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REVIEW

The Nrf2-Keap1-ARE Toxicity Pathway as a Cellular Sensor for Skin Sensitizers—Functional Relevance and a Hypothesis on Innate Reactions to Skin Sensitizers

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With the tight deadlines set both by the public and by the regulatory authorities to replace animal tests for toxicological endpoints relevant to the development of cosmetic products, a large number of research projects have recently focused on cellular endpoints affected by skin sensitizing compounds. The general aim stated in these projects was to find "markers" for skin sensitizers, be it at the level of the transcriptome or at the protein level. Rather than talking of "cellular markers," the new paradigm "Toxicity testing in the 21st century" formulated by the National Academy of Sciences in the United States focuses on "Toxicity pathways." A specific marker for any given toxicological endpoint can only exist, if specific toxicity pathways, comprising specific sensors, are linked to this endpoint. In the context of skin sensitization, one has to ask whether there is an innate cellular signaling pathway activated by skin sensitizers. Here a significant body of evidence, mainly accumulated in the last 20 months, is reviewed, indicating that indeed the Nrf2-Keap1-ARE regulatory pathway is such a toxicity pathway activated by cysteine-reactive skin sensitizers. Whereas first indications on the *in vivo* relevance are available, key questions remain open and can now specifically be addressed. A minority of sensitizers, more specifically reacting with lysine residues, appears not to activate the Nrf2-Keap1-ARE pathway and might trigger yet another unknown toxicity pathway.

Key Words: **skin sensitizers; Nrf2; antioxidant response element; toxicity pathway; in vitro testing; chemical reactivity.**

INTRODUCTION

To defend the body against invading pathogens, the immune system is able to react with innate immune reactions before specificity toward the pathogen has evolved by the expansion of antigen-specific T- and B-cell clones. This early innate reactions are triggered by signaling cascades in which Toll-like receptors (TLRs) act as sensors. These receptors are able to detect the "unifying structural features" between different pathogens (e.g., double-stranded RNA for viruses and lipopolysacharides, flagellin, or lipopeptides for bacteria) (Miller *et al*., 2005).

In the immune response toward small reactive molecules in the skin (skin sensitizers), there appears also to be an early innate reaction, which is triggered in the absence of specificity conveyed by specific T-cell clones. Thus, application of skin sensitizers to naive animals triggers maturation and migration toward the lymph nodes of dendritic cells residing in the skin (Kimber *et al*., 2004), a process which is regulated by different cytokines (Antonopoulos *et al*., 2008; Kimber *et al*., 2004). This observation has led to continued efforts in developing cell-based assays, which would specifically react to sensitizers and which could then be used in the prospective identification of skin sensitizers. Such alternative tests are currently urgently needed due to regulatory restrictions on animal testing. Yet, only if indeed a sensitizer-specific innate reaction is functioning *in vivo*, there is any hope that an *in vitro* assay based on a single cell type and in the absence of a diverse array of T-cell clones is able to identify different sensitizers.

In order for such a cell-based assay to be able to selectively detect all sensitizers but not the irritants and at the same time for it to be unselective for different structural classes of sensitizers, it needs to be able to detect the "unifying feature" of sensitizers. In other words, we should know the functional analog of a "TLR for skin sensitizers", which acts as a sensor to trigger the unspecific induction of the sensitization reaction/dendritic cell migration.

Based on the vast structural diversity of skin sensitizers, a classical lock-and-key receptor event can *ab initio* be excluded to be involved in the unspecific detection of different skin sensitizers. With (1) the high diversity in size and structure of sensitizers and (2) the close structural relationship of some sensitizers with some nonsensitizers (see Fig. 1), the

