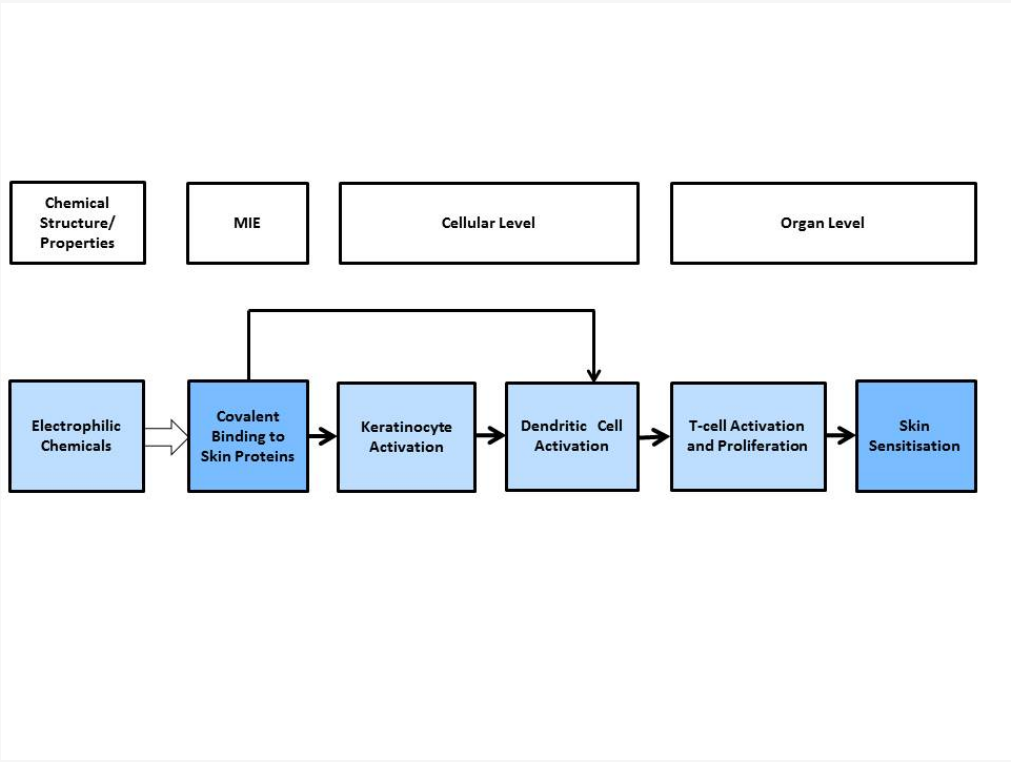
AOP ID and Title:

AOP 40: Covalent Protein binding leading to Skin Sensitisation
Short Title: Skin Sensitisation AOP

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment	WPHA/WNT Endorsed	1.1	Included in OECD Work Plan

Abstract

Skin sensitisation is a term used to denote the regulatory hazards known as human allergic contact dermatitis or rodent contact hypersensitivity, an important health endpoint taken into consideration in hazard and risk assessment of chemicals. Skin sensitisation is an immunological process that is described in two phases: the induction of sensitisation and the subsequent elicitation of the immune reaction. The first phase includes a sequential set of events which are described in this Adverse Outcome Pathway (AOP). The molecular initiating event (MIE) is covalent binding to skin proteins (specifically, to cysteine and/or lysine residues) which leads to keratinocytes' activation, a key event (KE) at cellular level. Another key event at cellular level is activation of dendritic cells, which is caused by hapten-protein complexes as well as by signalling from activated keratinocytes. Dendritic cells subsequently mature and migrate out of the epidermis to the local lymph node where they display major histocompatibility complex molecules, which include part of the hapten-protein complex to naive T-lymphocytes (T-cells). This induces differentiation and proliferation of allergen chemical-specific memory T-cells. This signifies the consecutive KE resulting in the acquisition of sensitisation, the adverse outcome on organ level. A sensitised subject has the capacity then to mount a more accelerated secondary response to the same chemical. Thus, if exposure occurs again, at the same or a different skin site, an aggressive immune response will be elicited resulting in allergic contact dermatitis.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	396	Covalent Binding, Protein	Covalent Binding, Protein
2	KE	826	Activation, Keratinocytes	Activation, Keratinocytes
3	KE	398	Activation, Dendritic Cells	Activation, Dendritic Cells
4	KE	272	Activation/Proliferation, T-cells	Activation/Proliferation, T-cells
5	AO	827	sensitisation, skin	sensitisation, skin

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Covalent Binding, Protein	adjacent	Activation, Keratinocytes	High	
Covalent Binding, Protein	adjacent	Activation, Dendritic Cells	High	
Activation, Keratinocytes	adjacent	Activation, Dendritic Cells	Moderate	
Activation, Dendritic Cells	adjacent	Activation/Proliferation, T-cells	High	
Activation/Proliferation, T-cells	adjacent	sensitisation, skin	High	

Overall Assessment of the AOP

1. Concordance of dose-response relationships

While no specific citations were found, an examination of the experimental data for selected compounds (e.g. 1-chloro-2,4-dinitrobenzene) reveals general agreement among the dose-response relationships both within and between intermediate endpoints (see Annex 1[1]). With exceptions, there is agreement between sensitisers initiated by covalent binding to proteins and non-sensitisers tested in mice, guinea-pigs, and humans; this is especially the case for extreme and strong sensitisers but lesser so for weak and non-sensitisers. One problem is that earlier results, especially with the guinea-pig, were not dose response experiments. Chemical reactivity data show very good concordance of dose-response relationships regardless of the method. In general, available data from *in vitro* assays are fragmentary and often qualitative (i.e., yes/no).

2. Temporal concordance among the key events and adverse effect;

There is good agreement between the sequences of biochemical and physiological events leading to skin sensitisation (see[2];[3];[4];[5];[6];[7]).

3. Strength, consistency, and specificity of association of adverse effect and initiating event

There is excellent strength, as well as good consistency and high specificity, of the association between *in vivo* skin sensitisation and *in chemico* protein binding. This is especially true for reactions that have thiol as the preferred molecular target. Based on linear regression analyses, there is excellent interlaboratory/protocol correlations within and between nucleophile depletion and adduct formation methods[8].

4. Biological plausibility, coherence, and consistency of the experimental evidence

The *in chemico*, *in vitro*, and *in vivo* experimental evidence is logical and consistent with the mechanistic plausibility proposed by covalent reactions based on the protein binding theory ([2];[3];[7]). In selected cases, (e.g. 1-chloro-2,4-dinitrobenzene) where the same compound has been examined in a variety of assays (see Annex 1[1]), the coherence and consistency of the experimental data is excellent.

