Keratinocytes, Innate Immunity and Allergic Contact Dermatitis - Opportunities for the Development of *In Vitro* Assays to Predict the Sensitizing Potential of Chemicals

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1. Introduction

Allergic contact dermatitis (ACD) is the most prevalent form of immunotoxicity in humans characterized by clinical manifestations such as red rashes, itchy skin and blisters. The disease is caused by skin sensitizers which are allergenic low-molecular weight chemicals. ACD is an important occupational disease that gives problems at different workplaces, including hair dressers, metal workers, construction workers, and cleaners. In addition, ACD can develop in the general population as well, since several consumer products contain skin sensitizers. Important skin sensitizers are metals (nickel, chromium), fragrances, hair dye ingredients and preservatives (Kimber et al., 2002a; Vandebriel & van Loveren, 2010).

ACD is a typical type IV (delayed-type) hypersensitivity response that develops in two phases, the initiation phase in which the immune system is sensitized and the elicitation phase in which the clinical symptoms develop. At initiation, the low-molecular weight and polarity of skin sensitizers allow for penetration of the stratum corneum of the skin. In addition, protein reactivity is a hallmark of skin sensitizers. After binding to proteins in the skin, hapten-carrier complexes are formed (Kimber et al., 2002a; Berard et al., 2003; Vandebriel & van Loveren, 2010). The formation of these hapten-protein complexes is crucial, since the chemicals themselves are not immunogenic and priming of T cells can only occur after formation of these complexes. After taking up these hapten-carrier complexes, immature Langerhans cells and dendritic cells start to migrate to the draining lymph node and become potent T cell activators through the upregulation of costimulatory molecules (Gaspari, 1997; Kimber et al., 2002a; Berard et al., 2003; Vocanson et al., 2009; Vandebriel & van Loveren, 2010). Hapten specific T cells are activated through a combination of haptenized peptide presentation on major histocompatibility complex (MHC) molecules and costimulatory molecules, such as CD54, CD80 and CD86. Activated T cells undergo clonal expansion, thereby generating skin homing CD8+ Tc1/Tc17 and CD4+ Th1/Th17 effector T

cells that enter the blood circulation (Kimber et al., 2002a; Freudenberg et al., 2009 ; Vocanson et al., 2009; Vandebriel & van Loveren, 2010). After being sensitized, subsequent skin contact with the hapten will elicit skin symptoms within 48 hours caused by the hapten specific T cells that migrate into the skin. Here they recognize the protein/hapten complex presented by either dendritic cells or keratinocytes. Upon recognition, the T cells become activated and start to produce typical Th1 and Th17 response cytokines, such as IFN-γ, IL-12, IL-17, and IL-23 (Kimber & Dearman, 2002; Zhao et al., 2009).

The identification of chemicals with skin sensitizing capacity is of great importance to ensure the safety of industrial chemicals and cosmetic ingredients. The current testing methods for skin sensitization are animal tests, either using guinea pigs (Guinea Pig Maximization Test or the Buehler Test) or mice (the murine Local Lymph Node Assay) (Kimber et al., 1994; Gerberick et al., 2007a). Recently, the pressure to develop non-animal testing strategies has increased due to public and political influence. The 7th amendment to the European Union (EU) Cosmetics Directive forbids the use of animal tests and the sensitizing potential of a great number of chemicals will have to be evaluated within the framework of Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH). Therefore, there is a great demand for in vitro alternative test methods that can replace the currently used animal assays. Knowledge on physical-chemical properties of haptens together with insight in the immunological mechanisms that lead to sensitization need to be applied in the alternative methods that are currently being developed or validated. The elucidation of pathways that play a role in the initiation phase of skin sensitization have long been subject of investigations. Several *in vitro* and *in vivo* studies have shown that pathways linked to innate immunity and oxidative stress are important in the first phase of skin sensitization (Natsch, 2009; Vandebriel et al., 2010; Martin et al., 2011). Activation of these pathways induces cell stress and damage, and production of pro-inflammatory cytokines and chemokines. In this way, 'danger' signals are produced in the skin, which are considered to be required for further development of an adaptive immune response (Kimber et al., 2002b; Martin et al., 2011).

In the skin, keratinocytes are abundantly present and these cells are the first to encounter haptens that penetrate through the skin. Keratinocytes are considered to be key players in the initiation phase of skin sensitization for several reasons (Figure 1). Keratinocytes contain enzymes with metabolic activity required for the conversion of prohaptens into biologically active haptens, thereby facilitating binding to proteins (Van Pelt et al., 1990; Gelardi et al., 2001). In addition, keratinocytes have been shown to express chemotactic factors upon exposure to sensitizers, including chemokines (CXCL8, CXCL9, CXCL10, CXCL11) and adhesion molecules (ICAM-1). These attract more immune cells to the exposed skin area, thereby strengthening the immune response (Gaspari, 1997; Albanesi, 2010). Keratinocytes are important in the elicitation phase of ACD as well, since they are able to present antigen to the surroundings through both MHC class I and MHC class II molecules (Albanesi et al., 2005; Nestle et al., 2009). In addition, after being targeted by IFN-γ, keratinocytes upregulate costimulatory molecules such as CD80 and are able to function as antigen presenting cells and facilitating activation of hapten specific T cells (Gaspari, 1997; Albanesi, 2010). Hence, keratinocytes are important in the sensitization and elicitation phase of ACD and for that reason these cells are often used for the development of *in vitro* assays for skin sensitization testing (Van Och et al., 2005; Corsini et al., 2009; Vandebriel et al., 2010; Galbiati et al., 2011).

