

## ***In Vitro* Approaches for Detection of Chemical Sensitization**

Erwin L. Roggen

Novozymes AS, Bagsvaerd, Denmark

(Received 19 November 2013; Accepted 14 January 2014)

**Abstract:** Concerns, legislation and research needs have precipitated developments such as the *mode of action* concept, the *Tox21* strategy, the concept of *pathways of toxicity* and the *adverse outcome pathway* framework. New technologies and paradigms are currently transforming these concepts into applicable animal-free toxicity testing systems. The adverse outcome pathway framework provides a structure for collecting, organizing and evaluating the available data that describe the compound and the events resulting in an adverse outcome at a biological level of organization. The current chapter intends to provide a non-exhaustive review of (i) our current understanding of the molecular mechanisms driven the key events of the mode of action for sensitization induction by chemicals, (ii) the tools that were developed on the basis of the available knowledge and (iii) the major gaps that need to be filled.

The adverse outcome pathway (AOP) framework provides a structure for collecting, organizing and evaluating the data that describe the compound and the events resulting in an adverse outcome at a biological level of organization with relevance for risk assessment. Several AOPs are currently developed and evaluated for a variety of human toxicity end-points [1,2]. The suggested mode of action (MOA) pathway formed the basis for the recently published flow diagram of the AOP for skin sensitization induction (Fig. 1) [3,4]. An AOP for respiratory sensitization is currently being discussed.

### ***In vivo* Reference Data Reflect ‘Organ Responses’ and ‘Organism Responses’**

Predictive assessment of the contact allergenic potential of chemicals is performed in guinea pigs, mice and human beings [5]. With the mouse local lymph node assay (LLNA) as the exception, *in vivo* models for skin sensitization assess sensitization induction by monitoring clinical ‘organism responses’ (Fig. 1), such as erythema, oedema and ear swelling, elicited by challenging the exposed individuals to the test compound or a vehicle control. The LLNA assesses the allergenic potential of chemicals by use of the induction phase only, as determined by cell proliferation in draining lymph nodes (‘organ responses’). The LLNA is currently the gold standard for potency assessment when human data are missing. Based upon historical data, the ability of animal tests to predict the potential of chemicals to induce contact allergens in human beings seems not to exceed 80%.

Author for correspondence: Erwin L. Roggen, Novozymes AS, Kroghshøjvej 36, 2880 Bagsvaerd, Denmark (e-mail elro@novozymes.com).

The currently available animal approaches for assessing respiratory sensitization were developed primarily to acquire mechanistic understanding and not for the purpose of broad hazard assessment [6]. The potential of a compound to trigger respiratory sensitization is determined by monitoring pulmonary reactions and allergic antibody production (‘organ responses’) triggered in guinea pigs, mice or rats after repeated exposures to chemicals or proteins by inhalation or intratracheal instillation.

### **Available Animal-Free Testing Approaches Cover the MOA for Skin Sensitization**

While the available animal models determine organ and organism responses to assess the sensitization potential of chemical, animal-free approaches capture the key events describing the MOA pathway for skin sensitization proposed by Adler *et al.* [3].

*Bioavailability: the compounds acquire access to the system.*

*The current understanding.* The bioavailability of a potential skin sensitizer and its metabolites is defined by its molecular weight, physicochemical characteristics, adsorption to macromolecules, concentration ( $\mu\text{g/ml}$ ), dose ( $\mu\text{g/cm}^2$ ), bioactivation, reactivity rate and contact time [7]. Several studies correlate pulmonary bioavailability to lipophilicity, molecular polar surface area and hydrogen bond donor counts of the chemical [8].

*Tools for animal-free testing.* The bioavailability of compounds can be assessed by a combination of *in vitro* and *in silico* modelling approaches. Useful *in vitro* methodologies

