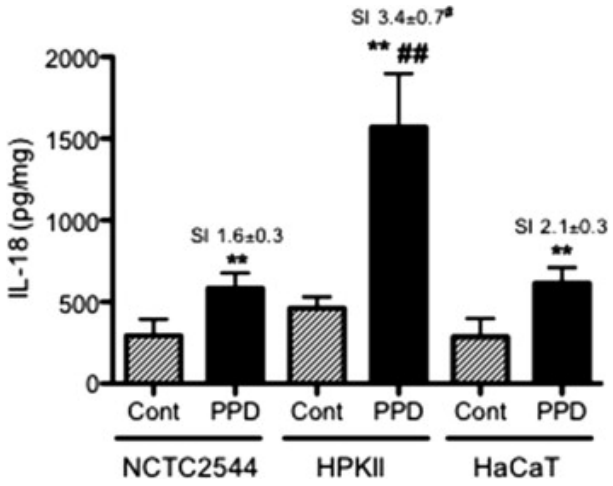


Fig. 5. Effect of PPD on intracellular IL-18 in three keratinocyte cell lines. NCTC 2544, HPKII and HaCaT cells lines were treated for 24 h with PPD (60 μg/ml) or DMSO (0.2% final concentration) as vehicle control. Results are expressed as mean ± SD. Statistical analysis was performed with Tukey's multiple comparison test, with $^{**}p < 0.01$ versus vehicle treated cells (0). SI, stimulation index. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs NCTC 2544 or HaCaT cells treated with PPD.

Signal transduction pathway

Finally, we wanted to investigate the signal transduction pathway involved in p-phenylenediamine (PPD)-induced IL-18 production. For this purpose, several selective inhibitors were used: GF109203X (5 μM) to inhibit protein kinase C, PDTC (100 μM) and Bay 11-7085 (1 μM) to inhibit NF-κB and SB203580 (100 nM) to inhibit p38 MAPK. As show in Fig. 6A, PPD induced intracellular IL-18 was not modulated by GF109203X, excluding the involvement of protein kinase C. On the contrary, PDTC, Bay 11-7085 and SB203580, significantly modulated PPD-induced intracellular IL-18, indicating a role for oxidative stress, NF-κB and p38 MAPK activation. (Fig. 6B–D). The ability of PPD to activate p38 MAPK and NF-κB was confirmed by Western blot analysis (Fig. 7). Cells were treated with PPD or DMSO

as vehicle control for 5, 15, 30 and 60 min. As shown in Fig. 7, p38 MAPK was clearly activated after 15 min, whereas NF-κB was activated at later time points.

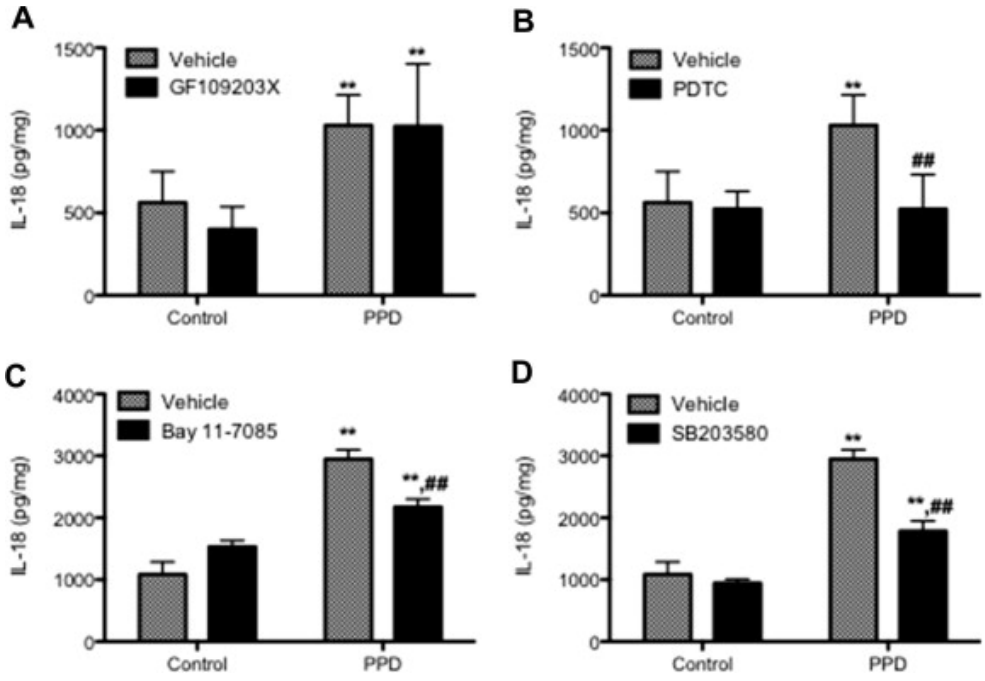


Fig. 6. Role of oxidative stress, p38 MAPK and NF-κB activation in PPD-induced IL-18 production. NCTC 2544 were treated with GF109203X (5 μM), PDTC (100 μM), Bay 11-7085 (1 μM) and SB203580 (100 nM) for 1 h and after with PPD (60 μg/ml) or DMSO (0.2% final concentration) as vehicle control for 24 h. Results are expressed as mean ± SD. Statistical analysis was performed with Tukey's multiple comparison test, with $^{**}p < 0.01$ versus vehicle treated cells (0). $^{\#\#}p < 0.01$ vs cells treated with PPD alone.

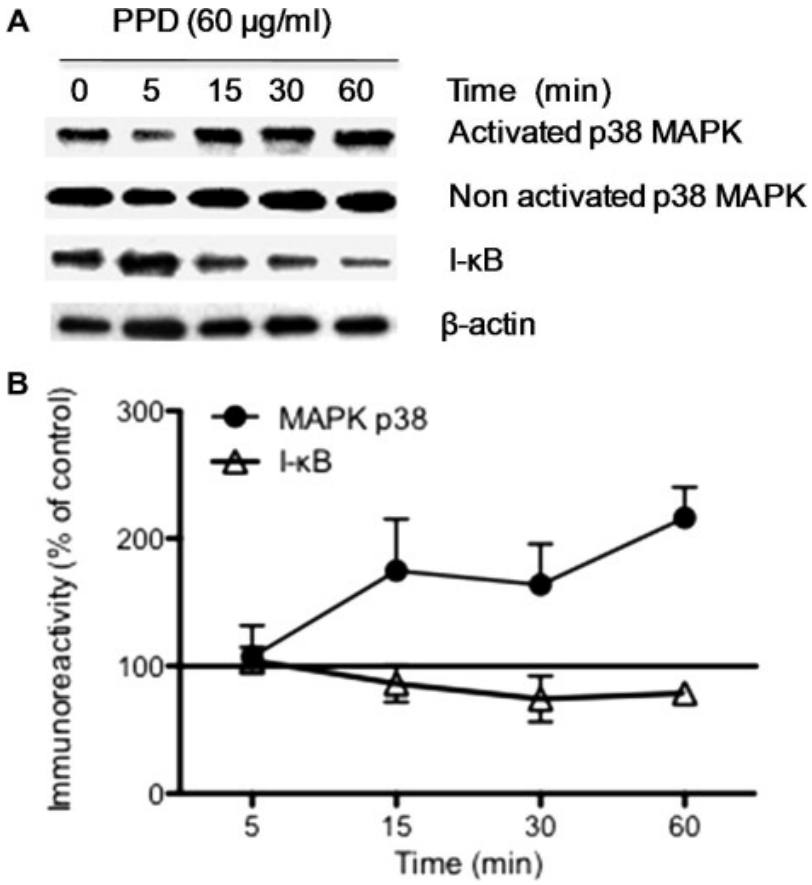


Fig. 7. PPD activated p38 MAPK and NF-B in NCTC 2544 cells. Cells were seeded in 6-well plate at a cell density of 2.5×10^5 cells/ml (2 ml/well). After 24 h of adherence, cells were treated with PPD (60 µg/ml) or DMSO as vehicle control for different times (5–60 min). Panel A, representative Western blots. Panel B, relative densitometric analysis. Each value represents the mean ± SD of 4 independent experiments.

Discussion

In the present manuscript, the 'NCTC 2544 IL-18 assay' developed to identify in vitro contact allergens, was further characterized. The experimental conditions were optimized to clarify the cell density issue, a high throughput assay was developed, the performance of other available keratinocyte cell lines was tested, and the signal transduction pathways involved in

PPD-induced IL-18 production were characterized.

In our previous manuscript (Corsini et al., 2009), and also during technology transfer of the assay to other laboratories, a critical point was identified in the cell density. If cells reach confluence at the moment of treatment, the ability to discriminate contact allergens from skin irritants and respiratory sensitiser is lost. Due to the use of different foetal calf serum, a different cellular doubling time can be expected in the different laboratories. Therefore, as the cells number deeply influences the outcome, each laboratory should carefully check for the optimal cell density, using the positive control PPD as a reference compound. We found that a possible alternative is to treat the cells the same day of seeding after two hours of adherence. Under this condition, the selective upregulation of intracellular IL-18 by contact allergens is maintained and the cell density issue is overcome.

Next, in order to further develop the assay and to increase its screening capacity, a 96-well format assay was developed. To speed up the assay and reduce cell manipulation (no cell washing steps and no protein determination), after the treatment, cells were lysed in culture medium directly adding Triton X-100 (final concentration 0.5%). In this case the total IL-18 (intracellular plus released) was assessed. Also under this experimental condition, a selective increase in total IL-18 was observed only following treatment with contact allergens, whereas both irritants and respiratory allergens failed, indicating the possibility to use total IL-18 to identify contact allergens. A total of eight contact allergens, namely 2,4-dinitrochlorobenzene, p-phenylenediamine, isoeugenol, cinnamaldehyde, tetramethylthiuram disulfite, resorcinol, cinnamic alcohol and eugenol were tested. All, including pre- (i.e. PPD) and pro-haptens (i.e. cinnamic alcohol and eugenol), induced a

statistically significant increase in total IL-18. Whereas the respiratory allergens, namely hexachloroplatinate, diphenylmethane diisocyanate, trimellitic anhydride, as well as the irritants phenol, sodium laurel sulphate, lactic acid and salicylic acid did not affect the total IL-18 content. The main limitation of assessing total versus intracellular IL-18 is the lost, in many cases, of a dose–response curve, which may hamper the possibility to use the induction of IL-18 for potency classification. Indeed, as previously shown (Corsini et al., 2009), contact allergens induced a dose-related increase in intracellular IL-18 and by calculation of the concentration of allergen that induced a 1.4 increase in cell-associated IL-18 a significant correlation with the in vivo LLNA EC3 values was obtained ($R = 0.754$, $p = 0.0098$). The main advantage of the 96-well format is, of course, time saving, less compound needed and high throughput, which allow a more rapid screening of chemicals for their contact sensitization potential. The lack of a clear dose–response may be due to a different effect, depending on the concentration, of the sensitiser on the release of IL-18 vs the effect on the intracellular IL-18 content, or to a possible interference of the chemicals with the ELISA. Indeed, by adding recombinant IL-18 to cell lysates, as an internal standard, a 20–40% reduction in its recovery was observed in cells treated with resorcinol and eugenol.

The ability of NCTC 2544 to identify contact allergens is not peculiar of this human KC cell lines, as previously demonstrated (Corsini et al., 2009) with primary human KC, similar results were also obtained using other human KC cell lines, i.e. HPKII and HaCaT, further confirming the relevance of the proposed model and the possibility to use different source of KC. Among the three KC cell lines tested, at least for PPD, a higher stimulation index was obtained with HPKII. Further chemicals, however, must be tested to prove the better

performance of this cell line.

Finally, we aimed to investigate the signal transduction pathways involved in contact allergen-induced IL-18 production. KC form an interface between the body and the environment, they are important guardians for the detection of danger signals and the consecutive initiation of an inflammatory response. The transduction of extracellular signals into cellular response is mediated by an array of different kinases, that includes protein kinase C and mitogen activated protein kinases (MAPK). Both kinases have been involved in the induction of allergic contact dermatitis. It has been reported in a mouse model of contact hypersensitivity, that pretreatment with SB202190, a p38 MAPK inhibitor abrogated DNFB-induced contact hypersensitivity, indicating the important role of this kinase (Lisby et al., 1995). The ability of allergens to activate p38 MAPK has been also demonstrate in in vitro studies in both keratinocyte cell line A431 (Mehrotra et al., 2007) and dendritic cells (Aiba et al., 2003, Arrighi et al., 2001, Boisleve et al., 2005 and Mitjans et al., 2008). Also protein kinase C modulation has been associated with reduction in both irritant and allergic contact dermatitis (Jacobson et al., 1995). Several selective inhibitors were therefore used together with PPD, as reference allergen, to investigate the role of protein kinase C, oxidative stress, NF-B and p38 MAPK in PPD-induced IL-18. In particular, GF109203X a selective inhibitor for the α and β1 protein kinase C isoforms was used to investigate the role of protein kinase C; PDTC, an inhibitor of NF-B and an anti-oxidant to investigate the role of NF-B activation and oxidative stress; Bay 11-7085 as an irreversible inhibitor IκBα phosphorylation to assess the role of NF-B activation, and finally, SB 203580 as inhibitor of p38 MAPK activation to investigate the role of MAPK. Results obtained indicated a role for oxidative stress, NF-B and p38 MAPK activation in PDD-induced IL-18 production. No

modulation was observed inhibiting protein kinase C activation, suggesting that its activation is not involved in IL-18 production. The results obtained with the inhibitors of NF-B and p38 MAPK are consistent with an inhibition of IL-18 transcription as in 5' upstream region of the human IL-18 gene AP-1, PU.1, NF-B and SP-1 transcription binding sites have been identified (Takeuchi et al., 1999).

Oxidative stress may be the starter point, as it leads to the activation of transcription factors and signalling pathways, including NF-B and p38 MAPK, which leads to the release of cytokines and chemokines (Gloire et al., 2006, Kim and Choi, 2010 and Rahman and Biswas, 2004). Reactive oxygen species plays also an important role in the activation of the NLRP3/NALP3 inflammasome (Bryant and Fitzgerald, 2009 and Martinon et al., 2009), which is required to direct the proteolytic maturation of inflammatory cytokines such as IL-1 and IL-18 (Martinon, 2010). The ability of contact sensitisers to induce the oxidative stress pathway in KC has been recently confirmed by several authors (Natsch and Emter, 2008 and Vandebriel et al., 2010).

In conclusion, total IL-18 production by NCTC 2544, the induction of IL-18 in other keratinocyte cell lines; all give the possibility to identify potential contact allergens in vitro. The proposed test may be used as a stand alone assay or as a pre-screen before the LLNA (i.e. the LLNA can be used to confirm a negative finding), or it can be used complementary to other non-animal test (i.e. QSAR, peptide binding assay, etc.), allowing for a rapid and easy discrimination of contact sensitisers from respiratory allergens and skin irritants. Due to the complexity of contact sensitization and the limited number of chemicals tested in the NCTC 2544 assay (only the SENS-IT-IV list has been so far tested), it is, however, likely that a

combined analysis of more biomarkers rather than analysis of a single biomarkers will give more satisfactory results. The NCTC 2544 intracellular IL-18 assay has currently entered a prevalidation stage sponsored by SENSTIV and the Dutch Governmental association ZonMW. It is hoped to have a fully validated assay implemented in time to meet the upcoming European regulatory deadlines.

3.3 ESTABLISHMENT OF AN *IN VITRO* PHOTOALLERGY TEST USING NCTC2544 CELLS AND IL-18 PRODUCTION

Abstract

Differentiation between photoallergenic and phototoxic reactions induced by low molecular weight compounds represents a current problem. The use of keratinocytes as a potential tool for the detection of photoallergens as opposed to photoirritants is considered an interesting strategy for developing in vitro methods. We have previously demonstrated the possibility to use the human keratinocyte cell line NCTC2455 and the production of interleukin-18 (IL-18) to screen low molecular weight sensitisers. The purpose of this work was to explore the possibility to use the NCTC2544 assay to identify photoallergens and discriminate from phototoxic chemicals.

First, we identified suitable condition of UV-irradiation (3.5 J/cm^2) by investigating the effect of UVA irradiation on intracellular IL-18 on untreated or chloropromazine (a representative phototoxic compound)-treated NCTC2544 cells. Then, the effect of UVA-irradiation over NCTC2544 cells treated with increasing concentrations of 15 compounds including photoallergens (benzophenone, 4-ter-butyl-4-methoxy-dibenzoylmethane, 2-ethylexyl-p-methoxycinnamate, ketoprofen, 6-methylcumarin); photoirritant and photoallergen (4-aminobenzoic acid, chlorpromazine, promethazine); photoirritants (acridine, ibuprofen, 8-methoxypsoralen, retinoic acid); and negative compounds (lactic acid, SDS and p-phenilendiamine) was investigated. Twenty-four hours after exposure, cytotoxicity was

evaluated by the MTT assay or LDH leakage, while ELISA was used to measure the production of IL-18. At the maximal concentration assayed with non-cytotoxic effects (CV80 under irradiated condition), all tested photoallergens induced a significant and a dose-dependent increase of intracellular IL-18 following UVA irratiation, whereas photoirritants failed. We suggest that this system may be useful for the in vitro evaluation of the photoallergic potential of chemicals.

Establishment of the irradiation conditions

The dose of UVA irradiation was assessed exposing cells to increasing UVA doses. Under our experimental conditions, as shown in Fig. 1 A, the dose of 3.5 J/cm^2 represents the optimal UVA dose. As at this dose, no effects of UVA alone was observed, while UVA dose of 7 J/cm^2 resulted in a loss of IL-18 production and LDH leakage (cytotoxicity), compared to not irradiated cells.

The photoallergen/photoirritant chloropromazine was then used to establish the optimal irradiation conditions in the presence of potential phototoxic compound. Initially, as highest concentration the CV80 calculated in non-irradiated condition was used (Fig. 1 B, C), hoping to use the same SOPs developed for the NCTC2544 IL-18 assay. In this condition, however, following UVA irradiation a dramatic cytotoxicity was observed as assessed by LDH leakage, which resulted in the impossibility to assess the effect of irradiated chloropromazine on IL-18 production. Therefore, CV80 was assessed in irradiated condition. As shown in Fig. 1D, using the CV80 calculated in irradiated condition as the highest concentration, irradiated chloropromazine induced a dose-related increase in intracellular IL-18. To appreciate the

effect of the combination of chemical + UVA, the ratio of IL-18 SI in irradiated vs non-irradiated conditions is reported as dotted line (Fig. 1D).

Based on these experiments, the UVA dose of 3.5 J/cm^2 was selected, and as highest concentration the CV80 calculated in irradiated condition was used.

