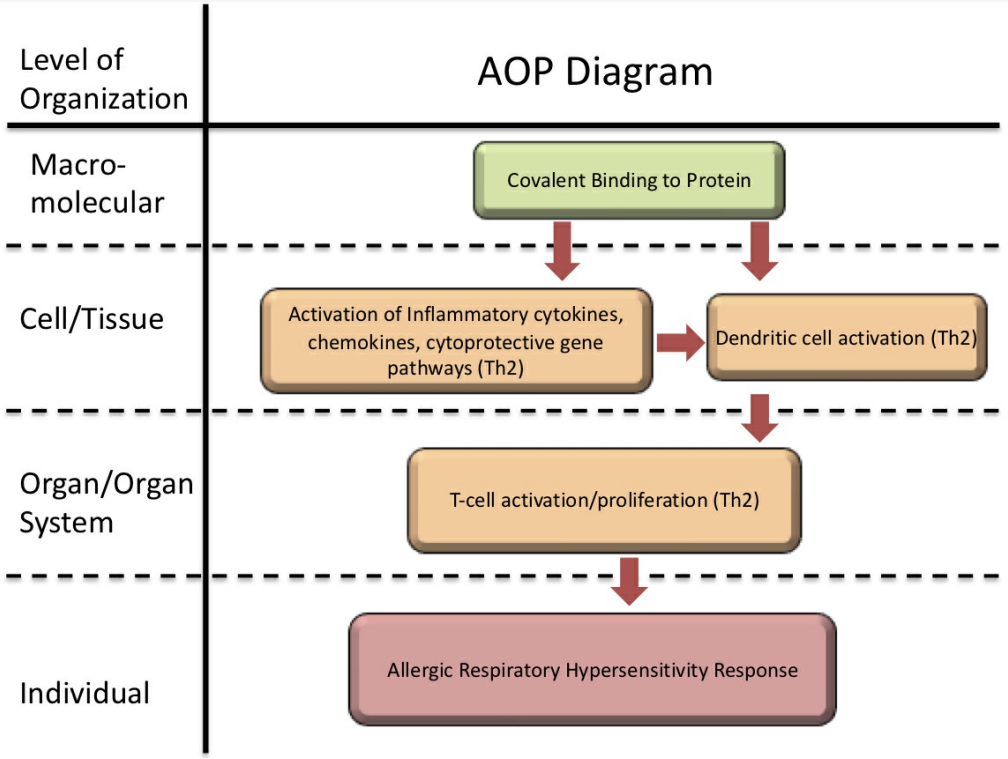
AOP ID and Title:

AOP 39: Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response
Short Title: Covalent binding to proteins leads to Respiratory Sensitisation/Sensitization/Allergy

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under Development: Contributions and Comments Welcome	Under Development	1.20	Included in OECD Work Plan

Abstract

The assessment of xenobiotics for potential to induce an allergenic response in the respiratory tract is of great regulatory and industrial interest. Ongoing work in this area has hypothesized some differences between the dermal and respiratory sensitisation pathways; however in some cases a lack of strong empirical evidence on a variety of chemistries to test these hypothesis. This AOP represents the currently available data with the aim of identifying knowledge gaps which may be filled with directed research. (Sullivan, et al., 2017)

Sensitization of the respiratory tract is an important occupational health challenge. Here we build on a previously published skin sensitization AOP (AOP 40), relying on literature evidence linked to low-molecular-weight organic chemicals and excluding other known respiratory sensitizers acting via different molecular initiating events. The established key events (KEs) are as follows: (1) covalent binding of chemicals to proteins, (2) activation of cellular danger signals (inflammatory cytokines and chemokines and cytoprotective gene pathways), (3) dendritic cell activation and migration, (4) activation, proliferation, and polarization of T cells, and (5) sensitization of the respiratory tract. There is some evidence that respiratory sensitizers bind preferentially to lysine moieties, whereas skin sensitizers bind to both cysteine and lysine, however this observation may be biased by the limited number of chemicals investigated. Furthermore, exposure to respiratory sensitizers seems to result in cell behavior for KEs 2 and 3, as well as the effector T cell response, in general skewing toward cytokine secretions predominantly associated with T helper 2 (Th2) response.

Background

Sensitization of the respiratory tract by chemicals is the first stage in the development of chemical respiratory allergy, an immune-mediated hypersensitivity reaction to an exogenous low-molecular-weight chemical, which can result in asthma and rhinitis on repeated exposure and is an important occupational health problem. (Mapp et al., 2005) Due to the severity and irreversibility of the adverse effect, identifying chemical respiratory allergens is of considerable regulatory, industrial, and socioeconomic importance. (Boverhof et al., 2008) Efforts to outline a framework for assessment of potential respiratory-sensitizing chemicals are underway. (North et al., 2016) Currently, however, there are no standardized, validated, and regulatory-accepted models for detecting these chemicals or discriminating them from skin sensitizers, potentially due to remaining gaps within the literature as to the exact mechanistic steps leading to respiratory allergy. (Kimber et al., 2011)

Another important issue in the development of predictive test methods is the route of exposure in the sensitization phase. Inhalation exposure is perhaps the most common exposure route of concern for many substances, but there is evidence that sensitization of the respiratory tract can be induced via skin exposure as well. (Tarlo and Malo, 2006, Heederik et al., 2012, Redlich and Herrick, 2008, Kimber and Dearman, 2002) This knowledge has implications for both the mechanistic understanding of the pathway and the potential test methods that may be used to detect respiratory sensitizers. Therefore, this AOP will include information from models using skin and lung exposure (in vivo) and with dermal and respiratory cells and tissues (in vitro/ex vivo).

The outlines of this pathway follow the already-published skin sensitization AOP 40. However, the divergent AOs of the two pathways reflect differences in the effector response (T helper 1 [Th1] vs. T helper 2 [Th2]) and other mechanistic details of at least some KEs;(Kimber et al., 2014) these differences are the focus of this effort. Therefore, the primary evidence relied on to build this AOP must relate directly to known low-molecular-weight organic chemicals to the exclusion of chemicals that act via other mechanisms and therefore require a separate AOP, for example, chloroplatinates.

In brief, the AOP can be summarized as beginning with covalent protein binding, potentially preferentially to lysine nucleophiles in the lung or skin after respiratory or dermal exposure to a low-molecular-weight organic chemical. This protein binding causes the activation of stress response pathways and cellular danger signals, including oxidative stress, cytokines, and chemokines released by epithelial and other cells, leading to dendritic cell (DC) maturation and migration to the draining lymph nodes (DLN). Haptens can also contribute to DC activation directly. Th2-skewed DCs in the DLN signal activation and maturation of T cells, which characterize the sensitization phase, resulting in chemical respiratory allergy. Consistent with regulatory practice, sensitization is considered the AO. (Vandebriel et al., 2011)

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
	KE	1496	Increased, secretion of proinflammatory mediators	Increased proinflammatory mediators
3	KE	398	Activation, Dendritic Cells	Activation, Dendritic Cells
4	KE	272	Activation/Proliferation, T-cells	Activation/Proliferation, T-cells
5	AO	313	Increase, Allergic Respiratory Hypersensitivity Response	Increase, Allergic Respiratory Hypersensitivity Response

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Covalent Binding, Protein	adjacent	Increased, secretion of proinflammatory mediators	High	Not Specified
Covalent Binding, Protein	adjacent	Activation, Dendritic Cells	High	Not Specified
Increased, secretion of proinflammatory mediators	adjacent	Activation, Dendritic Cells	Low	Low
Activation, Dendritic Cells	adjacent	Activation/Proliferation, T-cells	High	Not Specified
Activation/Proliferation, T-cells	adjacent	Increase, Allergic Respiratory Hypersensitivity Response	High	Not Specified
Covalent Binding, Protein	non-adjacent	Increase, Allergic Respiratory Hypersensitivity Response	High	Low

Stressors

Name	Evidence
Toluene diisocyanate	

Overall Assessment of the AOP

1. Concordance of dose-response relationships

There is fairly sparse evidence wth more than a few respiratory sensitizers that can offer confidence in dose-response concordance. In BALB/c mice, toluene diisocyanate (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) provides a detailed dose–response analysis of TDI-induced protein binding, colocalization of immune messenger cells, and migration to Delphian lymph nodes.

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

(Nayak et al., 2014) also provides a detailed temporal analysis of TDI-induced protein binding, colocalization of immune messenger cells, and migration to Delphian lymph nodes.

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

There are clear connections from chemicals with certain reactivity and binding profiles to cellular- and individual-level downstream Th2-related effects leading to respiratory sensitization. Though the number of chemicals studied is quite low, consistent patterns are identified. A better understanding of how differences in haptenation by these chemicals contribute to distinct cellular and immune-system-level responses, and how early DC gene changes contribute (or not) to the expression of maturation markers, will help to increase the specificity of the available test methods.

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

Each of the hypothesized KERs is supported by evidence from studies with at least one, and sometimes a few, known respiratory sensitizers. The events fit with what is known in general for sensitization, and the basic KEs outlined here are consistent with established biological knowledge. However, further research is needed to understand, for a larger number of chemicals, the steps leading to a skewing of the effector response toward Th2 and sensitization of the respiratory tract; therefore, the WoE is considered to be “moderate.”

5. Alternative mechanisms

Currently, there are about 80 chemicals identified as respiratory allergens. Exposure occurs primarily in occupational settings. AOs are asthma and rhinitis. The biological mechanisms are often Th2 mediated leading to the production of IgE and eosinophilic inflammation. However, this may not always be the case. For example, human studies reveal PPD to be a respiratory sensitizer, (Helaskoski et al., 2014) but it does not cause a Th2 cytokine response in mice. (Rothe et al., 2011) Specific IgE is induced in some subjects, but not in others, particularly for diisocyanate sensitization. Thus, it is unclear whether IgE is mandatory or not.

Notably, it has to be mentioned that for protein-induced respiratory allergy, the clinical understanding of the disease has been changing dramatically during the last years. For many years, asthma has been considered as a single disease with a defined phenotype. It was assumed that the biology of sensitization is based on Th2-mediated IgE production, migration of mast cells, and subsequent eosinophilic infiltration. Nevertheless, clinical studies of cohort revealed that only about 50% of all patients show a Th2-driven eosinophilic inflammation of the airways. It also covers Th17-driven neutrophilic airway inflammation—an asthmatic phenotype that also can be observed with chemical allergens.

Nowadays, asthma is considered as an umbrella disease with multiple heterogeneous phenotypes, depending on the underlying immunology, pathology, symptoms, and the time of elicitation during lifetime. Furthermore, the concept takes other environmental and genetic influences into consideration. The development of animal models reflecting the heterogeneity of asthma phenotypes is still ongoing and shows in particular the (i) irritant properties of the allergen, (ii) the route of exposure during sensitization and elicitation, and (iii) the dose levels of allergen define whether a Th2 or Th17 phenotype develops.

For chemical allergens, less is known about the influence of atopy, viral infections, and indoor and outdoor environmental pollutants such as cigarette smoke. Of interest is the influence of an additional coexposure to irritant if the chemical allergen is present at low dose. Genetic susceptibility is also a variable of interest. (Yucesoy et al., 2012) and (Wisnewski et al., 2008) among others, have determined factors that may affect the potential for a person's sensitization potential to diisocyanates, including genetic variants in antioxidant defense genes and PRRs.

A number of studies have looked into the sensitization of transition metal complexes, including one which outlines the evidence for these complexes initiating sensitization not through covalent bond formation, but rather through coordination complexes. (Chipinda et al., 2011) The authors provide evidence that these coordination complexes are not stable enough to survive the antigen processing that a covalent hapten undergoes. Instead an alternative MIE is outlined in which these complexes bind to cell surface proteins like MHC, bypassing the intracellular antigen process. This initiating event fits in with the observed cross-reactivity that appears to transcend the trends one would expect based on the periodic table (for example, complexes of Cr, a group 6 metal, cross sensitizing with complexes of Co, a group 9 metal). (Templeton, 2004) It is thought that the surface protein chelates the metal complex and presents it to T-cells directly, requiring a separate AOP from chemicals acting via covalent binding to proteins.