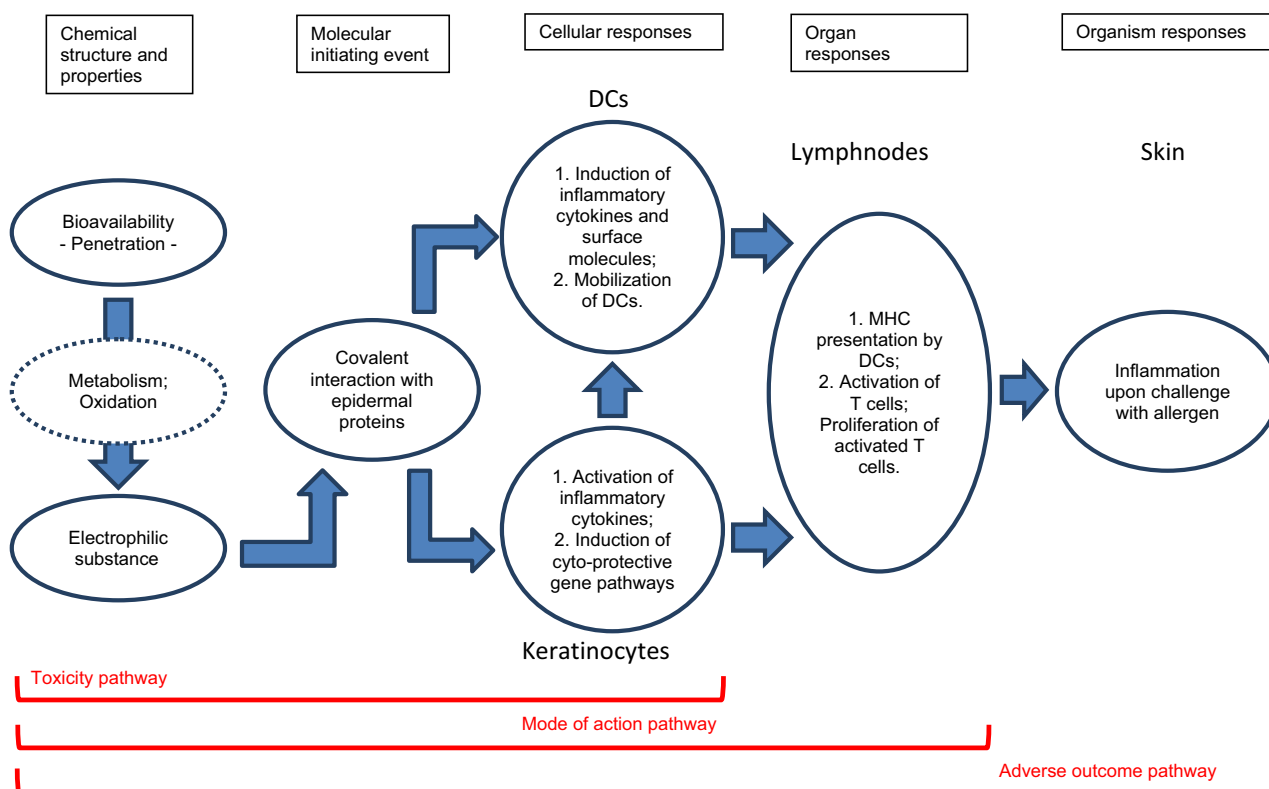


Fig. 1. OECD flow diagram of the pathways associated with skin sensitization. The Adverse Outcome Pathway (AOP) framework provides a structure for collecting, organizing and evaluating the available data that describe the compound and the events resulting in an adverse outcome at a biological level of organization [4].

for assessing skin absorption and systemic availability of chemicals exist [9]. *In vitro* and *in silico* tools for predicting effective *in vivo* lung doses were developed with apparent success [8].

**Important gaps.** With the methodologies available, efforts should now go to the development and implementation of methods for quantification of compound disposition in skin and lung, to obtain information on kinetics, potential tissue bioaccumulation and actual exposure at cellular level.

**Haptenation:** *the chemical reacts covalently with a 'carrier protein'.*

**The current understanding.** The majority of sensitizing chemicals are reactive, electrophilic chemicals that form covalent bonds with nucleophilic nuclei on proteins (haptens). Occasionally, chemicals require activation by the host cytochrome P450 oxidase system or peroxidases (pro-haptens) or by oxidative derivatization (pre-haptens) to acquire sufficient electrophilicity. Non-electrophilic binding occurs through disulfide exchange or co-ordination bonds [10]. The reaction rate and mechanism by which the hapten is reacting with the nucleophilic groups on the protein influence its allergenic potency [11,12]. *In vitro* studies suggest that the specificity of the covalent modification is time dependent and

dose dependent and that the target proteins *in vitro* become more general and non-discriminative over time and with increasing concentrations of chemical [13].

**Tools for animal-free testing.** The direct peptide reactivity assay (DPRA) is based on measurement of the reactivity of the hapten with two different peptides containing, respectively, lysine and cysteine. This choice allows for the detection of the majority of reactive chemicals [14]. On a set of 145 chemicals, the DPRA was concordant with *in vivo* data in eight of 10 cases (sensitivity: 82%; specificity: 74%) [15]. Adding an incubation step with horseradish peroxidase and hydrogen peroxide provides a straightforward approach for detecting the peptide reactivity of pro-haptens [16]. The available data indicate that chemical reactivity with lysine appears to drive a Type I sensitization response while reactivity with cysteine results in a Type IV response [17].

**Important gaps.** A better understanding is required of the features (if any) on both chemical and targeted protein that make the resulting hapten-protein complex a sensitizer, determine potency and drive T helper cell type 1 (Th1)-Th2 skewing. This will help the development of *in vitro* protein haptenation assays that provide a more complete data set on the tested chemicals.

*Inflammation: innate recognition followed by activation of innate immunity.*

*The current understanding.* There is increasing evidence underpinning the central role of innate immune responses and inflammation in both skin and respiratory sensitization.

*Haptens engage Toll-like receptors (TLR) 2 and 4.* The available evidence strongly points at TLR2 and TLR4 as crucial for activation of the Th1 responses in skin sensitization and allergic contact dermatitis [18]. While still unclear for chemical respiratory sensitizers, mechanistic studies on allergenic proteins implicate TLR2 and TLR4 signalling in dendritic cell (DC) activation and subsequent induction of Th2 responses leading to respiratory sensitization and allergy [19,20]. TLR signalling is linked to the lipid-binding properties of, for example, *Dermatophagoides* group 2 allergens [21]. The intrinsic adjuvant activity of these allergens appears to compensate for the low expression of endogenous myeloid differentiation factor-2 (MD-2) by human airway epithelium [22].

*Reactive oxygen species (ROS) play a role in allergen-induced sensitization induction.* Fig. 2 shows the processes believed to induce skin sensitization [23] in the context of the 'oxidative interface' [24]. Evidence for a role of ROS in respiratory sensitization induction and allergic airway inflammation is provided by pollen allergens exerting [NAD(P)H] oxidase activity, an enzyme activity found in mitochondria and driving intracellular ROS production [25].

*ROS production results in degradation of endogenous hyaluronic acid (HA), and TLR2 and TLR4 activation.* Changes in HA expression and fragmentation into LMW-HA breakdown products are associated with oxidative stress and skin sensitization induction [26]. Direct evidence for the role of LMW-HA generation by keratinocytes (KCs) in the induction of skin inflammation by contact sensitizers is provided by studies in germ-free mice and by *ex vivo* studies performed with human skin. LMW-HA fragments induce the expression of a variety of genes coding for signalling proteins, including tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-18 and interferon

(IFN)- $\gamma$ , which are *in vivo* directly responsible for driving the effector phases of chemical skin sensitization [16,24]. Similar mechanisms seem to play in lung inflammation [27].