Determination of 80% cell viability

The concentration of chemical resulting in 80% of viability (CV80) respect to vehicle treated cells 24 h was calculated for all chemicals in both non-irradiated and irradiated conditions. Results are presented in Table 1. For many chemicals, including photoallergens and photoirritants, a lower CV80s were observed in irradiated condition.

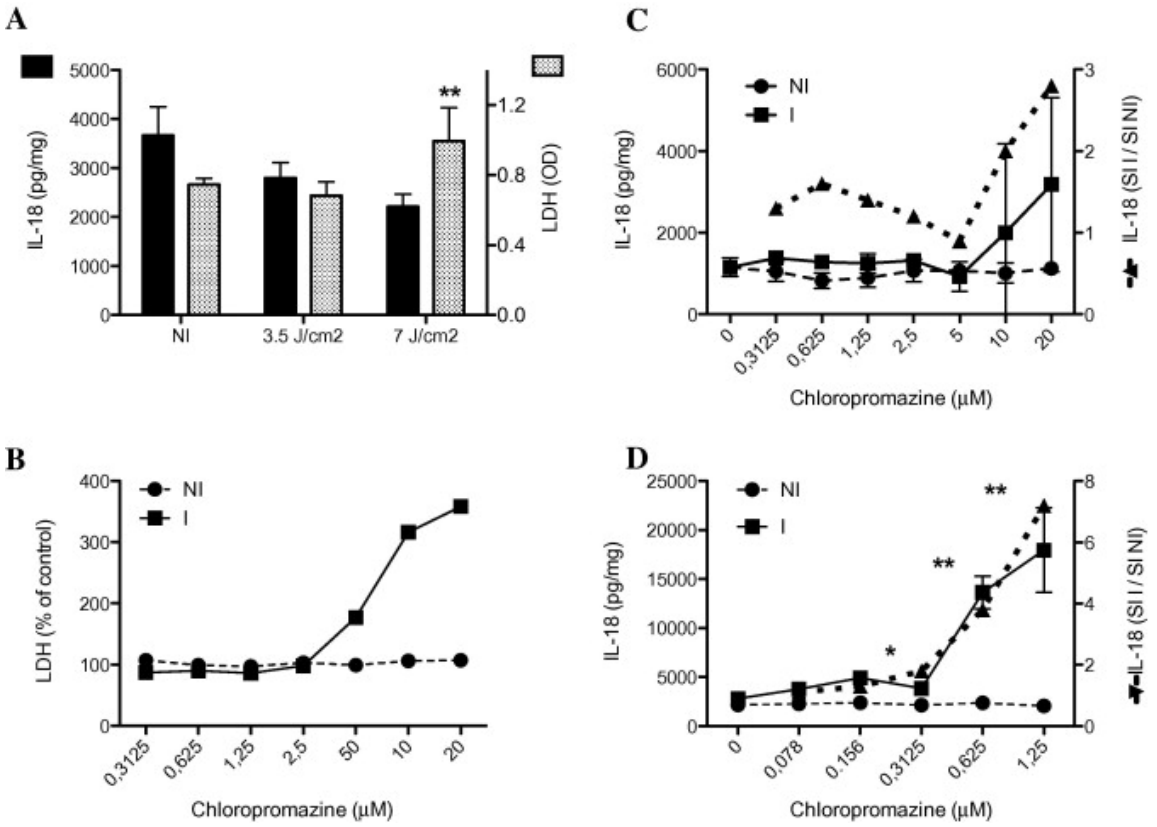


Fig. 1. Effects of UVA irradiation on intracellular IL-18 and viability of NCTC2544 cells. (A) Cells were irradiated at the UVA doses of 3.5 and 7 J/cm^2. Intracellular IL-18 and LDH leakage were assessed 24 h later. Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{**}p < 0.01$ vs non-irradiated cells (NI). Dose response effects of chloropromazine on cell viability (B) and cell-associated IL-18 (C). NCTC2544 cells were irradiated (I) at the UVA dose of 3.5 J/cm^2 or non-irradiated (NI) in the presence of increasing concentrations of chloropromazine, using the CV80 calculated under non-irradiated condition as highest concentration. (D) Dose response effects of chloropromazine on cell-associated IL-18, using the CV80 calculated under irradiation condition as highest concentrations. Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{*}p < 0.05$ and $^{**}p < 0.01$ vs vehicle treated cells (0).

Table 1. Concentration (μM) of the tested chemicals that induced 80% viability (CV_{80}) in non-irradiated and irradiated conditions (3.5 J/cm^2), and their classification.

Compound	CV_{80} Non-irradiated	CV_{80} irradiated	Category
2-Ethylhexyl-4-methoxycinnamate	3400	9.8	Photoallergen
6-Methylcoumarin	>1000	10	Photoallergen
Avobenzone	90	90	Photoallergen
Benzophenone	>1000	31.2	Photoallergen
Ketoprofen	3000	4.9	Photoallergen
4-Aminobenzoic acid (PABA)	7300	7300	Photoallergen/photoirritant
Chlorpromazine	20	1.25	Photoallergen/photoirritant
Promethazine	125	1	Photoallergen/photoirritant
8-Methoxypsoralen	700	2	Photoirritant
Acridine	2116	0.05	Photoirritant
Ibuprofen	>1000	>1000	Photoirritant
Retinoic acid	50	20	Photoirritant
p-Phenylenediamine (PPD)	55	27.5	Allergen
Lactic acid	>8300	>8300	Irritant
Sodium dodecyl sulfate (SDS)	100	100	Irritant

Selective induction of intracellular IL-18 by photoallergens

NCTC2544 cells were treated for 24 h with the selected photoallergens (Fig. 2), photoallergens/photoirritants (Fig. 3), photoirritants (Fig. 4), and non-phototoxic compounds (Fig. 5). Following treatment and UVA irradiation, the intracellular IL-18 content was assessed as described in the Materials and Methods section. Results are reported as the ratio of IL-18 SI in irradiated vs non-irradiated conditions (IL-18 SI (I/NI)).

As shown in Fig. 2 and Fig. 3, all irradiated photoallergens and photoallergens/photoirritants, induced a statistical significant increase in intracellular IL-18, with an IL-18 SI (I/NI) up to 7 for chloropromazine. The lowest inductions were observed for avobenzone and benzophenone. In all figures, a dotted line was set at a SI of 1.3, as provisional criteria for identification of photoallergens and discriminate them from photoirritants.

We then tested the effect of the photoirritants ibuprofen, acridine, retinoic acid and 8-methoxypsoralene (Fig. 5). Irradiated ibuprofen and acridine failed to induced IL-18 production, while a slight increase, below or close to IL-18 SI (I/NI) of 1.3, was observed at the highest concentration of 8-methoxypsoralene and retinoic acid.

Finally, we assessed the effects of not phototoxicants, namely PPD, lactic acid and SDS on intracellular IL-18 production. Following UVA irradiation, none of them was able to increase intracellular IL-18 (Fig. 5), confirming the selective up-regulation of IL-18 by irradiated photoallergens.

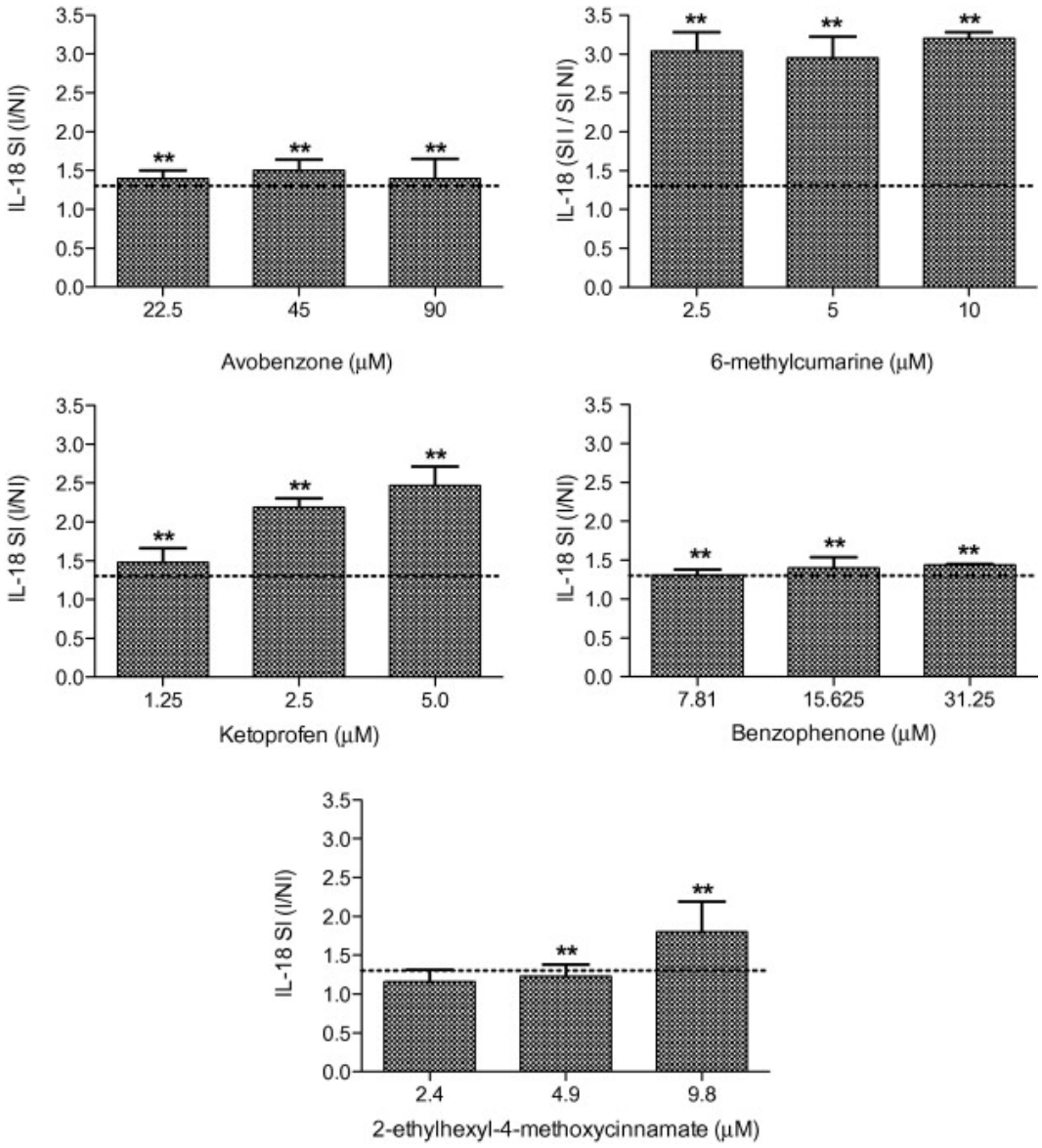


Fig. 2. Effects of the selected photoallergens on cell-associated IL-18. NCTC2544 cells were irradiated (I) at the UVA dose of 3.5 J/cm^2 or non-irradiated (NI) in the presence of increasing concentrations of the selected photoallergens, using the CV80 calculated under irradiated condition as highest concentration. Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each data is mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{*}p < 0.05$ and $^{**}p < 0.01$ vs vehicle treated cells.

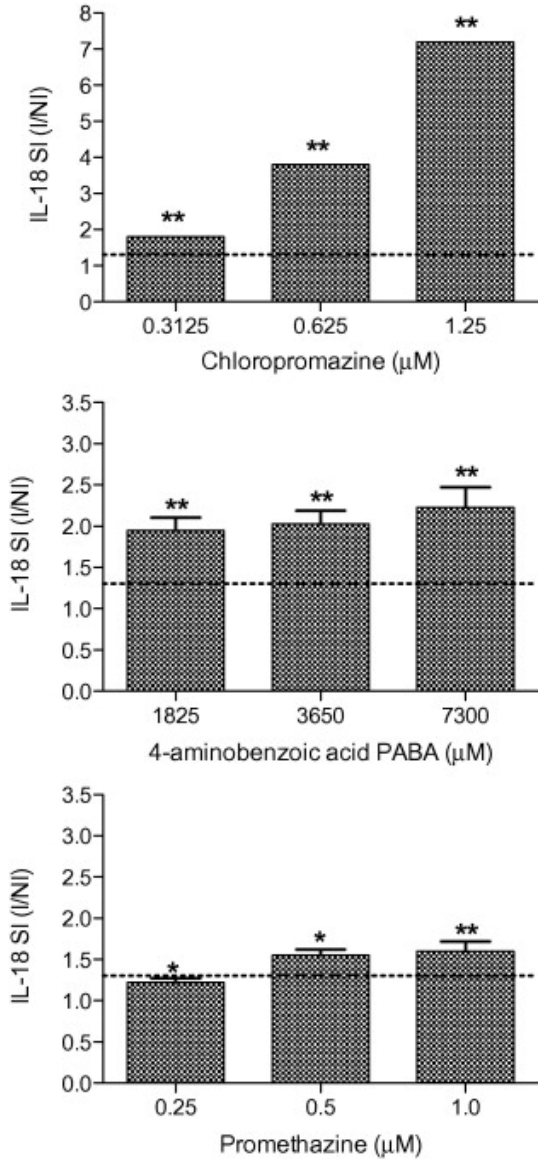


Fig. 3. Effects of the selected photoallergens/photoirritants on cell-associated IL-18. NCTC2544 cells were irradiated (I) at the UVA dose of 3.5 J/cm^2 or non-irradiated (NI) in the presence of increasing concentrations of the selected photoallergens/photoirritants, using the CV80 calculated under irradiated condition as highest concentration. Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each data is mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{*}p < 0.05$ and $^{**}p < 0.01$ vs vehicle treated cells.

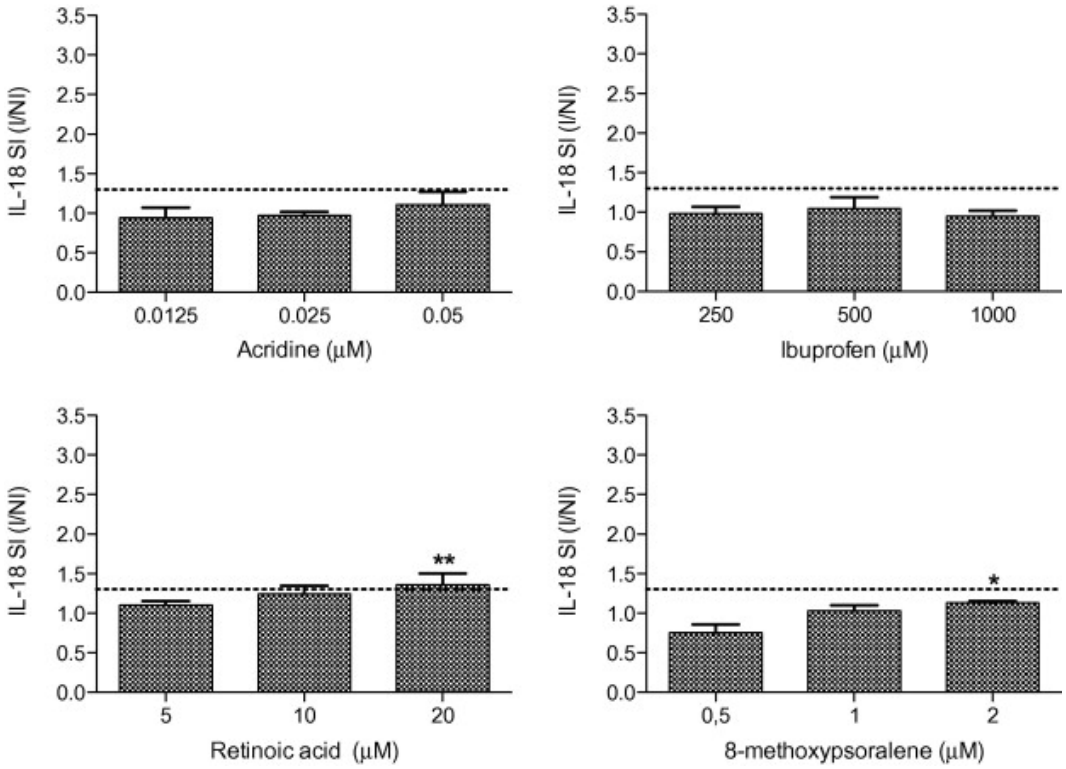


Fig. 4. Effects of the selected photoirritants on cell-associated IL-18. NCTC2544 cells were irradiated (I) at the UVA dose of 3.5 J/cm^2 or non-irradiated (NI) in the presence of increasing concentrations of the selected photoirritants, using the CV80 calculated under irradiated condition as highest concentration. Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each data is mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{*}p < 0.05$ and $^{**}p < 0.01$ vs vehicle treated cells.

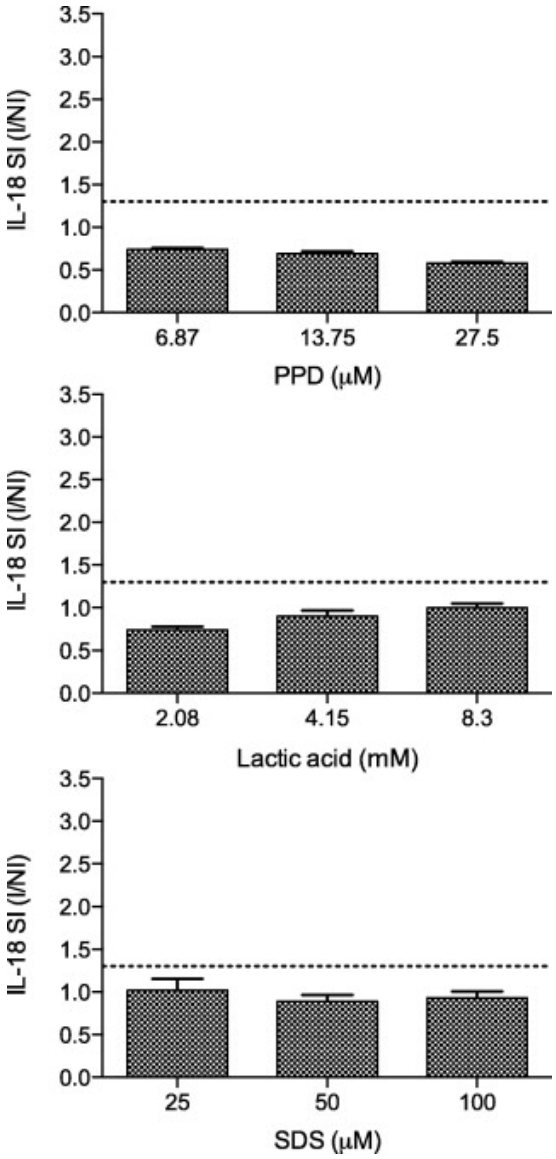


Fig. 5. Effects of the selected non-phototoxic compounds on cell-associated IL-18. NCTC2544 cells were irradiated (I) at the UVA dose of 3.5 J/cm^2 or non-irradiated (NI) in the presence of increasing concentrations of the selected non-phototoxic compounds, using the CV80 calculated under irradiated condition as highest concentration. Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each data is mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test.