6. Uncertainties, inconsistencies and data gaps.

A better understanding of how differences in haptenation by these chemicals contribute to distinct cellular responses, and how early DC gene changes contribute (or not) to the expression of maturation markers, will help to increase the specificity of the available test methods. A better understanding of human response and population variability is also needed, along with a better quantitative understanding of the linkages between KEs. Additional studies using human cells and tissues are recommended.

Furthermore, as noted in the evaluation section, efforts to fully understand this pathway and develop toxicological test methods and strategies are hampered by a spare data portfolio, as well as a lack of a robust set of harmonized reference chemicals clearly identified as respiratory sensitizers. Previous authors have gathered preliminary chemical sets with supporting rationale, and collating this information and building a set of harmonized reference chemicals, which can be used to optimize and characterize potential test methods or strategies, are the clear next steps. (Enoch et al., 2010, Cochrane et al., 2015, Enoch et al., 2009)

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Sensitizers which do not fit into this AOP:

There have been a number of studies into the sensitisation (and toxicity) of transition metal complexes; key amongst these is a recent study outlining the evidence for these complexes initiating sensitisation via the formation of co-ordination complexes rather than covalent bond formation. (Chipinda et al., 2011) The authors of this study present the evidence that these co-ordination complexes are not stable enough to survive the antigen processing that a covalent hapten undergoes, thus cannot sensitise via this MIE. Instead an alternative MIE is outlined in which these complexes bind to cell surface proteins like MHC, bypassing the intracellular antigen process. This MIE fits in with the observed cross-reactivity that appears to transcend the trends one would expect based on the periodic table (for example, complexes of Cr, a group 6 metal, cross sensitising with complexes of Co, a group 9 metal). (Templeton, 2004) It is thought that the surface protein chelates the metal complex and presents it to T-cells directly. Therefore, transition metals would require a separate AOP from chemicals acting via covalent binding to proteins.

Essentiality of the Key Events

	Defining Question	High (Strong)	Moderate	Low (Weak)
		Direct evidence	Indirect	

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.	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.
MIE (KE1): Covalent Binding, Protein	Moderate	There is extensive evidence in the literature for haptenation being the MIE for respiratory sensitization. In general, haptenation can be divided into five types of chemistry, so-called mechanistic domains. These being acylation, aliphatic nucleophilic substitution (SN1/SN2), aromatic nucleophilic substitution (SNAr), Michael addition, and Schiff base formation. (Enoch et al., 2011, Aptula et al., 2005, Aptula and Roberts, 2006, Lalko et al., 2011, Landsteiner and Jacobs, 1935, Landsteiner and Jacobs, 1936, Hopkins et al., 2005)			
KE2: Activation of Inflammatory Signaling	High	Strong evidence exists for the essential nature of cellular danger signals in respiratory sensitization. (Silva et al., 2014) found that HDI increased ROS by inhibiting superoxide dismutase (SOD1) in THP-1 cells. This inhibition may further encourage a redox environment via matrix metalloproteinase (MMP reduction). Increased ROS also led to extracellular signal-related kinase (ERK) signaling pathway phosphorylation and the transcription of cytoprotective and maturation pathways (HMOX1 and CD83). Coincubation with the antioxidant n-acetyl cysteine and SOD decreased ERK phosphorylation.			
KE3: Dendritic cells activation	High	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. Increased IL-4 and IL-10 were detected in the draining lymph nodes of mice after TMA exposure, and DC migration to the DLN was confirmed. Anti-IL-10 antibody ameliorated this response to TMA. (Holden et al., 2008, Cumberbatch et al. 2005)			
KE4: T-cells, activation and proliferation	High	In humans, support for the Th2-skewing being associated with sensitization of the respiratory tract rather than the skin comes from studying the responses of individuals who already have an immune response skewed in one direction or the other. (Holden et al., 2008, Newell et al., 2013, Ouyang et al., 2013)			

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	Extensive understanding of the KER based on previous	KER is plausible based on analogy to,accepted biological	Empirical support for association between KEs, but the structural or

	consistent with established biological knowledge?	documentation and broad acceptance.	relationships, but scientific understanding is incomplete.	functional relationship between them is not understood.
MIE => KE2: Covalent Binding, Protein leads to Activation of Inflammatory Signaling	High	It has been demonstrated with lung cell lines that exposure to haptenated human serum albumin increases reactive oxygen species. (Hur et al., 2009) It is well accepted and experimentally proved in lung cell lines, 3D human airway epithelial cell cultures, and human lung slices that exposure to haptens induces proinflammatory cytokine and chemokine (e.g. IL-1α, TNF-α, IL-6, IL-8, CCL2, CXCL1, CCL5, etc.) release. (Huang et al., 2013, Lauenstein et al., 2014, Verstraelen et al., 2009)		
MIE => KE3: Covalent Binding, Protein leads to Dendritic Cells Activation	High	It is well-accepted and experimentally proven that dendritic cells represent the most important antigen-presenting cells in the lung. Immature DCs are distributed above and beneath the basal membrane of the lung epithelium and sample antigens by extending dendrites into the airway lumen. Immature cells mature after encountering antigen, an essential event in the activation of immune response. (Lambrecht and Hammad, 2010, Lambrecht and Hammad, 2003, Lambrecht and Hammad, 2009, Holt et al., 1994)		
KE2 => KE3: Activation of Inflammatory Signaling leads to Dendritic Cells Activation	Low	DCs express receptors for, and respond to, constitutive and inflammatory chemokines and other chemoattractants, such as platelet-activating factor and formyl peptides. 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., 2011) The relevance of these studies for respiratory sensitization is not known.		
KE3 => KE4: Dendritic Cells Activation leads to T-cells, activation and proliferation	High	It is well-accepted and experimentally proven that a Th2-type T cell polarization is associated with respiratory sensitization. (Hopkins et al., 2005, Huang et al., 2013)		
KE4 => AO: T-cells, activation and proliferation leads to Sensitisation of the Respiratory Tract	High	It is well-demonstrated that clonal expansion of Th2 cells leads to the production of Th2 cytokines that induce Ig class-switching, with clonal expansion of B cells producing antigen-specific IgE. (Dearman et al., 2003)		

	Defining Question	**High (Strong)**	**Moderate**	**Low (Weak)**
	Does empirical evidence	Multiple		

Empirical Support for KERs	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?	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 => KE2: Covalent Binding, Protein leads to Activation of Inflammatory Signaling	Moderate	Haptenated peptides generated in vitro can be quantified after 15 minutes. (Hettick, et al., 2009) Most in vitro cellular assay protocols quantify inflammatory readouts after 24 – 48 hours of exposure. TMA induced increased production of IL-10 when incubated with precision cut lung slices (PCLS) for 24 hours. (Lauenstein et al., 2014)
MIE => KE3: Covalent Binding, Protein leads to Dendritic Cells Activation	Moderate	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.
KE2 => KE3: Activation of Inflammatory Signaling leads to Dendritic Cells Activation	Low	(Silva et al., 2014) found that HDI increased ROS by inhibiting superoxide dismutase (SOD1) in THP-1 cells. Increased ROS also led to extracellular signal-related kinase (ERK) signaling pathway phosphorylation and the transcription of cytoprotective and maturation pathways (HMOX1 and CD83).
KE3 => KE4: Dendritic Cells Activation leads to T-cells, activation and proliferation	Low	There is little known about many aspects of antigen processing, such as uptake pathway, peptide generation, and MHC peptide complex stability and density, in chemical sensitization of the respiratory tract. Differences may exist in how skin and respiratory sensitizers are processed that may provide key insight into how to distinguish such chemicals. (Hopkins et al, 2005) found increased expression of type 2 cytokines n mouse lymph node cells after topical exposure to TMA and FITC.
KE4 => AO: T-cells, activation and proliferation leads to Sensitisation of the Respiratory Tract	Low	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 (Vocanson et al., 2009)

Quantitative Consideration

Frequency of exposures to toluene diisocyanate exceeding 3 ppb in the time-weighted average (8 hrs, TWA-8) without respiratory protection were found to be associated with incidence. In this study, TWA-8 values above 3 ppb were indicative of peak exposure

events, i.e. spills. (Plehiers et al., 2020a and 2020b) This is consistent with a prior report by (Collins et al., 2017) which found a significant link between peak exposure and asthma incidence.

Considerations for Potential Applications of the AOP (optional)

The construction of an AOP for this endpoint would allow the following: (1) organization of available information to identify remaining uncertainties and prioritize further research, (2) highlighting of differences and similarities between skin and respiratory sensitization pathways, and (3) improvement of existing or identification of novel predictive models that, alone or in an integrated approach, could be used to identify respiratory sensitizers.

Given the available (WoE) outlined above, we propose that the AOP for sensitization of the respiratory tract outlined here allows the identification of gaps in knowledge, research needs, and potential test methods that may be developed further using a larger set of respiratory sensitizers.

References

APTULA, A. O., PATLEWICZ, G. & ROBERTS, D. W. 2005. Skin sensitization: reaction mechanistic applicability domains for structure-activity relationships. Chem Res Toxicol, 18, 1420-6.

APTULA, A. O. & ROBERTS, D. W. 2006. Mechanistic applicability domains for nonanimal-based prediction of toxicological end points: general principles and application to reactive toxicity. Chem Res Toxicol, 19, 1097-105.

BOVERHOF, D. R., BILLINGTON, R., GOLLAPUDI, B. B., HOTCHKISS, J. A., KRIEGER, S. M., POOLE, A., WIESCINSKI, C. M. & WOOLHISER, M. R. 2008. Respiratory sensitization and allergy: current research approaches and needs. Toxicol Appl Pharmacol, 226, 1-13.

CHIPINDA, I., HETTICK, J. M. & SIEGEL, P. D. 2011. Haptenation: chemical reactivity and protein binding. J Allergy (Cairo), 2011, 839682.

COCHRANE, S. A., ARTS, J. H. E., EHNES, C., HINDLE, S., HOLLNAGEL, H. M., POOLE, A., SUTO, H. & KIMBER, I. 2015. Thresholds in chemical respiratory sensitisation. Toxicology, 333, 179-194.

COLLINS, J. J., ANTEAU, S., CONNER, P. R., CASSIDY, L. D., DONEY, B., WANG, M. L., KURTH, L., CARSON, M., MOLENAAR, D., REDLICH, C. A. & STOREY, E. 2017. Incidence of Occupational Asthma and Exposure to Toluene Diisocyanate in the United States Toluene Diisocyanate Production Industry. Journal of occupational and environmental medicine, 59 Suppl 12, S22-S27.

ENOCH, S. J., ELLISON, C. M., SCHULTZ, T. W. & CRONIN, M. T. 2011. A review of the electrophilic reaction chemistry involved in covalent protein binding relevant to toxicity. Crit Rev Toxicol, 41, 783-802.

ENOCH, S. J., ROBERTS, D. W. & CRONIN, M. T. 2009. Electrophilic reaction chemistry of low molecular weight respiratory sensitizers. Chem Res Toxicol, 22, 1447-53.

ENOCH, S. J., ROBERTS, D. W. & CRONIN, M. T. 2010. Mechanistic category formation for the prediction of respiratory sensitization. Chem Res Toxicol, 23, 1547-55.

HEEDERIK, D., HENNEBERGER, P. K. & REDLICH, C. A. 2012. Primary prevention: exposure reduction, skin exposure and respiratory protection. Eur Respir Rev, 21, 112-24.

HELASKOSKI, E., SUOJALEHTO, H., VIRTANEN, H., AIRAKSINEN, L., KUULIALA, O., AALTO-KORTE, K. & PESONEN, M. 2014. Occupational asthma, rhinitis, and contact urticaria caused by oxidative hair dyes in hairdressers. Ann Allergy Asthma Immunol, 112, 46-52.