In the development of *in vitro* assays, human keratinocyte cell lines, primary keratinocytes, and 3D skin models are used. In order to base read-outs of these assays on toxicological relevant pathways it is important to understand the underlying mechanisms of skin sensitization. In this chapter, an overview of current knowledge on the role of innate immune and oxidative stress pathways in skin sensitization will be provided together with the relevance of these pathways for the development of *in vitro* assays using keratinocytes.
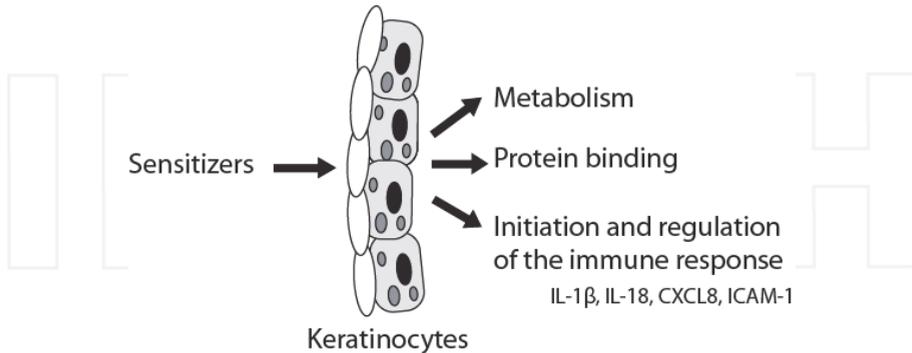

Fig. 1. Keratinocyte responses to sensitizers

2. Innate immune responses: Toll-like receptors and other pattern recognition receptors

The induction of innate signaling pathways by skin sensitizers in keratinocytes is believed to be a crucial factor in skin sensitization and a requirement for activation of Langerhans cells and dendritic cells and subsequent T cell priming (Martin et al., 2011). Studies have demonstrated that human primary keratinocytes express mRNA for Toll-like receptors (TLRs), such as TLR 1, 2, 3, 4, 5 and 9 (Kollisch et al., 2005; Son et al., 2006), which are important receptors of the innate immune system. Upon TLR activation, keratinocytes are able to produce a range of cytokines and chemokines, which allows for attraction of other immune cells, such as dendritic cells (Lebre et al., 2007).

TLRs are the first family to be identified of the germ-line encoded pattern recognition receptors (PRR). These receptors are involved in the first line of defense against pathogens. To date, 10 different TLRs have been identified in humans (Boehme & Compton, 2004). The TLRs are known to recognize various pathogen associated molecular patterns (PAMPs), which are conserved and essential molecules of pathogens. Some well-known examples are lipopolysaccharide (LPS) that is recognized by TLR4, double-stranded RNA recognized by TLR3 and lipopeptides recognized by TLR2 (Hosogi et al., 2004; Lebre et al., 2007; Kumar et al., 2009). The primary function of TLRs is the initial recognition of pathogenic microorganisms and subsequent activation of the innate immune response. Most pathogens express multiple PAMPs and are thus recognized by multiple TLRs. Activated TLRs will promote the phagocytosis of pathogens in innate immune cells such as macrophages and induce a respiratory burst, production of ROS and RNS, to neutralize pathogens. Combined, this promotes the presentation of pathogen specific peptides to cells of the adaptive immune system. Furthermore, the secreted reactive oxygen species can act as signaling molecules

and have been identified to be important pro-inflammatory mediators. Upon TLR activation, cells start to produce pro-inflammatory cytokines, attracting more immune cells to the site of infection (Kawai & Akira, 2009). In addition, TLRs are able to recognize endogenous danger signals, known as danger-associated molecular patterns (DAMPs). These molecules are released under cellular stress and include components of the extracellular matrix, such as hyaluronic acid and biglycan, heat shock proteins and uric acid crystals (Seong & Matzinger, 2004; Wheeler et al., 2009; Kawai & Akira, 2010; Martin et al., 2011).

Another important family of pattern recognition receptors are the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These intracellular receptors recognize PAMPs with the same leucine-rich repeat domains that can be found in TLRs. NLRs are activated by PAMPs such as bacterial RNA, flagellin and breakdown products of peptidoglycan (Feldmeyer et al., 2010). In addition, the NLRs are able to recognize DAMPs such as ATP and uric acid (Kawai & Akira, 2009). Upon activation, NOD receptors can activate NF-κB and large protein complexes called inflammasomes (Stutz et al., 2009; Feldmeyer et al., 2010; Latz, 2010). The inflammasomes are required to activate and secrete several cytokines that are expressed as nonfunctional proteins after NF-κB activation. The most important of these are IL-1β and IL-18, which are expressed as pro-IL-1β and pro-IL-18, respectively (Nestle et al., 2009).

2.1 Mechanisms of TLR activation

Several TLRs are located at the cell membrane, while others are in endosomal membranes (Figure 2). All TLRs consist of three domains: a leucine rich repeat domain that recognizes PAMPs, an anchoring transmembrane domain and a cytoplasmatic Toll/interleukin-1 receptor (TIR) domain involved in signal transduction (Kumar et al., 2009; Olaru & Jensen, 2010). Adaptor proteins need to bind to the TIR domain to ensure continuation and amplification of the signal transduction. The majority of TLRs use the myeloid differentiation primary-response gene 88 (MyD88) as adaptor, except TLR3 which uses the TIR-domain containing adaptor protein inducing IFN-β (TRIF). TLR4 can use both MyD88 and TRIF for signal transduction, with MyD88 being involved in the majority of TLR4 mediated processes (Hosogi et al., 2004; Lebre et al., 2007; Trinchieri & Sher, 2007; Kumar et al., 2009).