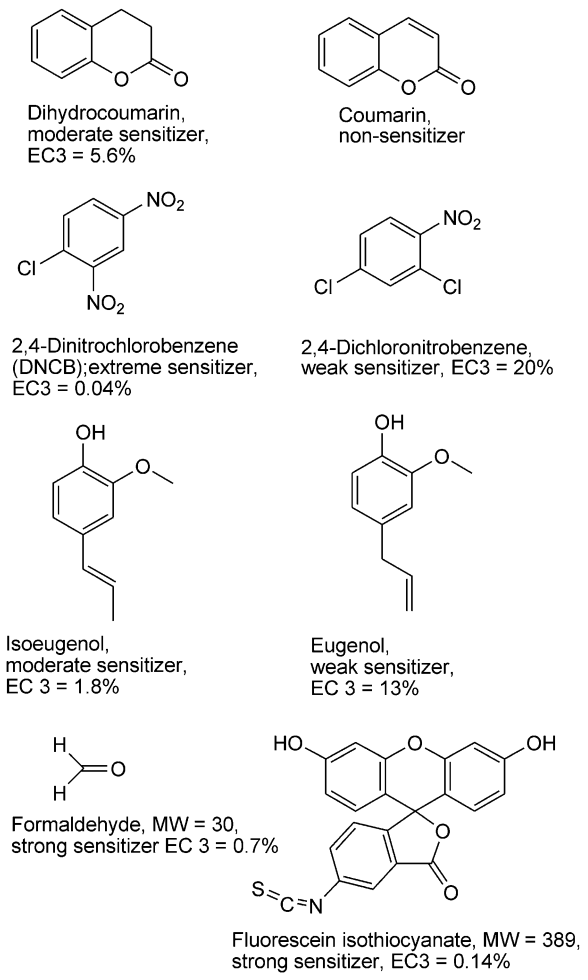

FIG. 1. Structurally similar molecules with differing reactivity give dissimilar sensitization results, whereas molecules of greatly differing molecular size and structure (e.g., formaldehyde and fluorescein isothiocyanate) must similarly activate the innate reactions, thus excluding a lock-and-key receptor event in the triggering of early innate responses. The sensitizing capacity of the molecules is expressed as their EC3 value in the LLNA, indicating the concentration that induces a threefold enhanced cellular proliferation in the draining lymph nodes after repeated topical application of the test compound onto the ears of mice (Basketter *et al.*, 2002).

recognition event cannot be based on the spatial structure as is the case for TLRs and most other biological receptors. But how else should this sensor work? There is one generally accepted unifying feature for skin sensitizers: Skin sensitizers are protein-reactive molecules (or can be metabolically converted into reactive molecules) (Roberts *et al.*, 2007) and thus the receptor should, on purely theoretical ground, respond to reactivity of molecules rather than to their three-dimensional structure.

Based on this reasoning, we have recently investigated the antioxidant response element (ARE)-dependent gene induction by skin sensitizers and reported data showing that the majority of skin sensitizers do induce genes regulated by this regulatory element (Natsch and Emter, 2008). This practical approach focused on the induction of this pathway as an *in vitro* approach to detect skin sensitizers, and its use appears to be a step forward in the development of an alternative assay (Basketter, 2008). Yet, this correlation did not yet indicate that there is also functional relevance for the *in vivo* situation. However, in the last 20 months, a number of new studies have appeared, which allow first conclusions to be drawn on the *in vivo* relevance of these data, and by combining insights from cell-based assays, peptide reactivity studies, and *in vivo* reports, one may try to define at least some "missing links" to identify further research needs.

SIX LINES OF EVIDENCE

The Keap1-Nrf2-ARE Regulatory Pathway

The sensor protein Keap1 (Kelch-like ECH–associated protein 1) contains highly reactive Cys residues (Wakabayashi *et al.*, 2004). In uninduced conditions, Keap1 targets the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2) for Cul3-mediated ubiquitinylation and proteolytic degradation in the proteasome (for review see Motohashi and Yamamoto, 2004). Covalent modification of the reactive Cys residues of Keap1 by small molecules leads to dissociation of Keap1 from the transcriptional regulator Nrf2, which then accumulates in the nucleus and activates genes (mainly genes coding for phase II detoxifying enzymes) having an ARE (also called EpRE, electrophile response element) in their promoter sequence (Dinkova-Kostova *et al.*, 2005; Wakabayashi *et al.*, 2004). Thus, reactivity toward specific Cys residues triggers the induction of a battery of genes, and Keap1 thus serves specifically as a cellular "reactivity sensor" or, more specifically, as a "cysteine-reactivity sensor." A simplified view of the Keap1-Nrf2-ARE regulatory pathway is depicted in Figure 2.

Induction of the Keap1-Nrf2-ARE Pathway by Skin Sensitizer

In Vitro

The well-established role of the Keap1-Nrf2-ARE regulatory pathway in the detection of electrophiles led us to the straightforward hypothesis that this pathway could be used to develop a cell-based alternative assay to replace animals for skin sensitization testing. Indeed, out of a total of 84 tested sensitizers, 66 induced a luciferase gene under the control of ARE elements. If restricting the analysis to the chemicals for which human evidence on the sensitization risk exists, 30 of 34 tested sensitizers were correctly identified by the test (Natsch *et al.*, 2009). In this simplified reporter gene assay, the evidence is somewhat indirect, as the different steps in the signaling cascade are not separately investigated (binding of the molecule to Keap1, nuclear accumulation of Nrf2, and binding to the consensus ARE sequence). This gap has partly been filled by the work of Ade *et al.* (2009) and Megherbi *et al.* (2009). These groups had shown by Western blotting that in