HETTICK, J.M., RUWONA, T.B. & SIEGEL, P.D. 2009. Structural elucidation of isocyanate-peptide adducts using tandem mass spectrometry. *J Am Soc Mass Spectrom* 20, 1567–1575.

HOLDEN, N. J., BEDFORD, P. A., MCCARTHY, N. E., MARKS, N. A., IND, P. W., JOWSEY, I. R., BASKETTER, D. A. & KNIGHT, S. C. 2008. Dendritic cells from control but not atopic donors respond to contact and respiratory sensitizer treatment in vitro with differential cytokine production and altered stimulatory capacity. Clin Exp Allergy, 38, 1148-59.

HOLT, P. G., HAINING, S., NELSON, D. J. & SEDGWICK, J. D. 1994. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. J Immunol, 153, 256-61.

HUANG, S., WISZNIEWSKI, L., CONSTANT, S. & ROGGEN, E. 2013. Potential of in vitro reconstituted 3D human airway epithelia (MucilAir™) to assess respiratory sensitizers. Toxicol In Vitro, 27, 1151-6.

HUR, G. Y., KIM, S. H., PARK, S. M., YE, Y. M., KIM, C. W., JANG, A. S., PARK, C. S., HONG, C. S. & PARK, H. S. 2009. Tissue transglutaminase can be involved in airway inflammation of toluene diisocyanate-induced occupational asthma. J Clin Immunol, 29, 786-94.

KAROL, M. H. & STOLIKER, D. 1999. Immunotoxicology: past, present, and future. Inhal Toxicol, 11, 523-34.

KIMBER, I., BASKETTER, D. A., GERBERICK, G. F., RYAN, C. A. & DEARMAN, R. J. 2011. Chemical allergy: translating biology

into hazard characterization. Toxicol Sci, 120 Suppl 1, S238-68.

KIMBER, I. & DEARMAN, R. J. 2002. Chemical respiratory allergy: role of IgE antibody and relevance of route of exposure. Toxicology, 181-182, 311-5.

KIMBER, I., DEARMAN, R. J., BASKETTER, D. A. & BOVERHOF, D. R. 2014. Chemical respiratory allergy: reverse engineering an adverse outcome pathway. Toxicology, 318, 32-9.

LALKO, J. F., KIMBER, I., DEARMAN, R. J., API, A. M. & GERBERICK, G. F. 2013. The selective peptide reactivity of chemical respiratory allergens under competitive and non-competitive conditions. J Immunotoxicol, 10, 292-301.

LALKO, J. F., KIMBER, I., DEARMAN, R. J., GERBERICK, G. F., SARLO, K. & API, A. M. 2011. Chemical reactivity measurements: potential for characterization of respiratory chemical allergens. Toxicol In Vitro, 25, 433-45.

LAMBRECHT, B. N. & HAMMAD, H. 2003. Taking our breath away: dendritic cells in the pathogenesis of asthma. Nature Reviews Immunology, 3, 994-1003.

LAMBRECHT, B. N. & HAMMAD, H. 2009. Biology of Lung Dendritic Cells at the Origin of Asthma. Immunity, 31, 412-424.

LAMBRECHT, B. N. & HAMMAD, H. 2010. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. Lancet, 376, 835-43.

LANDSTEINER, K. & JACOBS, J. 1935. STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS. J Exp Med, 61, 643-56.

LANDSTEINER, K. & JACOBS, J. 1936. STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS. II. J Exp Med, 64, 625-39.

LANGE, R. W., DAY, B. W., LEMUS, R., TYURIN, V. A., KAGAN, V. E. & KAROL, M. H. 1999. Intracellular S-glutathionyl adducts in murine lung and human bronchoepithelial cells after exposure to diisocyanatotoluene. Chem Res Toxicol, 12, 931-6.

LANTZ, R. C., LEMUS, R., LANGE, R. W. & KAROL, M. H. 2001. Rapid reduction of intracellular glutathione in human bronchial epithelial cells exposed to occupational levels of toluene diisocyanate. Toxicol Sci, 60, 348-55.

LAUENSTEIN, L., SWITALLA, S., PRENZLER, F., SEEHASE, S., PFENNIG, O., FÖRSTER, C., FIEGUTH, H., BRAUN, A. & SEWALD, K. 2014. Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS). Toxicol In Vitro, 28, 588-99.

MAPP, C. E., BOSCHETTO, P., MAESTRELLI, P. & FABBRI, L. M. 2005. Occupational asthma. Am J Respir Crit Care Med, 172, 280-305.

NATSCH, A., RYAN, C. A., FOERTSCH, L., EMTER, R., JAWORSKA, J., GERBERICK, F. & KERN, P. 2013. A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. J Appl Toxicol, 33, 1337-52.

NAYAK, A. P., HETTICK, J. M., SIEGEL, P. D., ANDERSON, S. E., LONG, C. M., GREEN, B. J. & BEEZHOLD, D. H. 2014. Toluene diisocyanate (TDI) disposition and co-localization of immune cells in hair follicles. Toxicol Sci, 140, 327-37.

NEWELL, L., POLAK, M. E., PERERA, J., OWEN, C., BOYD, P., PICKARD, C., HOWARTH, P. H., HEALY, E., HOLLOWAY, J. W., FRIEDMANN, P. S. & ARDERN-JONES, M. R. 2013. Sensitization via healthy skin programs Th2 responses in individuals with atopic dermatitis. J Invest Dermatol, 133, 2372-2380.

NORTH, C. M., EZENDAM, J., HOTCHKISS, J. A., MAIER, C., AOYAMA, K., ENOCH, S., GOETZ, A., GRAHAM, C., KIMBER, I., KARJALAINEN, A., PAULUHN, J., ROGGEN, E. L., SELGRADE, M., TARLO, S. M. & CHEN, C. L. 2016. Developing a framework for assessing chemical respiratory sensitization: A workshop report. Regul Toxicol Pharmacol, 80, 295-309.

OUYANG, B., BERNSTEIN, D. I., LUMMUS, Z. L., YING, J., BOULET, L. P., CARTIER, A., GAUTRIN, D. & HO, S. M. 2013. Interferon-γ promoter is hypermethylated in blood DNA from workers with confirmed diisocyanate asthma. Toxicol Sci, 133, 218-24.

OUWEHAND K, SPIEKSTRA SW, WAAJIMAN T, SCHEPER RJ, DE GRUJIL TD, GIBBS S. 2011. Technical advance: Langerhans cells derived from a human cell line in a full-thickness skin equivalent undergo allergen-induced maturation and migration. J Leukoc Biol. 290(5):1027-33.

PLEHIERS, P. M., CHAPPELLE, A. H. & SPENCE, M. W. 2020a. Practical learnings from an epidemiology study on TDI-related occupational asthma: Part I-Cumulative exposure is not a good indicator of risk. Toxicol Ind Health, 36, 876-884.
PLEHIERS, P. M., CHAPPELLE, A. H. & SPENCE, M. W. 2020b. Practical learnings from an epidemiology study on TDI-related occupational asthma: Part II-Exposure without respiratory protection to TWA-8 values indicative of peak events is a good indicator of risk. Toxicol Ind Health, 36, 885-891.

REDLICH, C. A. & HERRICK, C. A. 2008. Lung/skin connections in occupational lung disease. Curr Opin Allergy Clin Immunol, 8, 115-9.

REMY, S., VERSTRAELEN, S., VAN DEN HEUVEL, R., NELISSEN, I., LAMBRECHTS, N., HOOYBERGHS, J. & SCHOETERS, G.

2014. Gene expressions changes in bronchial epithelial cells: markers for respiratory sensitizers and exploration of the NRF2 pathway. Toxicol In Vitro, 28, 209-17.

ROTHE, H., SARLO, K., SCHEFFLER, H. & GOEBEL, C. 2011. The hair dyes PPD and PTD fail to induce a T(H)2 immune response following repeated topical application in BALB/c mice. J Immunotoxicol, 8, 46-55.

SILVA, A., NUNES, C., MARTINS, J., DINIS, T. C., LOPES, C., NEVES, B. & CRUZ, T. 2014. Respiratory sensitizer hexamethylene diisocyanate inhibits SOD 1 and induces ERK-dependent detoxifying and maturation pathways in dendritic-like cells. Free Radic Biol Med, 72, 238-46.

SULLIVAN, K.M., ENOCH, S.J., EZENDAM, J., SEWALD, K., ROGGEN, E.L., COCHRANE, S. 2017. An Adverse Outcome Pathway for Sensitization of the Respiratory Tract by Low-Molecular-Weight Chemicals: Building Evidence to Support the Utility of In Vitro and In Silico Methods in a Regulatory Context. *Appl In Vitro Tox,* 3:3, 213-226

TARLO, S. M. & MALO, J. L. 2006. An ATS/ERS report: 100 key questions and needs in occupational asthma. Eur Respir J, 27, 607-14.

TEMPLETON, D. 2004. Mechanisms of immunosensitization to metals (IUPAC Technical Report). Pure and Applied Chemistry - PURE APPL CHEM, 76, 1255-1268.

VANDEBRIEL, R., CALLANT CRANSVELD, C., CROMMELIN, D., DIAMANT, Z., GLAZENBURG, B., JOOS, G., KUPER, F., NATSCH, A., NIJKAMP, F., NOTEBORN, H., PIETERS, R., ROBERTS, D., ROGGEN, E., RORIJE, E., SEED, M., SEWALD, K., VAN DEN HEUVEL, R., VAN ENGELEN, J., VERSTRAELEN, S. & VAN LOVEREN, H. 2011. Respiratory sensitization: advances in assessing the risk of respiratory inflammation and irritation. Toxicol In Vitro, 25, 1251-8.

VERSTRAELEN, S., NELISSEN, I., HOOYBERGHS, J., WITTERS, H., SCHOETERS, G., VAN CAUWENBERGE, P. & VAN DEN HEUVEL, R. 2009. Gene profiles of a human alveolar epithelial cell line after in vitro exposure to respiratory (non-)sensitizing chemicals: identification of discriminating genetic markers and pathway analysis. Toxicol Lett, 185, 16-22.

VOCANSON M, HENNINO A, ROZIERES A, POYET G, NICOLAS JF. 2009. Effector and regulatory mechanisms in allergic contact dermatitis. Allergy. 64(12), 1699-714.

WISNEWSKI, A. V., LIU, Q., LIU, J. & REDLICH, C. A. 2008. Human innate immune responses to hexamethylene diisocyanate (HDI) and HDI-albumin conjugates. Clin Exp Allergy, 38, 957-67.

YUCESOY, B., JOHNSON, V. J., LUMMUS, Z. L., KISSLING, G. E., FLUHARTY, K., GAUTRIN, D., MALO, J. L., CARTIER, A., BOULET, L. P., SASTRE, J., QUIRCE, S., GERMOLEC, D. R., TARLO, S. M., CRUZ, M. J., MUNOZ, X., LUSTER, M. I. & BERNSTEIN, D. I. 2012. Genetic variants in antioxidant genes are associated with diisocyanate-induced asthma. Toxicol Sci, 129, 166-73.

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

Name

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

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 ([1];[19];[20]). 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 of[21]), the coherence and consistency of the experimental data is excellent. Alternative mechanism that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative mechanisms of action, if supported, require a separate AOP. 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*.

Earlier work on the molecular basis of skin sensitisation was reviewed by Lepoittevin et al. (1998)[22], since then our knowledge of skin sensitisation has continued to expand. Recent reviews (see[3];[9];[20];[22];[23];[24];[25]) repeatedly stress the same key steps leading to sensitisation. These events include hapten formation (i.e., the ability of a chemical to react with skin proteins).