Reproducibility of data

To appreciate the variability among independent experiments performed on different days over one year period, we reported in Fig. 6 the IL-18 SI (I/NI) obtained for chloropromazine 1.25 µM (15 experiments), ketoprofen 2.5 µM (4 experiments) and 6-methylcoumarin 10 µM (5 experiments). The reason for which we tested these chemicals repeatidly was to identify a positive control to be used routinely in the assay. We initially tought to use chloropromazine 1.25 µM, but due to variability in the SI, we later decided to use ketoprofen or 6-methylcoumarin. IL-18 SI (I/NI) for chloropromazine 1.25 µM ranged from 1.25 to 7.7 with an average of 3.16; for ketoprofen 2.5 µM from 1.28 to 1.42 with an average of 1.33, and for 6-methylcoumarin 10 µM from 1.2 to 3.20 with an average of 1.76. As for the NCTC2544 IL-18 assay (Corsini et al., 2009 and Galbiati et al., 2011), the differences observed in the absolute value of IL-18 may be due to many factors including the number of cells seeded and adhering to plate wells, to the use of a different cell batch, the performance of IL-18 ELISA and protein assay as well as to differences in the chemical concentration (fresh solutions are used) and slight variation in the irradiation conditions. Despite the variability, the discriminatory capacities based on defined prediction model are, however, preserved.

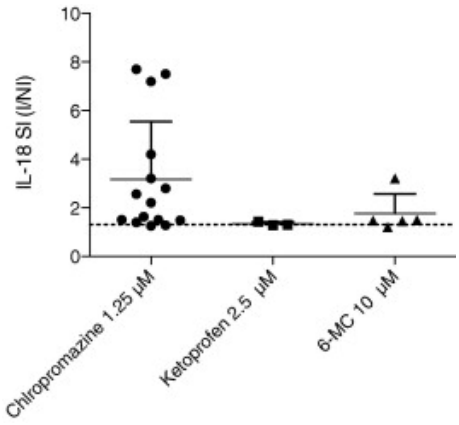


Fig. 6. Reproducibility of data. Results obtained in several independent experiments performed over one year period are reported. NCTC2544 cells were irradiated (I) at the UVA dose of 3.5 J/cm^2 or non-irradiated (NI) in the presence of chloropromazine 1.25 µM (15 experiments), ketoprofen 2.5 µM (4 experiments) and 6-methylcoumarin 10 µM (5 experiments). Results are expressed as IL-18 SI (I/NI). A dotted line was set at a SI of 1.3. Each dot represents an independent experiment. Mean ± SD is also reported.

Discussion

Given that there is no in vivo or in vitro methods developed which regulatory authorities to assess photosensitization induced by chemicals, in the present work, have adopted we describe the possibility to use the NCTC2544 IL-18 assay to identify in vitro photoallergens. We found that irradiated photoallergens induced a significant increase in intracellular IL-18 compared to photoirritants or not photoxic compounds. As provisional prediction model, based of the tested chemicals, an IL-18 SI (I/NI) > 1.3 was set as criteria for identification of photoallergens.

Due to their anatomical location and critical role in skin inflammatory and immunological reactions, the use of keratinocytes and skin organotypic culture as a simplified in vitro model to evaluate the potential toxicity of chemicals destined for epicutaneous application

is amply justified. In the present work, intracellular IL-18 was investigated after exposure of NCTC2544 cells to several photoallergens, and photoirritants. We showed that UVA irradiation of NCTC2544 cells treated with photoallergens results in a dose-related induction of intracellular IL-18, indicating the possibility to use IL-18 to specifically identify photoallergens and distinguish them from photoirritants.

As mentioned in the Introduction, IL-18 was chosen, as this cytokine is mainly associated with Th-1-mediated immune responses. Interestingly, it has been proposed that UV damage can be considered a "danger" signal. Recent data linking IL-1β and IL-18 production to "inflammasomes" supports this notion. IL-1β and IL-18 are normally expressed in human keratinocytes as an inactive precursor. Upon exposure to UV radiation, both precursors are cleaved by caspase-1 to yield active IL-1β and IL-18. Caspase-1 activation is dependent upon its recruitment to inflammasomes, which links UV-induced cytokine production to the innate immune system. Inflammasomes are composed of NOD-like receptor (NLR)-family proteins (Feldmeyer et al., 2007). These intracellular proteins contain a nucleotide-binding oligomerization domain called NACHT and several leucine rich repeat domains that may bind to microbial ligands similar to Toll-like receptor family proteins. The relationship between inflammasomes and UV-induced IL-1β and IL-18 production demonstrates that this pathway can also sense UV-induced tissue injury. Under our experimental condition, the UVA dose used alone didn't induce IL-18. Only the combination chemical (photoallergen) + UVA resulted in IL-18 induction. However, a similar mechanism of inflammasome activation is likely to be triggered by photoallergens. For allergens, we previously demonstrated a role for oxidative stress, NF-κB and p38 MAPK activation in PDD-induced IL-18 production (Galbiati et al., 2011), consistent with an inhibition of IL-18 transcription as in 5' upstream

region of the human IL-18 gene AP-1, PU.1, NF-κB and SP-1 transcription binding sites have been identified (Takeuchi et al., 1999).

A critical initial point in our study was the choice of UVA dose and chemical exposure conditions. Suitable irradiation conditions for the test were determined using chloropromazine as a representative photoallergen. This drug and several others related phenothiazines are known to cause both phototoxic and photoallergic reaction in the skin and eyes of patients. The original SOP developed for the NCTC2544 IL-18 assay had to be adapted to the UVA irradiation. A UVA dose of 3.5 J/cm^2 was chosen, and the CV80 calculated in irradiated condition was selected as the highest concentration.

We then investigated if commercially available photosensitisers could selectively up regulate IL-18 in human keratinocytes after UVA exposure. We could indeed demonstrate that compounds classified as photosensitisers or photoallergens/photoirritants have the ability to increase IL-18 intracellular levels in a dose related-manner, whereas photoirritants failed. A slight increase in IL-18 was observed with 8-methoxypsoralene and retinoic acid. It is important to mention that both chemicals are reported to possess photoallergenic effects. Recent literature and FDA-approved package for all-trans-retinoic acid products state explicitly that all-trans-retinoic acid is both a phototoxin and a photosensitiser (Fu et al., 2003, Moore, 2002, Tolleson et al., 2005 and Stein and Scheinfeld, 2007). Even if photoallergy to furocoumarins has rarely been reported, Bonamonte et al. (2010) clearly demonstrated photoallergic contact dermatitis to 8-methoxypsoralen. In the establishment of our prediction model, both compounds were, however, considered as photoirritants. Therefore, based on the effect on IL-18 observed for these two chemicals, the cut off of IL-

18 SI (I/NI) > 1.3 was set to identify and discriminate photoallergens from photoirritants. Based on human classification, an overall accuracy of 100 % of the proposed assay was obtained.

In conclusion, we propose a new reliable in vitro photosensitization assay using the keratinocyte cell line NCTC2544 and IL-18 production. The induction of IL-18 following UVA irradiation may represent a promising in vitro model for the screening and evaluation of potential photoallergens, reducing the risk of photoallergic reactions. Although we need to further confirm the applicability of the test evaluating a wider battery of chemicals, we consider that this assay may become a useful cell-based in vitro test for evaluating the photosensitizing potential of chemicals.

3.4 AN EPIDERMAL EQUIVALENT ASSAY FOR IDENTIFICATION AND RANKING POTENCY OF CONTACT SENSITISERS

Abstract

The purpose of this study was to explore the possibility of combining the epidermal equivalent (EE) potency assay with the assay which assesses release of interleukin-18 (IL-18) to provide a single test for identification and classification of skin sensitizing chemicals, including chemicals of low water solubility or stability. A protocol was developed using different 3D-epidermal models including in house VUMC model, epiCS® (previously EPICS® ™), MatTek EpiDerm™ and SkinEthic™ RHE and also the impact of different vehicles (acetone:olive oil 4:1, 1% DMSO, ethanol, water) was investigated. Following topical exposure for 24 h to 17 contact allergens and 13 non-sensitisers a robust increase in IL-18 release was observed only after exposure to contact allergens. A putative prediction model is proposed from data obtained from two laboratories yielding 95% accuracy. Correlating the in vitro EE sensitiser potency data, which assesses the chemical concentration which results in 50% cytotoxicity (EE-EC_{50}) with human and animal data showed a superior correlation with human DSA_{05} ($\mu g/cm^2$) data (Spearman r = 0.8500; P value (two-tailed) = 0.0061) compared to LLNA data (Spearman r = 0.5968; P value (two-tailed) = 0.0542). DSA_{05} = induction dose per skin area that produces a positive response in 5% of the tested population Also a good correlation was observed for release of IL-18 (SI-2) into culture supernatants with human DSA_{05} data (Spearman r = 0.8333; P value (two-tailed) = 0.0154). This easily transferable human in vitro assay appears to be very promising,

but additional testing of a larger chemical set with the different EE models is required to fully evaluate the utility of this assay and to establish a definitive prediction model.

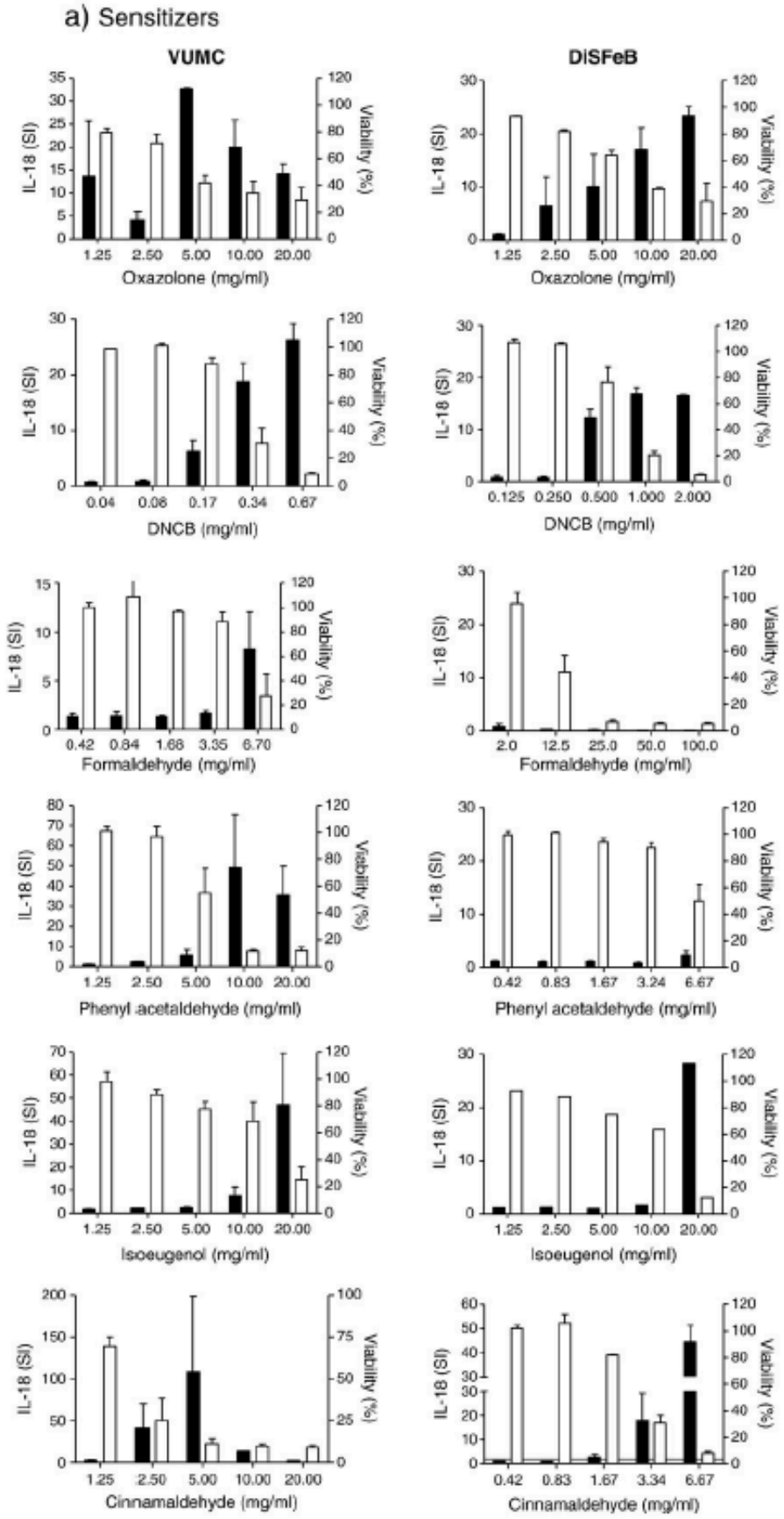
Dose dependent increase in IL-18 release by sensitisers but not non-sensitisers

A total of 17 sensitisers ranging from extreme, strong, moderate and weak and 10 non-sensitisers were selected for this study and tested using different EE models (Table 1). In the first series of experiments, Cell Systems epiCS® were exposed topically to 13 coded sensitisers and 9 non-sensitisers in a dose dependent manner for 24 h according to the EE potency assay protocol in two laboratories (VUMC, DiSFeB) (Teunis et al., 2013).

Chemical	CAS #	Category	Vehicle[a]			
			epiCS®	SkinEthic™ RHE	EpiDerm™	VUMC-EE
Oxazolone[b]	15646-46-5	Contact allergen	AOO			AOO
DNCB[b]	97-00-7	Contact allergen	AOO	EtOH/AOO/DMF	AOO/EtOH	DMSO
Formaldehyde[b]	50-00-0	Contact allergen	DMSO			
Isoeugenol[b]	97-54-1	Contact allergen	AOO			DMSO
Phenyl acetaldehyde [b]	122-78-1	Contact allergen	AOO			
Cinnamaldehyde[b]	104-55-2	Contact allergen	AOO		EtOH	DMSO
2-mercaptobenzothiazole[b]	149-30-4	Contact allergen	AOO		EtOH:DMSO 4:1	
α-hexylcinnamaldehyde[b]	101-86-0	Contact allergen	AOO			
Eugenol[b]	97-53-0	Contact allergen	AOO	EtOH/AOO/DMF	EtOH	DMSO
Benzocaine[b]	94-09-7	Contact allergen	AOO			
Citral[b]	5392-40-5	Contact allergen	AOO	EtOH/AOO/DMF		
Cobalt (II) chloride[b]	7646-79-9	Contact allergen	DMSO			
p-Phenylenediamine[b]	122-84-9 epiCS® 122-84-9 Epiderm™	Contact allergen	AOO		EtOH	
Cinnamic alcohol	104-54-1	Contact allergen			EtOH	
4-Nitro benzylbromide	100-11-8	Contact allergen			EtOH	
Glyoxal	107-22-2	Contact allergen			Water	
Resorcinol	108-46-3	Contact allergen			Water	
SDS	151-21-3	Irritant	DMSO		Water	
Octanoic acid	124-07-2	Irritant	AOO			
Lactic acid	50-21-5	Irritant	AOO		Water	
Phenol	108-95-2	Irritant	DMSO		Water	
Methyl salicylate	9041-28-5	Irritant	AOO			
Salicylic acid	69-72-7	Irritant	AOO			
Tween 20	9005-64-5	Irritant	AOO			
Chlorobenzene	108-90-7	Irritant	AOO			
Hydroxybenzoic acid	99-96-7	Irritant	AOO			
Glycerol	56-81-5	Non-irritant			None	

Table 1 Tested chemicals and vehicles. a The vehicles used in this study for dissolving chemicals before applying topically to EE. DMSO = 1% DMSO in culture medium; AOO = acetone: olive oil (4:1); EtOH = ethanol; Neat = 100%; Water = distilled H2O; n.d. = not determined; For details on vehicle and chemical suppliers, see Materials and Methods.b Tested coded at DiSFeB and VUMC in the EE potency assay.

Culture viability was determined by the MTT assay and IL-18 release into culture supernatants by ELISA. Of the 13 sensitisers and 9 non-sensitisers selected for this study, only 2 chemicals (cobalt II chloride and p-phenylenediamine (PPD)) had to be excluded due to their interference with the MTT colorimetric assay. For the remaining 11 sensitisers and 9 non-sensitisers, an increase in IL-18 release was generally observed only after exposure to sensitisers but not after exposure to non-sensitisers in both VUMC and DiSFeB laboratories (Figs. 1A and B). Results were further analyzed in order to identify a putative prediction model for identifying sensitisers.


a) Sensitizers
VUMC
DiSFeB
IL-18 (SI)
Viability (%)
Oxazolone (mg/ml)
DNCB (mg/ml)
Formaldehyde (mg/ml)
Phenyl acetaldehyde (mg/ml)
Isoeugenol (mg/ml)
Cinnamaldehyde (mg/ml)

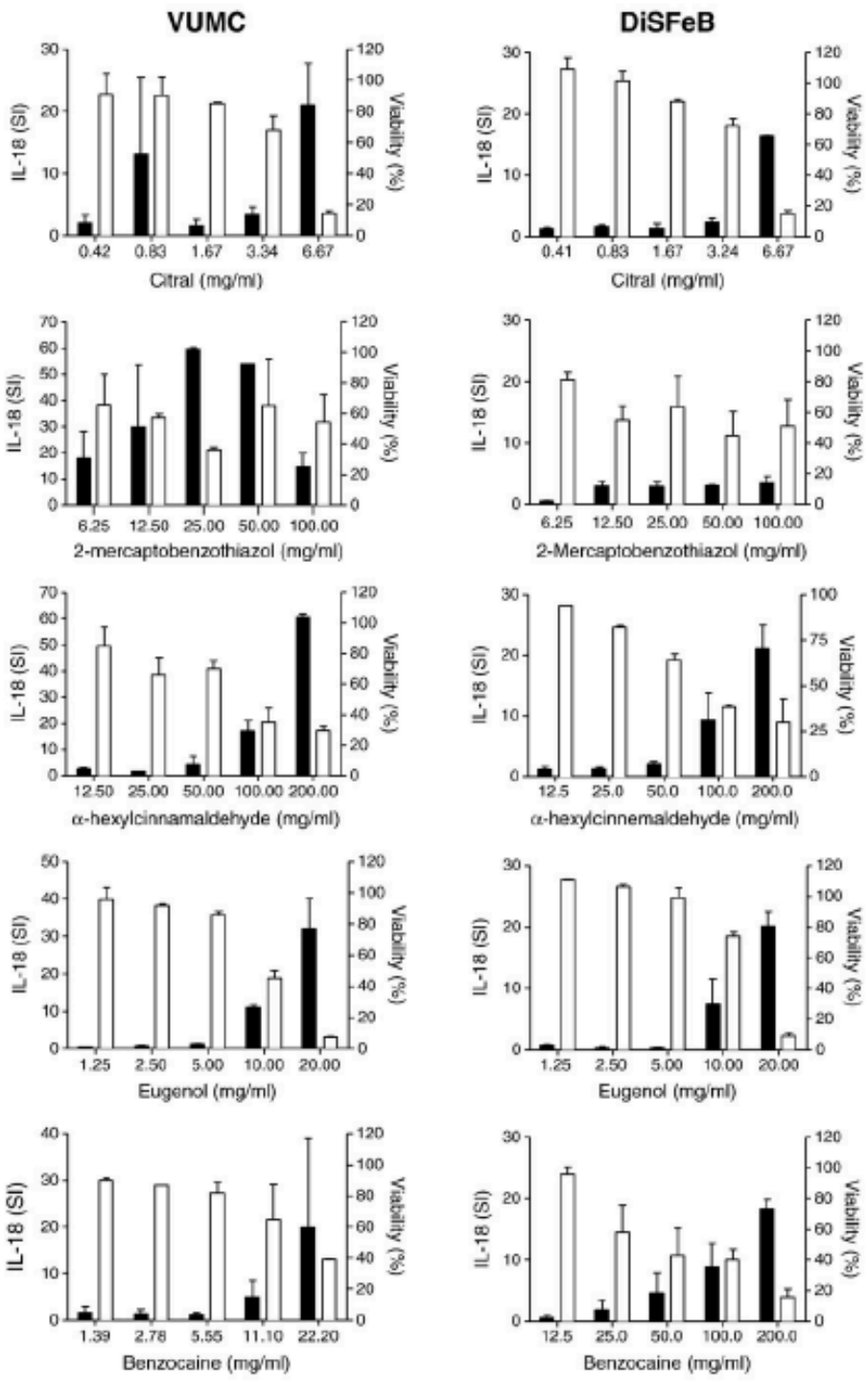


Fig. 1 (*continued*).