Through PAMP recognition, the TLRs homodimerize and enable the binding of the adaptor protein. An exception is the TLR2 molecule which forms heterodimers with either TLR1 or TLR6. Following activation of the MyD88 adaptor protein, a signaling cascade involving mitogen-activated protein kinases (MAPK) and ending with the ubiquitination of IκB occurs. This induces the release of NF-κB, resulting in the transcription of pro-inflammatory cytokines such as IFN-α, IL1-β and CXCL8. After activation of TRIF the resulting signaling cascade ends with the activation of IFN regulatory factors (IRFs) and the induction of type I IFNs. The expression pattern differs for each TLR. The activation of TLR3 by poly I:C generally leads to the production of the largest panel of cytokines and chemokines, including CCL2, CCL20, CCL27, CXCL8, CXCL9, CXCL10, and TNF-α. In comparison, activation of TLR4 using LPS leads to the production of CCL2, CCL20, CXCL8, and TNF-α (Lebre et al., 2007; Olaru & Jensen, 2010).
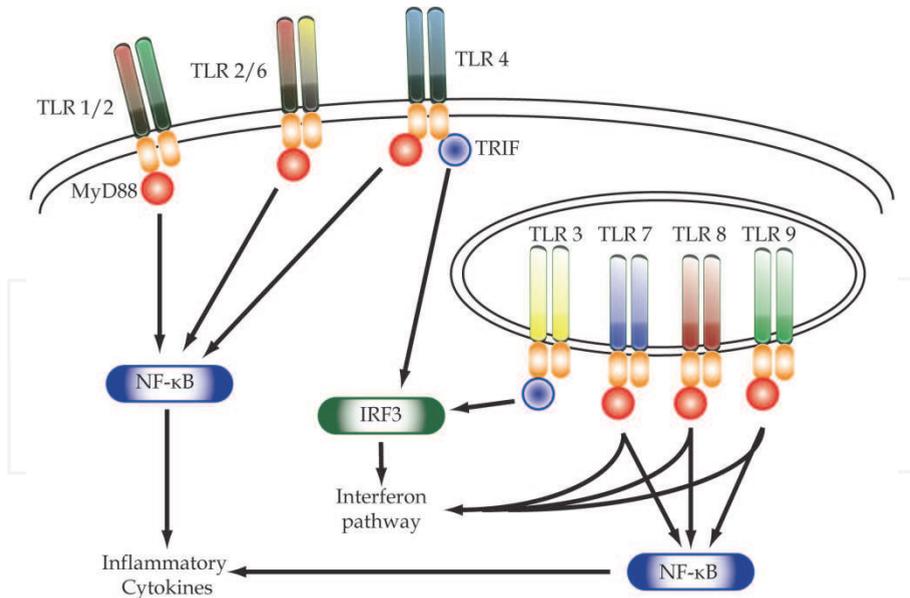
Fig. 2. Schematic presentation of TLR localization and signaling pathways (Boehme & Compton, 2004). TLR2 in combination with either TLR1 or TLR6, as well as TLR4, are expressed on the cell surface and use MyD88 as adaptor for cell signaling. This leads to NF-κB activation and inflammatory cytokine secretion. In addition to MyD88, TLR4 uses TRIF to activate IRF3 and the IFN pathway. TLRs 3, 7, 8, and 9 typically localize to endosomal membranes, where they detect a variety of nucleic acids. TLR3 utilizes TRIF to activate IRF3. TLRs 7, 8, and 9 trigger inflammatory cytokine secretion and the IFN pathway through MyD88.

3. The role of TLRs in skin sensitization

The importance of TLRs in skin sensitization has been shown *in vivo* by using different types of TLR knockout mice. In the majority of these experiments, the ear swelling response was measured as a read-out for contact hypersensitivity responses. Mice deficient of TLR2 had reduced ear swelling after challenge with the skin sensitizer oxazolone and these mice were unable to launch an effector Th1 response (Jin et al., 2009). The importance of TLR2 in skin sensitization was confirmed by Martin et al (2008), showing a reduced ear swelling in response to the skin sensitizers trinitrochlorobenzene (TNCB), oxazolone and fluorescein isothiocyanate (FITC). Skin sensitization to these compounds was completely prevented in the combined absence of TLR2 and TLR4, indicating that for full induction of sensitization both TLRs are required (Martin et al., 2008). More evidence for the importance of TLRs in skin sensitization was found in experiments in mice deficient for the TLR adaptor molecule MyD88. These mice were unable to mount an ear swelling response to dinitrofluorobenzene (DNFB), which was explained by an impaired upregulation of CD86 on dendritic cells, leading to a reduced activation of hapten specific T cells. In contrast, mice lacking TLR2, TLR4, TLR6 or TLR9 had no impaired ear swelling in response to DNFB. Mice deficient of

the adaptor molecule TRIF could be sensitized to DNFB, indicating that TLR3, using only TRIF for signal transduction, is not involved in skin sensitization (Klekotka et al., 2010).