The binding behavior of diisocyanates in particular has been well studied. Wisnewski et al.29,30 demonstrate that hexamethylene diisocyanate (HDI) and 4,4'-diphenylmethane diisocyanate (MDI) react with glutathione (GSH) across an in vitro physiologically relevant vapor/liquid-phase barrier to form conjugates, which may "shuttle," via a carbamoylating reaction, the chemical to bind with serum albumin. Diisocyanates (MDI) react with GSH across an in vitro physiologically relevant vapor/liquid-phase barrier to form conjugates, which may "shuttle," via a carbamoylating reaction, the chemical to bind with serum albumin.

In contrast to skin sensitization where cysteine and lysine are both key nucleophiles, experimental work has suggested that some respiratory sensitizers appear to preferentially bind to lysine; (Hettick et al., 2012, Lalko et al., 2012, Holsapple et al., 2006, Hopkins et al., 2005) however, an in chemico analysis of a larger set of respiratory sensitizers indicates lack of a simple division between the reactivity preferences of the two types of sensitizers, showing that certain classes displayed a lysine preference, for example, anhydrides, whereas others, such as diisocyanates, do not. (Dik et al., 2016)

While respiratory sensitizers and skin sensitizers can both bind to cellular and serum proteins in separate cultures, a study comparing the binding profiles of both classes in co-culture systems found that skin sensitizers preferentially bind cellular proteins, while respiratory sensitizers preferentially bind serum proteins. (Hopkins et al., 2005)

Domain of Applicability

Taxonomic Applicability

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

References

1. ↑ 1.0 1.1 1.2 1.3 Gerberick F, Aleksic M, Basketter D, Casati S, Karlberg AT, Kern P, Kimber I, Lepoittevin JP, Natsch A, Ovigne JM, Rovida C, Sakaguchi H and Schultz T. 2008. Chemical reactivity measurement and the predictive identification of skin sensitisers. Altern. Lab. Anim. 36: 215-242.
2. ↑ Karlberg AT, Bergström MA, Börje A, Luthman K and Nilsson JL. 2008. Allergic contact dermatitis- formation, structural requirements, and reactivity of skin sensitizers. Chem. Res. Toxicol. 21: 53-69.
3. ↑ 3.0 3.1 3.2 3.3 3.4 3.5 Schwöbel JAH, Koleva YK, Bajot F, Enoch SJ, Hewitt M, Madden JC, Roberts DW, Schultz TW and Cronin MTD. 2011. Measurement and estimation of electrophilic reactivity for predictive toxicology. Chem. Rev. 111: 2562-2596.
4. ↑ Hopkins JE, Naisbitt DJ, Kitteringham NR, Dearman RJ, Kimber I and Park BK. 2005. Selective haptenation of cellular or extracellular proteins by chemical allergens: Association with cytokine polarization. Chem. Res. Toxicol. 18: 375-381.
5. ↑ Lepoittevin JP. 2006. Metabolism versus chemical transformation or pro-versus prehaptens? Contact Dermatitis 54: 73-74.
6. ↑ Freidig AP and Hermens JLM. 2001. Narcosis and chemical reactivity QSARs for acute toxicity. Quant. Struct. Act. Rel. 19: 547-553.
7. ↑ Roberts DW, Aptula AO, Patlewicz G, Pease C. 2008. Chemical reactivity indices and mechanism-based read-across for non-animal based assessment of skin sensitisation potential. J.Appl. Toxicol. 28: 443-454.
8. ↑ Enoch SJ, Ellison CM, Schultz TW, Cronin MTD. 2011. A review of the electrophilic reaction chemistry involved on covalent protein binding relevant to toxicity. Crit. Rev. Toxicol. 41: 783– 802.
9. ↑ 9.0 9.1 OECD 2011. Report of the Expert Consultation on Scientific and Regulatory Evaluation of Organic Chemistry-based Structural Alerts for the Identification of Protein-binding Chemicals. OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 139. ENV/JM/MONO(2011).
10. ↑ Basketter DA, Pease C, Kasting G, Kimber I, Casati S, Cronin MTD, Diembeck W, Gerberick F, Hadgraft J, Hartung J, Marty JP, Nikolaidis E, Patlewicz G, Roberts DW, Roggen E, Rovida C, van de Sandt J. 2007. Skin sensitisation and epidermal disposition: The relevance of epidermal disposition for sensitisation hazard identification and risk assessment. The report of ECVAM workshop 59. Altern. Lab. Anim. 35: 137-154.
11. ↑ Schwöbel J, Wondrousch D, Koleva YK, Madden JC, Cronin MTD, Schüürmann G. 2010. Prediction of Michael type acceptor reactivity toward glutathione. Chem. Res. Toxicol. 23: 1576-1585.
12. ↑ Kato H, Okamoto M, Yamashita K, Nakamura Y, Fukumori Y, Nakai K, Kaneko H. 2003. Peptide-binding assessment using mass spectrometry as a new screening method for skin sensitization. J. Toxicol. Sci. 28: 19-24.
13. ↑ Schultz TW, Yarbrough JW, Woldemeskel M. 2005. Toxicity to Tetrahymena and abiotic thiol reactivity of aromatic isothiocyanates. Cell. Biol. Toxicol. 21: 181-189.
14. ↑ 14.0 14.1 Böhme A, Thaens D, Paschke A, Schüürmann G. 2009. Kinetic glutathione chemoassay to quantify thiol reactivity of organic electrophiles – Application to α, β-unsaturated ketones, acrylates, and propiolates, Chem. Res. Toxicol. 22: 742-750.
15. ↑ OECD. Test No 442C: In chemico skin sensitisation: Direct Peptide Reactivity Assay (DPRA). 2015. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects, OECD Publishing. Doi 10.1787/9789264229709-en.
16. ↑ EURL ECVAM DB-ALM. Protocol No154: Direct Peptide Reactivity Assay for skin sensitisation testing. Available on: http://ecvam-dbalm.jrc.ec.europa.eu/.
17. ↑ Yarbrough JW and Schultz TW. 2007. Abiotic sulfhydryl reactivity: A predictor of aquatic toxicity for carbonyl-containing α,β-unsaturated compounds. Chem. Res. Toxicol. 20: 558-562.
18. ↑ Roberts DW and Natsch A. 2009. High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitisation potency of Michael acceptor electrophiles. Chem. Res. Toxicol. 22: 592-603.
19. ↑ Karlberg AT, Bergström MA, Börje A, Luthman K, Nilsson JL. 2008. Allergic contact dermatitisformation, structural requirements, and reactivity of skin sensitizers. Chem. Res. Toxicol. 21: 53-69.
20. ↑ 20.0 20.1 Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Andersen KE, Angers-Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S, Coecke S, Corvi R, Cronin M, Daston G, Dekant W, Felter S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Leite SB, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhler S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, Tähti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM.2011. Alternative (non-animal) methods for cosmetics testing: current status and

future prospects-2010. Arch Toxicol.85(5):367-485.
21. ↑ OECD. 2012. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168.
22. ↑ 22.0 22.1 Lepoittevin JP, Basketter DA, Goossens A and Karlberg AT (eds) 1998. Allergic contact dermatitis: the molecular basis. Springer, Berlin.
23. ↑ Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF. 2009. Effector and regulatory mechanisms in allergic contact dermatitis. Allergy 64: 1699-1714.
24. ↑ Aeby P, Ashikaga T, Bessou-Touya S, Schapky A, Geberick F, Kern P, Marrec-Fairley M, Maxwell G, Ovigne JM, Sakaguchi H, Reisinger K, Tailhardat M, Martinozzi-Teisser S and Winkler P. 2010. Identifying and characterizing chemical skin sensitizers without animal testing; Colipa's research and methods development program. Toxicol. In Vitro 24: 1465-1473.
25. ↑ Basketter DA and Kimber I. 2010. Contact hypersensitivity. In: McQueen, C.A. (ed) Comparative Toxicology Vol. 5, 2nd Ed. Elsevier, Kidlington, UK, pp. 397-411.

BASKETTER, D., POOLE, A. & KIMBER, I. 2017. Behaviour of chemical respiratory allergens in novel predictive methods for skin sensitisation. Regul Toxicol Pharmacol, 86, 101-106.

DIK, S., RORIJE, E., SCHWILLENS, P., VAN LOVEREN, H. & EZENDAM, J. 2016. Can the Direct Peptide Reactivity Assay Be Used for the Identification of Respiratory Sensitization Potential of Chemicals? Toxicol Sci, 153, 361-71.

HETTICK, J. M. & SIEGEL, P. D. 2011. Determination of the toluene diisocyanate binding sites on human serum albumin by tandem mass spectrometry. Anal Biochem, 414, 232-8.

HETTICK, J. M., SIEGEL, P. D., GREEN, B. J., LIU, J. & WISNEWSKI, A. V. 2012. Vapor conjugation of toluene diisocyanate to specific lysines of human albumin. Anal Biochem, 421, 706-11.

HOLSAPPLE, M. P., JONES, D., KAWABATA, T. T., KIMBER, I., SARLO, K., SELGRADE, M. K., SHAH, J. & WOOLHISER, M. R. 2006. Assessing the potential to induce respiratory hypersensitivity. Toxicol Sci, 91, 4-13.

HOPKINS, J. E., NAISBITT, D. J., KITTERINGHAM, N. R., DEARMAN, R. J., KIMBER, I. & PARK, B. K. 2005. Selective haptenation of cellular or extracellular protein by chemical allergens: association with cytokine polarization. Chem Res Toxicol, 18, 375-81.

LALKO, J. F., KIMBER, I., GERBERICK, G. F., FOERTSCH, L. M., API, A. M. & DEARMAN, R. J. 2012. The direct peptide reactivity assay: selectivity of chemical respiratory allergens. Toxicol Sci, 129, 421-31.

NATSCH, A. & GFELLER, H. 2008. LC-MS-based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. Toxicol Sci, 106, 464-78.

WISNEWSKI, A. V., LIU, J. & REDLICH, C. A. 2013a. Connecting glutathione with immune responses to occupational methylene diphenyl diisocyanate exposure. Chem Biol Interact, 205, 38-45.

WISNEWSKI, A. V., MHIKE, M., HETTICK, J. M., LIU, J. & SIEGEL, P. D. 2013b. Hexamethylene diisocyanate (HDI) vapor reactivity with glutathione and subsequent transfer to human albumin. Toxicol In Vitro, 27, 662-71.

List of Key Events in the AOP

Event: 1496: Increased, secretion of proinflammatory mediators

Short Name: Increased proinflammatory mediators

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	Cytokine	increased
chemokine secretion	Chemokine	increased
complement activation		increased
	Interleukin	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis	KeyEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent

AOP ID and Name	Event Type
Aop:382 - Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis	KeyEvent
Aop:392 - Decreased fibrinolysis and activated bradykinin system leading to hyperinflammation	KeyEvent
Aop:409 - Frustrated phagocytosis leads to malignant mesothelioma	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:39 - Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	KeyEvent
Aop:237 - Substance interaction with lung resident cell membrane components leading to atherosclerosis	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Human, mouse, rat

Cytokines are the common pro-inflammatory mediators secreted following inflammogenic stimuli. Cytokines can be defined as diverse group of signaling protein molecules. They are secreted by different cell types in different tissues and in all mammalian species, irrespective of gender, age or sex. A lot of literature is available to support cross species, gender and developmental stage application for this KE. The challenge is the specificity; most cytokines exhibit redundant functions and many are pleotropic.