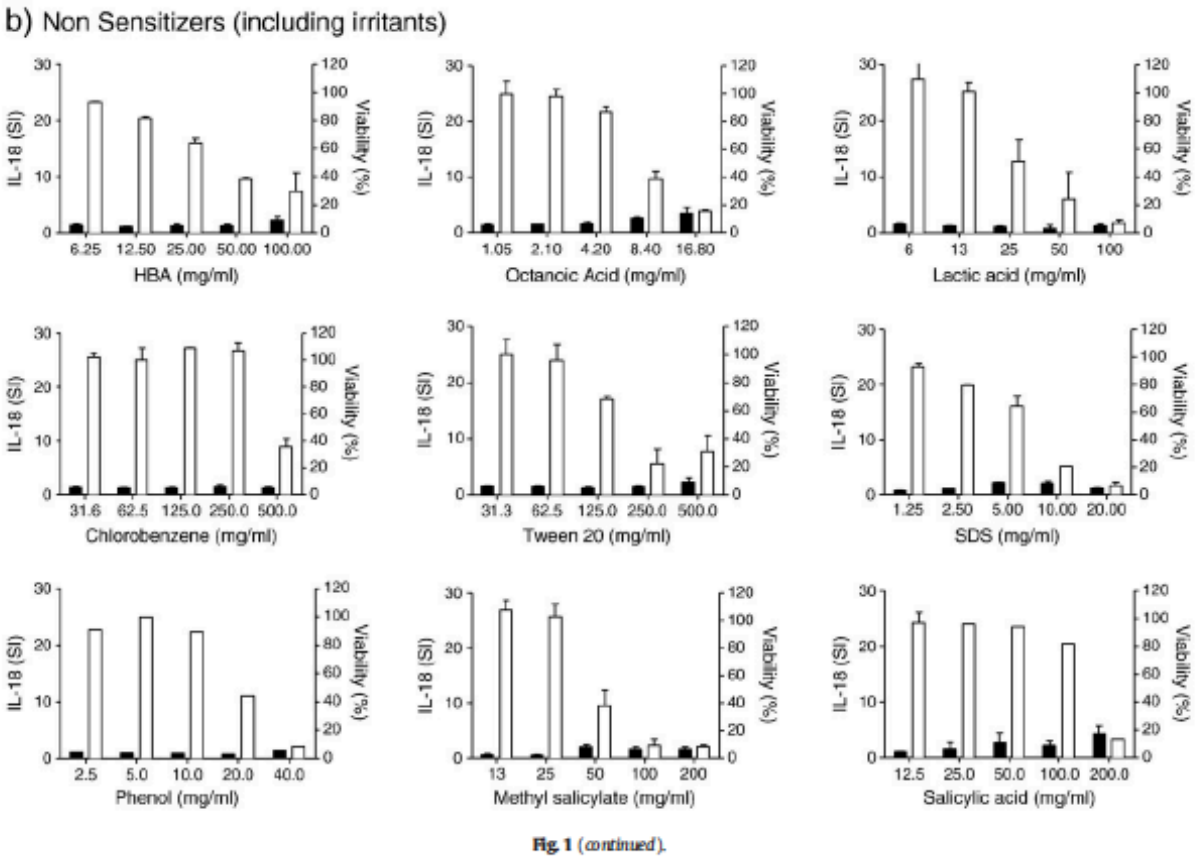


Fig. 1 (*continued*).

Fig. 1. Dose response effects of the selected sensitisers (A) and non-sensitisers (B) on IL-18 release and cell viability in the epiCS® EE model in VUMC and DiSFeB laboratories. EE were exposed to chemicals as described in Materials and Methods and Table 1. IL-18 was assessed by ELISA and results are expressed as stimulation index (SI) compared to vehicle alone. Cell viability was assessed by MTT reduction assay and is expressed relative to vehicle. Sensitisers were tested independently in duplicate in both laboratories. For non-sensitisers: SDS, lactic acid and methyl salicylate were tested at the VUMC laboratory whilst HBA, phenol, chlorobenzene, octanoic acid, tween 20 and salicyclic acid were tested at the DiSFeB laboratory.

Filled bars represent the IL-18 release, while open bars the viability. With the exception of isoeugenol in DiSFeB in which only 1 run was performed, results are expressed as average ± difference, n = 2 independent experiments using different batches of EE.

For epiCS®, three prediction models were tested (Table 2). Prediction model 1 (≥ 40% viability (EC40); ≥ 2 fold increase in IL-18): resulted in VUMC correctly identifying 10/11 sensitisers and DiSFeB correctly identifying 9/11 sensitisers. Only formaldehyde (VUMC and DiSFeB) and isoeugenol (DiSFeB) scored as a false negative in prediction model 1. With regards to the non-sensitisers VUMC correctly identified 2/4 chemicals and DiSFeB correctly identified 5/6 chemicals. Since it was noticed that in general IL-18 release was greater after sensitiser exposure at higher concentrations, prediction model 2 was next tested.

Chemical	Prediction model 1 IL-18 SI ≥ 2 at ≥ EC40		Prediction model 2 IL-18 SI ≥ 5 at ≥ EC5		Prediction model 3 IL-18 SI ≥ 5 at ≥ EC5 and ≤ EC40	
	VUMC	DiSFeB	VUMC	DiSFeB	VUMC	DiSFeB
Extreme						
oxazolone	4.1 ± 2.7	10.1 ± 8.7	32.7 ± 0.2	23.4 ± 2.4	32.7 ± 0.2	23.4 ± 2.4
DNCB	6.3 ± 2.9	12.3 ± 2.29	26.2 ± 4.2	16.8 ± 1.8	26.2 ± 4.2	16.8 ± 1.8
Strong						
formaldehyde	**1.7 ± 0.3**[a]	**0.35 ± 0.05**	8.2 ± 5.5	**0.9 ± 0.85**[a]	8.2 ± 5.5	**0.25 ± 0.05**
Moderate						
Phenyl acetaldehyde	5.8 ± 3.8	2.3 ± 1.1	35.6 ± 20.1	**2.3 ± 1.1**[b]	35.6 ± 20.1	(2.3 ± 1.1[b])
Isoeugenol	7.6 ± 5.4	**1.6**	47.0 ± 31.7	28.3	47.0 ± 31.7	28.3
Cinnamaldehyde	3.2 ± 1.12	2.9 ± 1.3	108 ± 128	44.4 ± 9.6	108 ± 128	44.4 ± 9.6
citral	3.4 ± 1.7	2.4 ± 0.9	21.0 ± 9.5	16.5 ± 0.1	21.0 ± 9.5	16.5 ± 0.1
2-mercaptobenzothiazol	54.0 ± 0.0	3.0 ± 1.3	59.7 ± 0.9	**3.5 ± 1.9**[b]	59.7 ± 0.9	(3.5 ± 1.9[b])
Weak						
α-hexylcinnamaldehyde	4.4 ± 4.3	2.1 ± 0.59	60.5 ± 2.0	21.1 ± 5.6	60.5 ± 2.0	21.1 ± 5.6
eugenol	11.1 ± 0.9	7.5 ± 5.7	32.1 ± 11.4	20.2 ± 3.4	32.1 ± 11.4	20.2 ± 3.4
Benzocaine	5.0 ± 4.9	8.9 ± 5.4	19.8 ± 27.2	18.4 ± 2.0	19.8 ± 27.2	18.4 ± 2.0
Non-sensitizers						
SDS	**2.2 ± 0.1**		2.2 ± 0.1		2.2 ± 0.1	
Octanoic acid		1.5 ± 0.8		3.4 ± 1.7		3.4 ± 1.7
Lactic acid	1.1 ± 0.3		1.5 ± 0.3		1.5 ± 0.3	
Phenol	**6.7 ± 3.1**	0.8 ± 0.0	**12.6 ± 5.6**	1.1 ± 0.1	**12.6 ± 5.6**	1.1 ± 0.1
Methylsalicylate	0.5 ± 0.2		2.1 ± 0.5		2.1 ± 0.5	
Salicylic acid		**2.2 ± 1.1**		4.3 ± 1.9		4.3 ± 1.9
Tween 20		1.2 ± 0.5		2.3 ± 1.0		2.3 ± 1.0
Chlorobenzene		1.5 ± 0.3		1.5 ± 0.3		1.5 ± 0.3
HBA		1.2 ± 0.3		2.3 ± 1.2		2.3 ± 1.2

Table 2. Prediction models for epiCS®. Highest SI level of IL-18 release observed within the defined criteria for the different prediction models (see also Fig. 1). For isoegenol, DiSFeB had data only from 1 run.

False positives and negatives in the predictionmodels are shown bold underlined. Values obtained from chemicals which do not fulfill the acceptance criteria of the predictionmodel are shown in brackets.

Predictionmodel 1: IL18 SI 2x at ≥ EC40, VUMChave 1 false negative and 2 false positives. DiSFeB has 2 false negatives and 1 false positive. ThemaximumIL-18 release (mg/ml) observed in the dose response (see Fig. 1) at a cell viability ≥ 40% relative to the vehicle is shown. Prediction model 2: IL-18 SI 5x at ≥ EC5,VUMC has in only 1 false positive. DiSFeB has 3 false negatives. ThemaximumIL-18 release (mg/ml) observed in the dose response (see Fig. 1) ata cell viability ≥ 5% relative to the vehicle is shown.

Predictionmodel 3: IL-18 SI 5 x at ≥ EC5 and ≤ 40, VUMChas only 1 false positive and DiSFeB has 1 false negative. ThemaximumIL-18 release (mg/ml) observed in the dose response (see Fig. 1) at a cell viability ≥ 5% and ≤ 40% relative to the vehicle is shown. With the exception of isoeugenol in DiSFeB in which only 1 run was performed, results are expressed as average ± difference, n = 2 independent experiments using different batches of EE.

a Viability ≥ 90%.

b Viability is N 50%, EC5 and EC40 = 5% and 40% cell viability respectively.

Prediction model 2 (≥ 5% viability (EC5); ≥ 5 fold increase in IL-18) resulted in the VUMC correctly identifying all sensitisers and DiSFeB correctly identifying 8/11 sensitisers. It should be noted though that the 2/3 false negatives in prediction model 2 in DiSFeB corresponded to dose response curves in which very little cytotoxicity was observed. EE were still > 40% viable after exposure to phenylacetone aldehyde and 2-mercaptobenzothiazol. Formaldehyde was the third false negative scored in DiSFeB even though high levels of cytotoxicity were reached. With regards to the non sensitisers VUMC correctly identified 3/4 non sensitisers and DiSFeB correctly identified all 6 non-sensitisers. These results led to the testing of prediction model 3 in which the greater release of IL-18 within a defined cytotoxicity range was tested.

Prediction model 3 (≥ 5% and ≤ 40% viability (EC5-40); ≥ 5 fold increase in IL-18 release: resulted in exclusion of the 2 false negatives from DiSFeB as critical cytotoxic levels were not reached and therefore all sensitisers with the exception of formaldehyde in DiSFeB were now correctly identified in both laboratories. With regards to non-sensitisers, the results were the same as those obtained in prediction model 2 namely VUMC correctly identified 3/4 non sensitisers and DiSFeB correctly identified all 6 non-sensitisers. Therefore for chemical exposures which fulfilled the criteria for prediction model 3, all but one chemical in each of the laboratories were correctly identified (phenol scored false positive in VUMC, and formaldehyde scored false negative in DiSFeB).

Prediction model 3 using epiCS® was therefore considered the most optimal putative prediction model since it showed 95% accuracy in both VUMC and DiSFeB laboratories. Of note, this prediction model is dependent on the chemical resulting in high cytotoxicity (5–40% viability; 60–95% cytotoxicity) in the EE.

In parallel, at MB Research Labs and MatTek Corp, 9 contact sensitisers (DNCB, NBB, PPD, CA, C-OH, GXL, RES, MBT, and EUG) and 3 non sensitisers (PHL, LAC, and GLC) were tested using the EpiDerm™ EE model (Fig. 2). Test chemicals were dissolved in a vehicle of either ethanol or water. Ethanol alone caused an IL-18 secretion of 12.5 and 15.1 pg/ml, and water caused secretion of 5.7 and 14.1 pg/ml, respectively for MB Research Labs and MatTek Corp. In support of the above data, sensitiser exposure resulted in a measurable increase of up to 40 fold in IL-18 release over vehicle controls. Using a 'Prediction Model 1.6x' (optimally between 5% and 50% viability (EC5-50); coupled with a ≥ 1.6 fold increase in IL-18 release as indicative of a positive sensitiser response), 9/9 sensitisers tested at MB Research Labs were correctly identified while at MatTek 8/9 sensitisers were correctly identified (RES was a false negative).

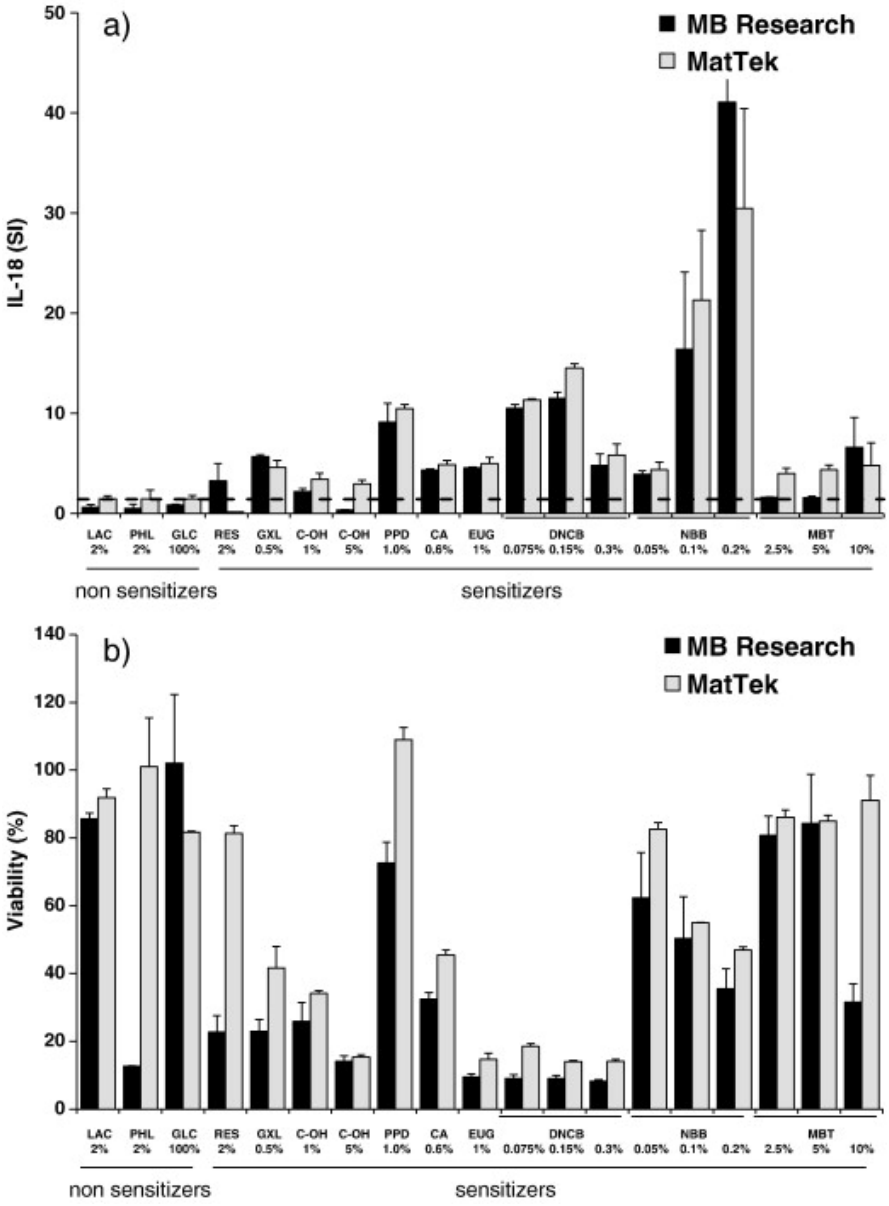


Fig. 2. IL-18 secretion and tissue viability using EpiDerm™.

EE were exposed to chemicals as described in Materials and Methods and Table 1. MatTek Corporation and MB Research Labs independently tested IL-18 secretion in duplicate EpiDerm™ tissues. Test chemicals were dissolved in a vehicle of either ethanol or water, with the exception of MBT which was dissolved in 25 ul of EtOH:DMSO 4:1. After 24 h topical exposure to chemicals, culture supernatant was analyzed by IL-18 ELISA, and an SI was calculated as a fold change above vehicle control (Panel A). An IL-18 SI value of 1.6 or above is the criterion for a positive response is indicated by the dotted line. Tissue viability was determined by MTT assay (Panel B) The figures depict average ± difference of duplicate EE within a single experiment in each laboratory.