5. Uncertainties, inconsistencies and data gaps.

Uncertainties include the structural and physicochemical cut-offs between theoretical and measured reactivity[8], the significance of the preferred amino acid target (e.g., cysteine versus lysine)[9], the significance of Th1 or type 1 (IFN-γ) versus Th2 or type 2 (IL-2, IL-4, IL-13) cytokine secretion profiles[10], and sensitisation measurements in different *in vivo* models.

Inconsistencies within the reported data are seen. There are differences between *in vitro* responses for highly similar chemicals (see[11];[12]). There are differences within and between *in vivo* test results for highly similar chemicals (see Annex C[13]). Highly hydrophobic chemicals, which are *in vivo* sensitisers, are not active in aquatic-based *in chemico* or *in vitro* assays. The specific nature of the relationship between irritation and sensitisation has yet to be elucidated.

Data gaps: Based on the more than 50 chemical reactions associated with covalent binding to thiol or primary amine moieties[9] *in vitro* data for keratinocytes, dendritic cells, and T-cell assays, as well as *in vivo* sensitisation data, is incomplete in that it does not cover the chemical spaces associated with many of these chemical reactions; *in chemico* data is also incomplete, especially for reactions that favour amino acid targets other than cysteine.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	

The molecular initating event of the present AOP is the hapten-protein binding. While covalent reactions with thiol groups and to lesser extent amino groups, are clearly supported by the proposed AOP, reactions targeting other nucleophiles may or may not be supported by the proposed AOP. Limited data on chemical reactivity shows that two competing reactions are possible, the faster reaction dominates. However, this has yet to be proven *in vitro* or *in vivo*.

Essentiality of the Key Events

Since the 1930's, there has been growing evidence that the main potency-determining step in skin sensitisation of industrial organic compounds is the formation of a stable hapten-protein conjugate (see[2];[3];[37]). Consequently, the molecular initiating event leading to skin sensitisation is postulated in this AOP to be covalent binding of electrophilic chemical species with selected nucleophilic molecular sites of action in skin proteins ([2];[3]). Protein binding reactions are a means of identifying different chemical structures associated with skin sensitisation, which may or may not lead to different expressions in other key events along the AOP.

	Defining Question	High (Strong)	Moderate	Low (Weak)
Support for Essentiality of KEs	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from experimental studies illustrating essentiality for at least one of the important KEs.	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE.	No or contradictory experimental evidence of the essentiality of any of the KEs.
KE1: Keratinocytes activation	Strong	When production of IL-1β and IL-18 from keratinocytes was inhibited, it resulted in impaired DC migration[29];[30];[19].		

KE2: Dendritic cells activation	Strong	A study performed in mice showed than when both Langerhans cells and Langerin+ dermal dendritic cells are depleted using DTR KI- mice (in which diphtheria toxin receptor is inserted into the Langerin locus) and subsequently administration of diphtheria toxin (allowing Langerin+ cells to be ablated), the contact hypersensitivity response is abrogated. In contrast, in the bacterial artificial chromosome (BAC)-transgenic mice (in which the diphtheria toxin subunit A (DTA) is cloned into the human Langerin locus, resulting in mice devoid of Langerhans cells) that lack only epidermal Langerhans cells but have normal number of dendritic cells, the contact hypersensitivity is unaffected[38]. Kim et al (2013) showed that exposition of murine dendritic cells to bisabolangelone (inhibitor of dendritic cell functions) attenuated the production of pro-inflammatory cytokines including IL-12, IL-1β, and TNF-alpha, migration to macrophage inflammatory protein-3 beta, and all-T cell activating ability of dendritic cells[39].
KE3: T-cells, activation and proliferation:	Strong	The use of ACY-1215, an histone deacetylase, prevented the development of contact hypersensitivity in mice in vivo by modulating CD8 T-cell activation and functions[40]. Another study showed that trichomide A exerts immunosuppressive activity against activated T lymphocytes and in an in vivo experiment they demonstrated that trichlomide A significantly ameliorate picryl chloride (PCI)-induced contact hypersensitivity in mice[41].

Weight of Evidence Summary

	Defining Question	High (Strong)	Moderate	Low (Weak)
Support for Biological Plausibility of KERs	Is there a mechanistic relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding of the KER based on previous documentation and broad acceptance.	KER is plausible based on analogy to,accepted biological relationships, but scientific understanding is incomplete.	Empirical support for association between KEs, but the structural or functional relationship between them is not understood.
MIE => KE1:	Strong	It is well accepted and experimentally proved that upon hapten application, keratinocytes are activated and produce various chemical mediators (e.g. TNFa, IL-1β, and prostaglandin E2) [14];[15].		
MIE => KE2:	Strong	It is accepted and experimentally proved that during skin sensitisation process,immature epidermal and dermal dendritic cells recognize and internalize the hapten-protein complex formed during covalent binding and subsequently mature and migrate to the local lymph nodes. [16];[17];[18].		
KE1 => KE2:	Moderate	Keratinocyte response activates multiple events, including the release of pro-inflammatory cytokines (e.g. IL-18) and the induction of cyto-protective cellular pathways. Under the influence of fibroblast- blood endothelial- and lymph endothelial-chemokines (e.g. CCL19, CCL21) and epidermal cytokines (e.g. IL-1α, IL-1β, IL-18, tumour necrosis factor alpha (TNFα)) maturing dendritic cells migrate from the epidermis to the dermis of the skin and then to the proximal lymph nodes. [19];[20].		
KE2 => KE3:	Strong	It is well accepted and experimentally proved that in the local lymph node, maturedendritic cells present the hapten-protein complex to T-cells via a majorhistocompatibility complex molecule (MHC)[20];[19]. T-cells are typically affected by protein-hapten complexes presented by dendritic cells on MHC molecules. The T-cell will be then activated to form a memory T-cell, which subsequently proliferates[4].		
KE3 => AO:	Strong	It is well known, recognised and experimentally proved that skin sensitisation is a T-cell mediated immune response. [4]		
MIE => AO:	Strong	Haptenation is widely accepted as molecular initiating event for skin sensitisation. In the form of a modified protein [21], the haptenation provides a source of antigen recognised by the immune system as non-self[22];[23];[24].		