Key Event Description

Pro-inflammatory mediators are the chemical and biological molecules that initiate and regulate inflammatory reactions. Pro-inflammatory mediators are secreted following exposure to an inflammogen in a gender/sex or developmental stage independent manner. They are secreted during inflammation in all species. Different types of pro-inflammatory mediators are secreted during innate or adaptive immune responses across various species (Mestas and Hughes, 2004). Cell-derived pro-inflammatory mediators include cytokines, chemokines, and growth factors. Blood derived pro-inflammatory mediators include vasoactive amines, complement activation products and others. These modulators can be grouped based on the cell type that secrete them, their cellular localisation and also based on the type of immune response they trigger. For example, members of the interleukin (IL)

family including IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, IL-3, IL-5 and Granulocyte-macrophage colony stimulating factor (GM-CSF) are involved in the adaptive immune responses. The pro-inflammatory cytokines include IL-1 family (IL-1α , IL-1β, IL-1rα, IL-18, IL-36α, IL-36β, IL-36γ, IL-36Rα, IL-37), IL-6 family, Tumor necrosis factor (TNF) family, IL-17, and Interferon gamma (IFN-γ) (Turner et al., 2014). While IL-4 and IL-5 are considered T helper (Th) cell type 2 response, IFN-γ is suggested to be Th1 type response.

Different types of pro-inflammatory mediators are secreted during innate or adaptive immune responses across various species (Mestas and Hughes, 2004). However, IL-1 family cytokines, IL-4, IL-5, IL-6, TNF-α, IFN-γ are the commonly measured mediators in experimental animals and in humans. Similar gene expression patterns involving inflammation and matrix remodelling are observed in human patients of pulmonary fibrosis and mouse lungs exposed to bleomycin (Kaminski, 2002).

Literature evidence for its perturbation:

Several studies show increased proinflammatory mediators in rodent lungs and bronchoalveolar lavage fluid, and in cell culture supernatants following exposure to a variety of carbon nanotube (CNT) types and other materials. Poland et al., 2008 showed that long and thin CNTs (>5 µm) can elicit asbestos-like pathogenicity through the continual release of pro-inflammatory cytokines and reactive oxygen species. Exposure to crystalline silica induces release of inflammatory cytokines (TNF-α, IL-1, IL-6), transcription factors (Nuclear factor kappa B [NF-κB], Activator protein-1 [AP-1]) and kinase signalling pathways in mice that contain NF-κB luciferase reporter (Hubbard et al., 2002). Boyles et al., 2015 found that lung responses to long multi-walled carbon nanotubes (MWCNTs) included high expression levels of pro-inflammatory mediators Monocyte Chemoattractant Protein 1 (MCP-1), Transforming growth factor beta 1 (TGF-β1), and TNF-α (Boyles et al., 2015). Bleomycin administration in rodents induces lung inflammation and increased expression of pro-inflammatory mediators (Park et al., 2019). Inflammation induced by bleomycin, paraquat and CNTs is characterised by the altered expression of pro-inflammatory mediators. A large number of nanomaterials induce expression of cytokines and chemokines in lungs of rodents exposed via inhalation (Halappanavar et al., 2011; Husain et al., 2015a). Similarities are observed in gene programs involving pro-inflammatory event is observed in both humans and experimental mice (Zuo et al., 2002).

How it is Measured or Detected

The selection of pro-inflammatory mediators for investigation varies based on the expertise of the lab, cell types studied and the availability of the specific antibodies.

Real-time reverse transcription-polymerase chain reaction (qRT-PCR) – will measure the abundance of cytokine mRNA in a given sample. The method involves three steps: conversion of RNA into cDNA by reverse transcription method, amplification of cDNA using the PCR, and the real-time detection and quantification of amplified products (amplicons) (Nolan T et al., 2006). Amplicons are detected using fluorescence, increase in which is directly proportional to the amplified PCR product. The number of cycles required per sample to reach a certain threshold of fluorescence (set by the user – usually set in the linear phase of the amplification, and the observed difference in samples to cross the set threshold reflects the initial amount available for amplification) is used to quantify the relative amount in the samples. The amplified products are detected by the DNA intercalating minor groove-binding fluorophore SYBR green, which produces a signal when incorporated into double-stranded amplicons. Since the cDNA is single stranded, the dye does not bind enhancing the specificity of the results. There are other methods such as nested fluorescent probes for detection, but SYBR green is widely used. RT-PCR primers specific to several pro-inflammatory mediators in several species including mouse, rat and humans, are readily available commercially.

Enzyme-linked immunosorbent assays (ELISA) – permit quantitative measurement of antigens in biological samples. The method is the same as described for the MIE. Both ELISA and qRT-PCR assays are used in vivo and are readily applicable to in vitro cell culture models, where cell culture supernatants or whole cell homogenates are used for ELISA or mRNA assays. Both assays are straight forward, quantitative and require relatively a small amount of input sample.

Apart from assaying single protein or gene at a time, cytokine bead arrays or cytokine PCR arrays can also be used to detect a whole panel of inflammatory mediators in a multiplex method (Husain et al., 2015b). This method is quantitative and especially advantageous when the sample amount available for testing is scarce. Lastly, immunohistochemistry can also be used to detect specific immune cell types producing the pro-inflammatory mediators and its downstream effectors in any given tissue (Costa et al., 2017). Immunohistochemistry results can be used as weight of evidence; however, the technique is not quantitative and depending on the specific antibodies used, the assay sensitivity may also become an issue (Amsen and De Visser, 2009).

Cell models - of varying complexity have been used to assess the expression of pro-inflammatory mediators. Two dimensional submerged monocultures of the main fibrotic effector cells – lung epithelial cells, macrophages, and fibroblasts – have routinely been used *in vitro* due to the large literature base, and ease of use, but do not adequately mimic the *in vivo* condition (Sundarakrishnan *et al.,* 2018, Sharma *et al.,* 2016). Recently, the EpiAlveolar *in vitro* lung model (containing epithelial cells, endothelial cells, and fibroblasts) was used to predict the fibrotic potential of MWCNT, and researchers noted increases in the pro-inflammatory molecules TNF-α, IL-1β, and the pro-fibrotic TGF-β using ELISA (Barasova *et al.,* 2020). A similar, but less complicated co-culture model of immortalized human alveolar epithelial cells and idiopathic pulmonary fibrosis patient derived fibroblasts was used to assess pro-fibrotic signalling, and noted enhanced secretion of Platelet derived growth factor (PDGF) and Basic fibroblast growth factor (bFGF), as well as evidence for epithelial to mesenchymal transition of epithelial cells in this system (Prasad et al., 2014). Models such as these better capitulate the *in vivo* pulmonary alveolar capillary, but have lower reproducibility as compared to traditional submerged mono-culture experiments.

References

1. Amsen D, de Visser KE, Town T. Approaches to determine expression of inflammatory cytokines. Methods Mol Biol. 2009;511:107-42. doi: 10.1007/978-1-59745-447-6_5.

2. Barosova H, Maione AG, Septiadi D, Sharma M, Haeni L, Balog S, O'Connell O, Jackson GR, Brown D, Clippinger AJ, Hayden P, Petri-Fink A, Stone V, Rothen-Rutishauser B. Use of EpiAlveolar Lung Model to Predict Fibrotic Potential of Multiwalled Carbon Nanotubes. ACS Nano. 2020 Apr 28;14(4):3941-3956. doi: 10.1021/acsnano.9b06860.

3. Boyles MS, Young L, Brown DM, MacCalman L, Cowie H, Moisala A, Smail F, Smith PJ, Proudfoot L, Windle AH, Stone V. Multi-walled carbon nanotube induced frustrated phagocytosis, cytotoxicity and pro-inflammatory conditions in macrophages are length dependent and greater than that of asbestos. Toxicol In Vitro. 2015 Oct;29(7):1513-28. doi: 10.1016/j.tiv.2015.06.012.

4. Costa PM, Gosens I, Williams A, Farcal L, Pantano D, Brown DM, Stone V, Cassee FR, Halappanavar S, Fadeel B. Transcriptional profiling reveals gene expression changes associated with inflammation and cell proliferation following short-term inhalation exposure to copper oxide nanoparticles. J Appl Toxicol. 2018 Mar;38(3):385-397. doi: 10.1002/jat.3548.

5. Halappanavar S, Jackson P, Williams A, Jensen KA, Hougaard KS, Vogel U, Yauk CL, Wallin H. Pulmonary response to surface-coated nanotitanium dioxide particles includes induction of acute phase response genes, inflammatory cascades, and changes in microRNAs: a toxicogenomic study. Environ Mol Mutagen. 2011 Jul;52(6):425-39. doi: 10.1002/em.20639.

6. Hubbard AK, Timblin CR, Shukla A, Rincón M, Mossman BT. Activation of NF-kappaB-dependent gene expression by silica in lungs of luciferase reporter mice. Am J Physiol Lung Cell Mol Physiol. 2002 May;282(5):L968-75. doi: 10.1152/ajplung.00327.2001.

7. Husain M, Kyjovska ZO, Bourdon-Lacombe J, Saber AT, Jensen KA, Jacobsen NR, Williams A, Wallin H, Halappanavar S, Vogel U, Yauk CL. Carbon black nanoparticles induce biphasic gene expression changes associated with inflammatory responses in the lungs of C57BL/6 mice following a single intratracheal instillation. Toxicol Appl Pharmacol. 2015a Dec 15;289(3):573-88. doi: 10.1016/j.taap.2015.11.003.

8. Husain M, Wu D, Saber AT, Decan N, Jacobsen NR, Williams A, Yauk CL, Wallin H, Vogel U, Halappanavar S. Intratracheally instilled titanium dioxide nanoparticles translocate to heart and liver and activate complement cascade in the heart of C57BL/6 mice. Nanotoxicology. 2015b;9(8):1013-22. doi: 10.3109/17435390.2014.996192.

9. Kaminski N. Microarray analysis of idiopathic pulmonary fibrosis. Am J Respir Cell Mol Biol. 2003 Sep;29(3 Suppl):S32-6.

10. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol. 2004 Mar 1;172(5):2731-8. doi: 10.4049/jimmunol.172.5.2731.

11. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006;1(3):1559-82. doi: 10.1038/nprot.2006.236.

12. Park SJ, Im DS. Deficiency of Sphingosine-1-Phosphate Receptor 2 ($S1P_2$) Attenuates Bleomycin-Induced Pulmonary Fibrosis. Biomol Ther (Seoul). 2019 May 1;27(3):318-326. doi: 10.4062/biomolther.2018.131.

13. Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A, Stone V, Brown S, Macnee W, Donaldson K. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotechnol. 2008 Jul;3(7):423-8. doi: 10.1038/nnano.2008.111.

14. Prasad S, Hogaboam CM, Jarai G. Deficient repair response of IPF fibroblasts in a co-culture model of epithelial injury and repair. Fibrogenesis Tissue Repair. 2014 Apr 29;7:7. doi: 10.1186/1755-1536-7-7.

15. Sharma M, Nikota J, Halappanavar S, Castranova V, Rothen-Rutishauser B, Clippinger AJ. Predicting pulmonary fibrosis in humans after exposure to multi-walled carbon nanotubes (MWCNTs). Arch Toxicol. 2016 Jul;90(7):1605-22. doi: 10.1007/s00204-016-1742-7.

16. Sundarakrishnan A, Chen Y, Black LD, Aldridge BB, Kaplan DL. Engineered cell and tissue models of pulmonary fibrosis. Adv Drug Deliv Rev. 2018 Apr;129:78-94. doi: 10.1016/j.addr.2017.12.013.

17. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta. 2014 Nov;1843(11):2563-2582. doi: 10.1016/j.bbamcr.2014.05.014.

18. Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, Lollini L, Morris D, Kim Y, DeLustro B, Sheppard D, Pardo A, Selman M, Heller RA. Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. Proc Natl Acad Sci U S A. 2002 Apr 30;99(9):6292-7. doi: 10.1073/pnas.092134099.