With regards to the 3 non-sensitisers, at the single chemical concentration tested (LAC 2%, PHL 2% and GLC 100%), no chemical exposure resulted in a ≥ 1.6-fold increase in IL-18, and therefore all non-sensitisers at these concentrations were correctly predicted as negative. Taken together our results highlight the advantages of the dose response and demonstrate the ability of the IL-18 endpoint to identify sensitisers in the epiCS® and EpiDerm™ 3D-epidermal models.

Epidermal equivalent EC_{50} values rank sensitiser potency

Next it was determined whether the EC_{50} values identified from the 11 coded sensitisers in the dose response experiments, which were used to quantify IL-18 release in the VUMC and DiSFeB laboratories, could be used to rank sensitiser potency. Table 3 shows that both laboratories obtained EC_{50} values of similar orders of magnitude. Extreme/strong sensitisers classified according to LLNA generally had a lower EC_{50} value than weak sensitisers. EC_{50} values for the moderate sensitisers generally ranged between the weak and strong sensitisers (Table 3). When results of both laboratories were combined and a correlation was performed with the LLNA and human DSA_{05} data, ranking according to the EE potency assay was found to significantly correlate to the ranking obtained by the LLNA (Spearman r = 0.5968; P value (two-tailed) = 0.0542) and to human DSA_{05} data (Spearman r = 0.8500; P value (two-tailed) = 0.0061) (Fig. 3A; Table 3). Of note, correlation between the EE-EC_{50} data and human DSA_{05} data was clearly better than between EE-EC_{50} data and LLNA data.

Next we determined whether the IL-18 SI-2 and SI-5 could also be used to rank sensitiser potency. As can be seen in Figs. 3B and C, the correlation of our in vitro human data was

again better with human data than with LLNA data. Indeed the correlation SI-2 with human DSA_{05} data was Spearman r = 0.8333; P value (two-tailed) = 0.0154 and the correlation SI-5 with human DSA_{05} data was Spearman r = 0.6267; P value (two-tailed) = 0.0603 indicating that IL-18 release may supply an additional potency parameter in this assay.

Fig. 3. Correlation of EE assay with $LLNA_{EC3}$ and human DSA_{05}. A) Correlation of EE-EC_{50} data with $LLNA_{EC3}$ and human DSA_{05}. B) Correlation of IL-18 SI-2 data with $LLNA_{EC3}$ and human $DSA_{05.}$ C) Correlation of IL-18 SI-5 data with $LLNA_{EC3}$ and human DSA_{05}.

$LLNA_{EC3}$ and human DSA data is extracted from Table 3. In vitro data is obtained by linear regression analysis based on changes in metabolic activity (MTT) (A) or IL-18 fold release into culture supernatant (B, C). Statistical significances were determined by Kruskal–Wallis (nonparametric, one way ANOVA) using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant for $p < 0.05$.

EE–EC_{50} data represents the average of the combined data for epiCS® obtained from 2 laboratories (VUMC and DiSFeB) n = 4 ± SD: dotted line shows visual line of equality.

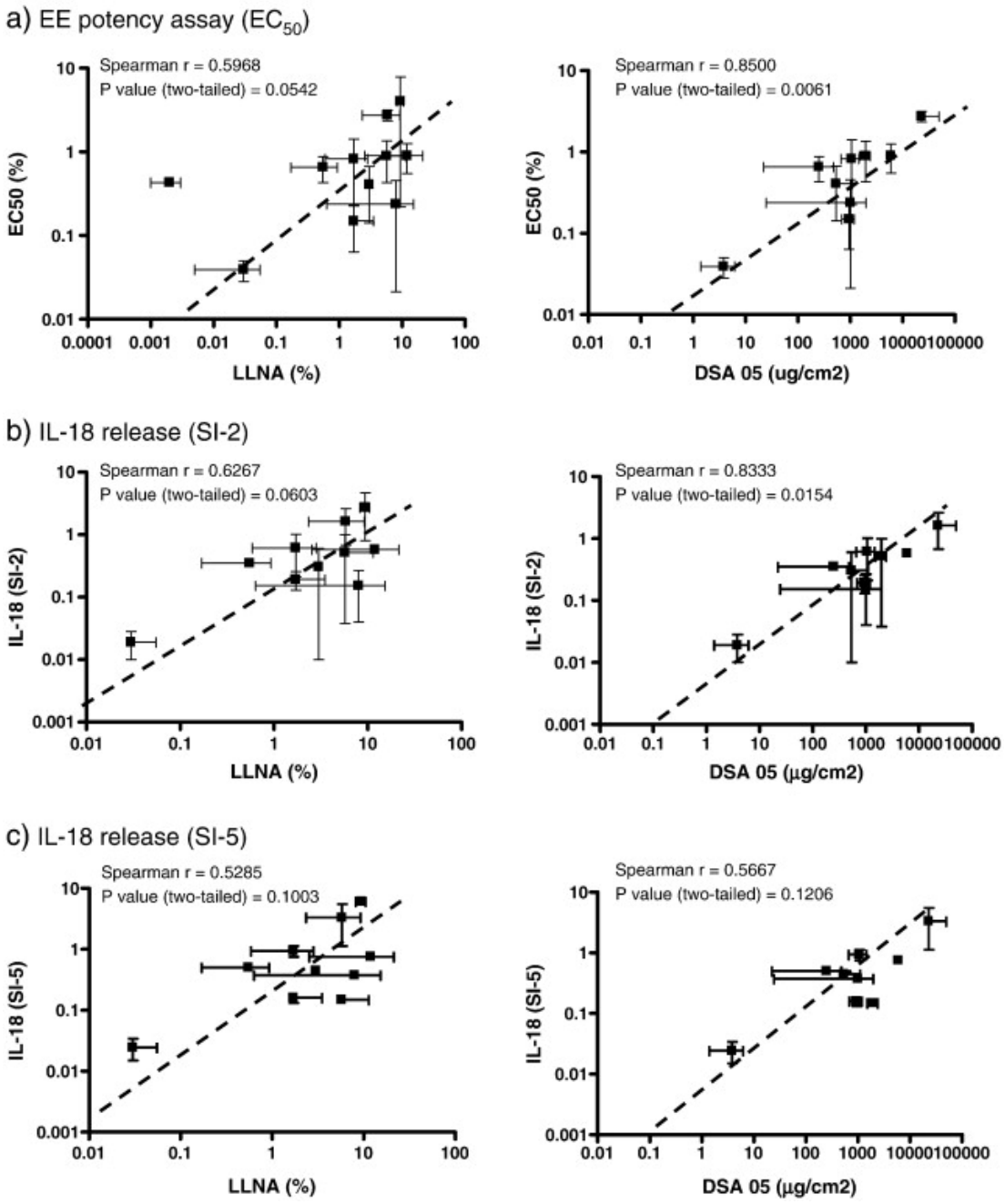

a) EE potency assay (EC50)
Spearman r = 0.5968
P value (two-tailed) = 0.0542
EC50 (%)
LLNA (%)
Spearman r = 0.8500
P value (two-tailed) = 0.0061
EC50 (%)
DSA 05 (ug/cm2)
b) IL-18 release (SI-2)
Spearman r = 0.6267
P value (two-tailed) = 0.0603
IL-18 (SI-2)
LLNA (%)
Spearman r = 0.8333
P value (two-tailed) = 0.0154
IL-18 (SI-2)
DSA 05 (µg/cm2)
c) IL-18 release (SI-5)
Spearman r = 0.5285
P value (two-tailed) = 0.1003
IL-18 (SI-5)
LLNA (%)
Spearman r = 0.5667
P value (two-tailed) = 0.1206
IL-18 (SI-5)
DSA 05 (µg/cm2)

Figure 3

Substance	$LLNA_{EC3}$ (%)	HumanDSA_{05} (μg/cm²)	EE–EC_{50} (mg/ml)			
			VUMC-EE	epiCS®		
				VUMC	DiSFeB	average
Extreme						
oxazolone	0.001–0.003	–	13.1	1.5 ± 0.2	7.4 ± 1.0	4.45 ± 2.95
dinitrochlorobenzene	0.012–0.047	2.1–5.5	1.13	0.3 ± 0.0	0.7 ± 0.2	0.5 ± 0.2
4-nitrobenzylbromide	0.05	–	0.70			
Strong						
p-phenylenediamine	0.11–2.5	6.9–345	NR	NR	NR	
Formaldehyde	0.28–0.82	89–411		5.7 ± .9.0	11.8 ± 3.52	8.76 ± 3.05
Phenyl acetaldehyde	3	133–938		5.7 ± 1.7	7.7 ± .5.0	6.7 ± 1.0
Cinnamaldehyde	0.42–3.0	157–1111	19.9	1.9 ± 0.1	2.8 ± 0.4	2.35 ± 0.45
Cobalt chloride	–	172–453		NR	NR	
Moderate						
Citral	2.75–13.2	310–1691		4.4 ± 0.6	4.7 ± 0.2	4.55 ± 0.15
Glyoxal	0.60–1.4	345	120.7			
Isoeugenol	0.92–2.50	775–1333	5.5	15.8 ± 1.3	28.6 ± 16.5	22.2 ± 6.4
2-bromo-2-(bromomethyl) pentanedinitrile	1.3	–	7.6			
2-mercatobenzothiazol	1.7–9.7	1642–2269	No EC_{50}	16.6 ± 1.4	13.8 ± 4.4	15.2 ± 1.4
Tetramethylthiuram disulfide	5.2–5.92	3832–5388	No EC_{50}			
Resorcinol	5.5	–	26.4			
Weak						
Eugenol	5.3–18.16	5926	44.6	8.6 ± 2.1	16.4 ± 0.5	12.5 ± 3.9
Cinnamic alcohol	21	625–13122	8.6			
Benzocaine	3.37–22	3831–41667	–	14.8 ± 7.0	48.1 ± 41.5	31.45 ± 16.65
α-hexylcinnamaldehyde	8.6–10.14	–		80.0 ± 15.5	76.9 ± 2.8	78.45 ± 1.55

Table 3 comparison of LLNAEC3, human DSA05 and EE-EC50 values for all sensitisers tested in EE potency assay using VUMC-EE and epiCS®.Table 3 shows all EC50 data obtained until present for the EE potency assay derived from this manuscript and dos Santos et al. (2011). Human DSA05 (**μ**g/cm2) = induction dose per skin area (DSA) that produces a positive response in 5% of the tested population.

The LLNAEC3 values expressed as% and its relative potency classification in parenthesis are reported. According to Basketter et al. (1999), potency classification is based on themathematical estimation of the concentration of chemical necessary to obtain a threshold positive response (SI = 3); this is termed the EC3 value. Chemicals with an EC3 value (%) N 10 to b 100 are classified as weak, N1 to b 10 moderate, N0.1 to b 1 strong, b0.1 extreme. For LLNA and human data: – indicates no data available; for EE–EC50: NR = indicates interference with MTT assay therefore no reliable EC50 obtained; No EC50 = due to lowsolubility of the chemical no EC50 was obtained (dos Santos et al., 2011).

Sensitisers are ranked taking into account ranges obtained from both LLNA and human DSA05 data from the following studies: (Danneman et al., 1983; Friedmann et al., 1983; Gerberick et al., 2005; Griem et al., 2003; ICCVAM, 2011a, 2011b, 2011c; Jordan and King, 1977; Kligman, 1966a, 1966b; Marzulli and Maibach, 1976; Rees et al., 1989; Schneider and Akkan, 2004; Steltenkamp et al., 1980).

Transferability of EE potency assay to different EE models and effect of different vehicles

Transferability of EE potency assay to different EE models

In this study, epiCS® was extensively used to further investigate the EE potency assay using coded sensitisers and to determine whether this assay could be combined with an IL-18 readout to identify sensitisers. In order to determine how well the results from one EE type correlate to the results obtained from another EE type, the results from this EE potency study were superimposed onto our previously described results using the VUMC-EE. The results shown in Fig. 4 indicate that very similar LLNA correlations are obtained for both EE derived from primary human keratinocytes. Oxazolone was a typical outlier when using either EE model. An overall ranking using the combined results of the both EE and 16 chemicals (including oxazolone) was found to significantly correlate to the ranking obtained by the LLNA (Spearman r = 0.52; P value (two-tailed) = 0.02) and was similar to that obtained by either EE alone (epiCS®: Spearman r = 0.60; P value (two-tailed) = 0.06; VUMC-EE: Spearman r = 0.58; P value (two-tailed) = 0.08). This indicates not only that the assay is fully transferable to different EE models but that the results obtained from all different EE may be superimposed onto a single sensitiser ranking correlation graph if identical methodology is used.

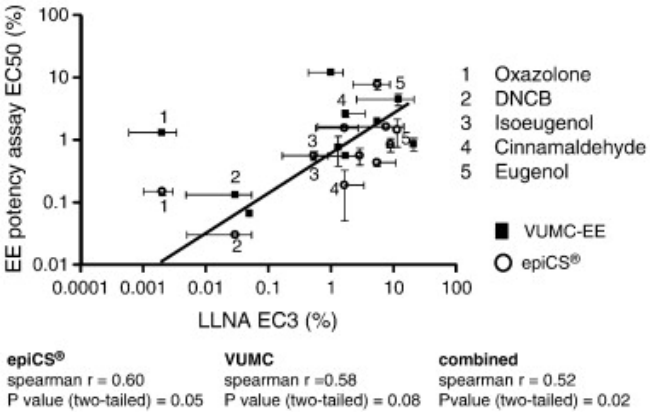


Fig. 4. Correlation EE–EC_{50} from VUMC-EE and epiCS® with $LLNA_{EC3}$ (%).
Data obtained from this study using epiCS® are plotted together with previous published data using VUMC-EE (dos Santos et al., 2011) and correlated to LLNA data. Five chemicals are indicated which were tested in both VUMC-EE and epiCS®.

The effect of vehicles on different EE viability

Next we compared the effect of three different commonly used vehicles on three different commercially available EE (epiCS®, SkinEthic™ RHE, MatTek EpiDerm™) and one in house EE (VUMC-EE). As can be seen from Fig. 5A, epiCS® and SkinEthic™ RHE produced very similar results after a 24 h exposure to AOO resulting in less than 10% decrease in cell viability and DMSO resulting in approximately 20% decrease in cell viability. For the VUMC-EE less than 10% decrease in cell viability was observed after a 24 h topical exposure to either water, AOO or DMSO. For EpiDerm™ approximately 10% decrease in cell viability was observed after a 24 h topical exposure to either water or AOO. For all EE, the potential cytotoxic effect of the vehicles was within the acceptance criteria of < 30% previously proposed for the EE potency assay (Teunis et al., 2013) again supporting the ease of technology transfer of the assay.

Effect of different vehicles and application methods on sensitiser induced IL-18 release

Finally we determined whether different vehicles and application methods influenced the IL-18 readout of the assay. DNCB (150 µg) was topically applied to EpiDerm™ directly in 25 µl of ethanol or 100 µl of AOO (1:20), or by use of a filter disc (25 µl in AOO 4:1). 50 µg of SDS (100 µl in water or 25 µl on a filter paper in water) was applied as reference irritant (Fig. 5B). EpiDerm™ were then incubated for 24 h. At similar cytotoxicity (empty columns), DNCB induced a higher release of IL-18 compared to tissues treated with SDS (SI > 15) as expected independent of the method used for application. However the IL-18 release induced by DNCB was higher if AOO (either directly applied on EE or on filters) was used as vehicle when compared to EtOH. This was later further investigated by MB research using SkinEthic™ RHE with three different sensitisers (DNCB, citral and eugenol) and 3 different vehicles (EtOH, AOO and DMF) (Fig. 5C). In SkinEthic™ RHE, release of IL-18 was similar when either EtOH or AOO was used for vehicle for a given sensitiser. However when DMF was used as vehicle, IL-18 secretion was clearly less. This observation was independent of the degree of cytotoxicity since EtOH and DMF exhibited similar cytotoxicity for each of the sensitisers. AOO appeared to have the least cytotoxic effect on EE when used as vehicle for citral and eugenol. Taken together these results indicate that the choice of vehicle may influence both the amount of IL-18 secretion and the cytotoxicity induced by the chemical together with a vehicle.

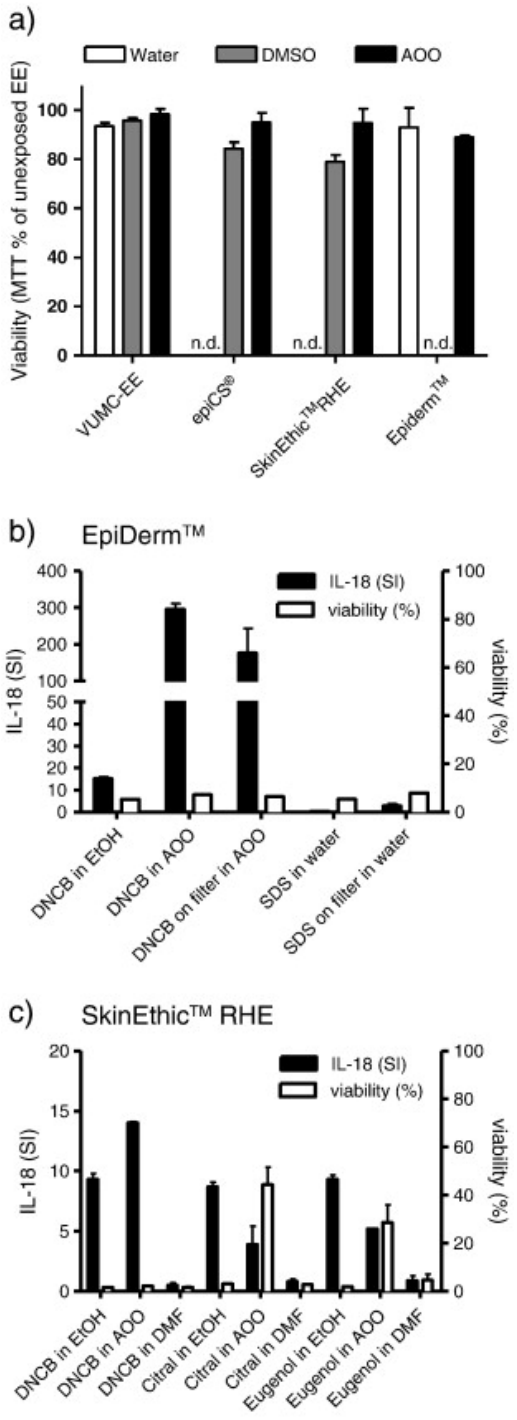

a)
Water
DMSO
AOO
Viability (MTT % of unexposed EE)
n.d.
n.d.
n.d.
VUMC-EE
epiCS®
SkinEthic™RHE
Epiderm™
b)
EpiDerm™
IL-18 (SI)
viability (%)
DNCB in EtOH
DNCB in AOO
DNCB on filter in AOO
SDS in water
SDS on filter in water
c)
SkinEthic™ RHE
DNCB in EtOH
DNCB in AOO
DNCB in DMF
Citral in EtOH
Citral in AOO
Citral in DMF
Eugenol in EtOH
Eugenol in AOO
Eugenol in DMF

Figure 5

Fig. 5. (p. 149) Effects of different vehicles on cell viability and IL-18 release in different EE models. (A) Effects of water, DMSO and AOO (applied in filter paper disc) on cell viability after 24 h of exposure in different EE models, namely VUMC-EE, epiCS®, SkinEthic™ RHE and EpiDerm™. (VUMC-EE: Water n = 15; DMSO n = 20; AOO n = 17; epics®: DMSO n = 14; AOO n = 19; SkinEthic™ RHE: DMSO n = 13; AOO n = 7; mean ± SEM is shown). (B) Comparison of the effects of different vehicles (AOO, ethanol, water) and mode of application (with or without filter paper disc) on DNCB and SDS-induced IL-18 release and cell viability in EpiDerm™ EE model. Results are expressed as average ± difference, n = 2 independent experiments. (C) Comparison of the effects of different vehicles (AOO, ethanol, water, DMF) on 0.15% DNCB, 0.3% citral and 1.0% eugenol induced IL-18 release and cell viability in SkinEthic™ RHE EE model performed at MB Research. Results are expressed as average ± difference, n = 2 replicates from a single experiment.