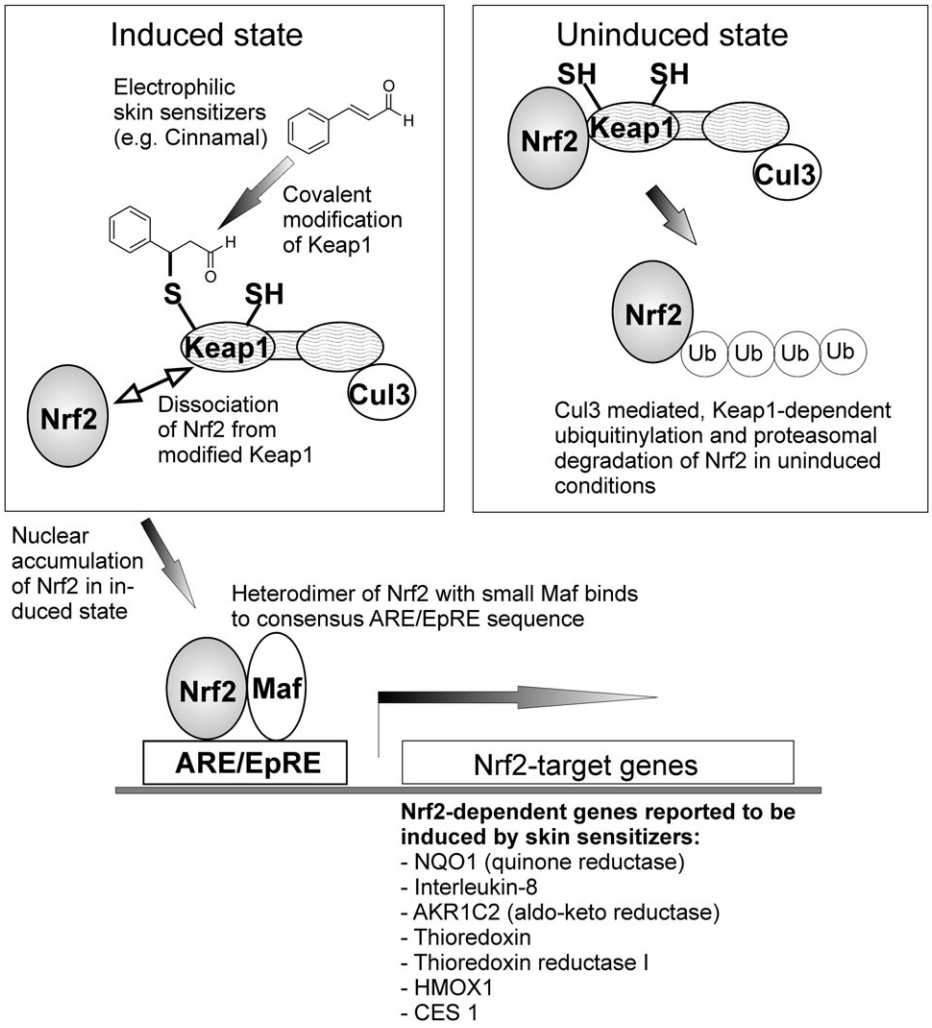

FIG. 2. A general view of the induction of the Nrf2 pathway by skin sensitizers.

dendritic cells the Nrf2 level is indeed enhanced after treatment with sensitizers.