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 GARD for assessing chemical respiratory sensitizers using transcriptional readouts of a genomic biomarker signature indicated 80% accuracy. (Forreryd, et al., 2015)

There are several in vitro assays available to assess DC maturation; the most advanced is the h-CLAT, which determines changes in CD86 and CD54 levels on THP-1 cell.(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 emerged over 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 LC migration. 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 toward CXCL12 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
h-CLAT	draft TG under discussion at OECD	[1]		X	
	DB-ALM	[2]			
	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

References

1. ↑ Ryan CA, Gerberick GF, Gildea LA, Hulette BC, Bettis CJ, Cumberbatch M, Dearman RJ, Kimber I. 2005. Interactions of contact allergens with dendritic cells: opportunities and challenges for the development of novel approaches to hazard assessment. Toxicol. Sci. 88: 4-11.
2. ↑ Ryan CA, Kimber I, Basketter, DA, Pallardy M, Gildea LA, Gerberick GF. 2007. Dendritic cells and skin sensitisation.

Biological roles and uses in hazard identification. Toxicol. Appl. Pharmacol. 221: 384-394.
3. ↑ 3.0 3.1 3.2 3.3 3.4 3.5 dos Santos GG, Reinders J, Ouwhand K, Rustemeyer T, Scheper RJ, Gibbs S. 2009. Progress on the development of human *in vitro* dendritic cell based assays for assessment of skin sensitizing potential of compounds. Toxicol. Appl. Pharmacol. 236: 372-382.
4. ↑ 4.0 4.1 4.2 Kimber I, Basketter DA, Gerberick GF, Ryan CA, Dearman, R.J. 2011. Chemical allergy: Translating biology into hazard characterization. Toxicol. Sci. 120(S1): S238-S268.
5. ↑ 5.0 5.1 Antonopoulos C, Cumberbatch M, Mee JB, Dearman RJ, Wei XQ, Liew FY, Kimber I, Groves RW. 2008. IL-18 is a key proximal mediator of contact hypersensitivity and allergen induced Langerhans cell migration in murine epidermis. J. Leukoc. Biol. 83: 361-367.
6. ↑ Ouwehand K, Santegoets SJAM, Bruynzeel DP, Scheper RJ, de Gruijl TD, Gibbs S. 2008. CXCL12 is essential for migration of activated Langerhans cells for epidermis to dermis. Eur. J. Immunol. 38: 3050-3059.
7. ↑ Vandebriel RJ and van Loveren H. 2010. Non-animal sensitisation testing: State-of-the-art. Crit. Rev. Toxicol. 40: 389-404.
8. ↑ Trompezinski S, Migdal C, Tailhardat M, Le Varlet B, Courtellemont P, Haftek M and Serres M. 2008. Charaterization of early events involved in human dendritic cell maturation induced by sensitizers: cross talk between MAPK signalling pathways. Toxicol. Appl. Pharmacol. 230: 397-406.
9. ↑ Lambrechts N, Vanheel H, Hooyberghs J, De Boever P, Witters H, Van Den Heuval R, Van Tendeloom V, Nelissen I, Schoeters G. 2010. Gene markers in dendritic cells unravel pieces of the skin sensitisation puzzle. Toxicol. Letters 196: 95-103.
10. ↑ Migdal C, Tailhardat M, Courtellemont P, Haftek M, Serres M. 2010. Responsiveness of human monocyte-derived dendritic cells to thimerosal and mercury derivatives. Toxicol. Appl. Pharmacol. 246: 66-73.
11. ↑ Aeby P, Ashikaga T, Bessou-Touya S, Schapky A, Geberick F, Kern P, Marrec-Fairley M, Maxwell G, Ovigne JM, Sakaguchi H, Reisinger K, Tailhardat M, Martinozzi-Teisser S, Winkler P. 2010. Identifying and characterizing chemical skin sensitizers without animal testing; Colipa's research and methods development program. Toxicol. In Vitro 24: 1465-1473.
12. ↑ Hooyberghs J, Schoeters E, Lambrechts N, Nelissen I, Witters H, Schoeters G, Van Den Heuvel R. 2008. A cell-based in vitro alternative to identify skin sensitizers by gene expression. Toxicol. Appl. Pharmacol. 231: 103-111.
13. ↑ Borrebaeck CA and Wingren C. 2009. Design of high-density antibody microarrays for disease proteomics: key technological issues. J. Proteomics 72: 928-935.
14. ↑ Groux H and Sabatier JM. 2010. Polypeptides for the *in vitro* assessment of the sensitising potential of a test compound. International Application Patent No.: PCT/EP2010/055895.
15. ↑ Sakaguchi H, Ashikaga T, Miyazawa M, Kosaka N, Ito Y, Yoneyama K, Sono S, Itagaki H, Toyoda H, Suzuki H. 2009. The relationship between CD86/CD54 expression and THP-1 cell viability in an *in vitro* skin sensitisation test-human cell line activation test (h-CLAT). Cell Biol. Toxicol. 25: 109-126.
16. ↑ Ashikaga T, Sakaguchi H, Sono S, Kosaka N, Ishikawa M, Nukada Y, Miyazawa M, Ito Y, Nishiyama N, Itagaki H. 2010. 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. 38:275-84.
17. ↑ EURL ECVAM DB-ALM. Protocol No158: Human Cell Line Activation Test (h-CLAT) Available on: http://ecvam-dbalm.jrc.ec.europa.eu/.
18. ↑ Ade N, Martinozzi-Teissier S, Pallaardy M, Rousset F. 2006. Activation of U937 cells by contact sensitizers: CD86 expression is independent of apoptosis. J. Immunotoxicol. 3: 189-197.
19. ↑ Python F, Goebel C, Aeby P. 2007. Assessment of the U937 cell line for detection of contact allergens. Toxicol. Appl. Pharmacol. 220: 113-124.
20. ↑ Ovigne JM, Martinozzi-Teissier S, Verda D, Abdou D, Piroird C, Ade N, Rousset F. 2008. The MUSST for *in vitro* skin sensitisation prediction: Applicability domains and complementary protocols to adapt to the physico-chemical diversity of chemicals. Toxicology Letters, 180: Supplement 1, 5, S216.

ASHIKAGA T, YOSHIDA Y, HIROTA M, YONEYAMA K, ITAGAKI H, SAKAGUCHI H, MIYAZAWA M, ITO Y, SUZUKI H, TOYODA H. 2006. Development of an in vitro skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol. *Toxicol In Vitro.* 20(5), 767-73.

BASKETTER, D., POOLE, A., KIMBER, I., 2017. Behaviour of chemical respiratory allergens in novel predictive methods for skin sensitisation, *Reg Tox and Pharmacol. 86,*101-106,

DOS SANTOS, G. G., REINDERS, J., OUWEHAND, K., RUSTEMEYER, T., SCHEPER, R. J. & GIBBS, S. 2009. Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound. *Toxicol Appl Pharmacol,* 236**,** 372-82.

FORRERYD A, JOHANSSON H, ALBREKT AS, BORREBAECK CA, LINDSTEDT M. 2015. Prediction of chemical respiratory sensitizers using GARD, a novel in vitro assay based on a genomic biomarker signature. *PLoS One.*11;10(3):e0118808.

JOHANSSON H, LINDSTEDT M, ALBREKT AS, BORREBAECK CA. 2011. A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. *BMC Genomics.* 8;12:399.

REES B, SPIEKSTRA SW, CARFI M, OUWEHAND K, WILLIAMS CA, CORSINI E, MCLEOD J.D., GIBBS S. 2011. Inter-laboratory study of the in vitro dendritic cell migration assay for identification of contact allergens. *Toxicol In Vitro.* 25(8), 2124-34.

SAKAGUCHI H, ASHIKAGA T, MIYAZAWA M, YOSHIDA Y, ITO Y, YONEYAMA K, HIROTA M, ITAGAKI H, TOYODA H, SUZUKI H. 2006. Development of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). II. An inter-laboratory study of the h-CLAT. *Toxicol In Vitro.* 20(5), 774-84.

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

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

References

1. ↑ 1.0 1.1 1.2 Martin SF, Esser PR, Schmucker S, Dietz L, Naisbitt DJ, Park BK, Vocanson M, Nicolas JF, Keller M, Pichler WJ, Peiser M, Luch A, Wanner R, Maggi E, Cavani A, Rustemeyer T, Richter A, Thierse HJ, Sallusto F. 2010. T-cell recognition of chemical, protein allergens and drugs; toward the development of *in vitro* assays. Cell. Mol. Life Sci. 67: 4171-4184.
2. ↑ Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF. 2009. Effector and regulatory mechanisms in allergic contact dermatitis. Allergy 64: 1699-1714.
3. ↑ OECD 2010. Test No.429: Skin sensitization: Local Lymph Node Assay. OECD Guidelines for the Testing of Chemicals, Section 4: Health effects. OECD Publishing. Doi: 10.1787/9789264071100-en.
4. ↑ OECD 2010. Test No442A: Skin sensitization: Local Lymph Node Assay:DA. OECD Guidelines for the Testing of Chemicals, Section 4: Health effects. OECD Publishing. Doi: 10.1787/9789264090972-en.
5. ↑ OECD 2010. Test No.442B: Skin sensitization: Local Lymph Node Assay: BrdU-ELISA. OECD Guidelines for the Testing of Chemicals, Section 4: Health effects. OECD Publishing. Doi: 10.1787/9789264090996-en.

BERNSTEIN, J. A., GHOSH, D., SUBLETT, W. J., WELLS, H. & LEVIN, L. 2011. Is trimellitic anhydride skin testing a sufficient screening tool for selectively identifying TMA-exposed workers with TMA-specific serum IgE antibodies? J Occup Environ Med, 53, 1122-7.

HOLDEN, N. J., BEDFORD, P. A., MCCARTHY, N. E., MARKS, N. A., IND, P. W., JOWSEY, I. R., BASKETTER, D. A. & KNIGHT, S. C. 2008. Dendritic cells from control but not atopic donors respond to contact and respiratory sensitizer treatment in vitro with differential cytokine production and altered stimulatory capacity. Clin Exp Allergy, 38, 1148-59.

List of Adverse Outcomes in this AOP

Event: 313: Increase, Allergic Respiratory Hypersensitivity Response

Short Name: Increase, Allergic Respiratory Hypersensitivity Response

Key Event Component

Process	Object	Action
Respiratory Hypersensitivity		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:39 - Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	AdverseOutcome

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

lung

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
guinea pig	Cavia porcellus	Low	NCBI
rat	Rattus norvegicus	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Unspecific	High

The domain of applicability for respiratory sensitisation is primarily limited to humans. Guinea pigs, and to a lesser degree rats, are the only rodent species that exhibit respiratory hypersensitivity, but in guinea pigs, IgG1 is the driver antibody (Boverhof et al 2008). In mice, vascular hypersensitivity is observed rather than respiratory response (Boverhof et al 2008). Therefore investigations in mice are limited to the study of earlier key events, or elicitation of vascular hypersensitivity.

Key Event Description

The development of an allergic hypersensitivity reaction in the respiratory tract is a two-step process, first requiring induction of the immune response (Boverhof et al, 2008). Subsequent single or multiple exposures to the same substance result in elicitation of an allergic hypersensitivity reaction, characterized by breathlessness and wheezing, airflow obstruction, bronchoconstriction, and tightness of the chest (Lauenstein et al, 2014). Reactions can be acutely life threatening or lead to chronic occupational asthma (Boverhof et al, 2008).

How it is Measured or Detected

Clinical signs described above can be objectively assessed in humans to confirm diagnosis of respiratory hypersensitivity. Airflow obstruction and bronchoconstriction are measured using serial measures of airflow (usually FEV1) about 12 hours after exposure to test for immediate or delayed bronchoconstriction (Beckett, 2008). However, to differentiate between irritant and allergic asthma, additional testing to confirm immune involvement must also be conducted.

Allergen-specific IgE detection and measurement techniques include skin tests (intradermal and subcutaneous skin prick testing) and blood testing using immune assays such as ELISAs and commercially available tests such as ImmunoCAP™. For example, Bernstein et al. investigated the ability of TMA skin testing to identify sensitized workers and found that skin prick testing was positive in 8 of 11 workers with serum-specific IgE and intradermal testing in a further two (Bernstein et al., 2011). It is important to note, however, that there are technical challenges associated with detection and measurement of specific IgE and IgG to chemical respiratory allergens, including production of the correct protein conjugate and timing of measurement (Kimber et al., 2014, Quirce, 2014). Immune assays such as ELISA or ImmunoCAP are also used to investigate allergen-specific antibody isotype profiles (Movérare et al., 2017). Investigations into direct and indirect class switching involve transcriptomic analyses of IgE heavy chain transcripts and are challenging due to the scarcity of IgE-switched B cells in human blood (Davies et al., 2013).