Discussion

The goal of this study was to determine whether i) IL-18 released into the culture supernatant of EE models could be used to distinguish sensitisers from non-sensitisers and ii) whether the assay protocol could successfully rank potency of sensitisers. Results obtained in this study indicate that indeed this single in vitro human assay may be able to distinguish sensitisers from non-sensitisers as well as rank sensitisers according to potency. The use of reconstituted epidermis overcomes the limitation of chemical solubility and

stability in water solution, which are the major limitations of many in vitro methods based on the use of submerged cultures. Remarkably consistent results were obtained between the different 3D models, indicating the assays robustness and ease to transfer to other laboratories. It is probable that minor method optimization may be required for different types of commercially available EE and different vehicles.

Due to their anatomical location and critical role in skin inflammatory and immunological reactions, the use of the KC and skin organotypic cultures as a simplified in vitro model to evaluate the potential toxicity of chemicals destined for epicutaneous application is amply justified. The proposed method is mechanistically anchored, as IL-18 has been shown to play a key proximal role in the induction of allergic contact sensitization (Antonopoulos et al., 2008; Okamura et al., 1995). The possibility of using IL-18 for the in vitro identification of contact allergens is not novel; as we have previously demonstrated in a human keratinocyte cell line (NCTC2544) a selective up-regulation of intracellular IL-18 by contact allergens at cell viability $\geq$ 80%, whereas irritants and respiratory allergens failed. A sensitivity of 87%, specificity of 95% and an accuracy of 90% was reported (Corsini et al., 2009, Corsini et al., 2012 and Galbiati et al., 2011). In addition to requiring the analysis of intracellular IL-18 production rather than release of IL-18, the NCTC2544 assay is proving difficult to transfer to different laboratories since it is dependent on critical culture conditions of the NCTC cells (culture time after thawing, seeding density, degree of confluency) and has a cut-off for discriminating sensitisers from non-sensitisers at only 1.2 fold increase in intracellular IL-18 (Corsini et al., 2012). This indicates that considerable cell culture know-how is required to perform the assay and that each laboratory needs to optimize the assay independently. Replacing NCTC2544 cell line with a standardized commercially available EE as described in

this manuscript makes the assay extremely transferable and easy to perform for any laboratory with standard cell culture facilities.

In all EE models, we found a robust release of IL-18 only after treatment with contact allergens. Cytotoxicity was essential to observe the release of intracellular IL-18 into culture supernatants. At a similar degree of cytotoxicity only contact allergens, and not irritants, generally induced a dramatic increase in IL-18 release. This is consistent with prior neosynthesis and intracellular accumulation only after sensitiser exposure, and is in line with our previous findings (Corsini et al., 2009). Our observation that cytotoxicity is required indicates that stored intracellular IL-18 is released into the supernatant rather than active secretion of IL-18 and again is in line with previous findings that it is intracellular production rather than secretion, which distinguishes sensitisers from non sensitisers. Increasing the chemical concentration to toxic concentrations enables intracellular protein to be released via membrane porosity rather having to introduce an alternative lysis method as is done in the NCTC assay.

In prediction model 2 a number of false negatives and positives were observed which led to the final proposed prediction model 3. In prediction model 2 (≥ 5% viability (EC5); ≥ 5 fold increase in IL-18) 3 false negatives were observed in DiSFeB and 1 false positive in VUMC. Two of the false negatives in DiSFeB corresponded to dose response curves in which very little cytotoxicity was observed. EE were still > 40% viable after exposure to phenyl acetaldehyde and 2-mercaptobenzothiazol. Formaldehyde scored as the third false negative in DiSFeB even though high levels of cytotoxicity were reached. A possible explanation for this may be that too much cytotoxicity may have resulted in fixation of the tissue preventing

IL-18 release, or in diffusion of formaldehyde into the culture supernatant resulting in denaturation of proteins which in turn may have affected the ELISA. The dose range in the DiSFeB dropped rapidly from 44.5 ± 17.53% viability to 7.15 ± 1.55% viability in which the first viability value may have been too high and the next viability value too low. Noticeably, the concentrations of formaldehyde used by DiSFeB were considerably higher than those used by VUMC. With regards to the non-sensitisers VUMC correctly identified 3/4 non-sensitisers and DiSFeB correctly identified all 6 non-sensitisers. The false positive phenol in the VUMC may possibly be due to extended storage of this chemical resulting in partial oxidation. These results taken together led to the testing of prediction model 3 in which a greater release of IL-18 within a defined cytotoxicity range was tested. Prediction model 3 (≥ 5% and ≤ 40% viability (EC5-40); ≥ 5 fold increase in IL-18 release) resulted in exclusion of the 2 false negatives from DiSFeB as critical cytotoxic levels were not reached. For chemical exposures which fulfilled the criteria for prediction model 3, all but one chemical in each of the laboratories were correctly identified (phenol scored false positive in VUMC, and formaldehyde scored false negative in DiSFeB) resulting in 95% accuracy in both VUMC and DiSFeB laboratories. Of note, this prediction model is dependent on the chemical resulting in high cytotoxicity (5–40% viability; 60–95% cytotoxicity) in the EE.

In this study, a previously defined standard operating procedure for the EE potency assay was used (Teunis et al., 2013). The procedure defines how a dose finding is performed for any unknown coded chemical (of unknown molecular mass) in order to obtain an EE-EC_{50} value. Our results show that by following this procedure not always similar dose responses were obtained between the two laboratories and when difference were observed this generally corresponded with the variation in IL-18 release between the laboratories (e.g.

formaldehyde and phenyl acetaladehyde). Minor modifications to the procedure will be expected to remedy this in the future.

Correlation of the EE-EC_{50} values obtained from the 11 chemicals in two different laboratories showed very good and statistically significant correlation with both LLNA and human DSA_{05} data. Interestingly the correlation was much better with the human data (Spearman r = 0.8500; P value of 0.0061) than with mouse LLNA data (Spearman r = 0.5968; P value of 0.0542) indicating that our human in vitro EE assay ranks human sensitiser potency more accurately than the mouse assay. It should be mentioned though that these values may be biased slightly as no human data is available for correlation for the chemical oxazolone which clearly deviates in the LLNA correlation. However, our finding that human in vitro assays may prove superior to current animal LLNA in predicting human sensitization is further supported by the findings of Schneider and Akkan (2004) who reported that when comparing human and animal data for 46 substances a correlation of only r = 0.77 (HRPIT vs LLNA data) and r = 0.65 (HMT vs LLNA data) was obtained (Schneider and Akkan, 2004).

With regards to assessment of sensitiser potency in the NCTC2544 assay, there is a general trend for IL-18 induction at lower concentrations for extreme/strong sensitisers, whereas higher concentrations are required in the case of weak sensitisers (Corsini et al., 2012). In this study we show for the first time that in addition to the EE–EC_{50}, release of IL-18 into culture supernatants of EE may possibly also be used to assess sensitiser potency. Indeed our correlation results of IL-18 SI-2 and SI-5 with human data strongly warrant further testing with extended chemical panels.

Importantly the EE-potency assay is not only transferable between laboratories but also

between different types of EE. Results obtained from the $LLNA_{EC3}$–EE–EC_{50} correlation in this study using epiCS® could be superimposed onto the correlation graph obtained with VUMC-EE (dos Santos et al., 2011). Even the combined correlation showed a significant Spearman r = 0.52; P value (two-tailed) = 0.02 with the limited number of chemicals tested including the outlier oxazolone. Further experiments are required to determine whether indeed a single correlation graph can be used in the future independent of the type of EE used to assess sensitiser potency. However it is possible that at least minor differences in exposure times and/or concentrations will need to be optimized among different EE models. For example, EpiDerm™ will be expected to perform slightly differently than epiCS® or SkinEthic™ RHE due to differences in the properties of the stratum corneum barrier between these models. This is illustrated in the OECD validated skin irritation assay where the treatment time for inducing irritation is 15 min followed by 42 h post-incubation in the EpiSkin model whereas the protocol required optimization for SkinEthic™ RHE (42 min followed by a 42 h post-incubation) and EpiDerm™ (60 min followed by a 42 h post-incubation) (Alepee et al., 2010, Kandarova et al., 2009 and Kidd et al., 2007) (see also OECD TG 439; Adopted: 22 July 2010).

In addition to comparing different EE, different vehicles and application procedures were compared. Our preliminary results indicate that the choice of vehicle can greatly influence the result obtained. A lower IL18 secretion was observed when the vehicle ETOH was used rather than AOO. This is an area which clearly requires further investigation using the single protocol developed in this study with epiCS® and is also mirrored in the results reported from in vivo studies. The broad range of $LLNA_{EC3}$ values and human DSA_{05} values shown in Table 3 are due in part to the different vehicles used in the different studies. The vehicle

effect is thought to differ due to the following two reasons: 1) the vehicle will influence the ability of the test substance to traverse the stratum corneum and reach the viable epidermis and 2) the vehicle will influence the efficiency of Langerhans cell migration from the skin (Basketter et al., 2001, Lea et al., 1999, McGarry, 2007 and Wright et al., 2001). The former of these two points is of particular relevance to the EE assay described in this manuscript. In contrast, the means by which the chemical was applied: direct chemical application or filter paper application did not appear to influence the result.

In conclusion, using a single standard operating procedure we have developed a putative prediction model in two different laboratories (VUMC and DiSFeB) using two different types of EE (epiCS® and VUMC-EE). Our results indicate that only minor modifications in the assay methodology and prediction model are required to optimize the assay read-out for the different commercially available EE (eg. SkinEthic™ RHE, EpiDerm™). Factors which may vary between different EE are the optimal duration and method of chemical exposure (direct vs via filter paper disc), the type of vehicle used, the optimal cytotoxicity margin to insure maximum release of IL-18 and the IL-18 induction factor to distinguish a sensitiser from a non sensitiser. Our first results do show that this simple, easily transferable EE assay may enable sensitisers to be distinguished from non-sensitisers (IL-18 release: ELISA) and also rank sensitisers according to potency better than the current LLNA (EC_{50} value: MTT assay; IL-18 SI-2: ELISA). The assay is easy to implement in any laboratory with standard cell culture and ELISA capabilities.

3.5 ROLE OF ROS AND HMGB1 IN CONTACT ALLERGEN–INDUCED IL-18 PRODUCTION IN HUMAN KERATINOCYTES

Abstract

Keratinocytes have a key role in all phases of allergic contact dermatitis. We have recently identified the possibility to use IL-18 production for the *in vitro* identification of contact allergens. The purpose of this study was to characterize the molecular mechanisms underlying allergen-induced IL-18 production, in order to identify the cellular source of reactive oxygen species (ROS) and the danger signals involved. The NCTC2544 cell line was exposed to three contact allergens, namely *p*-phenylenediamine (PPD), 2,4-dinitrochlorobenzene (DNCB), and citral, in the presence or absence of diphenylene iodonium (DPI), allopurinol, and rotenone to identify the source of ROS, and to anti-Toll-like receptor 4 antibody and glycirrizic acid to characterize the danger-associated molecular pattern molecules. In the case of PPD, the induction of IL-18 can be modulated by rotenone, allopurinol, and DPI. In the case of DNCB, rotenone completely prevents the induction of IL-18, whereas for citral, DPI completely prevents the induction of IL-18. We demonstrated the ability of all allergens tested to induce the release of high-mobility group protein B1 (HMGB1). Its sequester by glycirrizic acid significantly modulates PPD-induced IL-18 production and completely prevents DNCB- and citral-induced IL-18. We found that different intracellular sources of ROS are triggered by contact allergens, and an important role for HMGB1 in chemical allergen-induced IL-18 production was demonstrated.

Role of rotenone and allopurinol in allergen-induced IL-18 production

As reference allergens, PPD, 2,4-dinitrochlorobenzene (DNCB), and citral were used. The concentrations used were selected from previously published data (Corsini *et al.*, 2009; Galbiati *et al.*, 2011; Corsini *et al.*, 2013).

To investigate the intracellular source of ROS, specific inhibitors of the three main cellular sources of ROS, namely rotenone (mitochondrial electron transport), diphenylene iodonium (nicotinamide adenine dinucleotide phosphate oxidase and nitric oxide synthetase), and allopurinol (xanthine oxidase), were used. The list of inhibitors used, the concentrations used, cytotoxicity, and the effect on IL-18 are reported in Table 1. At the concentrations used, none of the inhibitors were cytotoxic or affected IL-18 content.

Chemicals	Concentrations	Cell viability[1]	Intracellular IL-18[2]
Allopurinol	10 μM	109±10	0.98±0.01
Anti-TLR4 antibody	2 μg ml^{-1}	97±2	1.10±0.08
DPI	5 μM	99±2	1.04±0.13
Glycirrizic acid	100 μM	98±5	0.82±0.06
Rotenone	20 μM	101±2	0.94±0.05
Z-VAD-FMK	10 μM	100±1	1.05±0.07

Abbreviations: DPI, diphenylene iodonium; TLR4; Toll-like receptor 4.

Cells were treated for 24 hours with the selected inhibitors. Cell viability was assessed by the MTT reduction assay and intracellular IL-18 by ELISA. Each value represents the mean±SD, *n*=4.

[1] Expressed as % of viable cells versus vehicle-treated cells (100%).

[2] Expressed as SI (stimulation index).

As shown in Figure 1, all sources appear to be involved to some degree in allergen-induced IL-18, as all inhibitors were able to significantly modulate allergen-induced IL-18. However, we found that the different allergens tested seem to preferentially trigger ROS production from the different intracellular sources. In particular, a central role of mitochondria could be demonstrated for DNCB and of nicotinamide adenine dinucleotide phosphate oxidase for citral, whereas for PPD a main source of ROS could not be identified as all inhibitors equally modulated the induction of IL-18.

Figure 1. Role of mitochondria, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and xanthine oxidase in allergen-induced IL-18 production. NCTC2544 cells were treated with rotenone (20 μm), allopurinol (10 μm), and diphenylene iodonium (DPI) (5 μm) for 1 hour and then with *p*-phenylenediamine (PPD) (30 μg ml^{-1}), 2,4-dinitrochlorobenzene (DNCB) (2 μg ml^{-1}), citral (20 μg ml^{-1}), or DMSO (0.2% final concentration) as vehicle control for 24 hours. Results are expressed as mean±SD, *n*=4. Statistical analysis was performed with analysis of variance followed by Bonferroni post-tests, with ***P*<0.01 versus vehicle-treated cells; ##*P*<0.01 versus cells treated with inhibitors alone.

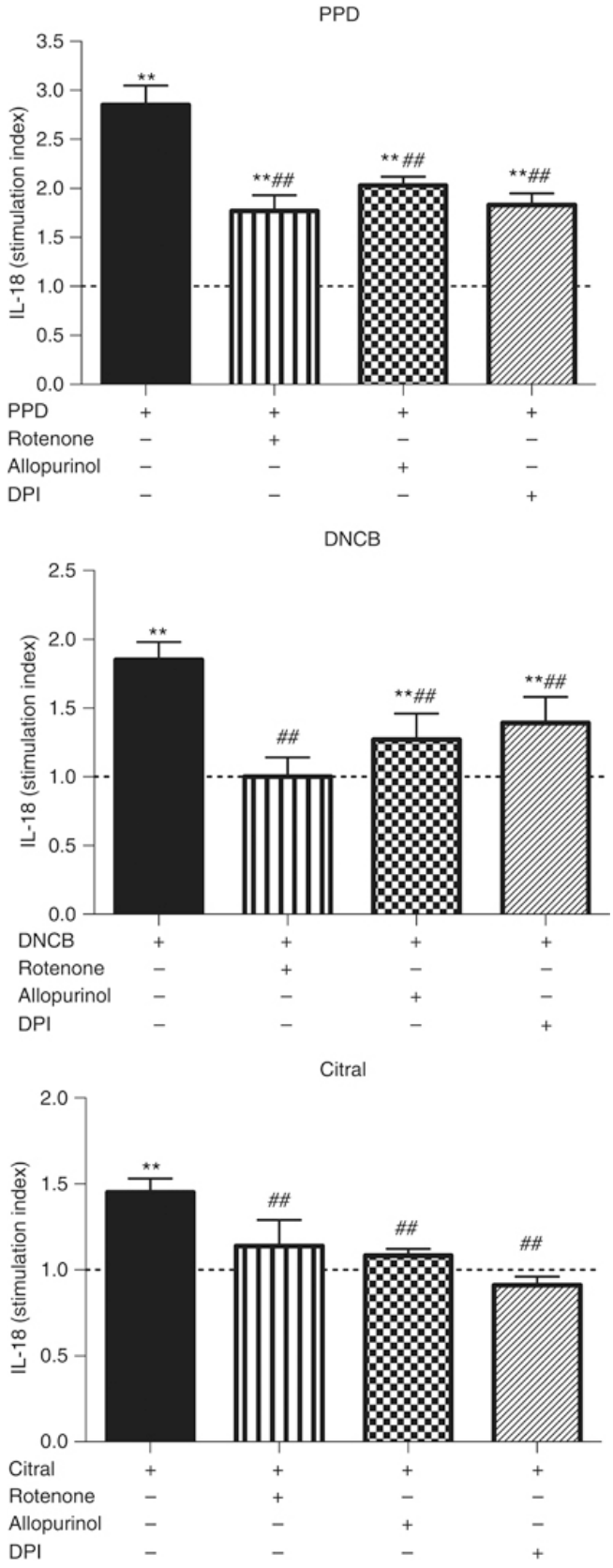

PPD
IL-18 (stimulation index)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**##
**##
**##
PPD + + + +
Rotenone − + − −
Allopurinol − − + −
DPI − − − +
DNCB
IL-18 (stimulation index)
2.5
2.0
1.5
1.0
0.5
0.0
**
##
**##
**##
DNCB + + + +
Rotenone − + − −
Allopurinol − − + −
DPI − − − +
Citral
IL-18 (stimulation index)
2.0
1.5
1.0
0.5
0.0
**
##
##
##
Citral + + + +
Rotenone − + − −
Allopurinol − − + −
DPI − − − +

Figure 1

The effects of different sources of ROS

To investigate the direct effect of an exogenous font of ROS on cytokines production, H_2O_2 was used. Dose-response experiments were performed starting from H_2O_2 1 μm as the highest concentration. PPD (30 μg ml−1) was used as a positive control. We determined IL-18, IL-1α, and IL-8 production. Cell viability was evaluated in parallel with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

As shown in Figure 2, for IL-18 (panel a) and IL-1α (panel b) only at the two highest concentrations of H_2O_2 (1 μm and 0.5 μm) a statistical significant increase in cytokine production was observed. These concentrations were associated with high cytotoxicity (about 50% of mortality). Otherwise, H_2O_2 was able only to induce a statistically significant IL-8 release at a concentration of 0.1 μm (panel c).