Besides the results of these targeted approaches, focused directly on the Keap1-Nrf2-ARE pathway, evidence from several gene-chip studies also indicates that ARE-regulated genes are induced in different cell types after sensitizer challenge. The genes coding for thioredoxin and thioredoxin reductase I were significantly upregulated by a sensitizer in the study of Ryan *et al*. (2004), and these genes were shown to be under the control of Nrf2 in a gene-chip study comparing expression changes after addition of an ARE inducer to wild-type and Nrf2-deficient mice (Kwak *et al*., 2003). Another gene whose enhanced expression was identified by gene-chip–based screening and reverse transcriptase-polymerase chain reaction (RT-PCR) (Gildea *et al*., 2006; Ryan *et al*., 2004) as a robust marker for sensitizers is *AKR1C2*, coding for an aldo-keto reductase. Interestingly, this gene also contains a functional ARE sequence in its promoter (Lou *et al*., 2006). In the gene-chip study of Ryan *et al*. (2004), interleukin (IL)-8 messenger RNA (mRNA) was also found to be increased by a sensitizer both when detected with gene-chip and RT-PCR analysis; several authors had reported enhanced levels of IL-8 mRNA in sensitizer-treated dendritic cells (Bergström *et al*., 2007; Python *et al*., 2007). Interestingly, IL-8 formation appears to be under the control of Nrf2 (Zhang *et al*., 2005). Finally and most recently, Python *et al*. (2009) reported a gene-chip study on the effects of the contact sensitizer cinnamic aldehyde in blood-derived dendritic cells and the dendritic cell line MUTZ-3. Interestingly, among the 2472 genes upregulated by cinnamic aldehyde on either of the two cell types, there were only 72 genes common to both the primary cells and the stable cell line, a frequency which is even lower as one might expect by chance. This fact highlights the difficulties of having a good model cell line reflecting the effects in primary cells. Nevertheless, among the four robust markers induced by sensitizers in both models (as verified by RT-PCR), there were the two Nrf2-regulated genes *CES1* and *NQO1*, indicating the reproducible induction of the Nrf2 pathway by sensitizers in

different cell types. The evidence summarized above indicates that many cellular markers responding to sensitizer challenge are under control of the Keap1-Nrf2-ARE pathway.

Skin Sensitizers Which Do Not, or Only Weakly, Induce the Keap1-Nrf2-ARE Pathway In Vitro

At this stage, it is valuable to have a closer look at the data and especially at the false negatives and the outliers. (Here we discuss only the moderate, strong and extreme sensitizers: The database of weak sensitizers includes some irritating chemicals, for which the positive local lymph node assay [LLNA] results may come from both the irritation and/or the sensitization reaction, which makes interpretation more difficult.) In our recent data compilation (Natsch *et al*., 2009), 57 moderate to extreme sensitizers were included. Among these, the following seven chemicals did not induce Nrf2-dependent luciferase activity: phthalic anhydride, trimellitic anhydride, 3,4-dihydrocoumarin, ethylenediamine, *trans*-anethole, diethyl-sulfate, and creosol.

Ethylenediamine, *trans*-anethole, and creosol may be viewed as typical pro-haptens, for which a metabolic activation step was postulated (Roberts *et al*., 2007). Yet phthalic anhydride, trimellitic anhydride (Gerberick *et al*., 2007), and 3,4-dihydrocoumarin (Aleksic *et al*., 2009) are some of the few chemicals that in peptide reactivity assays directly and exclusively react with lysine-containing peptides and not with cysteine residues. In terms of outliers when looking at potency, fluorescein-5-isothiocyanate and oxazolone are the two chemicals among the strong and extreme sensitizers which only at relatively high concentrations induce the ARE-regulated reporter gene (Natsch *et al*., 2009). These two chemicals not only appear to react with Cys residues but also have a particularly high reactivity toward Lys residues (Gerberick *et al*., 2007). These differences in reactivity toward different nucleophiles may be of importance when we are trying to see "the greater picture" from a molecular and mechanistic point of view.

Effect of Sensitizers in Nrf2$^{-/-}$ Knockout Mice

Most important evidence for the *in vivo* relevance of the Nrf2 pathway for skin sensitization comes from a recent study with Nrf2-knockout mice (Kim *et al*., 2008). Older Nrf2-knockout mice (but not young ones) showed a reduced but not abolished reaction to the sensitizers oxazolone and 2,4-dinitrofluorobenzene (DNFB), when increases in ear thickness were taken as the endpoint. The allergen-induced formation of the T_H1 cytokine interferon-γ (IFN-γ) was completely abolished in the knockout mice, whereas the T_H2 cytokine IL-4 was not affected. These data indicate that Nrf2 is essential for a full sensitization to be expressed, and especially it is a prerequisite for the allergen-induced IFN-γ formation and type 1 T-cell response. Two chemicals were tested in this study: oxazolone and DNFB. Regarding reactivity, oxazolone (Gerberick *et al*., 2007) and DNFB (Tingle *et al*., 1990) were shown to covalently react with both Cys and Lys. This fact may be important to explain the differential effects on cytokine induction and ear swelling as highlighted below.