*ROS signal the 'nucleotide-binding domain and leucine-rich repeat containing family' protein 3 (NLRP3) inflammasome resulting in IL-1 $\beta$ , IL-18 and IL-33 activation.* In skin sensitization, cells are stressed to release adenosine triphosphate (ATP) leading to NLRP3 inflammasome activation, and processing and secretion of IL-1 $\beta$ , IL-18 and IL-33, among others. Especially, IL-18 is consistently found to play a central role in skin sensitization induction, but not irritant contact dermatitis [28]. Respiratory exposure causes the release of ATP leading to uric acid production, activation of the NLRP3 inflammasome complex, processing and secretion of IL-1 $\beta$ , release of IL-6 and chemokines and enhanced differentiation of a specific IL-17-producing T-cell subpopulation (Th17) [29]. Uric acid may in this context play an important role in Th2 skewing [30].

*Tools for animal-free testing.* The acquired knowledge about the mechanisms driving allergic inflammation has resulted in several assays performed with human primary KCs or keratinocyte cell lines.

*Assessing oxidative stress:* The relevance of 'nuclear factor erythroid 2-related factor 2' (Nrf2) – 'Kelch-like ECH-associated protein 1' (Keap1) pathway to skin sensitization is explained by the direct reactivity of most sensitizing materials to key cysteine residues of Keap1, an Nrf2 repressor protein. Test chemicals that exclusively react with lysine should therefore be considered outside the chemical applicability domain [31,32]. The KeratinoSens<sup>TM</sup> assay (Givaudan Schweiz AG, Dübendorf, Switzerland) is a cell-based reporter gene assay for screening substances with a full dose–response assessment. The induction of a luciferase gene under the control of the antioxidant response element (ARE) is determined. An extensive evaluation of the test on 145 chemicals revealed a sensitivity of 79%, specificity of 72% and accuracy of 77% [15]. SenCeeTox<sup>®</sup> (CeeTox, Kalamazoo, MI, USA) combines markers for

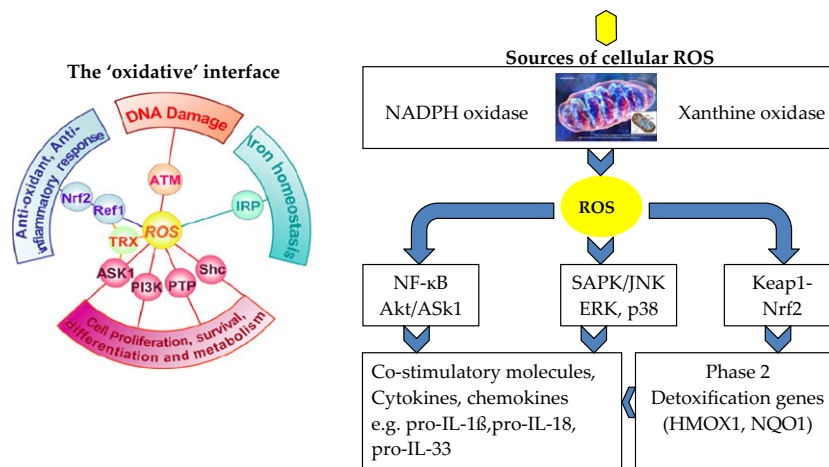


Fig. 2. Schematic overview of the role of ROS in sensitization induction. Reactive oxygen species (ROS) play a role in allergen-induced sensitization induction. Figure shows the oxidative interface between ROS and the processes believed to induce sensitization [23] the context of the 'oxidative interface' [24].

cell viability, Nrf2/ARE gene expression and direct reactivity over concentration and time in a proprietary algorithm. An unpublished blinded study on 67 chemicals demonstrated a good sensitivity (81%) and specificity (92%) (<http://www.cee-tox.com>). An external evaluation of the predictive capacity on a set of 40 blinded compounds was concordant for seven of 10 compounds (sensitivity: 62%; specificity: 79%) and revealed poor potency prediction (62.5%) (<http://www.wc8.loreal.com/medias/4-theme/EVALUATION%20OF%20SENSCEETOX.pdf>, 2011). SENS-IS<sup>®</sup> (Immunosearch, Le Plan de Grasse, France) is based on the quantitative analysis of specific biomarkers for irritation and ARE gene expression in combination with a proprietary gene set expressed in 3-dimensional reconstructed epidermis (Episkin<sup>®</sup>, SkinEthics, Lyon, France) upon exposure to chemicals. A blinded evaluation of the predictive capacity of this approach on a set of 40 compounds revealed a good sensitivity (91%), specificity (83%) and concordancy (87%). This model failed to accurately subcategorize the test chemicals (63%) ([http://www.skinethic.com/iso\\_album/evaluation\\_of\\_sens-is.pdf](http://www.skinethic.com/iso_album/evaluation_of_sens-is.pdf), 2011).

**Assessing IL-18 levels:** IL-18 plays a proximal role in skin sensitization induction, but not in irritant contact dermatitis or asthma, by enhancing the secretion of pro-inflammatory mediators and by favouring Th-1 type immune response [28,29]. The NCTC 2544 IL-18 test was shown to be potentially useful for identification of skin sensitizers. When tested on 33 chemicals, 32 were correctly classified (concordance: 97%). Respiratory sensitizers (n = 7) and nine of 10 irritants were consistently negative in this assay (specificity: 94%) [33]. In combination with a 3D-reconstructed human epidermis (RHE) irritation test, a correct subcategorization was obtained for nine of 10 skin sensitizers [34]. The RHE IL-18 potency test is a RHE test system for identification and classification of skin-sensitizing chemicals, including chemicals of low water solubility or stability. Concordant results were obtained for all 20 chemicals. Comparison of the *in vitro* potency data with human DSA<sub>05</sub> (µg/cm<sup>2</sup>) data revealed a good correlation (Spearman r = 0.8500; *p* > 0.01) [35]. DSA<sub>05</sub> is the induction dose per skin area that produces a positive response in 5% of the tested population.