Figure 2. Effects of different sources of reactive oxygen species (ROS). NCTC2544 cells were treated with increasing concentrations of H_2O_2 or with *p*-phenylenediamine (PPD) (30 μg ml^{-1}) as positive control. After incubation for 24 hours, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, whereas IL-18 (**a**), IL-1α (**b**), and IL-8 (**c**) were assessed by ELISAs. Results are expressed as mean±SD, *n*=3. Statistical analysis was performed with analysis of variance, followed by Bonferroni post-tests, with **P*<0.05 and ***P*<0.01 versus vehicle-treated cells. In panel **d**, cells were treated with PPD 30 μg ml^{-1} in pink, 2,4-dinitrochlorobenzene (DNCB) 2 μg ml^{-1} in green, and citral 20 μg ml^{-1} in orange for 5 minutes. The purple histogram represents control cells.

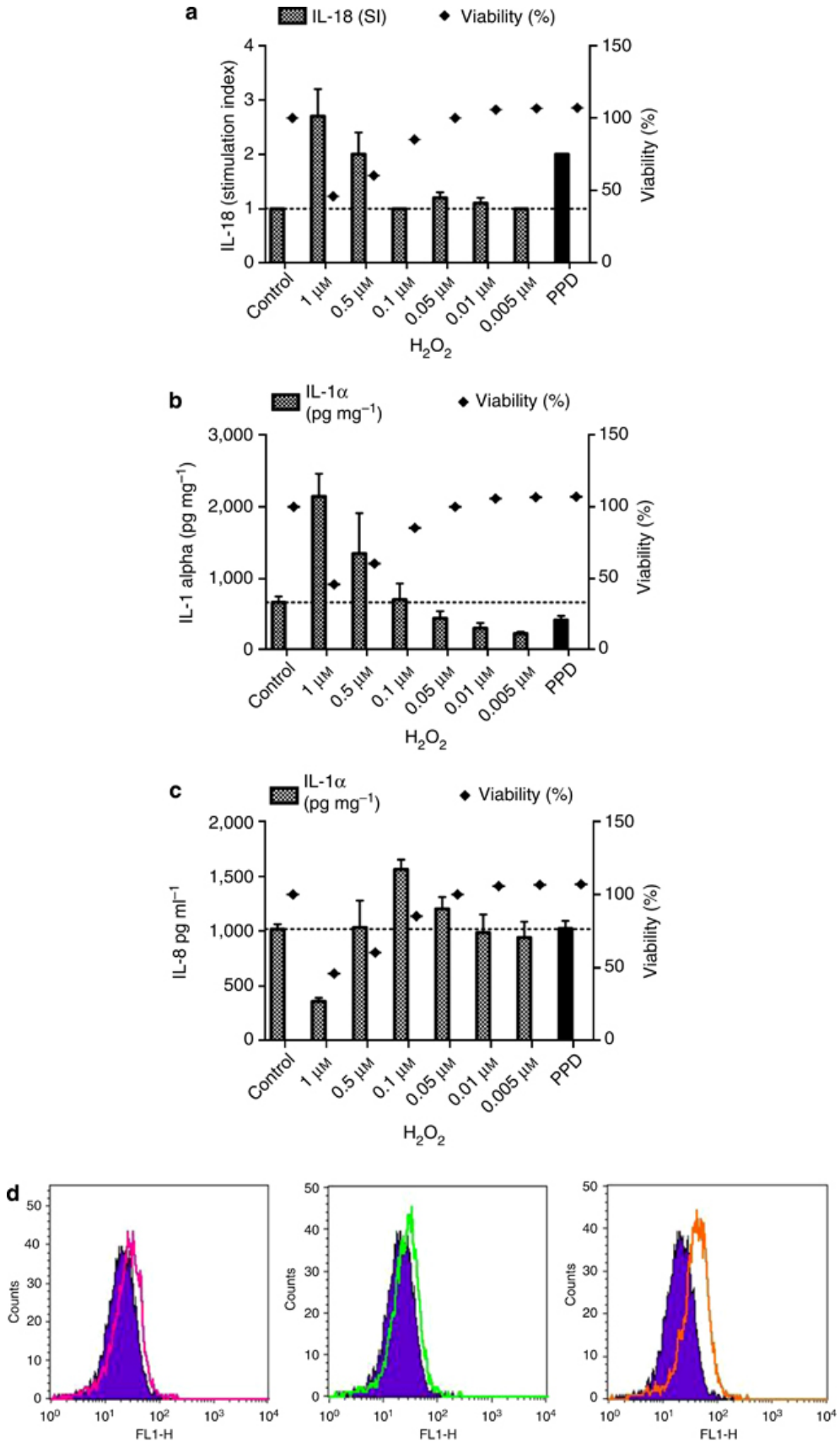

a
IL-18 (SI)
Viability (%)
IL-18 (stimulation index)
Viability (%)
Control
1 μM
0.5 μM
0.1 μM
0.05 μM
0.01 μM
0.005 μM
PPD
H2O2
b
IL-1α (pg mg−1)
Viability (%)
IL-1 alpha (pg mg−1)
Viability (%)
Control
1 μM
0.5 μM
0.1 μM
0.05 μM
0.01 μM
0.005 μM
PPD
H2O2
c
IL-1α (pg mg−1)
Viability (%)
IL-8 pg ml−1
Viability (%)
Control
1 μM
0.5 μM
0.1 μM
0.05 μM
0.01 μM
0.005 μM
PPD
H2O2
d
Counts
FL1-H
Counts
FL1-H
Counts
FL1-H

Figure 2

The results obtained suggest that KCs react differently to exogenous or to endogenous oxidative stresses. In particular, we found that an external font of ROS, such as H_2O_2, was able to induce IL-18 and IL-1α production only at cytotoxic concentrations, whereas contact allergens induced a dose-related IL-18 production at noncytotoxic concentrations (viability >80%).

To estimate the expression of ROS (H_2O_2), we used the dichlorofluorescein diacetate assay. As shown in Figure 2, panel d, and in agreement with literature data, to a different extent all allergens tested induce an oxidative stress in KCs.

Role of caspase-1 and TLR4 in allergen-induced IL-18 production

Z-VAD-FMK, a cell-permeant pan caspase inhibitor that irreversibly binds to the catalytic site of caspases, and a neutralizing anti-TLR4 antibody were used to investigate the role of the inflammasome and TLR4 in allergen-induced IL-18 production. DNCB- and citral-induced IL-18 were completely prevented by Z-VAD-FMK (Figure 3a) and by anti-TLR4 antibody (Figure 3b), whereas PPD-induced IL-18 was significantly reduced but completely not abrogated, possibly indicating the involvement of other pathways.

DAMPs

We focused our attention on HMGB1 as possible DAMP associated with allergen-induced IL-18. By measuring the release of HMGB1 (Figure 4a) and using its direct inhibitor glycirrizic acid (Figure 4c), we could demonstrate that all allergens tested induced HMGB1 release and its sequestration by glycirizzic acid prevented IL-18 induction, demonstrating the role of HMGB1 in allergen-induced IL-18. In addition, the release of HMGB1 can be significantly reduced by Z-VAD-FMK (Figure 4b). This is consistent with published data, which showed

that the secretion of HMGB1 requires inflammasome and caspase activation, probably associated with the translocation of HMGB1 from the nucleus to the cytoplasm (Apetoh *et al.*, 2007; Yamada *et al.*, 2011; Lu *et al.*, 2012).

Data presented show the involvement of this DAMP in allergen-induced KC activation. This is not limited to the NCTC2544 cell line, as we also observed the release of HMGB1 in human reconstituted epidermis treated with increasing concentrations of PPD, DNCB, and citral (Figure 4d), demonstrating the release of this protein not only in the KC cell line but also in a more complex *in vitro* system.

Finally, to investigate the role of HMGB1 in allergen-induced IL-8 and IL-1α production, cells were pretreated with glycirrizic acid and then with PPD. As shown in Table 2, glycirizzic acid was able to prevent the intracellular IL-1α production induced by PPD, without affecting IL-8 production.

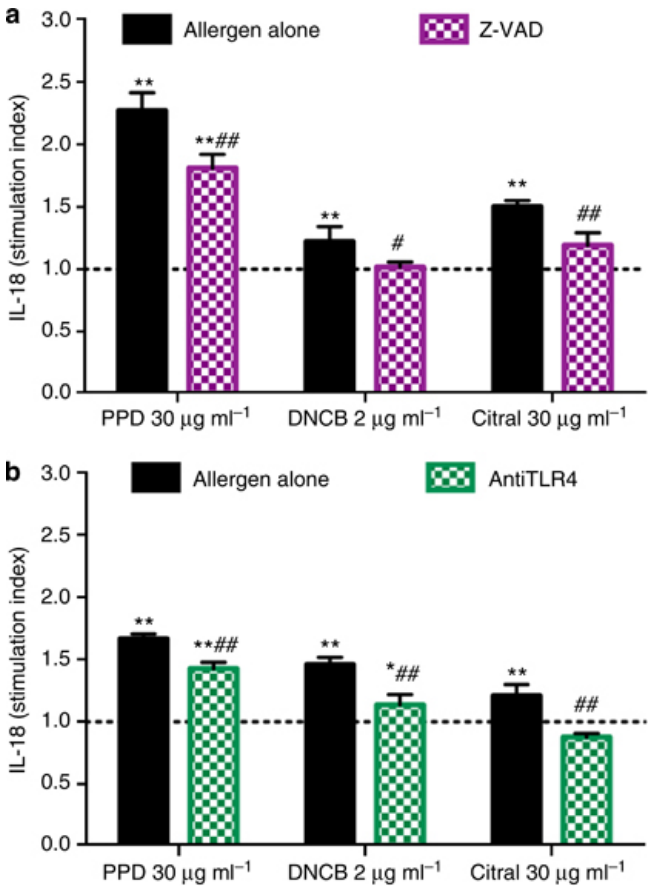


Figure 3. Role of the inflammasome and Toll-like receptor 4 (TLR4) in allergen-induced IL-18 production. NCTC2544 cells were treated with Z-VAD-FMK (10 µm, (**a**) in purple) and anti-TLR4 (2 µg ml^{-1}, (**b**) in green) for 1 hour and then with *p*-phenylenediamine (PPD) (30 µg ml^{-1}), 2,4-dinitrochlorobenzene (DNCB) (2 µg ml^{-1}), citral (30 µg ml^{-1}), or DMSO (0.2% final concentration) as vehicle control for 24 hours. Results are expressed as mean±SD, *n*=4. Statistical analysis was performed with analysis of variance followed by Bonferroni post-tests, with *P<0.05 and **P<0.01 versus vehicle-treated cells; #P<0.05 and ##P<0.01 versus cells treated with inhibitors alone.

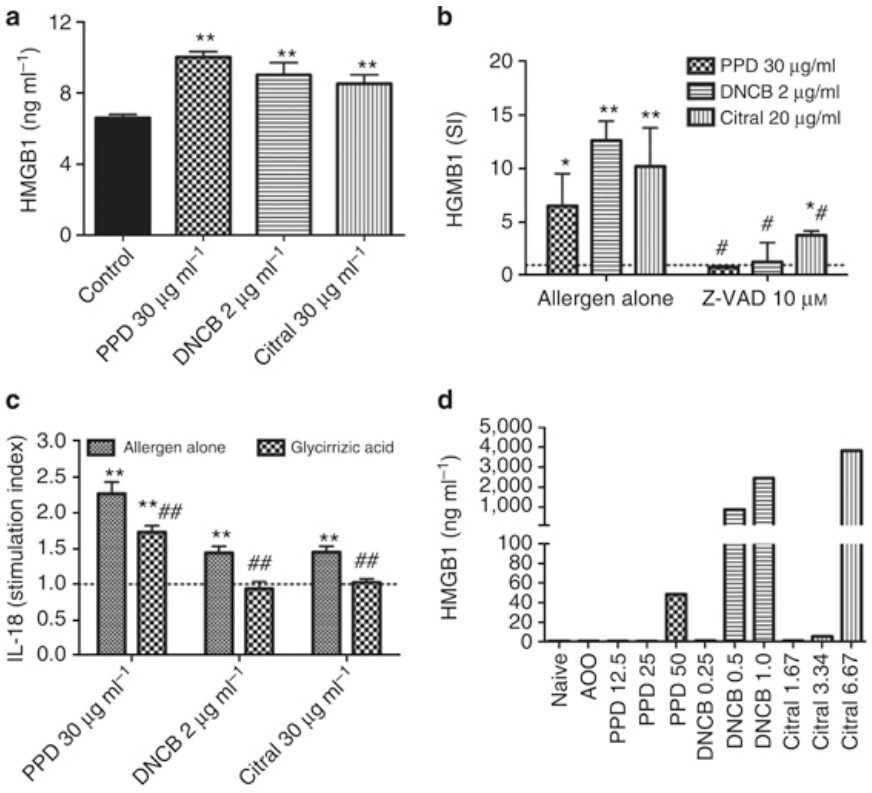


Figure 4. Role of high-mobility group protein B1 (HMGB1) in allergen-induced IL-18. (**a**) NCTC2544 cells were treated for 24 hours with *p*-phenylenediamine (PPD) (30 µg ml^{-1}), 2,4-dinitrochlorobenzene (DNCB) (2 µg ml^{-1}), citral (30 µg ml^{-1}), or DMSO. (**b**) Cells were treated with Z-VAD-FMK 10 µm, and then selected allergens were added for 24 hours. (**c**) Cells were treated for 1 hour with glycirrizic acid (100 µm) and then contact allergens were added for 24 hours. (**d**) HMGB1 release in EPICS® model. After topical exposure to chemicals, HMGB1 release was assessed by ELISA. Results are expressed as mean±SD, *n*=4. Statistical analysis was performed with two-way analysis of variance followed by Bonferroni post-tests, with **P*<0.05 and ***P*<0.01 versus vehicle-treated cells and ##*P*<0.01 versus cells treated with inhibitors alone.

Treatment	Intracellular IL-1 α[1]		IL-8[2]		*n*
	Vehicle	Glycirrizic acid	Vehicle	Glycirrizic acid	
Control	979±66	986±134	849±34	723±40	3
p-Phenylenediamine 30 µg ml−1	2,036±138	1,624±254	809±54	789±85	3

Cells were treated for 24 hours with *p*-phenylenediamine 30 µg ml^{-1}. Cytokines were assessed by ELISA. Each value represents the mean±SD, *n*=3. [1] Expressed as pg per mg. [2] Expressed as pg per ml.

Discussion

We found that contact allergens, acting as danger signals, stress the cells inducing oxidative stress and DAMP release, and stimulate the assembly of the inflammasome, resulting in IL-18 neosynthesis and release. Different intracellular sources of ROS, including mitochondria, nicotinamide adenine dinucleotide phosphate oxidase, and xanthine oxidase, seem to be involved, as allergen-induced IL-18 can be modulated to a different extent by different selective inhibitors.

Danger signals are typically divided into pathogen-associated molecular patterns and DAMPs. The majority of contact allergens have the ability to deliver both antigenic and danger signals (McFadden *et al.* 2013). In this case, the danger signals are related to the irritant capacity of the chemical allergens, resulting in cell and tissue trauma and the elaboration of DAMPs, which are the key events for successful contact hypersensitivity. Among the possible DAMPs, here, we demonstrated the ability of contact allergens to induce the release of HMGB1, which via TLR4 induces IL-18 neosynthesis in KCs. HMGB1 is a major mediator of endotoxin shock (Wang *et al.*, 2002), and it acts on several immune cells to trigger inflammatory responses as DAMPs (Scaffidi *et al.*, 2002). HMGB1 can induce dendritic cell maturation via upregulation of CD80, CD83, CD86, and CD11c; it can induce production of proinflammatory cytokines in myeloid cells (i.e., IL-1, TNF alpha, IL-6 ,and IL-8), as well as upregulate the expression of cell adhesion molecules (i.e., ICAM-1, VCAM-1) on endothelial cells (Ferrari *et al.*, 1996; Gardella *et al.*, 2002; Scheibner *et al.* 2006). Extracellular HMGB1 can bind to TLR2/4 and RAGE on effector cells in order to induce inflammation, chemotaxis, and repair responses (Vande Walle *et al.*, 2011). We could

demonstrate the ability of contact allergens to induce the release of HMGB1 in KCs and its role in IL-18 production.