In Vivo *Gene Induction in the LLNA by Sensitizers: Recent Gene-Chip Studies*

Two recent studies have investigated the induction of genes in local lymph nodes in mice treated with sensitizers. Ku *et al*. (2008) performed the LLNA with the sensitizing test chemicals oxazolone, toluene-2,4-diisocyanate, and 2,4-dinitro-chlorobenzene (DNCB) and compared reactions to the irritants croton oil and nonanoic acid. They recorded gene expression changes with a complete mouse oligo DNA-chip and found a significant number of genes upregulated by all three sensitizers. They followed this up with RT-PCR and found four genes significantly upregulated by all three sensitizers but not the irritants. Interestingly, these genes included IFN-γ- and IFN-regulated genes. As indicated above, oxazolone and DNCB have mixed reactivity with Cys and Lys (Gerberick *et al*., 2007), and isocyanates have a rapid reversible reactivity toward cysteine and a slow irreversible binding to lysine (Nakamura *et al*., 2009).

The second study of Boverhof *et al*. (2009) applied trimellitic anhydride to the mouse ears and examined gene expression changes in the local lymph nodes. Anhydrides such as trimellitic anhydride and phthalic anhydride do exclusively react with lysine residues (Gerberick *et al*., 2007). Boverhof *et al*. examined dose-response curves for gene induction, to calculate EC3 values for gene induction similar to the EC3 values classically calculated for cell proliferation. The most sensitive genetic marker identified was the T_H2 cytokine IL-4, with an EC3 of 0.12%, the EC3 for cell proliferation being 0.11%. Interestingly, no induction of the IFN-γ gene was observed in this study on a Lys-reactive chemical, although the IFN-γ gene was specifically included as a control in the set of genes whose expression was confirmed with RT-PCR.

Differential Cytokine Induction by Skin Sensitizers and Respiratory Sensitizers

There is a large body of evidence on differential cytokine induction in the lymph nodes by either respiratory or skin sensitizers (Dearman *et al*., 2000; Van Och *et al*., 2002). These studies have mainly tested different anhydrides and isocyanates as respiratory sensitizers and compared them to DNCB and other prototypic skin sensitizers. As a general conclusion, the respiratory sensitizers (most of which are also skin sensitizers) did preferentially induce T_H2 cytokines such as IL-4 and IL-10, whereas the exclusive skin sensitizers predominantly induced T_H1 cytokines such as IFN-γ. This observation is called "cytokine polarization." Again we may ask the question, whether it is linked to the fact that the tested respiratory sensitizers, especially the anhydrides and the isocyanates, have a particularly high reactivity with Lys residues, whereas the skin sensitizers tested have a mixed reactivity and are more reactive with Cys residues. Hopkins *et al*. (2005) tested