More evidence for the importance of TLR in skin sensitization has recently been found for nickel, one of the most prevalent human sensitizers. Remarkably, nickel is a false-negative in the mouse LLNA. This paradox was recently explained by the discovery that nickel interacts directly with non-conserved histidine residues in human but not mouse TLR4, thereby activating the innate immune system and driving the development of ACD (Schmidt et al., 2010). Taken together, these data show that especially TLR 2 and 4 play an important role in mounting a full immune response to skin sensitizers.

Evidence for an involvement of the signaling pathway p38 MAPK in skin sensitization further underpin the relevance for TLR. p38 MAPK are enzymes that play an important role in the signal transduction of TLR (Mehrotra et al., 2007). In mice treated with the specific p38 MAPK inhibitor SB202190 the ear swelling in response to DNFB was impaired (Takanami-Ohnishi et al., 2002). Further evidence for the importance of p38 MAPK was found in *in vitro* studies. In dendritic cells exposed to DNFB, DNCB or nickel, the signaling pathways p38 MAPK and extracellular signal-regulated kinase (ERK) were activated (Matos et al., 2005; Miyazawa et al., 2008). In the keratinocyte cell line NCTC2544 IL-18 production induced by skin sensitizers was greatly decreased after addition of a specific p38 MAPK inhibitor, SB203580 (Galbiati et al., 2011). Similar results were obtained in an experiment using the monocytic THP-1 cell line (Mitjans et al., 2010). These signaling pathways are essential for the further maturation of dendritic cells and the activation of hapten-specific T cells, since they trigger the production of cytokines such as TNF-α, IL-6, IL-12 and IL-18 in keratinocytes or dendritic cells and are essential in the upregulation of costimulatory molecules on the surface of the dendritic cells (Antonios et al., 2009; Antonios et al., 2010).

It is not clear which ligands trigger TLR activation after exposure to skin sensitizers. It has been shown that ACD can develop in germ-free mice; hence danger signals from pathogenic microbes are not required for sensitization. This indicates that the presence of haptens is sufficient for TLR activation and presumably endogenous TLR ligands are involved (Martin et al., 2008). Several endogenous danger signals can be formed by the breakdown of extracellular matrix under influence of oxidative stress induced by sensitizers. These fragments can be recognized by TLR and initiate the innate immune response. In addition, NF-κB regulates hyaluronidases that degrade hyaluronic acid. Transcription of these enzymes can therefore be a result of earlier TLR activation, eventually strengthening the TLR activation in the skin (Martin et al., 2011). Another possible endogenous danger signal is uric acid, which can be released due to damage to the skin. Mice that were exposed to a combination of TNCB, uric acid crystals and an uricase inhibitor have increased sensitization. Uric acid has been shown to activate the NLRP3 inflammasome, thereby facilitating cytokine production (Liu et al., 2007). Other endogenous danger signals that have been linked to skin sensitization include the heat-shock proteins 27 and 70, which are also recognized by TLR4. Neutralizing antibodies for these heat shock proteins resulted in an impaired ear swelling in response to DNFB. In addition, the cytokine profile shifted from a Th1 to a Th2 repertoire (Yusuf et al., 2009).

3.1 The role of Nod-like receptors and the inflammasome in skin sensitization

TLRs are not the only PRR family members that play a role in the development of ACD, NOD-Like receptors (NLR) have also been implicated, especially their ability to form or activate inflammasomes containing caspase-1 is important. Ear swelling responses to DNFB or oxazolone were decreased significantly in caspase-1 knock out mice when compared to wild type mice (Antonopoulos et al., 2001). In addition, migration of Langerhans cells was evaluated in wildtype and caspase-1 deficient mice. It was shown that in the absence of caspase-1 the migration of Langerhans cells was impaired, but the maturation of Langerhans cells was not affected. In these mice, Langerhans cells migration could be restored when IL1-β, but not TNF-α were intradermally injected, indicating that these mice were unable to produce IL1-β, which is dependent on caspase-1 (Antonopoulos et al., 2001; Cumberbatch et al., 2001). IL1-α has been shown to have a marked effect on skin sensitization as well, since ear swelling in response to TNBS was impaired in IL1-α deficient mice and not in IL1-β deficient mice (Nakae et al., 2001). Whereas IL1-β is mainly produced by Langerhans cells, keratinocytes are the main source of IL1-α. These studies show that IL1-α is required in the induction of skin sensitization, whereas IL1-β plays an important role in Langerhans migration.

The NLRP3 inflammasome can be assembled due to the activation of the $P2X_7$ receptor. This receptor on the cell membrane recognizes extracellular ATP, which is a damage-associated molecular pattern. Mice deficient of $P2X_7$ had impaired ear swelling responses after exposure to TNCB and oxazolone. The ear swelling response was restored when a potent $P2X_7$-independent NLRP3 activator was applied. To determine whether the triggering of NLRP3 via $P2X_7$ is specific to sensitizers remains to be determined (Weber et al., 2010).

Importantly, the activation of inflammasomes seems to be an effect that is not specific to sensitizers. Other chemicals, such as irritants can also induce cellular stress and activate the inflammasome. The same stress that causes activation of inflammasomes could also lead to the degradation of hyaluronic acid and thus activation of TLR. Hence, this illustrates that NLRP3 inflammasome activation is not limited to skin sensitization and that this pathway cannot be used for the identification of skin sensitizing properties.