The inflammasome induces the activation of caspase-1, an enzyme that is central to the development of skin sensitization (Ainscough *et al.*, 2013). Using the caspase inhibitor Z-VAD-FMK, we could demonstrate the involvement of the inflammasome in allergen-induced IL-18 production in KCs. It has been demonstrated that a multitude of diverse stimuli can activate NLRP3, including adenosine triphosphate, silica, asbestos, uric acid, and several bacterial products (Pedra *et al.*, 2009). Among the processes associated with toxin- and crystal-mediated NLPR3 activation is the production of ROS (Dostert *et al.*, 2008). Evidence indicates that allergic and inflammatory skin diseases are mediated by oxidative stress (Okayama, 2005; Byamba *et al.*, 2007). The disturbance of the antioxidant systems, both enzymatic and nonenzymatic, can lead to increased ROS, which can damage cellular macromolecules (Byamba *et al.*, 2007). Many *in vitro* studies have revealed that ROS production is induced by contact allergens (Corsini *et al.*, 2013). The majority of chemical allergens bind to the thiol group of cysteine (Divkovic *et al.*, 2005), which may lead to glutathione depletion and oxidative stress, tissue damage, and increased inflammation. We have previously demonstrated that PPD-induced IL-18 production in KCs can be abrogated by the antioxidant and NF-κB inhibitor pyrrolidine dithiocarbamate (Galbiati *et al.*, 2011), supporting the important role of ROS in KC activation. It is proposed that mitochondria may have a central role in NLRP3 activation (Zhou *et al.*, 2010), as these organelles are believed to be the main source of cellular ROS (Tait and Green, 2012). Engagement of certain TLRs (TLR 1, 2, and 4) leads to mitochondrial translocation of the signaling adaptor TRAF6. At the mitochondria, TRAF6 interacts with ECSIT, a protein implicated in the assembly of complex I,

leading to its ubiquitylation, which results in increased ROS production. Finally, among the protein carbonylated by contact allergens, mitochondrial adenosine triphosphate synthase was identified (Je *et al.*, 2008), further supporting the central role of mitochondria in the pathophysiology of contact allergy. Using selective inhibitors of the main intracellular sources of ROS, namely mitochondria, NAPDH oxidase, and xanthine oxidase, we could demonstrate that all these three sources appear to be involved in allergen-induced IL-18 production, with a different contribution depending on the allergen.

Among the several assays available to measure the levels of ROS, we used the dichlorofluorescein diacetate assay. This assay is sensitive to oxidation by peroxynitrite and hydrogen peroxide, whereas it is not suitable to measure nitric oxide, hypochlorite, or superoxide in biological systems as it does not distinguish between the different types of ROS (Byamba *et al.*, 2007). Furthermore, during our experiments, we had noticed some "interference problem" with the pro-hapten PPD. PPD is a white solid that becomes dark owing to air oxidation, and we suppose that this color change could interfere with the flow cytometry laser. As explained in the Results section, KCs react differently to internal or to external oxidative stimuli. Taken together, these evidence suggest that external sources of ROS (i.e., H_2O_2), not induced by contact allergens, are not able to modulate the IL-18 and IL-1α response, hence indicating that this response is specific to contact allergens.

In addition to the NLR, other pathogen-associated molecular pattern/DAMP receptors exist, including TLRs, c-type lectin receptors, prostanoid receptors, and neuropeptide receptors (Trinchieri and Sher, 2007), many of which have been implicated in the development of skin sensitization. On blocking TLR4 with a specific antibody, a significant reduction in IL-18

production was observed, demonstrating that allergen-induced IL-18 production is dependent upon TLR4 activation.

Among the DAMPs that are able to activate TLR4, we demonstrated the involvement of HMGB1 in allergen-induced IL-18 production. He *et al.* (2012) speculated that HMGB1 could increase the synthesis of pro-IL-1β and pro-IL-18 in THP-1 cells. We used glycirrizic acid, as it has been demonstrated to be a direct inhibitor of HMGB1 (Girard, 2007; Mollica *et al.*, 2007). Our data indicated that glycirrizic acid significantly reduces allergen-induced IL-18 and IL-1α production, supporting the involvement of HMGB1 in the induction of these two cytokines. Otherwise, glycirrizic acid does not affect the IL-8 levels, suggesting that HMGB1 is not involved in the release of this cytokine following allergenic stimulation.

Oxidative stress, the inflammasome, and TLR4 activation are strictly cross-linked and they have a key role in the innate immune and stress responses. TLR4, after a danger signal, can activate NF-κB and MAPK pathways, inducing the release of inflammatory cytokines such as IL-1β and IL-18. In addition, TLR4 has the ability to further induce ROS, which have a triple role: (1) they are able to directly stimulate inflammatory and cytotoxic responses; (2) they can activate the NF-κB and MAPK pathways; and (3) they can interact with the inflammasome leading to IL-1β and IL-18 release.

On the basis of our results, the following scenario can be imagined: chemical sensitisers can induce oxidative stress owing to their electrophilicity, which in turn activates the inflammasome and HMGB1 release (and possibly other DAMPs), which can activate TLR4. Activation of TLR4 will result in NF-κB and p38 MAPK activation and in the neosynthesis of IL-18.

More importantly, a similar scenario has been demonstrated *in vivo*. In a mouse model of contact hypersensitivity the ability of contact allergens to induced DAMPs and stimulation of TLR4, trigger the production of proinflammatory cytokines, which in turn promote the acquisition of skin sensitization, and subsequently the elicitation of allergic contact dermatitis (Martin *et al.*, 2011).

In conclusion, we demonstrated a role of oxidative stress, inflammasome activation, HMGB1 release, and TLR4 activation in contact allergen–induced IL-18 production in human KC.

Chapter 4

CONCLUSIONS AND FUTURE PERSPECTIVES

Allergic contact dermatitis is a complex disease, and a relevant challenge for public health and immunology. Workplace exposure, age, sex, use of consumer products and genetic predispositions were identified as the most important risk factors. In Europe about 20% of the general population suffers from contact allergy to at least one contact allergen. Most common are allergies to nickel, fragrances and preservatives. Allergic reactions to chromate and p-phenylenediamine (PPD) are generally less common but occur frequently in occupationally exposed subgroups of the population (Peiser at al., 2011).

For skin sensitisers, the local lymph node assay was the first method to be fully and independently validated, as well as the first to offer an objective end point with a quantitative measure of sensitizing potency (in addition to hazard identification). Fifteen years later, it serves as the primary standard for the development of *in vitro/in chemico/in silico* alternatives. (Basketter et al.,2014).

There is still a lack of comparable *in vitro* methods, and substantial efforts have been, and are being, made world-wide to develop alternative assays to achieve a full replacement of animals use. The main challenge is to address the required level of integration of the molecular and cellular processes that are underlying skin sensitisation.

Four goals have been identified for a full replacement of skin sensitisation animal data:

1. Hazard identification: prediction of potential sensitiser (yes/no answer)
2. Classification and labelling (i.e. GHS, EU-CLP): more than yes/no answer, i.e. some potency determination
3. Hazard characterization: prediction of sensitiser and its potency, i.e. non-sensitiser, weak, moderate, strong, extreme (dose-response information).
4. Risk assessment: accurate evaluation of relative skin sensitizing potency to support effective risk assessment

Several European Union legislations request the use of *in vitro* methods for toxicological evaluations, including sensitization, in order to increase consumer safety but also to reduce the use of animals.

In this context, the NCTC 2544 assay (results described in Chapter 3.1), based on the selective induction of IL-18 in human keratinocytes by contact allergens, was developed within the SENS-IT-IV project sponsored by the European Union. The assay proved to be useful in the ***identification and discrimination of contact allergens from respiratory sensitisers and irritants*** (Corsini et al., 2009). Exposure of NCTC 2544 cells to contact allergens results in a dose-related induction of intracellular IL-18, whereas exposure to respiratory allergens and irritants fails to induce IL-18 production, indicating the possibility to use IL-18 to specifically identify contact allergens and distinguish them from respiratory allergens and irritants.

About one year later the experimental conditions were ***optimized*** to clarify the cell density issue, a high throughput assay was developed, the performance of other available KC cell lines was tested, and the signal transduction pathways involved in PPD-induced IL-18

production were characterized, as described in Chapter 3.2 (Galbiati et al., 2011). Critical points identified in the performance of the NCTC 2544 assay are the cell density and the time cells have been cultured before use in experiments. If cells reach confluence at the moment of treatment, the ability to identify contact allergens is lost. In our hands, a cell density of 2.5×10^5 cells/ml gave optimal stimulation. Regarding time after thawing, cells should be used between 3 weeks after thawing, up to 5 months after.

The influence of cell density on the discriminatory capacity of the NCTC 2544 assay can be attributed to a decrease in cellular reactivity once cells reach confluence. We have demonstrated that PPD-induced IL-18 production is dependent upon oxidative stress, and it has been demonstrated that the capacity of KC to generate reactive oxygen species decrease as the cells reached confluence (Turner et al., 1998), which offers a logical explanation for the effect observed.

During my thesis in order to further develop the assay and to increase its screening capacity, a 96-well format assay was developed. The purpose of this 96-well format was to speed up the assay and reduce cell manipulation (no cell washing steps and no protein determination). In this case the total IL-18 (intracellular plus released) was assessed and also under these experimental conditions, a selective increase in total IL-18 was observed only following treatment with contact allergens, whereas both irritants and respiratory allergens failed (Galbiati et al., 2011).

Another important issue in sensitisations field is the ***differentiation between photoallergens and phototoxic reactions*** induced by low molecular weight compounds. The use of KCs as a potential tool for the detection of photoallergens as opposed to

photoirritants is considered an interesting strategy for developing *in vitro* methods. As part of my thesis (see Chapter 3.3 - Galbiati et al., 2013) was to explore the possibility to use the NCTC2544 IL-18 assay to identify photoallergens and discriminate from phototoxic chemicals. We found that irradiated photoallergens induced a significant increase in intracellular IL-18 compared to photoirritants or no phototoxic compounds.

In addition to being able to determine whether or not a chemical is a sensitiser (labelling) it is also equally important to ***determine the potency of a sensitiser*** (classification) in order to identify a maximum safe concentration for human exposure (risk assessment). The assessment of risk is the deciding point in the toxicology of skin sensitization. A primary step in the risk assessment process is determination of the relative skin sensitizing potency of the contact allergens. (Basketter 1998). As part of my thesis work in collaboration with Prof. Sue Gibbs of VUMC (Netherland), we developed "An Epidermal equivalent assay for identification and ranking potency of contact sensitisers". As described in Chapter 3.4, the possibility of combining the epidermal equivalent (EE) potency assay with the assay, which assesses release of IL-18 to provide a single test for identification and classification of skin sensitizing chemicals, including chemicals of low water solubility or stability is reported.
A putative prediction model is proposed from data obtained from two laboratories yielding 95% accuracy. Results obtained in this study indicate that indeed the single in vitro human assay may be able to distinguish sensitisers from non-sensitisers as well as rank sensitisers according to potency. The use of reconstituted epidermis overcomes the limitation of chemical solubility and stability, which are the majors limitations of many in vitro methods based on the use of traditional submerged cultures. Remarkably consistent results were

obtained between the different 3D-models, indicating that assays robustness and easy to transfer to other laboratories. In all EE models, a robust release of IL-18 was observed only after treatment with contact allergens. This is consistent with prior neosynthesis and intracellular accumulation only after sensitiser exposure, and is in line with our previous findings (Corsini et al., 2009; Galbiati et al., 2011).

Next it was determined whether the EC_{50} values identified from the sensitisers in the dose response experiments could be used to rank sensitiser potency. Extreme/strong sensitisers classified according to LLNA generally had a lower EC_{50} value than weak sensitisers. EC_{50} values for the moderate sensitisers generally ranged between the weak and strong sensitisers. It was found a significant correlation with the ranking obtained by the LLNA (Spearman r = 0.5968; P value (two-tailed) = 0.0542) and to human DSA_{05} data (Spearman r = 0.8500; P value (two-tailed) = 0.0061). We also determined whether the IL-18 SI-2 and SI-5 could be used to rank sensitiser potency. The correlation of *in vitro* human data suggested that IL-18 release might supply an additional potency parameter in this assay.

Finally, the purpose of the last part of my thesis was to ***characterize the molecular mechanism underlying allergen-induced IL-18 production***, to identify the cellular sources of reactive oxygen species and the danger signals involved.

Contact allergens acting as danger signals, stress the cells inducing oxidative stress and DAMP release, and stimulate the assembly of inflammasome, resulting in IL-18 neosynthesis and release. Different intracellular sources of ROS, including mitochondria, NADPH oxidase, and xanthine oxidase, seem to be involved, as allergen-induced IL-18 can be modulated to a

different extent by different selective inhibitors. In addition, to prove the specificity of response of keratinocytes to contact allergens we can demonstrate that KCs reacts differently to internal or external oxidative stimuli. These evidences suggest that external sources of ROS (i.e.H_2O_2), not induced by contact allergens, are not able to modulate the IL-18 response, hence indicating that this response is specific to contact allergens.

Furthermore, we demonstrated, among the DAMPs that are able to activate TLR4, the involvement of HMBG1 in allergen-induced IL-18 production. Glycirrizic acid was used as direct inhibitor of HMGB1 production. Our data indicated that glycirrizic acid significantly reduces allergen-induced IL-18 and IL-1α production, supporting the involvement of HMGB1 in the induction of these two cytokines. The direct involvement of TLR4 in allergen-induced IL-18 production was demonstrated by blocking this receptor with a specific neutralizing antibody.

On the basis of results obtained, the following scenario can be imagined (see Fig.12): chemicals sensitisers can induce oxidative stress owing to their electrophilicity, which in turn activates the inflammasome and HMGB1 release (and possible other DAMPs), which can activate TLR4. Activation of TLR4 will result in NF-κB and p38 MAPK activation and in the neosynthesis of IL-18.

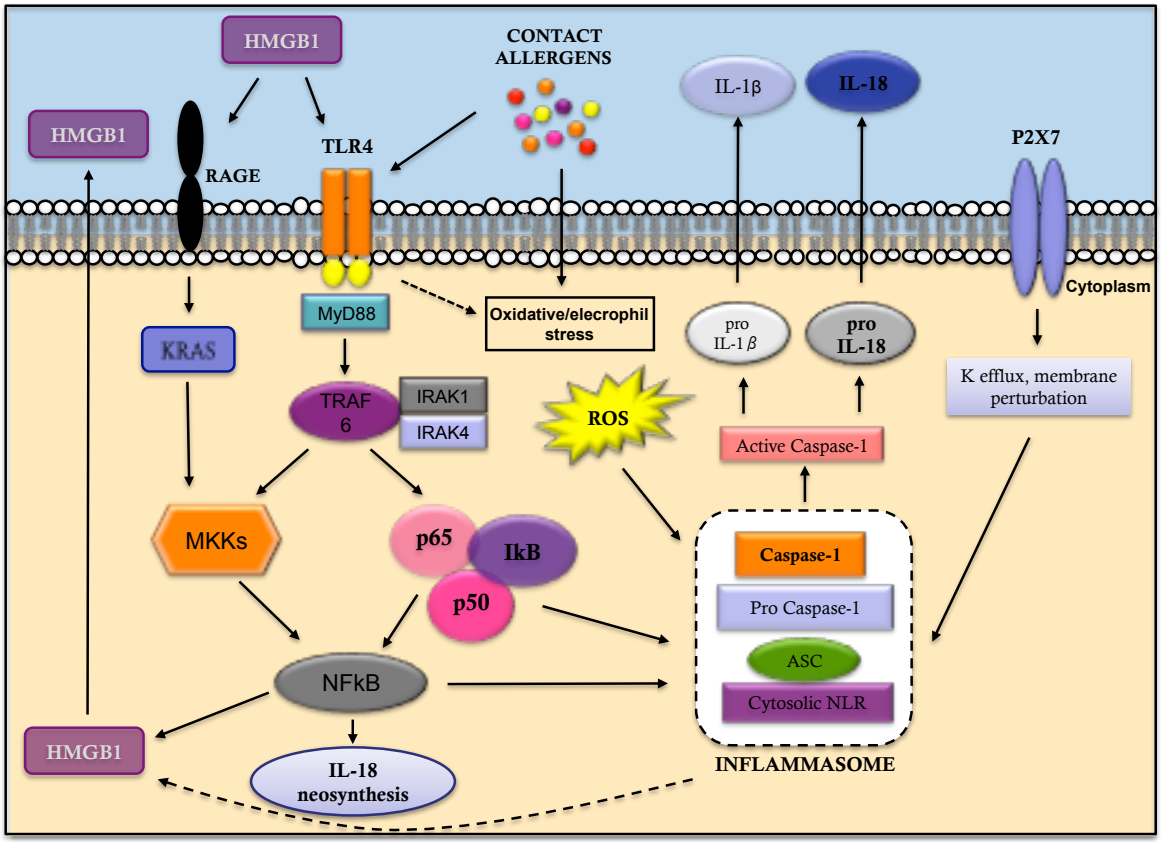


Fig. 12 – Signal transduction pathways

In conclusion, the NCTC2544 IL-18 assay optimized and characterized as part of my thesis is an *in vitro* method able to discriminate contact allergens and photoallergens from irritants/photoirritants and also from respiratory allergens.

The combination of two *in vitro* methods (EE and IL-18), both "born" within the SEN-IT-IV project, has lead to the development of a putative prediction model to identify and rank sensitisers potency.

Finally, signal transduction pathways involved in contact allergens-induced IL-18 production in KCs was investigated. Oxidative stress, the inflammasome, and TLR4 activation are strictly

linked: after a danger signal released following allergen-induced oxidative stress, TLR4 can trigger to NF-κB and MAPK pathways activation, necessary to the neosynthesis of inflammatory cytokines such as IL-1β and IL-18. In addition, TLR4 has the ability to further induce ROS, which have a triple role: (1) they are able to directly stimulate inflammatory and cytotoxic responses; (2) they can activate the NF-κB and MAPK pathways; and (3) they can activate the inflammasome leading to IL-1β and IL-18 processing and release.

As future perspectives the following experiments can be foreseen:

- To further study the signal transduction pathways involved in allergen-induced IL-18 production, the role played by the purinergic receptor (P2RX7) and ATP should be investigated;
- The correlation between ROS, TXNIP (Thioredoxin interacting protein) and NLRP3 in allergen-induced IL-18 production should also be addressed;
- Finally, a better understanding of the molecular mechanisms underlying potency is mandatory to achieve a full replacement of animal's models in contact-hypersensitivity. All this regards the opposite role of NLRP12 and Blimp-1 in the activation of the inflammasome may offer the possibility to discriminate between weak and strong sensitisers.

List of publications

1. ***Galbiati V***, Papale A, Galli CL, Marinovich M, Corsini E. (2014). Role of ROS and HMGB1 in Contact Allergen-Induced IL-18 Production in Human Keratinocytes. J Invest Dermatol.
2. Galbiati V1, Bianchi S, Martínez V, Mitjans M, Corsini E. (2014). NCTC 2544 and IL-18 production: a tool for the in vitro identification of photoallergens. Toxicol In Vitro.
3. Gibbs S, Corsini E, Spiekstra SW, ***Galbiati V***, Fuchs HW, Degeorge G, Troese M, Hayden P, Deng W, Roggen E. (2013). An epidermal equivalent assay for identification and ranking potency of contact sensitizers. Toxicol Appl Pharmacol
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