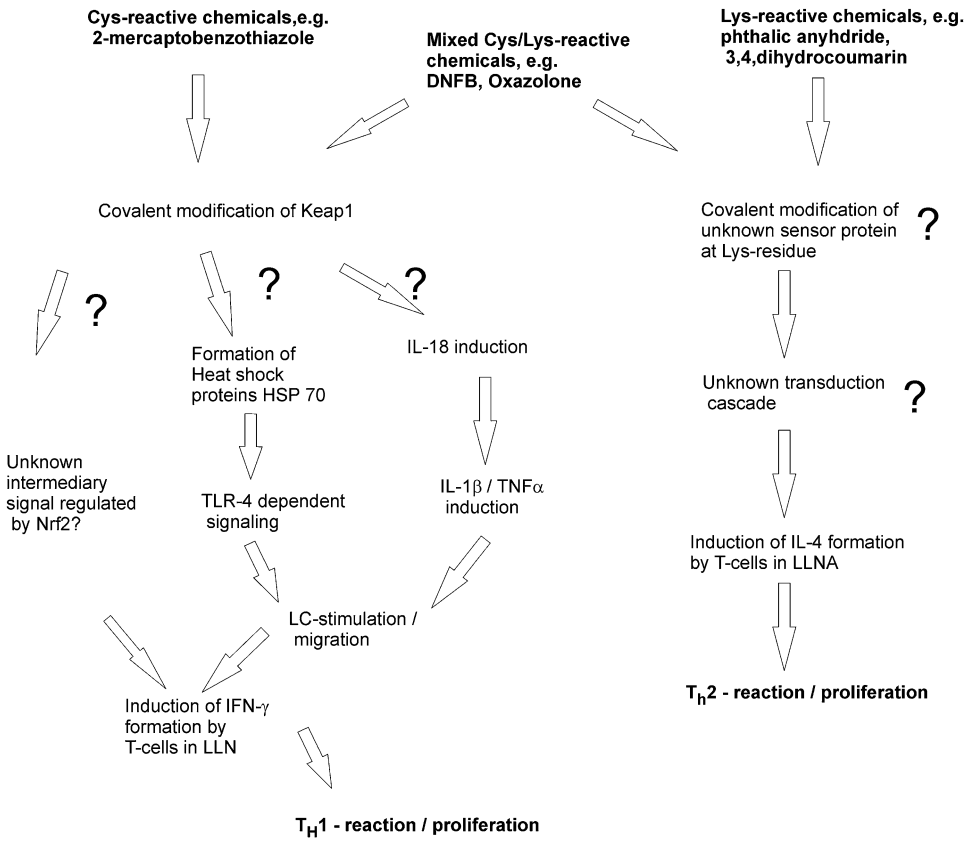

FIG. 3. Hypothetical model with two separate signal cascades for –SH- and –NH_2-reactive chemicals ultimately leading to T_H1 and T_H2 responses. The hypothetical steps are indicated by a question mark and indicate the potential further research directions.

cytokine polarization and protein binding by the skin sensitizers DNCB, DNFB, and the respiratory sensitizers fluorescein isothiocyanate, trimellitic anhydride, and dinitrobenzenesulfonyl chloride (DNBSCl). The chemicals inducing a type 1 reaction preferentially bound to intracellular proteins, whereas the type 2 cytokine-inducing chemicals were rather binding to serum proteins. The authors discussed the possibility that this could be due to a selective binding of DNCB and DNFB to Cys residues and binding of the other chemicals to Lys residues. Yet their conclusion was that the difference is rather due to a preferential binding to either intracellular or extracellular proteins *per se* and not due to a residue specificity since DNBSCl is known to selectively bind to tryptophan and not to Lys residues. Yet binding to intracellular versus extracellular proteins and specificity versus Cys or Lys maybe linked as the oxidizing extracellular environment contains few reduced Cys residues, which are abundant in the reducing intracellular environment.

AN INTEGRATED HYPOTHESIS LEADING TO FURTHER RESEARCH QUESTIONS

From the, at first sight disparate, six lines of evidence summarized above, we may start to draw a picture, incomplete in its very nature, but potentially helpful in formulating further research questions. To understand how chemicals may trigger cellular signals of the innate immune reaction and to interpret results, we should talk not only about "sensitizers" versus "nonsensitizer" but also keep in mind their reaction mechanisms and their specific reactivity, thus the "biological view" and the "chemical view" of skin sensitization have to meet:

(1) Sensitizing chemicals with a significant reactivity toward Cys residues may covalently modify the highly reactive Cys residues in the Keap1 protein and thereby trigger activation of ARE-regulated genes. The induction of IFN-γ, and thus the induction of the type 1 T-cell response by sensitizers, appears to be dependent on this process, and it is therefore likely that Keap1 is the sensor for Cys-reactive chemicals, which leads to a specific sensitizer-induced IFN-γ production and the type 1 T-cell response. Yet the clear signaling pathway for this process is unknown, as IFN-γ has not been shown to be directly regulated by Nrf2. The detailed link still needs to be established.

(2) Chemicals with an exclusive reactivity toward Lys residues such as anhydrides do not induce the Keap1 pathway and thus fail to induce IFN-γ formation. These chemicals induce IL-4, which is independent of the Nrf2 pathway. As IL-4 induction has been shown for different chemicals with Lys

reactivity, we may postulate a second sensor mechanism by which reactivity of a chemical with a Lys residue on an hitherto unknown sensor protein (and by an unknown transduction mechanism) finally triggers the induction of IL-4. Activation (or inactivation) of a regulator protein by spontaneous reaction with reactive molecules at Lys residues is to my knowledge not known, yet the enzymatic acetylation of Lys residues in regulatory proteins to activate or silence gene expression is well established (see e.g., Ikenoue *et al*., 2008).

As pointed out above, many mechanistic studies on sensitizers are performed with extreme sensitizers, such as DNCB, DNFB, and oxazolone, which have a mixed reactivity toward Cys and Lys residues and which may often also induce a mixed type 1 and type 2 response. This would explain the results from the Nrf2-knockout study, where only a partial effect was seen on the ear thickness response but very clear-cut results were obtained on IFN-γ by the knockout and no effect at all on IL-4. If the above model is valid, repetition of the study in $Nrf2^{-/-}$ mice with an exclusively Lys-reactive chemical such as phthalic anhydride would have no effect on the ear swelling as compared to wild-type mice, whereas an exclusively Cys-reactive chemical such as 2-mercaptobenzothiazole or a weak Michael acceptor would result in a complete loss in ear swelling in the knockout (unless tested at irritant dose) as it does for IFN-γ formation. This hypothesis along with the open questions is schematically illustrated in Figure 3.