**Important gaps.** The involvement of TLR2, TLR4 and ROS signalling, and NLRP3 inflammasome assembly in both skin and lung sensitization induction seems to exclude a role in Th1–Th2 skewing. A better understanding is required about the subtle balance between danger signals (e.g. IL-18 *versus* uric acid) or intracellular interactions promoting distinct immune phenotypes. Furthermore, it is imperative to understand how reactivity rate and mechanisms of haptentation affect this balance. This will help the development of *in vitro* epidermal inflammation assays that provide a more complete data set on the tested chemicals.

**Dendritic cell activation: from innate responses to DC maturation.**

**The current understanding.** It is generally accepted that activation of DCs results in mature cells having changed phenotype [36]. The most prominent changes include

Table 1.

A selection of the most prominent phenotypic changes resulting from DC activation and applied for assessing the sensitization potential of chemicals.

Property	Immature	Mature
MHC class I	+	+++
CD44	±	+++
CD54	±	+++
CD58	±	+++
CD80	+	+++
CD83	+	+++
CD86	+	+++
11-12	+	+++
CCR1,2,5,6	+++	+
CCR7	+	+++
CXCR4	+	+++
E-cadherin	+++	+
Ag capture	+++	+
Ag processing	+++	+
Ag presentation	+	+++

antigen-presenting capacity, enhanced levels of MHC class I and co-stimulatory molecules such as cluster of differentiation (CD)54, CD80 and CD86, and receptors that are essential for migration (table 1). Extensive genomic analysis of MUTZ-3 cells has identified genes describing eight dominating functions. The primary pathways of skin sensitization induction involve signalling through transcription factors Nrf2 and aryl hydrocarbon receptor (AHR), and protein ubiquitination [37]. Monocyte-derived dendritic cells (Mo-DCs) and human monocytic leukaemia cell line (THP-1) exposed to haptens induce the Nrf2 pathway when exposed to chemicals exerting cysteine and cysteine/lysine reactivity. Lysine-reactive chemicals were less efficient [32]. By stratifying the sensitizing chemicals into chemical reactivity groups, a number of canonical pathways known to be involved in the biology of sensitization were confirmed. In addition, novel pathways were identified [12]. Sensitizers with different reactivity mechanisms or potency were further shown to engage different pathways, indicating that the biological end-point of T-cell priming is achieved through different upstream mechanisms (Fig. 3).

**Tools for animal-free testing.** Functional and transcriptional analysis of various myeloid cell lines has clearly demonstrated the significance of the MUTZ-3 cell line as a model for functional studies of inflammatory responses [38]. The genomic allergen rapid detection (GARD) test is a MUTZ-3-based assay for assessing chemical sensitizers utilizing genomic biomarker prediction signatures to generate prediction calls of unknown chemicals as skin sensitizers, respiratory sensitizers or non-sensitizers, including irritants [37]. The predictive performance of the skinGARD was assessed in an *in-house* validation study. In total, 33 of 37 coded chemicals were correctly classified, yielding 90% concordancy with available *in vivo* data (sensitivity: 92%; specificity: 83%). The test data set contained chemicals that are prone to be misclassified in both *in vivo* and *in vitro* assays (Malin Lindstedt, personal communication). The respGARD for assessing chemical respiratory sensitizers was

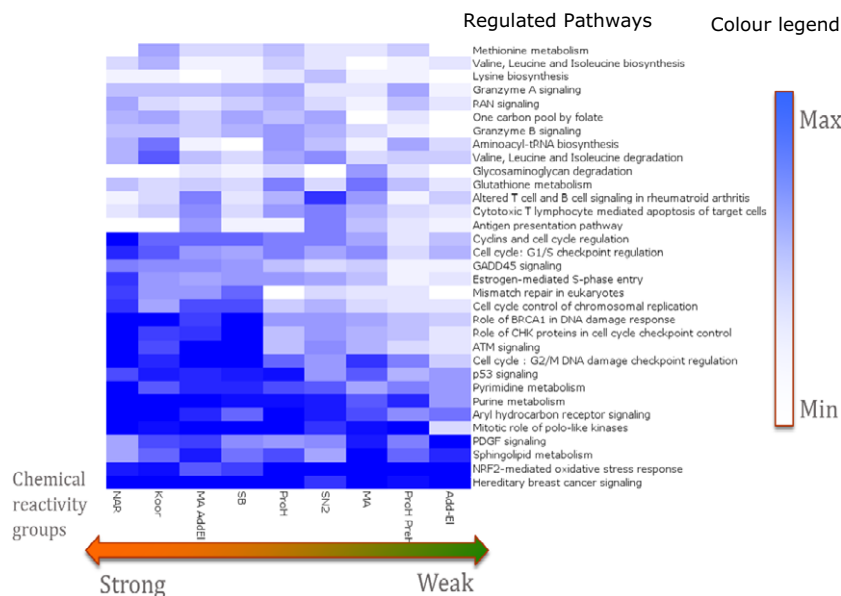


Fig. 3. Pathways affected by a sensitizer correlate with its chemical reactivity and the potency. Albrekt *et al.* [12] confirmed canonical pathways known to be involved in the biology of sensitization and identified novel pathways. Subsets of sensitizers seem to engage different pathways, suggesting that the biological end-point of T-cell priming is achieved through different upstream mechanisms. Abbreviations: Add: Addition to carboxylic acid derivatives; El: Elimination from carboxylic acid derivatives; KOOR: Co-ordination bonds; MA: Michael addition or 1,4 addition to  $\alpha/\beta$  unsaturated carbonyl; NAR: nucleophilic aromatic substitution; PreH: pre-hapten; ProH: pro-hapten; SB: Schiff's base reaction; SN2: bimolecular nucleophilic substitution.

found to build upon a gene pool that is different from the gene pool used by the skinGARD (Malin Lindstedt, personal communication).