	Defining Question	High (Strong)	Moderate	Low (Weak)
Empirical Support for KERs	Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses, earlier time points, and higher in incidence than KEdown ? Inconsistencies?	Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. No or few critical data gaps or conflicting data.	Demonstrated dependent change in both events following exposure to a small number of stressors. Some inconsistencies with expected pattern that can be explained by various factors.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor; and/or significant inconsistencies in empirical support across taxa and species
MIE => KE1:	Strong	Using a series of thiol-reactive cages fluorescent haptens (i.e. bromobimanes) deployed in combination with two photon fluorescence microscopy, immunohistochemistry, and proteomics, Simonson et al. (2011) identified the possible hapten targets in proteins in human skin. Key target found were the basal keratinocytes and the keratins K5 and K14[25]. In a review about murine contact sensitivity, Honda et al[14] reported that haptens can activate keratinocytes in an NLR-dependent manner. Among the NLR family, NLRP3 controls the production of proinflammatory cytokines through activation of caspase-1. Without NLRP3 or its adaptor protein ASC[26];[27];[28], the production of IL-1β and IL-18 from keratinocytes was inhibited[29];[30];[19].		
MIE => KE2:	Strong	Using an flow-cytometric assay, the influence of contact sensitisers on endocytic mechanisms in murine Langerhans cells was measured. Epidermal cell suspensions were labelled with a monoclonal antibody directed to MHC class II molecules and pH-sensitive fluorochrome-coupled second step reagents. Study reported that stimulation with well-known sensitising compounds resulted in a partial conservation of the fluorescence intensity due to the internalisation of the labelled complexes into less acidic compartments. For untreated Langerhans cells or in the presence of irritants a significant quenching of fluorescence intensity due to the internalization of the MHC-antibody complexes into acidic compartments was noticed[31]. In the h-CLAT assay measuring the expression of CD86 and CD54 protein markers on the surface of the human monocytic leukemia cell line THP-1, the cell exposure to known non sensitisers does not increase cell biomarker expression. On the contrary, exposure to well-known sensitisers leads to an increase of the CD86 and CD54 expression[32];[33].		
KE1 => KE2:	Moderate	Matjeka et al. (2012) exposed HaCaT cell line used as a model of human keratinocytes to skin sensitisers for one hour and then, after washed off, cocultured them with dendritic cells. Data showed that exposure of dendritic cells to chemically treated HaCaT cells led to the activation of dendritic cells measured by CD83 and CD86 upregulation[34].		
KE2 => KE3:	Strong	A recent study showed in mice model that dendritic cells coordinate the interactions that are necessary to initiate polyclonal regulatory T cells proliferation[35].		
KE3 => AO:	Strong	Using dinitrofluorobenzene and mice models, it was shown that cutaneous contact with reactive antigen induces KC/CXC chemokine ligand 1 production and neutrophil infiltration in an antigen, dose-dependent manner. The intensity of neutrophil infiltration into cutaneous antigen challenge sites, in turn, controls the number of antigen-primed T cells recruited into the site and the magnitude of immune response elicited[36].		

Quantitative Consideration

The final aspect of the OECD approach to using the AOP concept is an assessment of the quantitative understanding of an AOP. This includes the evaluation of the experimental data and models used to quantify the molecular initiating event and other key events. It also includes transparent determination of thresholds and response-to-response relationships used to scale *in chemico* and *in vitro* effects to *in vivo* outcomes. **For skin sensitisation, a major hurdle is moving from a qualitative AOP to a quantitative AOP.** While the assessment of the experimental

evidence, empirical data and confidence in the AOP expressed by the Weight-of-Evidence clearly supports the qualitative AOP as a means to identify and characterize the potential for a chemical to be a sensitiser, these same assessments clearly reveal the current lack of ability to consistently predict relative potency. One aspect to be resolved is that of the *in vivo* data with which to scale the response-to-response ratios. Because the Local Lymph Node Assay (LLNA) can directly quantify the adverse outcome[42], public databases have recently been made available ([43];[44]). LLNA results are often compared with results from alternative methods (e.g.[33]). Such one-to-one comparisons may not be the best approach. As noted by Basketter et al.[42], the LLNA is not without limitations, including variability between EC3 values or any other value (i.e. ECx) within mechanistic classes with equal or near equal chemical reactivity. The specific nature of the *in vivo* relationship between irritation and sensitisation has yet to be elucidated.

Considerations for Potential Applications of the AOP (optional)

This AOP study[45] describing mechanistic knowledge has supported the development of a number of methods for assessing chemical sensitisation hazard potential or potency without the need for animal testing by measuring the impact of chemical sensitisers on the identified key events[46];[47]. This AOP also forms the mechanistic basis for the development of Integrated Approaches to Testing and Assessment (IATA)[48];[49]. Additionally, data-driven approaches for predicting sensitizer potency also have been developed[50];[51];[52].

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Appendix 1

List of MIEs in this AOP

Event: 396: Covalent Binding, Protein

Short Name: Covalent Binding, Protein

Key Event Component

Process	Object	Action
protein binding	electrophilic reagant	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:40 - Covalent Protein binding leading to Skin Sensitisation	MolecularInitiatingEvent
Aop:39 - Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	MolecularInitiatingEvent

Stressors

Name
1-CHLORO-2,4-DINITROBENZENE

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI
guinea pig	Cavia porcellus		NCBI

Term	Scientific Term	Evidence	Links
mouse	Mus musculus		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The OECD 2012 document does not indicate *in vivo* assays that measure covalent protein binding.

Key Event Description

The molecular initiating event is covalent binding of electrophilic chemical species with selected nucleophilic molecular sites of action in proteins generating immunogenic neoantigens through a process termed haptenisation[1];[2]. In contrast to receptor-mediated chemical interactions electrophiles are not specific with regard to their molecular target. Moreover, some chemicals are able to react with several different nucleophilic chemical substituents. Therefore, the identification of the specific target protein is not considered to be critical. Moreover, it is recognized that reactivity measured with a particular nucleophilic target or model nucleophile does not necessarily reflect a specific chemical reaction, as many reactions target the same chemical substituent[3]. For toxicological endpoints for which protein binding is important, the biological nucleophile is assumed to be selected amino acids. The exact extent of adduct formation to each amino acid is dependent on the relative hardness / softness of the electrophile and nucleophile[3]. The inability to identify the exact biological nucleophile is deemed less important than information regarding the electrophile. As noted in the hard-soft acid base theory, a soft electrophile will have a relative preference for a soft nucleophile; while a hard electrophile will have a relative preference for a hard nucleophile. As a consequence, for a series of electrophiles assigned to the same mechanistic cluster within a particular domain, the relative rates of reactivity between each electrophile and any nucleophile will remain the same. In other words, while absolute reactivity may vary with protocols, relative reactivity will usually not vary significantly[3]. Binding experiments with small model nucleophiles reveal that, within a particular reaction within a mechanism, the rate of reactivity varies markedly. Moreover, while some compounds appear to bind exclusively with thiol or amine, others bind to a variety of nucleophiles. However, an electrophile is most likely to exhibit a preference for a particular nucleophile. In more complex systems, nucleophilic target preferences may be masked by other factors. It is self-evident that the number of cysteine and lysine residues within a protein will impact target probability. For example, for serum albumin, a major serum protein, 10% of the amino acid residues are lysine but albumin has very few free cysteine residues. Also, it is self-evident that a target site (e.g. cysteine or lysine) which is located on an exposed surface of a protein is more likely to react with an electrophile than one that is located within a grove or fold of a protein. Such steric constraints are imposed by the primary structure (i.e. amino acid sequence) of the peptide or protein, as well as the secondary and tertiary structure of proteins imposed by disulfide bridges, and folding and coiling. Similarly, the microenvironment of the reaction site (e.g. hydrophilic versus hydrophobic) may affect the probability of a particular reaction. Free cysteine residues are more abundant in proteins in the aqueous cytosol than in the non- aqueous biomembranes [4]. An ancillary event in identifying protein-binding is metabolism and/or abiotic transformation (e.g. autoxidation)[5].