In cases where specific IgE cannot be identified, the Basophil Activation Test (BAT) can identify allergic response in patients within a year of the last allergen exposure. Basophils degranulate in response to IgE cross-links bound to the high-affinity IgE receptor, much like mast cells. In fresh blood samples (less than 24 hours old) this can be measured by the translocation of CD63 to the membrane using flow cytometry. A review of the use of BAT in diagnosing occupational asthma shows that BAT is a functional readout that works for a variety of allergens, including dust, latex, and small molecules such as ammonium persulfate, chlorhexidine, and beta-lactam antibiotics. However, 10 - 20% of people are estimated to be BAT non-responders in which this response is not detected (Vera-Berrios et al., 2019).

In vivo, alterations in breathing parameters such as respiratory rate, minute volume, tidal volume, peak expiratory flow, inspiratory and expiratory times and a flow-derived estimation of airflow restriction (enhanced pause, Penh) have been used for quantitative assessment of allergen induced airway hyperreactivity (Boverhof et al. 2008).

In rats, respiratory exposure to diisocyanites leads to immediate and delayed airway response. Elicitation is confirmed measuring PMN in bronchoalveolar lavage fluid (BAL) one day after inhalation challenge and exhaled NO (Pauluhn 2014).

Regulatory Significance of the AO

This adverse outcome is of high regulatory interest and relevance, though no test guideline is available. Regulatory agencies and industrial producers are interested in preventing the first step--induction of immune response. Importantly, induction of respiratory sensitisation can be obtained via skin exposure, which is consequential for potential exposure restrictions.

References

Beckett, W.S., 2005. Revised protocol: criteria for designating substances as occupational asthmagens on the AOEC List of Exposure Codes.

Boverhof DR, Billington R, Bhaskar Gollapudi B, Hotchkiss JA, Krieger SM, Poole A, Wiescinski CM, and Woolhiser MR. 2008. Respiratory sensitization and allergy: Current research approaches and needs. Tox Appl Pharm 226:1-13.

Dearman RJ, Betts CJ, Humphreys N, Flanagan BF, Gilmour NJ, Basketter DA, Kimber I. 2003. Chemical allergy: considerations for the practical application of cytokine profiling. Toxicol. Sci. 71, 137–145.

Lauenstein L, Switalla S, Prenzler F, Seehase S, Pfennig O, Förster C, Fieguth H, Braun A and Sewald K. 2014. Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS). Tox in Vitro 28:588–599.

OECD (2010) Test No. 429: Skin Sensitisation: Local Lymph Node Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264071100-en.

Pauluhn J. 2014. Development of a respiratory sensitization/elicitation protocol of toluene diisocyanate (TDI) in Brown Norway rats to derive an elicitation-based occupational exposure level. Toxicology 319: 10–22.

BERNSTEIN, J. A., GHOSH, D., SUBLETT, W. J., WELLS, H. & LEVIN, L. 2011. Is trimellitic anhydride skin testing a sufficient screening tool for selectively identifying TMA-exposed workers with TMA-specific serum IgE antibodies? J Occup Environ Med, 53, 1122-7.

DAVIES, J. M., PLATTS-MILLS, T. A. & AALBERSE, R. C. 2013. The enigma of IgE+ B-cell memory in human subjects. J Allergy Clin Immunol, 131, 972-6.

KIMBER, I., DEARMAN, R. J. & BASKETTER, D. A. 2014. Diisocyanates, occupational asthma and IgE antibody: implications for hazard characterization. J Appl Toxicol, 34, 1073-7.

MOVÉRARE, R., BLUME, K., LIND, P., CREVEL, R., MARKNELL DEWITT, Å. & COCHRANE, S. 2017. Human Allergen-Specific IgG Subclass Antibodies Measured Using ImmunoCAP Technology. Int Arch Allergy Immunol, 172, 1-10.

QUIRCE, S. 2014. IgE antibodies in occupational asthma: are they causative or an associated phenomenon? Curr Opin Allergy Clin Immunol, 14, 100-5.

VERA-BERRIOS, R. N., FEARY, J. & CULLINAN, P. 2019. Basophil activation testing in occupational respiratory allergy to low molecular weight compounds. Curr Opin Allergy Clin Immunol, 19, 92-97.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2649: Covalent Binding, Protein leads to Increased proinflammatory mediators

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	High

Sex Applicability

Sex	Evidence
Unspecific	High

Covalent protein binding leading to increased secretion of proinflammatory molecules is not limited to a specific sex or age.

Key Event Relationship Description

Covalent binding to proteins by electrophiles generates haptenated proteins which result in measurable increases in proinflammatory signaling molecules. As such, the induction and/or activation of a variety of proinflammatory mediators is a measurable result of stressors that covalently bind proteins.

Evidence Supporting this KER

Evidence mostly from in vitro studies show that low molecular weight electrophiles are able, via protein-chemical conjugates, to generate cellular danger signals, including proinflammatory cytokines and oxidative stress.

Biological Plausibility

Multiple cell types in the lung and skin express the necessary pattern recognition receptors for this KER, including epithelial cells, endothelial cells, macrophages, fibroblasts, and dendritic cells. It is likely that different cell types are involved in the process.

Empirical Evidence

It has been demonstrated in lung cell lines, 3D human airway epithelial cell cultures, and human lung slices that exposure to haptens induces proinflammatory cytokine and chemokine (e.g. IL-1α, TNF-α, IL-6, IL-8, CCL2, CXCL1, CCL5, etc.) release. (Huang et al., 2013, Lauenstein et al., 2014, Verstraelen et al., 2009)

Exposure of reconstituted three-dimensional (3D) human airway epithelia (MulcilAir™) to respiratory sensitizers trimellitic anhydride (TMA) and methylene diphenyl diisocyanate (MDI) elevated the levels of proinflammatory cytokines and chemokines interleukin (IL)-6, IL-8, monocyte chemoattractant protein-1 (MCP-1)/chemokine ligand (CCL)2, growth regulated oncogene-a (GRO-a)/C-X-C motif (CX)CL1, and regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5. (Huang et al., 2013) Similarly, typical respiratory sensitizers caused an elevation of proinflammatory cytokines IL-1a (TMA) and tumor necrosis factor (TNF)-a (glutaraldehyde) in precision-cut lung slices. (Lauenstein et al., 2014)

(Hur et al., 2009) found that toluene diisocyanate (TDI)-human serum albumin (HSA) conjugates increased reactive oxygen species (ROS) production in A549 cells. The significance of the Nrf2-Keap1 pathway in respiratory sensitization is not as extensively studied compared to skin sensitization, but in vitro data for a limited number of respiratory sensitizers showed that these are able to activate Nrf2-dependent genes both in airway and skin epithelium. (Emter et al., 2010, Natsch et al., 2013, Remy et al., 2014) Activation of Nrf2-Keap1 by skin sensitizers has been explained by covalent interaction of cysteine residues on Keap1 with cysteine-reactive chemicals, leading to Nrf2 association and transcriptional activation of genes. It is not fully understood how respiratory sensitizers activate this pathway. Although respiratory sensitizers are more likely to bind to hard nucleophiles such as lysine, (Enoch et al., 2010) in chemico studies show that cysteine binding occurs as well. (Lalko et al., 2011, 2013) Hence, Nrf2 activation may be a direct result of covalent interaction with cysteine residues or an indirect result of GSH depletion and an altered redox balance. The indirect activation of Nrf2-dependent genes was shown in THP-1 cells exposed to acid anhydrides, which had a preference to lysine in the direct peptide reactivity assay (DPRA) (Migdal et al., 2013); however, actual Nrf2 and heme oxygenase-1 proteins accumulated only minimally in the cells.

Uncertainties and Inconsistencies

Both qualitative and quantitative characterization of inflammatory pathway activation in response to haptenation remains an area of investigation for sensitization.

Respiratory Sensitizers
To elucidate which pathways respiratory sensitizers regulate, in vitro DNA microarray studies were performed in different human lung cell lines exposed to a limited set of respiratory sensitizers. These studies were not able to identify specific molecular pathways that were regulated by respiratory sensitizers. They could identify activation of genes, related to innate immune response. In human

alveolar epithelial cells (A549 cell line), for example, genes encoding for TLR2, TNF-a, IL-1 receptor, and cytokine signaling pathways were upregulated by hexamethylene diisocyanate (HDI) and TMA. (Verstraelen et al., 2009) NLRP3 has been demonstrated to be important in respiratory sensitization by proteins, (Besnard et al., 2012) but the involvement in the induction of respiratory sensitization by low-molecular-weight chemicals is unknown. In human keratinocytes, the respiratory sensitizers MDI and TMA failed to elevate intracellular proinflammatory IL-18 levels. (Corsini et al., 2009) Conflicting reports as to whether IL-18 is associated with a Th1 or Th2 immune response hamper interpretation of this result.

Additionally, the canonical phosphatase and tensin homolog (PTEN)-signaling pathway might be relevant for respiratory sensitization. (Verstraelen et al., 2009) This pathway regulates cell survival signaling pathways and plays a protective role in the pathogenesis of asthma. (Kwak et al., 2003) In a mouse model of TDI-induced asthma, the PTEN pathway was shown to play a protective role in asthma pathogenesis, because it was involved in the regulation of IL-17 induction and NF-kB activation. (Kim et al., 2007) A more recent in vitro study showed that the PTEN pathway was not consistently induced by all respiratory sensitizers, since maleic anhydride and 7-aminocephalosporanic acid failed to induce this pathway but another diisocyanate, HDI, did. (Remy et al., 2014)

There is some evidence to support the hypothesis that the binding behavior of respiratory sensitizers is related to the eventual Th2-skewed immune response, with binding to lysine on serum albumin in particular, as well as secretion of type 2 cytokines, being associated with known respiratory sensitizers. (Hopkins et al., 2005) The biological hypothesis that lysine is the primary nucleophile responsible for respiratory sensitization is supported by the preference for harder electrophiles compared with those that cause skin sensitization (lysine is a harder nucleophile than cysteine). (Enoch et al., 2010) This is evidenced by the difference in the coverage of the various mechanistic domains that show typical respiratory sensitizers to be chemicals acting via the acylation and Schiff base mechanisms rather than Michael addition (a significantly important mechanism for skin sensitization). Structure/activity analysis has shown the importance of electrophilicity and protein crosslinking for respiratory sensitization for low-molecular-weight organic chemicals. (Hopkins et al., 2005, Agius et al., 1991, 1994, Seed and Agius, 2010, 2017)

One proposed explanation (Kimber et al., 2018) for the association between peptide selectivity and deviation between respiratory and dermal sensitization is based on the observation that respiratory sensitizers, in a co-culture including both U937 cells and serum, preferentially react with serum proteins such as albumin, which has a high number of lysine residues. This behavior was observed for TMA, fluorescein isothiocyanate (FITC), and dinitrobenzenesulfonyl chloride (DNBSCl). Concordantly, skin sensitizers dinitrochlorobenzene (DNCB), dinitrofluorobenzene (DNFB) preferentially bound to cellular proteins in the same co-culture. (Hopkins et al., 2005) This is corroborated by the observation that serum albumin is a major target protein of the respiratory sensitizer hexahydropthalic anhydride in humans. (Johannesson et al., 2001) Further, this is a reasonable hypothesis for the biological mechanism of deviation between skin and respiratory sensitizers, particularly in the case of dermal exposure, as the distribution of antigen formation of chemical allergens in the in vitro model system segregates with the type (Th1- or Th2-activating) of cytokines secreted from activated lymph node cells in an in vivo mouse model.