The most advanced DC maturation test is the human cell line activation test (h-CLAT), which uses THP-1 cell [39]. When challenged by 117 chemicals, the test revealed a good concordance (85%) with the LLNA data (sensitivity: 88%; specificity: 75%). As compared to the human response data, these performance characteristics were 80%, 84% and 69%, respectively [40]. There are indications that the h-CLAT correlates with the LLNA and may have the potential to provide information about the potency of the test chemical.

**Important gaps.** A better understanding of how the early gene changes contribute (or not) to the expression of maturation markers may help to understand and resolve the reasons behind the low specificity of the available test methods. Furthermore, understanding better the association between pathway activity and chemical class will help the development of assays for subcategorization. Our understanding about the processes triggered in DC by chemical respiratory sensitizers and protein allergens, and how they affect (if at all) Th2 skewing, is only just emerging and needs to be expanded.

**Dendritic cell migration: translating the message into specific actions.**

**The current understanding.** The molecular mechanisms driving migration of DC to and from peripheral tissues were reviewed [41]. Fibroblasts play a key role both as advisors helping the KCs and Langerhans cells (LCs) to discriminate

irritants from sensitizers, which in many cases are irritants themselves, and as guides helping the LCs out of the epidermis into the dermis and further towards lymphatic vessels (Fig. 4) [42]. Using a full-thickness tissue-engineered skin model containing fully functional MUTZ-3-derived LCs (MUTZ-LC), the MUTZ-LCs were demonstrated to mature and to acquire the ability to migrate towards C-X-C motif ligand (CXCL)12 and C-C motif ligand (CCL)19/21 in a comparable manner with primary LCs in skin explants [43].

**Tools for animal-free testing.** The acquired knowledge has resulted in a DC-migration assay which is based on carboxyfluorescein succinimidyl ester (CFSE)-labelled MUTZ-3 cells. The discriminating feature of the assay is that irritant-induced migration is CCL5 dependent, while sensitizer-induced migration is CXCL12 dependent. The read-out of the test is the ratio between migration towards CXCL12 or to CCL5. In spite of its complexity, the assay seems to be relatively well transferable [44].

**Important gaps.** While the preliminary data on 12 chemicals are promising (no misclassification), further evaluation performed with more chemicals is required. The test is also expensive and rather complicated which may hamper its application by industry. More work is required to refine the test to make it more attractive for industrial use.

**T-cell priming and proliferation: the turning point.**

**The current understanding.** Primary T-cell responses in lymph nodes require contact-dependent information exchange

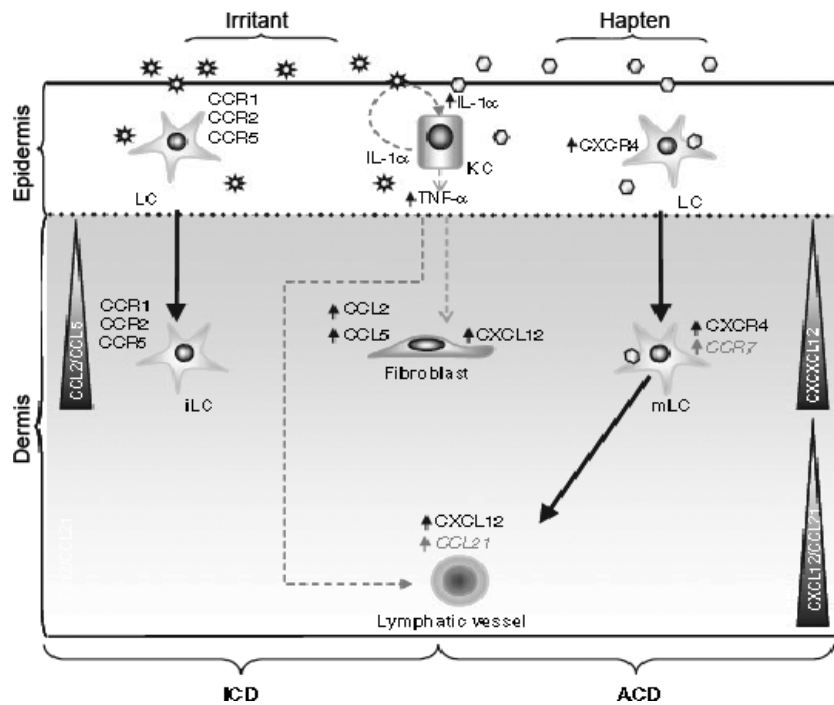


Fig. 4. Mechanisms of Langerhans cell (LC) migration in irritative *versus* allergic contact dermatitis. The molecular mechanisms driving migration of dendritic cells to and from peripheral tissues are reviewed extensively elsewhere [41]. Fibroblasts play a key role both as advisors helping the keratinocytes (KCs) and LCs to discriminate irritants from sensitizers, which in many cases are irritants themselves, and as guides helping the LCs out of the epidermis into the dermis and further towards lymphatic vessels [42].