How it is Measured or Detected

In silico models, including physiological-based pharmacokinetic models and traditional structure activity ones, as well as *in vitro* and *in vivo* experimental approaches exist.

In silico Methods

It is generally recognized that reaction-based methods, as opposed to other means of defining chemical similarity, allow for easier interpretation and provide greater confidence in their use[6]. Chemical reactions related to covalent protein binding have recently been reviewed[7];[8];[9]. Measurements and estimations of reactivity have also recently been reviewed[1];[3]. Computational or *in silico* techniques to predict chemical reactivity have been developed; they vary in complexity from the relatively simple approach of forming chemical categories from 2D structural alerts (i.e. SARs for qualitative identification of chemical sub-structures with the potential of being reactive), such as used in the Organisation for Economic Co-Operation and Development (OECD)QSAR Toolbox[10] to QSAR models (i.e. quantitative prediction of relative reactivity) as described by Schwöbel et al.[11].

In Chemico Protocols and Databases

While methionine, histidine, and serine all possess nucleophilic groups that are found in skin proteins, the –SH group

of cysteine and the ε-NH2 group of lysine are the most often studied. Soft electrophilic interactions involving the thiol group can be modelled with small molecules. Glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine) is the most widely used model nucleophile in soft electrophilic reactivity assays. Typically, chemicals are incubated with GHS and, after a defined reaction time, the concentration of free thiol groups is measured. Such depletion based assays assume adduct formation, which is typically not confirmed. Good relationships between GSH reactivity and toxicity have been demonstrated. Examples of this method can be found in the literature[3];[12];[13];[14]. Recently, OECD adopted the new Test Guideline (TG) No442C: *In chemico* skin sensitisation – Direct Peptide Reactivity Assay (DPRA). This method quantifies the reactivity of chemicals towards model synthetic peptides containing either lysine or cysteine[15]. The DPRA protocol can be found in the EURL ECVAM Database Service on Alternative Methods to animal experimentation (DB-ALM): Protocol No154 for Direct Peptide Reactivity Assay (DPRA) for skin sensitisation testing[16]. The importance of reaction chemistry for sensitisation indicates that identification of the reaction limited chemical spaces is critical for using the proposed AOP. Systematic databases for reaction-specific chemical spaces are being developed. For example, *in chemico* databases reporting measurements of reactive potency currently exist for Michael acceptors ([14];[17];[18]). The use of model nucleophiles containing primary amino (–NH2) groups, such as in the amino acids lysine are less well-documented, with the principle of measuring relative reactivity being the same as for thiol[1].

Respiratory Sensitizers

Both respiratory and skin sensitizers are detected by in vitro and in silico methods used to measure electrophilic binding to proteins and peptides. (Basketter et al., 2017) The rate of covalent binding can also be measured. (Natsch and Gfeller, 2008) Dik et al. modified the DPRA protocol to include two peptide depletion measurement time points, and added high-performance liquid chromatography mass spectrometry (MS) analysis of reaction products, which improved predictive capacity. (Dik et al., 2016) Other authors have worked to investigate the binding of diisocyanates in vapor and liquid phases with LC/MS, MS/MS, and ELISA, as well as, Western blot. (Wisnewski et al., 2013a, 2013b, Hettick et al., 2012, Hopkins et al., 2005, Hettick and Siegel, 2011)

Overview table: How it is measured or detected

Method(s)	Reference	URL	Regulatory Acceptance	Validated	Non Validated
Direct Peptide Reactivity Assay (DPRA)	TG 442C DB-ALM	[1] [2]	X	X	

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List of Key Events in the AOP

Event: 826: Activation, Keratinocytes

Short Name: Activation, Keratinocytes

Key Event Component

Process	Object	Action
keratinocyte activation		increased
cytokine production involved in inflammatory response		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:40 - Covalent Protein binding leading to Skin Sensitisation	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
keratinocyte

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI

A dose-dependent release of IL-18 has been shown following exposure of the murine keratinocyte cell line HEL-30 to sensitisers[4]. Moreover, a concentration-dependant increase in intracellular IL-18 at non-cytotoxic concentrations of chemicals was observed in the human keratinocyte cell line NCTC2455 following 24-h treatment[11].

Key Event Description

Keratinocytes are the major cell type of the epidermis of the skin. They are known to be the primary site of skin metabolism and play an important role in epithelial Dendritic Cells (DC) activation. Uptake of the hapten-protein complex formed during covalent binding by keratinocytes activates multiple events, including the release of pro-inflammatory cytokines (i.e. IL-18) and the induction of cyto-protective cellular pathways. Hapten-protein complexes can activate the inflammasome ([1];[2]) and in so doing induce IL-18 production. Activation of the pro-inflammatory cytokine IL-18 results from cleavage of inactive IL-18 precursor protein by inflammasome-associated caspase-1[3]. Intracellular Nod-like receptors (NLR) contain sensors for a number of cellular insults. Upon activation, NLRs oligomerise form molecular complexes (i.e. inflammasomes) that are involved in the activation of inflammatory-associated caspases, including caspase-1. Induction of intracellular levels of IL-18 exhibit responses upon exposure to hapten-protein complexes which can be used to establish potency[4]. Keratinocyte exposure to allergens also results in induction of antioxidant/electrophile response element ARE/EpRE-dependent pathways[5]. Briefly, reactive chemicals bind to Keap1 (Kelch-like ECH-associates protein 1) that normally inhibit the nuclear erythroid 2-related factor 2 (Nrf2). Released Nrf2 interacts with other nuclear proteins and binds to and activates ARE/EpREdependent pathways, including the cytoprotective genes NADPH-quinone oxidoreductase 1 (NQO1) and glutathione S-transferase (GSHST), among others ([5];[6]).

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Investigations have focused on the DNA antioxidant-response elements (ARE), also known as electrophile response

element. OECD TG 442D is the validated test guideline for measuring the activation of the antioxidant/electrophile response element (ARE) - dependant pathway[7]). Currently, the only in vitro ARE-Nrf2 luciferase test method covered by this Test Guideline is the KeratinoSensTM. This assay uses a luciferase reporter gene under control of a single copy of the ARE element of the human AKR1C2 gene stably inserted into immortalized human keratinocytes (HaCaT cells)[8]. The KeratinoSensTM protocol can be found in the EURL ECVAM Database Service on Alternative Methods to animal experimentation (DB-ALM): Protocol No155 for KeratinoSensTM[9]. The Keap1/Nrf2/ARE/EpRE cell signalling assay is also the mechanistic basis for the work on skin sensitisation chemicals at CeeTox Inc.[10]. This work includes quantitative realtime polymerase chain reaction measurements of the relative abundance of mRNA for eleven selected genes whose expression is controlled by one of the three following pathways: Keap1/Nrf 2/ARE/EpRE, ARNT/AhR/XRE, and Nrf1/MTF/MRE. Interestingly, both Emter et al.[8] and McKim et al.[10] combine their cell signalling results with chemical reactivity data in algorithms, which can be viewed as a first step in using the AOP in quantitative assessment.