3.2 Effects of co-exposure to PAMPs on skin sensitization

In the initiation of skin sensitization, innate immune responses play an important role and it has been shown that skin sensitizers are able to trigger this pathway via PRR members. In reality it is possible that humans with an existing skin inflammation are exposed to haptens. This inflammation leads to microbial danger signals in the skin which could possibly aggravate the immune response induced by skin sensitizers. Evidence for this was found in *in vivo* studies in which the effects of PAMPs on ACD development were studied in mice using TLR4, 7 and 9 ligands.

In C57BL6 and C3H/HeN mice that were prior to sensitization exposed to the TLR4 ligand LPS by intradermal ear injection the ear swelling response to DNFB was increased. In the TLR4 deficient C3H/HeJ strain, co-exposure with LPS did not enhance the ear swelling response, providing evidence for a crucial role of TLR4 activation (Yokoi et al., 2009). The dose required for sensitization to DNFB was reduced a 100-fold when mice were pretreated

with R-848, a TLR7 ligand (Gunzer et al., 2005). Pretreatment with the TLR9 ligand CpG ODN enhanced ear swelling in response to DNFB, but it was shown that the site of exposure to CpG ODN is important. When the site of hapten exposure was not the same as the CpG administration site, no effect on skin sensitization was observed, illustrating that co-existing inflammatory signaling in the same skin area is needed to enhance the response (Akiba et al., 2004). There is evidence that a reduced skin barrier function, caused by mutations in the filaggrin gene, increases the sensitization rates to nickel (Novak et al., 2008; Metz & Maurer, 2009). Possibly, the impaired barrier function leads to more pathogen exposure and increased TLR activation. On the other hand, this mutation might also lead to increased skin penetration of the haptens thereby increasing the bioavailability in the skin.

In general, when mice are exposed to a hapten together with PAMPs, the ACD response is enhanced, which is most likely due to the increased activation of the innate immune system. In the skin, multiple danger signals are produced in response to the PAMPs thereby facilitating the innate immune response and subsequent adaptive immune response. Therefore, it is possible that concurrent hapten and pathogen exposure leads to increased risk of sensitization.

4. The role of the Nrf2-KEAP1 pathway in skin sensitization

Besides triggering the innate immune response, *in vitro* studies have shown that exposure to skin sensitizers induced oxidative stress in keratinocytes and dendritic cells (Matsue et al., 2003; Mehrotra et al., 2005). Particularly the antioxidant response Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-Keap1 pathway has been identified to play an important role in sensitization. The importance of this pathway has been shown in several microarray studies, in which gene expression analysis revealed that genes downstream of Nrf2 were highly upregulated in sensitizer exposed keratinocytes and dendritic cells (Ade et al., 2009; Python et al., 2009; Vandebriel et al., 2010). The relevance of Nrf2 for skin sensitization has been shown in Nrf2 deficient mice. The ear swelling in response to DNFB and oxazolone was reduced but not completely prevented in old (but not young) Nrf2 deficient mice. Furthermore, in these old mice it was shown that IFN-γ but not IL-4 production was absent, indicating that Nrf2 plays a role in the type 1 T cell response (Kim et al., 2008).

Under physiological conditions, the transcription factor (Nrf2) is bound to the sensory protein Keap1 (Figure 3). This complex promotes Cul-3 mediated ubiquitination and subsequent degradation of Nrf2. In response to oxidative stress, the highly reactive Cys residues of Keap1 are activated and Nrf2 is released (Niture et al., 2009). The free Nrf2 translocates into the nucleus and forms a complex with small MAF (F, G and K) molecules. This complex then recognizes the antioxidant responsive elements (ARE) in the promoter region of several genes, such as the cytoprotective heme oxygenase 1 (HMOX1), the phase II detoxification protein NAD(P)H quinine oxidoreductase (NQO1) and several genes involved in glutathione regulation. It is thought that skin sensitizers, which are known to be reactive to cysteine, are able to bind to the cysteine residues of Keap1 and thereby facilitate the release of Nrf2 (Motohashi & Yamamoto, 2004; Natsch, 2009). Although the majority of sensitizers can indeed bind to the cysteine residues, there are some that preferentially bind to lysine residues and these do not activate Nrf2 (Gerberick et al., 2007b; Natsch, 2009).
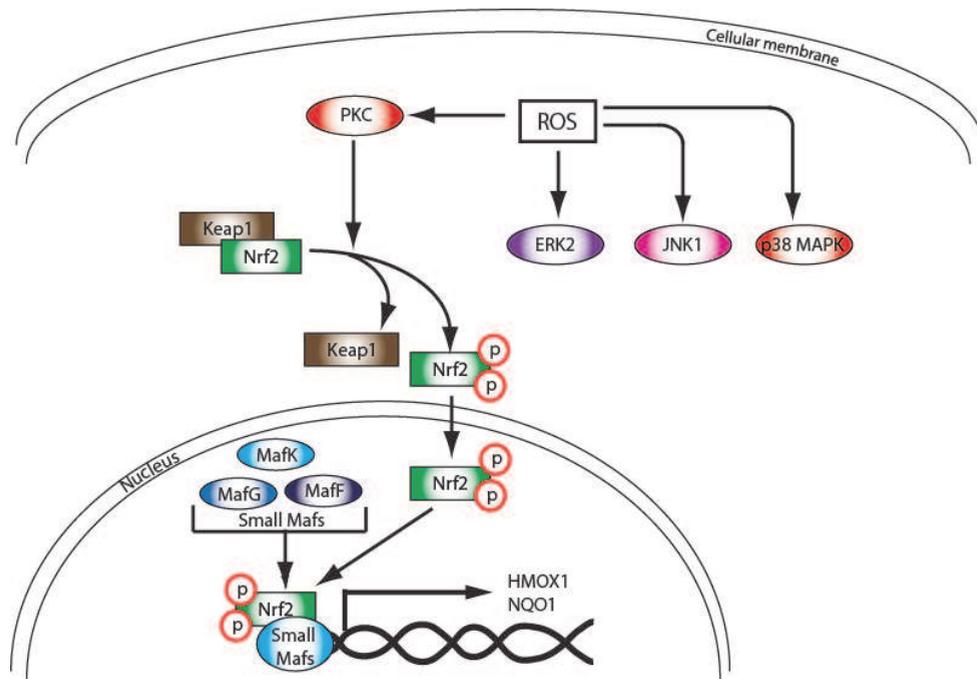
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Fig. 3. The Nrf2-Keap1 pathway (adapted from biocarta, available at:
http://www.biocarta.com/pathfiles/h_ARENRF2PATHWAY.asp)