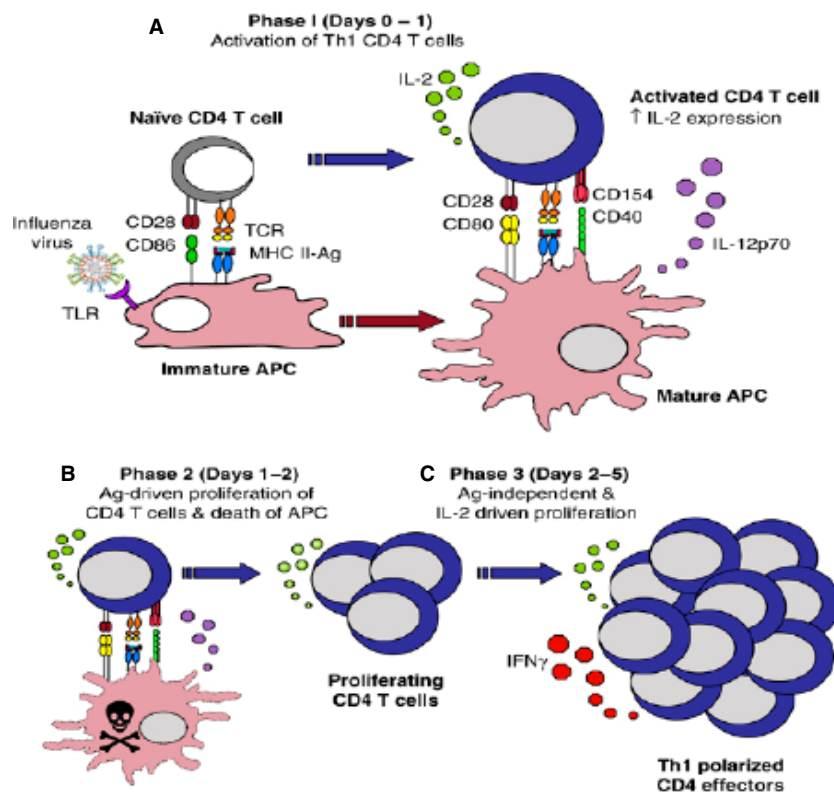


Fig. 5. Effector T cells develop over three phases. Primary T-cell responses in lymph nodes require contact-dependent information exchange between T cells and DCs. T-cell priming by DCs occurs in three successive stages. Transient serial encounters during the first activation phase (T-cell activation) are followed by a second phase of stable contacts culminating in cytokine production antigen-driven T-cell proliferation, which triggers a transition into a third phase of high motility and rapid proliferation (antigen-independent and IL-12-driven proliferation) [45,46].

between T cells and DCs. The available evidence indicates that T-cell priming by DCs occurs in three successive stages (Fig. 5). Transient serial encounters during the first activation phase (T-cell activation) are followed by a second phase of stable contacts culminating in cytokine production antigen-driven T-cell proliferation, which triggers a transition into a third phase of high motility and rapid proliferation (antigen-independent and IL-12-driven proliferation) [45,46]. Studies in mice have exposed the induction of two functionally polarized populations of T cells, distinguished by patterns of cytokine production [47]. Th17 cells were shown to play a crucial role in allergen-specific cellular and humoral immune responses through the activation of both contact hypersensitivity and airway hyper-responsiveness. It has been suggested that IL-17 has activities similar to the pro-inflammatory cytokines IL-1 and TNF- $\alpha$ , which are known to have crucial roles in the induction of other cytokines, chemokines and adhesion molecules. It is also known that IL-17 itself is a potent inducer of IL-1 and TNF- $\alpha$  acting on macrophages and keratinocytes [48,49]. It has been established that NLRP3 inflammasome activation and IL-1 $\beta$  production are required to develop allergic airway inflammation in mice and that IL-17 and IL-22 production by Th17 cells plays a critical role in established asthma [48,50].

*Tools for animal-free testing.* The human T-cell priming assay (hTCPA) is based on a co-culture system that measures the effect derived from the contact of freshly isolated T cells (CD45RA<sup>+</sup>, C-C motif (CCR)7<sup>+</sup>, CD45RO<sup>-</sup>, CD2<sup>-</sup>) with autologous DC cells previously activated and chemically modified by the test substance. The test is usually repeated on T cells derived from five different donors to minimize donor-to-donor variability. T cells are isolated again and re-stimulated with autologous DC and the same control chemicals to assess antigen specificity [51]. Comparison of the proliferation during stimulation and re-stimulation is an important parameter. The second, more reliable and robust read-out is the expression of IFN- $\gamma$ , which is efficiently produced by T cells in response to contact allergens. This parameter shows more effective results when TNF- $\alpha$  is measured simultaneously, as some donors fail to respond by IFN- $\gamma$  production. Preliminary results demonstrated the capability of this assay to effectively predict antigenicity of chemicals, including drugs. Currently, two protocols [51,52] are optimized and harmonized and the potential to predict sensitization potency using frequency and T-cell receptor repertoire analysis is investigated.

*Important gaps.* Our understanding of the T-cell populations that are activated by xenobiotics and proteinaceous allergens is increasing. It is, however, not clear yet how Th1–Th2 skewing and the balance between regulatory and effector T cells is controlled. T-cell stimulation is a pivotal event, being part of the sensitization induction phase as well as the clinical phase. Potency assessment performed with T-cell-based assays needs therefore to build on an in-depth understanding of mechanisms behind potency of sensitization

induction on one hand and severity of clinical symptoms on the other hand.

### Summary

During the last decade, several methods for assessing skin and respiratory sensitization have emerged. Some have entered the pre-validation process, and other less advanced assays generated interesting contributions to the molecular understanding of sensitization mechanisms. There are also promising animal-free strategies emerging, which in contrast to the *in vivo* studies distinguish between skin and respiratory sensitizers. Evaluation of the potency of chemical sensitizers with *in vitro* methods may become reality in the near future.

Our understanding of the molecular mechanisms driving skin sensitization and contact dermatitis is substantial, but the mechanisms driving chemical respiratory sensitization are still unclear.

While the most advanced tools can be used for classification, our understanding of the relation between reactivity rate, mechanism of haptation, protein target selection, pathway activation and T-cell skewing is still not sufficient to fully describe chemicals using animal-free testing methods. More efforts should be addressed to refine existing methods and to further develop new methods that lead to an improved awareness of the real mechanisms of a chemical in triggering a sensitization reaction in exposed human beings.