In vitro assays based on IL-18 induction in human keratinocytes (cell line NCTC 2544)[11] or IL-8 induction in THP-1 cells[12] have also been developed to identify allergens. Other studies have described chemokines (e.g. CCL2, CCL4) and receptor (e.g. CCR7) (see[13]).

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Event: 398: Activation, Dendritic Cells

Short Name: Activation, Dendritic Cells

Key Event Component

Process	Object	Action
cell activation		increased
MHC protein complex assembly		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:40 - Covalent Protein binding leading to Skin Sensitisation	KeyEvent
Aop:39 - Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

dendritic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

The main in vitro assays currently used and based on dendritic cells activation use human dendritic-cell-like cell lines (e.g. THP-1, U-937, MTZ-3)[3]. In addition to that some assays were performed on murine models[5].

Key Event Description

Immature epidermal dendritic cells, known as Langerhans cells, and dermal dendritic cells serve as antigen-presenting cells ([1];[2];[3];[4]). In this role, they recognize and internalize the hapten-protein complex formed during covalent binding leading to their activation. Subsequently, the dendritic cell loses its ability to seize new hapten-protein complexes and gains the potential to display the allergen-MHC-complex to naive T-cells; this process is often referred to as dendritic cell maturation. Simultaneously, under the influence of fibroblast- blood endothelial- and lymph endothelial chemokines (e.g. CCL19, CCL21) and epidermal cytokines (e.g. interleukin (IL), IL-1 α, IL-1β, IL-18, tumour necrosis factor alpha (TNF-α)) maturing dendritic cells migrate from the epidermis to the dermis of the skin and then to the proximal lymph nodes, where they can present the hapten-protein complex to T-cells via a major histocompatibility complex (MHC) molecule ([5];[6]). Dendritic cell activation, upon exposure to hapten-protein complexes also leads to functional changes in the cells. For example, there are changes in chemokine secretion, cytokine secretion and in the expression of chemokine receptors (see[3]). Additionally, during dendritic cell maturation MHC, co-stimulatory and intercellular adhesion molecules (e.g. CD40, CD86, and DC11 and CD54, respectively) are up-regulated (see[3];[4];[7]). Signal transduction cascades precede changes in expression of surface proteins markers and chemokine or cytokine secretion. In fact, there is evidence that during the response, hapten-protein complexes can react with cell surface proteins and activate mitogen-activated protein kinase signalling pathway. In particular, the biochemical pathway involving extracellulare signal-regulating kinases- the c-jun N-terminal kinases and the p38 kinases have been shown to be activated upon exposure to protein-binding chemicals[8]. These pathways are of particular importance in keratinocytes and dendritic cell response to protein-hapten complexes. Components of signal transduction pathways are kinases, which phosphorylate and dephosphorylate a variety of substrates in order to elicit a change in the expression or secretion of target molecules. As a result, components of the signal transduction cascade are thought to be biomarkers[9]. Investigations into the possible role of calcium influx as an early event in dendritic cell activation suggest that calcium influx is a second event following reactive oxygen species induction[10];[11].

How it is Measured or Detected

Omic studies

Genomic and proteomic studies also have the potential to reveal biomarkers in dendritic cell-based assays. Custom designed arrays or quantitative polymerase chain reaction (PCR) of selected genes have been used to highlight the reaction of dendritic cells (see[3]). VITOSENS, an assay that uses human CD34+ progenitor-derived dendritic cells

(CD34-DC), is based on the differential expression of the cAMP-responsive element modulator (CREM) and monocyte chemotactic protein-1 receptor (CCR2)[12]. Genomic signatures have been also developed for the identification of human sensitising chemicals: a biomarker signature, the Genomic Allergen Rapid Detection test (GARD) based on the human myelomonocytic cell line MUTZ-3[13] and a genomic platform, SENSIS, which consists of measuring the over-expression of 3 sets of genes, that may allow the *in vitro* assessment of the sensitising potential of a compound[14].

In Vitro Assays for Cell Surface Markers, Cytokines, and Chemokines

Alterations in intercellular adhesion molecules, cytokines, and chemokines are part of the immunology response which can serve as biomarkers. Since dendritic cell maturation upon exposure to hapten-protein complexes is accompanied by changes in surface marker expression, these surface markers are perceived as promising candidates as primary biomarkers of dendritic cell activation for the development of cell-based *in vitro* assays. While a variety of surface markers have been described to be up-regulated upon dendritic cell maturation, a review of the literature reveals that CD86 expression, followed by CD54 and CD40, are the most extensively studied intercellular adhesion and co-stimulator molecules to date. The human Cell Line Activation Test (h-CLAT) reported flow cytometry results for CD86 and CD54 expression in THP-1 cells[15];[16]. An OECD Test Guideline for the h-CLAT is currently under review. The h-CLAT protocol can be found in the EURL ECVAM Database Service on Alternative Methods to animal experimentation (DB-ALM): Protocol No158 for human Cell Line Activation Test (h-CLAT)[17]. Other studies with THP-1 cells include that of An et al. (2009). Another assay, the myeloid U937 skin sensitisation test (U-SENS), is based as well on the measurement of CD86 by flow cytometry[18];[19];[20]). In addition to that, a variety of cytokines have been studied in relationship to skin sensitizers[4]. IL-8 is a promising chemokine for distinguishing sensitisers from non-sensitisers. Quantification of IL-8 can be performed by Enzyme Linked Immunosorbent Assay, a technique that is far simpler and amenable to high throughput screening than the flow cytometry technique used to measure CD86 expression[3]. The expression of other cytokines linked to skin sensitisers include IL-1 α, IL-1β, IL-18, and TNF-α form the basis for other dendritic cell assays.