It can be hypothesized that Nrf2 plays different roles in skin sensitization. First, Nrf2 activation is a result of oxidative stress induced by sensitizer exposure. Second, Nrf2 is involved in the regulation of the immune response, since several genes that are under Nrf2 control have been shown to have immunological effects. For example, upregulation of HMOX1 has been shown to inhibit the maturation of dendritic cells, thereby reducing T cell activation. Furthermore, HMOX1 induces an increased expression of the anti-inflammatory cytokine IL-10 (Listopad et al., 2007). Nrf2 has been shown to be important in attenuating different inflammatory responses (Kim et al., 2010). In Nrf2 deficient mice, the severity of disease in a colitis model was aggravated and this was attributed to increased levels of IL1-β, IL-6, IL12p40, and TNF-α, (Khor et al., 2006). In old mice deficient of Nrf2, skin sensitization was less pronounced, meaning that Nrf2 is essential for the skin sensitization process (Kim et al., 2008). Hence, Nrf2 has an important role in regulating immune responses.

Since skin sensitization involves different cell types, signaling pathways, cytokines and chemokines it might be possible that there is a direct link between Nrf2 activation and TLR activation. Nrf2 is involved in protection against oxidative stress and the induction of antioxidants. Activation of this pathway could affect redox-sensitive factors associated with TLR activation, such as NF-κB (Kim et al., 2010) and chemokines (Sozzani et al., 2005). For example, in cells exposed to LPS or nickel, the levels of thioredoxin-1 (Trx-1), an enzyme that is regulated by Nrf2, were elevated. It is postulated that the production of reactive

oxygen species following TLR4 activation causes Nrf2 translocation and transcription of the target genes (Listopad et al., 2007; Rushworth et al., 2008). Evidence for a direct link between Nrf2 and TLR was found in *in vitro* studies in macrophages. It was shown that Nrf2 activation by LPS was dependent on MyD88 but independent of the production of reactive oxygen species, indicating a second induction mechanism for Nrf2. It was speculated that MyD88 dependent signaling induced via TLR4 leads to activation of Nrf2 in order to regulate the inflammatory response (Kim et al., 2011). It remains unclear if there is a link between Nrf2 and TLR activation in skin sensitization and more research is needed to better understand the underlying mechanisms of skin sensitization.
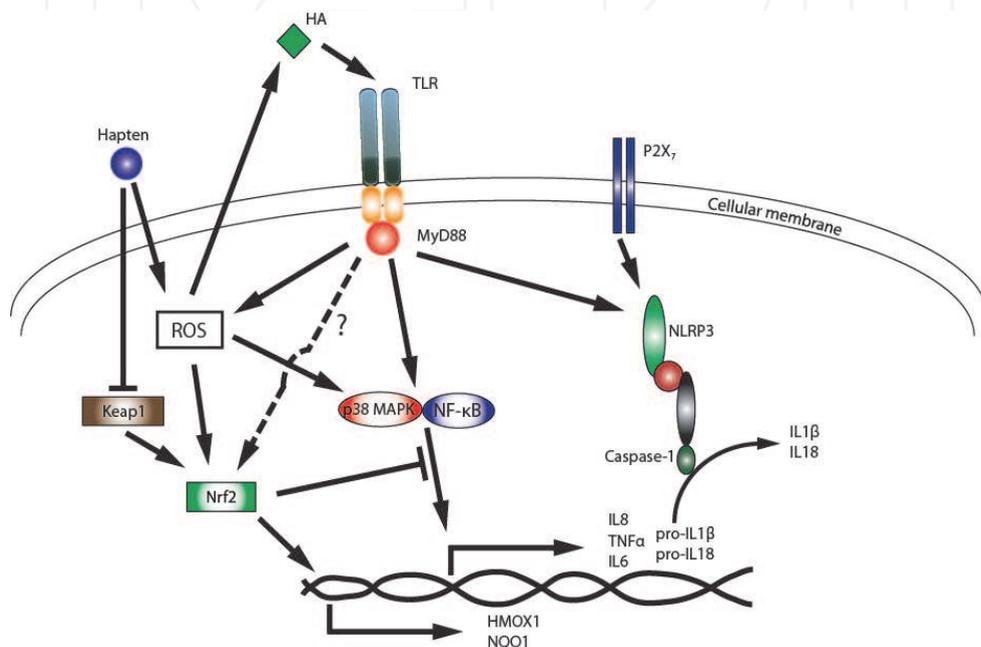