SPECIFIC FUTURE RESEARCH QUESTIONS

(1) With the current cell-based *in vitro* assays based on induction of ARE-dependent genes (Ade *et al*., 2009; Natsch and Emter, 2008), the majority of skin sensitizers can be recognized, yet a small number of exclusively Lys-reactive chemicals may escape detection. This can be complemented with a peptide reactivity assay measuring Lys reactivity either with a separate Lys-peptide (Gerberick *et al*., 2004) or with a peptide containing both Lys and Cys residues (Natsch and Gfeller, 2008; Nilsson *et al*., 2005). Yet if the goal is to recognize all sensitizers in a battery of purely cell-based assays, we should find the molecular sensor and transduction pathways reacting to Lys-reactive chemicals such as anhydrides, which are finally triggering IL-4 formation. Since Lys reactivity and IL-4 induction appears to be a feature that might be specific for respiratory sensitizers, identification of this potential mechanism might then also help to develop assays to specifically recognize respiratory sensitizers. Hopefully, by analyzing the genomic and proteomic data being generated in the European Sens-it-iv project (investigating both skin and respiratory sensitizers) and related projects, some insight will be gained. Thus, for example, Verstraelen *et al.* (2009) reported the phosphatase and tensin homolog (PTEN) pathway as the probably most specific signaling pathway in the context of respiratory sensitization. Interestingly, PTEN is regulated by enzymatic acetylation of critical Lys residues (Ikenoue *et al*., 2008; Okumura *et al*., 2006) and whether Lys-reactive chemicals specifically interfere with this process is a challenging question.

(2) As outlined above, testing further mechanism-specific chemicals in Nrf2-knockout mice may better define the *in vivo* importance of the Nrf2 pathway as a sensor for different chemical classes of sensitizers. Thus, specifically anhydrides should be compared versus specifically thiol-reactive chemicals in the $Nrf2^{-/-}$ mice.

(3) We should find the link between Nrf2 induction and IFN-γ formation. As reviewed above, induction of IFN-γ formation by sensitizers requires a functional Nrf2, yet the direct link is not known. Here, it will also be important to find out in which cell types Nrf2 induction is crucial: Nrf2 induction in the keratinocytes might induce specific cytokine secretion leading to dendritic cell activation. On the other hand, Nrf2 signaling in dendritic cells might directly lead to factors such as IL-12, finally triggering IFN-γ formation in T cells.

(4) Several new and sensitive biological markers for sensitizing chemicals have been described recently. Detailed gene-chip and RT-PCR studies have identified *CCR2* (Chemokine (C–C motif) receptor 2) and *CREM* (cyclic AMP-responsive element modulator) (Hooyberghs *et al*., 2008) or Notch 3 (Gildea *et al*., 2006). It will be interesting to investigate whether these genes are also under control of the Nrf2 pathway or whether yet another reactivity-specific sensor is involved in their regulation. The most investigated marker for sensitizers is CD86, whose expression on the surface of dendritic cells is upregulated by sensitizers (Sakaguchi *et al*., 2006). This process is dependent on TLR signaling and the mitogen activated protein kinase kinase p38 (Miyazawa *et al*., 2008), yet the sensor involved in triggering this response has also not been identified yet.

(5) How are dendritic cells activated by Cys-reactive chemicals activating the Nrf2 pathway? The hallmark of the innate immune reaction in the sensitization phase is the activation and emigration from the skin of Langerhans cells (dendritic cells). Thus, we come to the last and most crucial research question: Is this process directly regulated by Nrf2, and if so what are the possible links? Two ideas may be followed:

(i) IL-18 appears to have an important and decisive role in the sensitizer-induced formation of IL-1β and tumor necrosis factor-α in the skin (Antonopoulos *et al*., 2008) and in the induction of Langerhans cell migration. This cytokine can be produced both by keratinocytes and by dendritic cells. It appears not to be induced by irritants. IL-18 (formerly known as IFN-γ-inducing factor) is known for its ability to induce IFN-γ formation in T cells, a process that now appears to be under control of Nrf2. Thus one hypothesis to be tested is whether the Nrf2 pathway acts upstream of IL-18, which in turn activates dendritic cells and finally triggers IFN-γ formation in the lymph node (see Fig. 3). Both IL-1β and IL-18 are produced as inactive forms upon TLR signaling and are then processed by caspase-1 (contained in the

inflammasome) into the active form. This processing is induced by skin sensitizers (Watanabe *et al*., 2007). However, these processes are paralleled for IL-1β and IL-18 and also induced by irritants and ultraviolet light. Thus, how Nrf2 or another sensor could trigger the selective induction of IL-18 by reactive chemicals only cannot yet be predicted based on these studies.