While some respiratory sensitizers have been assessed, it is unclear whether this event is distinct between skin and respiratory sensitizers. (dos Santos et al., 2009) The genomic allergen rapid detection (GARD) test is an MUTZ-3-based assay for assessing chemical sensitizers utilizing genomic biomarker prediction signatures to generate prediction calls of unknown chemicals such as skin sensitizers,respiratory sensitizers, or nonsensitizers, including irritants. (Johannsen et al., 2011) Preliminary data on the performance of the GARDfor assessing chemical respiratory sensitizers using transcriptional readouts of a genomic biomarker signature indicated £0% accuracy. (Forreryd, et al., 2015)

There are several in vitro assays available to assessDC maturation; the most advanced is the h-CLAT, which determines changes in CD86 and CD54 levels on THP-1cell.(Ashikaga, et al., 2006, Sakaguchi, et al., 2006) However, only limited data are available substantiating its performance on chemical respiratory sensitizers. (Basketter, et al. 2017) Several assays similar to the h-CLAT have emergedover time and are currently in the process of being validated (e.g., the MUSST measuring CD86 responses by U937 cells),but again no or minimal information is available to assess assay performance in detecting respiratory sensitizers. The MUTZ-3 cell line is also being investigated for the potential to assess the capacity of a chemical to induce LCmigration. The discriminating feature of the assay is that irritant-induced migration is CCL5 dependent, while sensitizer-induced migration is CXCL12 dependent. The readout of the test is the ratio between migration towardCXCL12 or to CCL5. Despite its complexity, the assay seems to be relatively well transferable.(Rees et al., 2011)

Overview table: How it is measured or detected

Method(s)	Reference	URL	Regulatory Acceptance	Validated	Non Validated
	draft TG under discussion at OECD	[1]			
h-CLAT	DB-ALM	[2]		X	
	EURL ECVAM Recommendation	[3]			
	Ashiga et al., 2015	[4]			
Genomic Allergen Rapid Detection test (GARD)	Johansson et al., 2013	[5]			X
VitroSens	Hooyberghs et al., 2008	[6]			X

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Event: 272: Activation/Proliferation, T-cells

Short Name: Activation/Proliferation, T-cells

Key Event Component

Process	Object	Action
T cell activation	T cell	increased
cell proliferation	memory T cell	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:40 - Covalent Protein binding leading to Skin Sensitisation	KeyEvent
Aop:39 - Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

lymph node

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI

Some *in vitro* assays have been developed using human T cells[1]. Lymph node proliferation is the basis for the *in vivo* mouse LLNA.

Key Event Description

T-cells are typically affected by protein-hapten complexes presented by dendritic cells on Major Histocompatibility Complex (MHC) molecules. Molecular understanding of this process has improved in recent years (see[1]). Briefly, MHC molecules are membrane proteins which present the small peptide antigens placed in a “groove” of the MHC molecule during its intracellular synthesis and transport to the cell surface. In the context of the MHC molecular on the cell surface, the small peptide antigen is recognized via the T-cell receptors as self or non-self (e.g. foreign). If this peptide is a foreign peptide, such as part of a protein-hapten complex, the T-cell will be activated to form a memory T-cell, which subsequently proliferates. If reactivated upon presentation by skin dendritic cells, these memory T-cells will induce allergic contact dermatitis[2].

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Most protocols recognize the importance of the process of antigen-presentation, so *in vitro* T-cell-based assays are typically co-cultures of allergen-treated dendritic cells and modified T-lymphocytes with expression of selected biomarkers (e.g. interferon gamma), or T-cell proliferation being the reported outcome. Much of this work has been reviewed by Martin et al[1]. It should be remembered that lymph node cell proliferation is the basis for the *in vivo*

mouse Local Lymph Node Assay (LLNA). OECD TG 429 is the validated test guideline for the Skin Sensitisation: Local Lymph Node Assay[3] together with its two non-radioactive modifications (LLNA-DA TG442A[4] and LLNA-BrdU ELISA TG 442B[5]).

Human T cell proliferation and DC and T cell cytokine profiles produced in response to chemical respiratory stimuli have been measured in vitro. (Holden et al., 2008, Bernstein et al., 2011)

Overview table: How it is measured or detected

Overview					
Method(s)	Reference	URL	Regulatory Acceptance	Validated	Non Validated
Local Lymph Node Assay (LLNA)	TG 429 TG 442A LLNA:DA TG 442B LLNA: BrdU-ELISA	[1] [2] [3]	X	X	

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List of Adverse Outcomes in this AOP

Event: 827: sensitisation, skin

Short Name: sensitisation, skin

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:40 - Covalent Protein binding leading to Skin Sensitisation	AdverseOutcome

Biological Context

Level of Biological Organization

Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
guinea pig	Cavia porcellus	High	NCBI

In vivo studies remain the basis of assessing the sensitisation potential of chemicals (see[6]). As previously noted, human sensitisation testing is conducted with the HRIPT[4]. Other *in vivo* methods include the guinea-pig occluded patch test[6];[15], the Magnusson- Kligman guinea-pig maximization test[16] and the mouse LLNA[11];[12];[13].

Key Event Description

Skin sensitisation is an immunological process that is described in two phases: the induction of sensitisation and the subsequent elicitation of the immune reaction. A sensitised subject has the capacity to mount a more accelerated secondary response to the same chemical. Upon reaching an unknown threshold number of hapten-specific T cells an individual will be said to be sensitised and will elicit a T cell-mediated eczematous skin reaction (termed allergic contact dermatitis, ACD) at the site of sensitiser re-exposure. Above the threshold, the severity of the adverse effect is assumed to increase proportionally to the dose, so the total dose per area of skin (e.g. µg/cm2) is the critical exposure determinant. In this regard, animal data is consistent with human clinical data[1]. The allergic reaction causes inflammation of the skin manifested by varying degrees of erythema, oedema, and vesiculation. It takes up to one week or more for individuals to develop specific sensitivity to a new allergen following exposure. An individual who never has been sensitised to a substance may develop only a mild dermatitis 2 weeks following the initial exposure but typically develops severe dermatitis within 1-2 days of the second and subsequent exposures[2].

How it is Measured or Detected

[3]Human sensitisation testing is conducted with the Human Repeat Insult Patch Test (HRIPT), as described by McNamee et al.[4];[5]. Skin biopsy may help to confirm the diagnosis and exclude other disorders.

Animal models have been developed to assess the sensitisation potential of chemicals. Adler et al. (2011) have reviewed animal test methods for skin sensitisation[6]. Briefly, among these in vivo assays are the guinea-pig occluded patch test[7];[8], the Magnusson-Kligman guinea pig maximization test[7];[9];[10], and the murine Local Lymph Node Assay[11];[12];[13]. Using LLNA data, sensitisers can be grouped into potency groups (e.g. extreme, strong, moderate, weak and non-sensitisers). However, as noted by Basketter et al. [14], the LLNA is not without limitations.