### References

- 1 MacKay C, Davies M, Summerfield V, Maxwell G. From pathways to people: applying the adverse outcome pathway (AOP) for skin sensitization to risk assessment. *ALTEX Online* 2013; S1868696X1301251X.
- 2 Yozzo KL, McGee SP, Volz DC. Adverse outcome pathways during zebrafish embryogenesis: a case study with paraoxon. *Aquat Toxicol* 2013;**126**:346–54.
- 3 Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V *et al.* Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. *Arch Toxicol* 2011;**85**:367–485.
- 4 Organization for Economic Co-operation and Development. The adverse outcome pathway for skin sensitization initiated by covalent binding to proteins. Part 1: Scientific evidence. Series on Testing and Assessment No 168, 2013.
- 5 Thyssen JP, Giménez-Arnu E, Lepoittevin JP, Menné T, Boman A, Schnuch A. The critical review of methodologies and approaches to assess the inherent skin sensitization potential (skin allergies) of chemicals Part I. *Contact Dermatitis* 2012;**66**:11–24.
- 6 Boverhof DR, Billington R, Gollapudi BB, Hotchkiss JA, Krieger SM, Poole A *et al.* Respiratory sensitization and allergy: current research approaches and needs. *Toxicol Appl Pharmacol* 2008;**226**:1–13.
- 7 Smith CK, Hotchkiss SAM. *Allergic Contact Dermatitis: Chemical and Metabolic Mechanisms*. Taylor & Francis Ltd, London, UK, 2001: doi: 10.1034/j.1600-0536.2001.450224-2.x
- 8 Cooper A, Potter T, Luker T. Prediction of efficacious inhalation lung doses via the use of *in silico* lung retention quantitative structure-activity relationship models and *in vitro* potency screens. *Drug Metab Dispos* 2010;**38**:2218–25.
- 9 Basketter D, Pease C, Kasting G, Kimber I, Casati S, Cronin M *et al.* Skin sensitization and epidermal disposition: the relevance of

- epidermal disposition for sensitization hazard identification and risk assessment. *Altern Lab Anim* 2007;**35**:137–54.
- 10 Chipinda I, Hettick JM, Siegel PD. Haptenation: chemical reactivity and protein binding. *J Allergy* 2011;**2011**:839682.
- 11 Chipinda I, Ajibola RO, Morakinyo MK, Ruwona TB, Simoyi RH, Siegel PD. Rapid and simple kinetics screening assay for electrophilic dermal sensitizers using nitrobenzenethiol. *Chem Res Toxicol* 2010;**23**:918–25.
- 12 Albrekt AS, Johansson H, Börje A, Borrebaeck CAK, Lindstedt M. Differentially regulated signalling pathways in MUTZ-3 cells stimulated with skin sensitizers. *BMC Pharmacol Toxicol* 2014. In press.
- 13 Manchanda T, Hess D, Dale L, Ferguson SG, Rieder MJ. Haptenation of sulfonamide reactive metabolites to cellular proteins. *Mol Pharmacol* 2002;**62**:1011–26.
- 14 Gerberick F, Aleksic M, Basketter D, Casati S, Karlberg AT, Kern P *et al.* Chemical reactivity measurement and the predictive identification of skin sensitizers. The report and recommendations of ECVAM Workshop 64. *Altern Lab Anim* 2008;**36**:215–42.
- 15 Natsch A, Ryan CA, Foertsch L, Emter R, Jaworska J, Gerberick F *et al.* A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. *J Appl Toxicol* 2013;**33**:1337–52.
- 16 Gerberick F, Troutman J, Foertsch L, Vasallo JD, Quijano M, Dobson RLM *et al.* Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. *Toxicol Sci* 2009;**112**:164–74.
- 17 Lalko JF, Dearman RJ, Gerberick GF, Troutman JA, Api AM, Kimber I. Reactivity of chemical respiratory allergens in the peroxidase peptide reactivity assay. *Toxicol In Vitro* 2012;**27**:651–61.
- 18 Martin SF, Dudda JC, Bachtanian E, Lembo A, Liller S, Durr C *et al.* Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. *J Exp Med* 2008;**205**:2151–62.
- 19 Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;**15**:410–6.
- 20 Page K, Ledford JR, Zhou P, Wills-Karp M. A TLR2 agonist in German cockroach frass activates MMP-9 release and is protective against allergic inflammation in mice. *J Immunol* 2009;**183**:3400–8.
- 21 Thomas WR, Hales BJ, Smith WA. Structural biology of allergens. *Curr Allergy and Asthma Rep* 2005;**5**:388–93.
- 22 Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R *et al.* Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009;**457**:585–8.
- 23 Corsini E, Galbiati V, Nikitovic D, Tsatsakis AFM. Role of oxidative stress in chemical allergens induced skin cells activation. *Food Chem Toxicol* 2013;**61**:74–81.
- 24 Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signalling. *Cell Signal* 2012;**24**:981–90.
- 25 Boldogh I, Bacsı A, Choudhury BK, Dharajiyā N, Alam R, Hazra TK *et al.* ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J Clin Invest* 2005;**115**:2169–79.
- 26 Esser PR, Wölfle U, Dürr C, von Loewenich FD, Schempp CM, Freudenberg MA *et al.* Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PLoS ONE* 2012;**7**:e41340.
- 27 Monzon ME, Fregien N, Schmid N, Falcon NS, Campos M, Casalino-Matsuda SM *et al.* Reactive oxygen species and hyaluronidase 2 regulate airway epithelial hyaluronan fragmentation. *J Biol Chem* 2010;**285**:26126–34.
- 28 Antonopoulos C, Cumberbatch M, Mee JB, Dearman RJ, Wei X, Liew FY *et al.* IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis. *J Leukoc Biol* 2008;**83**:361–7.
- 29 Hartwig C, Tschering T, Mazzega M, Braun A, Neumann D. Endogenous IL-18 in experimentally induced asthma affects cytokine serum levels but is irrelevant for clinical symptoms. *Cytokine* 2008;**42**:298–305.
- 30 Kool M, Willart MA, van Nijmegen M, Bergen I, Pouliot P, Virchow JC *et al.* An unexpected role for uric acid as an inducer of T Helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity* 2001;**34**:527–40.
- 31 Emter R, van der Veen JW, Adamson G, Ezendam J, van Loveren H, Natsch A. Gene expression changes induced by skin sensitizers in the KeratinoSens™ cell line: discriminating Nrf2-dependent and Nrf2-independent events. *Toxicol In Vitro* 2013;**27**:2225–32.
- 32 Migdal C, Botton J, El Ali Z, Azoury ME, Guldemann J, Giménez-Arnau E *et al.* Reactivity of chemical sensitizers toward amino acids *in cellulo* plays a role in the activation of the Nrf2-ARE pathway in human monocyte dendritic cells and the THP-1 cell line. *Toxicol Sci* 2013;**133**:259–74.
- 33 Corsini E, Galbiati V, Mitjans M, Galli CL, Marinovich M. NCTC 2544 and IL-18 production: a tool for the identification of contact allergens. *Toxicol In Vitro* 2013;**27**:1127–34.
- 34 Dos Santos GG, Spiekstra SW, Sampat-Sardjoepersad SC, Reiniers J, Scheper RJ, Gibbs S. A potential in vitro epidermal equivalent assay to determine sensitizer potency. *Toxicol In Vitro* 2011;**25**:347–57.
- 35 Gibbs S, Corsini E, Spiekstra SW, Galbiati V, Fuchs HW, Degeorge G *et al.* An epidermal equivalent assay for identification and ranking potency of contact sensitizers. *Toxicol Appl Pharmacol* 2013;**272**:529–41.
- 36 Tan JKH, O'Neil HC. Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. *J Leukoc Biol* 2005;**78**:319–24.
- 37 Johansson H, Lindstedt M, Albrekt AS, Borrebaeck CAK. A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. *BMC Genomics* 2011;**12**:399–417.
- 38 Lundberg K, Albrekt AS, Nelissen I, Santegoets S, de Gruijl TD, Gibbs S *et al.* Transcriptional profiling of human dendritic cell populations and models - Unique profiles of in vitro dendritic cells and implications on functionality and applicability. *PLoS ONE* 2013;**8**:e52875.
- 39 Ashikaga T, Yoshida Y, Hirota M, Yoneyama K, Itagaki H, Sakaguchi H *et al.* Development of an in vitro skin sensitization test using human cell lines; human cell line activation test (h-CLAT). I. Optimization of the h-CLAT protocol. *Toxicol In Vitro* 2006;**20**:767–73.
- 40 Ashikaga T, Sakaguchi H, Sono S, Kosaka N, Ishikawa M, Nukada Y *et al.* A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). *Altern Lab Anim* 2010;**38**:275–84.
- 41 Villablanca EJ, Russo V, Mora JR. Dendritic cell migration and lymphocyte homing imprinting. *Histol Histopathol* 2008;**23**:897–910.
- 42 Ouwehand K, Scheper RJ, de Gruijl TD, Gibbs S. Epidermis-to-dermis migration of immature Langerhans cells upon topical irritant exposure is dependent on CCL2 and CCL5. *Eur J Immunol* 2012;**40**:2026–34.
- 43 Ouwehand K, Spiekstra SW, Waaijman T, Scheper RJ, de Gruijl TD, Gibbs S. Langerhans cells derived from a human cell line in a full-thickness skin equivalent undergo allergen-induced maturation and migration. *Tech Adv* 2011;**90**:1028–33.
- 44 Rees B, Spiekstra SW, Carfi M, Ouwehand K, Williams CA, Corsini E *et al.* Inter-laboratory study of the in vitro DC migration assay for identification of contact allergens. *Toxicol In Vitro* 2011;**25**:2124–34.