(ii) TLR4 signaling is essential for the sensitization reaction in absence of IL-12 receptors (Martin *et al*., 2008). In addition, recent studies had shown that endogenous ligands of TLRs are important in the induction of skin sensitization (Freudenberg *et al*., 2009), and in particular the heat-shock proteins Hsp27 and Hsp70 have been investigated (Yusuf *et al*., 2009). These Hsps act as endogenous ligands for TLRs and in particular TLR4 (Miyake, 2007). Antibodies against these proteins reduced the sensitization reaction, whereas the Hsp27 protein itself augmented the sensitization response. Lymph node cells from Hsp70/Hsp27-antibody–treated, DNFB-sensitized animals had a reduced capability to react *in vitro* with the formation of type 1 cytokines upon treatments with 2,4-dinitrobenzensulfonic acid treated dendritic cells. On the other hand, the capability of these cells to secrete IL-4 and IL-10 was enhanced. The action of the Hsps was TLR4 dependent (Yusuf *et al*., 2009). These data indicate that the Hsps are essential in the stimulation of dendritic cells by sensitizers, especially to mount the T_c1/T_H1-reaction. Several gene-chip studies compared the induction of genes by Nrf2-activating compounds in $Nrf2^{-/-}$ mice with wild-type mice. Genes activated by the activators only in wild-type but not knockout mice appear to be Nrf2 regulated. Interestingly, all these studies found different Hsps to be Nrf2-inducible (Hu *et al*., 2006; Kwak *et al*., 2003; Nair *et al*., 2007; Thimmulappa *et al*., 2002), and *Hsp70* was specifically upregulated by sulforaphane in mouse liver in wild-type but not knockout mice in one study (Hu *et al*., 2006). A regulation of Hsp70 by an ARE/EpRE sequence was also proposed in the work of Almeida *et al.* (in press). In addition, several studies reported a concomitant induction of Hsp70 and the Nrf2-regulated gene heme oxygenase-1 (*HMOX1*) by electrophiles (Thompson and Burcham, 2008), and the fact that the same inducers induce Hsp70 and Nrf2 led to the speculation that this could be due to a common mechanism (Calabrese *et al*., 2008). In conclusion, a model with Cys-reactive chemicals activating Nrf2, thereby inducing Hsp70 formation, which in turn activates dendritic cells by TLR4 signaling to induce a type 1 T-cell response appears plausible. However, the evidence from these gene-chip studies is somewhat circumstantial, and more data are needed to prove a model with direct activation of Hsp70 by Nrf2. As Hsps are often only induced close to cytotoxic concentrations and since Hsp induction has also been related to depletion of reduced cellular glutathione (Calabrese *et al*., 2000; Liu *et al*., 1996), the glutathione status (which is directly modified by Cys-reactive chemicals close to cytotoxic concentrations) could also be an alternative trigger for Cys-reactive chemical–dependent Hsp activation.

APPLICATION FOR HAZARD IDENTIFICATION AND RISK ASSESSMENT

The discussion in this work focused on the innate sensor mechanisms that may allow cells to react to electrophilic chemicals in order to finally trigger dendritic cell activation and immune reactions. With an enhanced understanding of the sensor mechanisms, by which cells can sense skin sensitizers in the unspecific induction phase of skin sensitization, improved sensor-based assays may be created and efficient high-throughput, nonanimal test systems may be developed, which fit the paradigm "Toxicity testing in the 21st century" formulated by the National Academy of Sciences in the United States focusing on "Toxicity pathways". Dissecting the regulatory pathways involved in the sensitizer-induced polarization toward either a type 1 or a type 2 T-cell response may also help to discriminate better between respiratory and skin sensitizers.

With high-throughput assays based on the specific sensors, one may measure detailed dose-response curves for individual chemicals and complete structure-activity datasets for classes of compounds. These data from biological tests can then also be integrated together with data from *in chemico* tests investigating the chemical reactivity of test chemicals (Gerberick *et al*., 2007; Jowsey *et al*., 2006; Natsch *et al*., 2009; Roberts and Natsch, 2009). Correlating this combined *in vitro* data to known evidence from human and animal tests will help to build models in order to finally predict sensitization potential of novel compounds. These models may be either local models for specific structural classes, so-called mechanistic applicability domains (Roberts *et al*., 2006, 2007), or more global models covering a wide range of novel substances.

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