Regulatory Significance of the AO

Skin sensitisation is an endpoint that needs to be assessed within:

- CLP Regulation (EC) No1272/2008 for "Classification, Labelling and Packaging of substances and Mixtures",

- REACH Regulation (EC) No1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals,

- PPP Regulation (EC) No1107/2009 concerning the placing of plant protection products on the market,

- Biocidal Products Regulation (BPR) (EU) No528/2012 concerning the making available on the market and use of biocidal products,

- Cosmetics Regulation (EC) No1223/2009.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 833: Covalent Binding, Protein leads to Activation, Keratinocytes

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Covalent Protein binding leading to Skin Sensitisation	adjacent	High	

Key Event Relationship Description

Uptake of the hapten-protein complex by keratinocytes activates multiple events, including the release of pro-inflammatory cytokines and the induction of cyto-protective cellular pathways. Activation of the pro-inflammatory cytokine IL-18 results from cleavage of inactive IL-18 precursor protein by inflammasome-associated caspase-1[1]. Hapten-protein complexes can activate the inflammasome ([2];[3]) and in so doing induce IL-18 production. Intracellular Nod-like receptors (NLR) contain sensors for a number of cellular insults. Upon activation (by a currently unknown mechanism), NLRs oligomerise form molecular complexes (i.e. inflammasomes) that are involved in the activation of inflammatory-associated caspases, including caspase-1. Keratinocyte exposure to hapten-protein complex also results in induction of antioxidant/electrophile response element ARE/EpRE-dependent pathways[4]. Briefly, reactive chemicals bind to Keap1 (Kelch-like ECH-associates protein 1) that normally inhibits the nuclear erythroid 2-related factor 2 (Nrf2). Released Nrf2 interacts with other nuclear proteins and binds to and activates ARE/EpREdependent pathways, including the cytoprotective genes NADPH-quinone oxidoreductase 1 (NQO1) and glutathione S-transferase (GSHST), among others ([4];[5]).

This KER description is based only on the OECD document 2012 and needs updating

Evidence Supporting this KER

Biological Plausibility

It is well accepted and experimentally proved that upon hapten application, keratinocytes are activated and produce various chemical mediators (e.g. TNF□, IL-1β, and prostaglandin E2) [6];[7].

Empirical Evidence

Using a series of thiol-reactive cages fluorescent haptens (i.e. bromobimanes) deployed in combination with two photon fluorescence microscopy, immunohistochemistry, and proteomics, Simonson et al. (2011) identified the possible hapten targets in proteins in human skin. Key target found were the basal keratinocytes and the keratins K5 and K14[8]. In a review about murine contact sensitivity, Honda et al.[6] reported that haptens can activate keratinocytes in an NLR-dependent manner. Among the NLR family, NLRP3 controls the production of proinflammatory cytokines through activation of caspase-1. Without NLRP3 or its adaptor protein ASC[2];[3];[9], the production of IL-1β and IL-18 from keratinocytes was inhibited[10];[11];[12].

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Relationship: 377: Covalent Binding, Protein leads to Activation, Dendritic Cells

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Covalent Protein binding leading to Skin Sensitisation	adjacent	High	
Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Key Event Relationship Description

Dendritic cells are activated directly by exposure to haptens in both skin and respiratory sensitization.

This portion of the KER description is based only on the OECD document 2012 and needs updating:

As noted in the AOP during allergen contact with the skin, immature epidermal dendritic cells, known as Langerhans cells, and dermal dendritic cells serve as antigen-presenting cells[1];[2];[3]. In this role, they recognize and internalize the hapten-protein complex formed during covalent binding. Subsequently, the dendritic cell loses its ability to seize new hapten-protein complexes and gains the potential to display the allergen-MHC-complex to naive T-cells; this process is often referred to as dendritic cell maturation.

Evidence Supporting this KER

Biological Plausibility

It is accepted and experimentally proved that during skin sensitisation process, immature epidermal and dermal dendritic cells recognize and internalize the hapten-protein complex formed during covalent binding and subsequently mature and migrate to the local lymph nodes[1];[2];[3].

Monocyte-derived DCs (Mo-DCs) and THP-1 cells exposed to haptens with cysteine, lysine, or cysteine/lysine reactivity induced the expression of Nrf2 pathway-related genes when exposed to chemical sensitizers having cysteine and cysteine/ lysine affinities, while lysine-reactive chemicals (phthalic anhydride [PA] and TMA) were less efficient. (Migdal et al., 2013) Also, these chemicals did not prod the Mo-DCs to produce maturation markers CD86 and CD83, while PA was able to modify THP-1 cells to produce CD86 and CD54 markers.

(Toebak et al., 2006) used Mo-DCs to investigate the polarization potential of TMA compared to contact and protein allergens. In contrast to 2,4-dinitrochlorobenzene (DNCB) and similarly to protein allergen Der p1, TMA led to a decreased IL-12p70/IL-10 ratio and did not induce TNF-a or CXCL10 production, a demonstration of Th2-skewing. TMA was also found to increase the production of the cytokines IL-10 and IL-13, another hallmark of Th2 response, in DCs enriched from human blood. (Holden et al., 2008) Finally, TMA induced increased production of IL-10 when incubated with precision cut lung slices (PCLS) for 24 hours. (Lauenstein et al., 2014)

In BALB/c mice, TDI applied to the skin led to TDI-haptenated protein (TDI-hp) (skin keratins and albumin) localization in the stratum corneum, hair follicles, and sebaceous glands within 3 hours, with intensity of staining following a dose–response relationship. (Nayak et al., 2014) Subsequently, CD11b+, Langerin (CD207)-expressing DCs, and CD103+ cells migrated to regions of TDI-hp staining. These cells are involved in antigen uptake and stimulation of effector T cells.

Migration depends on the expression of chemokine receptors and their respective CCLs, as well as on adhesion molecules, such as integrins. DCs express receptors for, and respond to, constitutive and inflammatory chemokines and other chemoattractants, such as platelet-activating factor and formyl peptides.

Empirical Evidence

There is good agreement between the sequences of biochemical and physiological events leading to skin sensitisation (see [4];[5];[6];[7];[8];[9]).

Using a flow-cytometric assay, the influence of contact sensitisers on endocytic mechanisms in murine Langerhans cells was measured. Epidermal cell suspensions were labelled with a monoclonal antibody directed to MHC class II molecules and pH-sensitive fluorochrome-coupled second step reagents. Study reported that stimulation with well-known sensitising compounds resulted in a partial conservation of the fluorescence intensity due to the internalisation of the labelled complexes into less acidic compartments. For untreated Langerhans cells or in the presence of irritants a significant quenching of fluorescence intensity due to the internalization of the MHC-antibody complexes into acidic compartments was noticed[10]. In the h-CLAT assay measuring the expression of CD86 and CD54 protein markers on the surface of the human monocytic leukemia cell line THP-1, the cell exposure to known non sensitisers does not increase cell biomarker expression. On the contrary, exposure to well-known sensitisers leads to an increase of the CD86 and CD54 expression[11];[12].