Fig. 4. Schematic overview of innate immune responses induced by sensitizers. Hapten exposure leads to degradation of hyaluronic acid (HA) in the skin and the HA fragments act as endogenous ligands for TLR activation. Downstream signaling pathways such as p38 MAPK are activated leading to NF-kB activation and release of pro-inflammatory cytokines (IL-6, IL-8, TNF-α) in the skin. At the same time, stress induced by haptens activates the NLRP3 inflammasome leading to caspase-1 activation and processing pro-IL-1β and pro-IL18 and subsequent release of IL-1β and IL-18. Together, this cascade of signaling pathways results in the essential factors needed for the development of an adaptive immune response to the skin sensitizers. On the other hand, the hapten exposure activates the Nrf2 pathway through generation of ROS and binding to Keap1, releasing Nrf2. Leading to production of antioxidants, affecting the redox balance. Redox-sensitive signaling pathways, such as p38 MAPK, NF-κB and chemokine production are attenuated in an attempt to reduce the inflammatory response in the skin and prevent skin sensitization (adapted from Martin et al., 2011).

5. Opportunities for the development of *in vitro* keratinocyte-based assays to predict the sensitizing potential of chemicals

In recent years many efforts have been made to develop cell-based assays able to identify skin sensitizers and to distinguish them from irritants. In keratinocyte-based assays, cytokine induction or gene expression profiles were assessed to find predictive biomarkers or pathways. In the human keratinocyte cell line HaCaT it was shown that intracellular IL-18 was significantly upregulated after exposure to four sensitizers and not after exposure to irritants (Van Och et al., 2005). Similarly, in the keratinocyte cell line NCTC2544 IL-18 was found to be predictive for skin sensitizers, and to distinguish them from respiratory sensitizers and irritants. Prohaptens require metabolic activation to become sensitizers were included in this assay and could be identified as well, indicating that these cells have sufficient metabolic activity (Corsini et al., 2009). IL-18 production is dependent on caspase-1 and requires the inflammasome activation. The relevance of signal transduction pathways in skin sensitizer induced IL-18 production was demonstrated using selective inhibitors and revealed a role for oxidative stress, NF-κB and p38 MAPK activation (Van Och et al., 2005; Corsini et al., 2009; Galbiati et al., 2011). In a reconstructed human epidermis model cytokine profiling was used to discriminate skin sensitizers from irritants. Five sensitizers and three irritants were tested in this 3D model and it was shown that sensitizers induce IL-8 production and secrete only low levels of IL-1α. In contrast, irritants induce high levels of IL-1α and only low levels of IL-8. With this limited number of substances the ratio of IL-8/IL-1α could be used to distinguish sensitizers from irritants. The benefits of using a 3D skin model are the possibilities to test topical formulations and compounds with low water solubility (Coquette et al., 2003) .

Tools that can be used to identify biomarkers for specific toxic effects, such as skin sensitization, include "omics" technologies, such as transcriptomics (measuring mRNA expression) and proteomics (measuring protein expression in tissues or cells). Transcriptomics has been used in the HaCaT cell line to find genes that are able to distinguish between sensitizers and irritants. After exposure to eight sensitizers and six irritants, pathway analysis showed that the Nrf2 pathway was significantly affected by sensitizers while it was not triggered by irritants. In addition, a set of 13 genes was identified that could predict the sensitizing potential of chemicals with 73% accuracy (Vandebriel et al., 2010). Further research is needed to confirm the accuracy of this gene list when more substances are used. The relevance of Nrf2 for skin sensitization was confirmed using primary keratinocytes exposed to two skin sensitizers (Yoshikawa et al., 2010)

The importance of Nrf2 in skin sensitization has resulted in the development of a reporter cell line from the HaCaT cell line, the KeratinoSens assay (Natsch, 2009). The promoter sequence of the Nrf2 dependent human gene AKR1C2, coding for an aldo-keto reductase, was placed before the gene encoding luciferase. The principle of this assay is that exposure to skin sensitizers leads to activation of the Nrf2 pathway and luciferase expression. In this assay, 67 substances (sensitizers, non-sensitizers and irritants) were tested. A fold increase of 1.5 in luciferase expression was used to identify skin sensitizers and it was shown that accuracy of this assay was 85.1% (Natsch, 2009; Emter et al., 2010). In a ring study with five laboratories it was shown that the KeratinoSens assay was easy transferable to other laboratories. The accuracy of the assay was tested with 28 blinded test compounds and the assay was reproducible between the laboratories (Natsch et al., 2011).

The limitation of these keratinocyte based *in vitro* assays is that they only provide a yes/no answer and are currently not able to predict the potency of compounds. For classification and labeling purposes, it is essential to have a test method that can predict both the potential and potency of skin sensitizing chemicals. Sensitizing potency has been assessed in an assay using an *in vitro* reconstructed human epidermal equivalent. This model has been developed to determine the potency of skin irritants (Spiekstra et al., 2009). To asses if this model could be used for skin sensitizing potency as well, viability and IL-1α secretion were assessed after 24 hour of exposure to 12 substances. It was shown that potency estimates could be derived, but only for sensitizers with irritant and cytotoxic properties. Furthermore, the endpoints chosen were not able to distinguish between sensitizers and irritants. It has been proposed that this 3D skin model could be used in a tiered approach where it is combined with an assay capable of making this distinction, for example measuring IL-18 in the NCTC2544 cell line. More substances need to be tested in order to establish if potency estimates can be made for skin sensitizers (dos Santos et al., 2011). Furthermore, in the development of *in vitro* tests more attention should be given to this by focusing on dose-response relations and establish if these correlate to human and LLNA potency values.