- 45 Jelley-Gibbs DM, Strutt TM, McKinsty KK, Swain SL. Influencing the fates of CD4 T cells on the path to memory: lessons from influenza. *Immunol Cell Biol* 2008;**86**:343–52.
- 46 Mempel TR, Henricksen SE, von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 2004;**8**:154–9.
- 47 Xu H, Dilulio NA, Fairchild RL. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing(Tc1) effector CD8<sup>+</sup> T cells and interleukin (IL) 4/IL-10 negative regulatory CD4<sup>+</sup> T cells. *J Exp Med* 1996;**183**:1001–12.
- 48 Albanesi C, Scarponi C, Cavani A, Federici M, Nasorri F, Girolomoni G. Interleukin-17 is produced by both Th1 and Th2 lymphocytes, and modulates interferon-gamma and interleukin-4-induced activation of human keratinocytes. *J Invest Dermatol* 2000;**115**:81–7.
- 49 Nakae S, Komiyama Y, Nambu A, Sudo K, Iwase M, Homma I *et al.* Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 2002;**17**:375–87.
- 50 Watanabe H, Gaide O, Petrilli V, Martinon F, Contassot E, Roques S *et al.* Activation of the IL-1 $\beta$ -processing inflammasome is involved in contact hypersensitivity. *J Invest Dermatol.* 2007;**127**:1956–63.
- 51 Richter A, Schmucker SS, Esser PR, Traska V, Weber V, Dietz L *et al.* Human T cell priming assay (hTCPA) for the identification of contact allergens based on naive T cells and DC-IFN- $\gamma$  and TNF- $\alpha$  readout. *Toxicol In Vitro* 2013;**27**:1180–5.
- 52 Vocanson M, Cluzel-Tailhardat M, Poyet G, Valeyrie M, Chavaqnac C, Levarlet B *et al.* Depletion of human peripheral blood lymphocytes in CD25<sup>+</sup> cells allows for the sensitive in vitro screening of contact allergens. *J Invest Dermatol* 2008;**128**:2119–22.