In BALB/c mice, topical application of TMA induced rapid cytokine secretion in the skin—namely IL-4 and IL-10, which was not the case for the skin sensitizer DNCB. Increased IL-4 and IL-10 were also detected in the DLN after TMA exposure, and DC migration to the DLN was confirmed, although delayed behind DNCB-caused migration. Anti-IL-10 antibody ameliorated this response to TMA. (Cumberbatch et al., 2005)

Uncertainties and Inconsistencies

The expression of other cytokines linked to skin sensitisers include IL-1 α, IL-1β, IL-18, and TNF-α form the basis for other dendritic cell assays. In general, an increase in cytokine/chemokine secretion or receptor expression is observed when sensitisers were tested but not when non-sensitisers were tested. However, there is currently only a limited number of chemicals evaluated in more than one assay and results are sometimes contradictory.

Much investigation has gone into assessing the specific mechanistic events involved in skin sensitizer-caused DC migration. Ex vivo studies with intact human skin, epidermal sheets, and MUTZ-3-derived Langerhans cells (LC) show that fibroblasts mediate migration of cytokine-matured LC via chemokines, including CXCL12, CXCR4, and dermis-derived CCL2 and CCL5. (Ouwehand et al., 2008, 2011, 2012) The relevance of these studies for respiratory sensitization is not known. Some evidence indicates that IL-10, upregulated by TMA, may block the migration of LC for a short period of time to allow a Th2 phenotype to develop.(Holden et al., 2008, Cumberbatch et al., 2005)

Quantitative Understanding of the Linkage

It is not known how much change in the first event is needed to impact the second.

Time-scale

Mo-DCs express maturation factors in a few hours following exposure, similar in time-scale to the activation of inflammatory responses. In vivo, DC migration to lymph nodes is typically measured 18 hours after exposure.

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Relationship: 834: Activation, Keratinocytes leads to Activation, Dendritic Cells

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Covalent Protein binding leading to Skin Sensitisation	adjacent	Moderate	

Key Event Relationship Description

Uptake of the hapten by keratinocytes activates multiple events, including the release of pro-inflammatory cytokines (e.g. IL-18) and the induction of cyto-protective cellular pathways. Under the influence of fibroblast- blood endothelial- and lymph endothelial-chemokines (e.g. CCL19, CCL21) and epidermal cytokines (e.g. interleukin (IL), IL-1 α, IL-1β, IL-18, tumour necrosis factor alpha (TNF-α)) maturing dendritic cells migrate from the epidermis to the dermis of the skin and then to the proximal lymph nodes, where they can present the hapten-protein complex to T-cells via a major histocompatibility complex molecule ([1];[2]).

This KER description is based only on the OECD document 2012 and needs updating.

Evidence Supporting this KER

Biological Plausibility

Keratinocyte response activates multiple events, including the release of pro-inflammatory cytokines (e.g. IL-18) and the induction of cyto-protective cellular pathways. Under the influence of fibroblast- blood endothelial- and lymph endothelial-chemokines (e.g. CCL19, CCL21) and epidermal cytokines (e.g. IL-1 α, IL-1β, IL-18, tumour necrosis factor alpha (TNF-α)) maturing dendritic cells migrate from the epidermis to the dermis of the skin and then to the proximal lymph nodes[1];[2].

Empirical Evidence

Matjeka et al. (2012) exposed HaCaT cell line used as a model of human keratinocytes to skin sensitisers for one hour and then, after washed off, cocultured them with dendritic cells. Data showed that exposure of dendritic cells to chemically treated HaCaT cells led to the activation of dendritic cells measured by CD83 and CD86 upregulation[3].

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Relationship: 379: Activation, Dendritic Cells leads to Activation/Proliferation, T-cells

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Covalent Protein binding leading to Skin Sensitisation	adjacent	High	
Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	adjacent	High	Not Specified

Key Event Relationship Description

Under the influence of fibroblast- blood endothelial- and lymph endothelial-chemokines (e.g. CCL19, CCL21) and epidermal cytokines (e.g. interleukin (IL), IL-1 α, IL-1β, IL-18, tumour necrosis factor alpha (TNF-α)) maturing dendritic cells migrate from the epidermis to the dermis of the skin and then to the proximal lymph nodes, where they can present the hapten-protein complex to T-cells via a major histocompatibility complex molecule ([1];[2]). T-cells are typically affected by protein-hapten complexes presented by dendritic cells on MHC molecules. Molecular understanding of this process has improved in recent years ([3]). Briefly, MHC molecules are membrane proteins which present the small peptide antigens placed in a “groove” of the MHC molecule during its intracellular synthesis and transport to the cell surface. In the context of the MHC molecular on the cell surface, the small peptide antigen is recognized via the T-cell receptors as self or non-self (e.g. foreign). If this peptide is a foreign peptide, such as part of a protein-hapten complex, the T-cell will be activated to form a memory T-cell, which subsequently proliferates ([4]). These observations are consistent with the immunological mechanism presented with this AOP, where it is assumed that for an adverse outcome to commence, a certain number of dendritic cells is required to be activated and to migrate to the nearest lymph node in order to instigate the further cascade of biological events (see[5]).

This KER description is based only on the OECD document 2012 and needs updating.

Evidence Supporting this KER

Biological Plausibility

It is well accepted and experimentally proved that in the local lymph node, mature dendritic cells present the hapten-protein complex to T-cells via a major histocompatibility complex molecule (MHC)[2];[1]. T-cells are typically affected by protein-hapten complexes presented by dendritic cells on MHC molecules. The T-cell will be then activated to form a memory T-cell, which subsequently proliferates[4].

Empirical Evidence

A recent study showed in mice model that dendritic cells coordinate the interactions that are necessary to initiate polyclonal regulatory T cells proliferation[6].

Quantitative Understanding of the Linkage

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)

Taylor et al. (2020) found single nucleotide polymorphisms (SNPs) associated with differences in biomarker levels following occupational exposure to 1,6-hexamethylene diisocyanate isocyanurate and 1,6-hexamethylene diisocyanate implicate the TGF-beta pathway regulating endothelial migration and proliferation as well as genes regulating chemokine-induced lymphocyte migration.

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Relationship: 835: Activation/Proliferation, T-cells leads to sensitisation, skin

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Covalent Protein binding leading to Skin Sensitisation	adjacent	High	

Key Event Relationship Description

After recognition of a non-self peptide (i.e. foreign) presented by dendritic cells, T-cells are activated and form memory T-cell, which proliferate. If reactivated upon hapten presentation by skin dendritic cells, these memory T-cells will induce allergic contact dermatitis[1]).

This KER description is based only on the OECD document 2012 and needs updating.

Evidence Supporting this KER

Biological Plausibility

It is well known, recognised and experimentally proved that skin sensitisation is a T-cell mediated immune response[1].

Empirical Evidence

Using dinitrofluorobenzene and mice models, it was shown that cutaneous contact with reactive antigen induces KC/CXC chemokine ligand 1 production and neutrophil infiltration in an antigen, dose-dependent manner. The intensity of neutrophil infiltration into cutaneous antigen challenge sites, in turn, controls the number of antigen-primed T cells recruited into the site and the magnitude of immune response elicited[2].

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