Keratinocyte-based *in vitro* assays appear to be promising for the identification of skin sensitizers, but represent only one aspect of the skin sensitization process. However, it is not foreseen that hazard identification can be accomplished with one single test, but that a battery approach combining several alternative test methods should be used. In such an integrated testing strategy (ITS), other alternative skin sensitization tests that could be included are *in silico* approaches, such as quantitative structure activity relationship (QSAR) models. Characteristic physical-chemical properties of skin sensitizers, such as electrophilic reactivity and hydrophobicity, can be assessed in this approach. The reaction mechanisms for skin sensitizers have been defined in five applicability domains (Roberts et al., 2008). A promising assay for evaluation of the protein binding capacity *in chemico* is the Direct Peptide Binding Reactivity Assay (DPRA), in which the binding of substances to synthetic peptides is measured by HPLC analysis. The accuracy of this assay is 89% and this assay is currently in prevalidation at the European Centre for the Validation of Alternative Methods (ECVAM) (Gerberick et al., 2007b; Gerberick et al., 2009). Besides keratinocyte based assays, *in vitro* assays using dendritic cells could be included in an ITS. The activation and maturation of dendritic cells can be analyzed using several assays, such as the Myeloid U937 skin sensitization test (MUSST) and the human cell line activation test (h-CLAT). Both assays apply chemicals to a monocytic cell line, U937 and THP-1 respectively, and measure the upregulation of CD86 protein on the cell surface. In addition to CD86, the upregulation of CD54 is measured in the h-CLAT assay (Sakaguchi et al., 2006; Sakaguchi et al., 2007; Sakaguchi et al., 2009; Ashikaga et al., 2010). Both assays are currently in ECVAM prevalidation. Finally, T cell proliferation induced by chemical exposure is important to demonstrate if a substance is immunogenic and is comparable to the endpoint measured in the LLNA. T cell activation is evaluated by first exposing dendritic cells to chemicals; the dendritic cells will load their MHC with haptenized peptides and upregulate costimulatory molecules. Next, these chemical-exposed activated dendritic cells are added to autologous peripheral blood mononuclear cells (PBMC) from which immune regulatory cells have been

removed. Naïve T cells that are able to recognize the hapten are activated and start to proliferate (Martin et al., 2010). The sensitivity of this assay has not been shown to date and this should be further explored.

Much progress is currently being made in the development of alternative (non-animal) test methods. However, it is as yet unclear how these different tests could be best combined in an ITS approach in order to make an accurate prediction of skin sensitizing potential and even more challenging: potency. An important step towards an ITS will be the development of a database in which all current outcomes per chemical and assay are listed in a matrix. In this database LLNA (or GPMT) data and human data should be included as well. This will allow evaluation of the sensitivity and specificity of a specific assay. In addition, such an approach can be applied to study the relationship between the various alternative assays and to get insight in the applicability domain of the individual assays. Another aspect is the relative importance of each assay for the outcome of the ITS. This will improve hazard identification as well as identify the key step(s) in the sensitization process (Vandebriel & van Loveren, 2010). In the integration of the results predefined weight factors for each assay should be summed and this sum is then used for prediction of skin sensitizing potential (Jowsey et al., 2006). To date, the applicability of ITS in this area has not been studied extensively. Natsch et al. (2009) integrated data of 116 chemicals from different *in vitro* and *in silico* assays. They used (1) peptide reactivity, (2) ARE induction in the KeratinoSens® assay, (3) calculated octanol-water partition coefficient (LogP) and (4) *in silico* TImes MEtabolism Simulator platform used for predicting Skin Sensitization (TIMES-SS), and compared the outcomes to LLNA data. It was shown that peptide reactivity and ARE induction similarly contributed to the model, whereas log$P$ had only negligible contribution (TIMES-SS was not included in this analysis). The prediction accuracy for the optimized ITS model was 87.9% (Natsch et al., 2009). In an interlaboratory validation of four assays tested with 23 chemicals it was shown that the accuracy of the individual assays (DPRA, MUSST, h-CLAT and KeratinoSens®) ranged from 83-91%. However, the accuracy increased when the different assays were combined and the combination of KeratinoSens® with the MUSST resulted in 100% accuracy for these 23 chemicals (Bauch et al., 2011). Although these data are promising, the way in which the individual assays were combined should be clarified, since this was not well described in the paper. This approach should therefore be further validated with additional chemicals and a predefined ITS approach.

In the future the safety evaluation of skin sensitizers might be possible without using animal tests. Current hurdles are the need for alternative test systems that can also provide potency estimates. Also, the concept of ITS should be further developed with a focus on formulating guidelines on which assays should be included in a strategy. The most logical way forward is that of a weight of evidence approach. In a recent expert meeting arranged by the EU, it was foreseen that replacement of the current animal test for the hazard identification skin sensitization would take at least 5 to 7 years, but that it is unknown when a more quantitative approach is possible without experimental animals (Adler et al., 2011).

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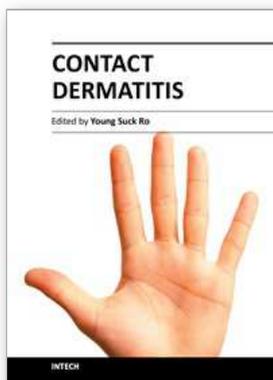
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