Quantitative Understanding of the Linkage

Time-scale

Haptenation is essentially instantaneous, and in order to protect the in vivo organism from further damage, local cellular responses to induce inflammation to haptens are rapid. As a result, relevant in vitro readouts to detect proinflammatory mediators (e.g. cytokine/chemokine secretion, redox responses) may be quantifiable within minutes to a few hours, but sensitivity and precision vary based on the assay detection method. Haptenated peptides generated in vitro can be quantified after 15 minutes. (Hettick, et al., 2009) Cellular assay protocols commonly quantify inflammatory secretion 24 – 48 hours after exposure.

Known modulating factors

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

Respiratory sensitizers without intrinsic electrophilic activity have been observed, and this is attributed to in situ generation of electrophilic activity. Pre-haptens and pro-haptens are converted from inactive molecules into active electrophiles by UV light and metabolic enzymes, respectively. (Aptula et al., 2007) Therefore incerases or decreases in UV exposure or metabolic gene expression can affect the downstream KEs.

(Taylor et al, 2020) found that single nucleotide polymorphisms (SNPs) in genes regulating inflammation, calcium regulation and endothelial function, and serine/threonine protein kinsase signaling were associated with differences in plasma and urine levels of two known haptens (1,6-hexamethylene diisocyanate monomer and 1,6-hexamethylene diisocyanate isocyanurate) following occupational exposure. This suggests a genetic component of inter-individual variation that could influence susceptibility for downstream KEs.

References

AGIUS, R. M., ELTON, R. A., SAWYER, L. & TAYLOR, P. 1994. Occupational asthma and the chemical properties of low molecular weight organic substances. *Occup Med (Lond),* 44**,** 34-6.

AGIUS, R. M., NEE, J., MCGOVERN, B. & ROBERTSON, A. 1991. Structure activity hypotheses in occupational asthma caused by

low molecular weight substances. *Ann Occup Hyg,* 35**,** 129-37.

APTULA, A. O., ROBERTS, D. W. & PEASE, C. K. 2007. Haptens, prohaptens and prehaptens, or electrophiles and proelectrophiles. *Contact Dermatitis,* 56**,** 54-56.

BESNARD, A. G., TOGBE, D., COUILLIN, I., TAN, Z., ZHENG, S. G., ERARD, F., LE BERT, M., QUESNIAUX, V. & RYFFEL, B. 2012. Inflammasome-IL-1-Th17 response in allergic lung inflammation. *J Mol Cell Biol,* 4**,** 3-10.

CORSINI, E., MITJANS, M., GALBIATI, V., LUCCHI, L., GALLI, C. L. & MARINOVICH, M. 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicol In Vitro,* 23**,** 789-96.

EMTER, R., ELLIS, G. & NATSCH, A. 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. *Toxicol Appl Pharmacol,* 245**,** 281-90.

ENOCH, S. J., ROBERTS, D. W. & CRONIN, M. T. 2010. Mechanistic category formation for the prediction of respiratory sensitization. *Chem Res Toxicol,* 23**,** 1547-55.

HETTICK, J.M., RUWONA, T.B. & SIEGEL, P.D. 2009. Structural elucidation of isocyanate-peptide adducts using tandem mass spectrometry. *J Am Soc Mass Spectrom* 20, 1567–1575.

HUANG, S., WISZNIEWSKI, L., CONSTANT, S. & ROGGEN, E. 2013. Potential of in vitro reconstituted 3D human airway epithelia (MucilAir™) to assess respiratory sensitizers. *Toxicol In Vitro,* 27**,** 1151-6.

HUR, G. Y., KIM, S. H., PARK, S. M., YE, Y. M., KIM, C. W., JANG, A. S., PARK, C. S., HONG, C. S. & PARK, H. S. 2009. Tissue transglutaminase can be involved in airway inflammation of toluene diisocyanate-induced occupational asthma. *J Clin Immunol,* 29**,** 786-94.

JOHANNESSON, G., ROSQVIST, S., LINDH, C. H., WELINDER, H. & JÖNSSON, B. A. 2001. Serum albumins are the major site for in vivo formation of hapten-carrier protein adducts in plasma from humans and guinea-pigs exposed to type-1 allergy inducing hexahydrophthalic anhydride. *Clin Exp Allergy,* 31**,** 1021-30.

KIM, S. R., LEE, K. S., PARK, S. J., MIN, K. H., LEE, K. Y., CHOE, Y. H., LEE, Y. R., KIM, J. S., HONG, S. J. & LEE, Y. C. 2007. PTEN down-regulates IL-17 expression in a murine model of toluene diisocyanate-induced airway disease. *J Immunol,* 179**,** 6820-9.

KIMBER, I., POOLE, A. & BASKETTER, D. A. 2018. Skin and respiratory chemical allergy: confluence and divergence in a hybrid adverse outcome pathway. *Toxicol Res (Camb),* 7**,** 586-605.

KWAK, Y. G., SONG, C. H., YI, H. K., HWANG, P. H., KIM, J. S., LEE, K. S. & LEE, Y. C. 2003. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest,* 111**,** 1083-92.

LALKO, J. F., KIMBER, I., DEARMAN, R. J., API, A. M. & GERBERICK, G. F. 2013. The selective peptide reactivity of chemical respiratory allergens under competitive and non-competitive conditions. *J Immunotoxicol,* 10**,** 292-301.

LALKO, J. F., KIMBER, I., DEARMAN, R. J., GERBERICK, G. F., SARLO, K. & API, A. M. 2011. Chemical reactivity measurements: potential for characterization of respiratory chemical allergens. *Toxicol In Vitro,* 25**,** 433-45.

LAUENSTEIN, L., SWITALLA, S., PRENZLER, F., SEEHASE, S., PFENNIG, O., FÖRSTER, C., FIEGUTH, H., BRAUN, A. & SEWALD, K. 2014. Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS). *Toxicol In Vitro,* 28**,** 588-99.

NATSCH, A., RYAN, C. A., FOERTSCH, L., EMTER, R., JAWORSKA, J., GERBERICK, F. & KERN, P. 2013. A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. *J Appl Toxicol,* 33**,** 1337-52.

REMY, S., VERSTRAELEN, S., VAN DEN HEUVEL, R., NELISSEN, I., LAMBRECHTS, N., HOOYBERGHS, J. & SCHOETERS, G. 2014. Gene expressions changes in bronchial epithelial cells: markers for respiratory sensitizers and exploration of the NRF2 pathway. *Toxicol In Vitro,* 28**,** 209-17.

SEED, M. & AGIUS, R. 2010. Further validation of computer-based prediction of chemical asthma hazard. *Occup Med (Lond),* 60**,** 115-20.

SEED, M. J. & AGIUS, R. M. 2017. Progress with Structure-Activity Relationship modelling of occupational chemical respiratory sensitizers. *Curr Opin Allergy Clin Immunol,* 17**,** 64-71.

TAYLOR, L. W., FRENCH, J. E., ROBBINS, Z. G., BOYER, J. C. & NYLANDER-FRENCH, L. A. 2020. Influence of Genetic Variance on Biomarker Levels After Occupational Exposure to 1,6-Hexamethylene Diisocyanate Monomer and 1,6-Hexamethylene Diisocyanate Isocyanurate. Front Genet, 11, 836.

VERSTRAELEN, S., NELISSEN, I., HOOYBERGHS, J., WITTERS, H., SCHOETERS, G., VAN CAUWENBERGE, P. & VAN DEN HEUVEL, R. 2009. Gene profiles of a human alveolar epithelial cell line after in vitro exposure to respiratory (non-)sensitizing chemicals: identification of discriminating genetic markers and pathway analysis. *Toxicol Lett,* 185**,** 16-22.

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.

References

1. ↑ 1.0 1.1 Ryan CA, Gerberick GF, Gildea LA, Hulette BC, Bettis CJ, Cumberbatch M, Dearman RJ and Kimber I. 2005. Interactions of contact allergens with dendritic cells: opportunities and challenges for the development of novel approaches to hazard assessment. Toxicol. Sci. 88: 4-11.
2. ↑ 2.0 2.1 Ryan CA, Kimber I, Basketter DA, Pallardy M, Gildea LA, Gerberick GF. 2007. Dendritic cells and skin sensitisation. Biological roles and uses in hazard identification. Toxicol. Appl. Pharmacol. 221: 384-394.
3. ↑ 3.0 3.1 Kimber I, Basketter DA, Gerberick GF, Ryan CA and Dearman RJ. 2011. Chemical allergy: Translating biology into hazard characterization. Toxicol. Sci. 120(S1): S238-S268.
4. ↑ Gerberick F, Aleksic M, Basketter D, Casati S, Karlberg AT, Kern P, Kimber I, Lepoittevin JP, Natsch A, Ovigne JM, Rovida C, Sakaguchi H and Schultz T. 2008. Chemical reactivity measurement and the predictive identification of skin sensitisers. Altern. Lab. Anim.36: 215-242.
5. ↑ Karlberg AT, Bergström MA, Börje A, Luthman, K, Nilsson JL. 2008. Allergic contact dermatitis- formation, structural requirements, and reactivity of skin sensitizers. Chem. Res. Toxicol. 21: 53-69.
6. ↑ Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF. 2009. Effector and regulatory mechanisms in allergic contact dermatitis. Allergy 64: 1699-1714.
7. ↑ Aeby P, Ashikaga T, Bessou-Touya S, Schapky A, Geberick F, Kern P, Marrec-Fairley M, Maxwell G, Ovigne J-M, Sakaguchi H, Reisinger K, Tailhardat M, Martinozzi-Teisser S, Winkler P. 2010. Identifying and characterizing chemical skin sensitizers without animal testing; Colipa's research and methods development program. Toxicol. In Vitro 24: 1465-1473.
8. ↑ Basketter DA and Kimber I. 2010. Contact hypersensitivity. In: McQueen, CA (ed) Comparative Toxicology Vol. 5, 2nd Ed. Elsevier, Kidlington, UK, pp. 397-411.

9. ↑ Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Ejner-Andersen K, Angers- Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S Coecke S Corvi R, Cronin M, Daston G, Dekant W, Felter S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Batista Leite S, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhler S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, Tahti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM. 2011. Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. Arch. Toxicol. 85: 367-485.
10. ↑ Lempertz U, Kühn U, Knop J and Becker D. 1996. An approach to predictive testing of contact sensitizers in vitro by monitoring their influence on endocytic mechanisms. Internat. Arch. Allergy Immunol. 111: 64-70.
11. ↑ Sakaguchi H, Ashikaga T, Miyazawa M, Kosaka N, Ito Y, Yoneyama K, Sono S, Itagaki H, Toyoda H, Suzuki H. 2009. The relationship between CD86/CD54 expression and THP-1 cell viability in an *in vitro* skin sensitisation test-human cell line activation test (h-CLAT). Cell Biol. Toxicol. 25: 109-126.
12. ↑ Ashikaga T, Sakaguchi H, Sono S, Kosaka N, Ishikawa M, Nukada Y, Miyazawa M, Ito Y, Nishiyama N, Itagaki H. 2010. 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. 38:275-84.

CUMBERBATCH, M., CLELLAND, K., DEARMAN, R. J. & KIMBER, I. 2005. Impact of cutaneous IL-10 on resident epidermal Langerhans' cells and the development of polarized immune responses. *J Immunol,* 175**,** 43-50.

HOLDEN, N. J., BEDFORD, P. A., MCCARTHY, N. E., MARKS, N. A., IND, P. W., JOWSEY, I. R., BASKETTER, D. A. & KNIGHT, S. C. 2008. Dendritic cells from control but not atopic donors respond to contact and respiratory sensitizer treatment in vitro with differential cytokine production and altered stimulatory capacity. *Clin Exp Allergy,* 38**,** 1148-59.

MIGDAL, C., BOTTON, J., EL ALI, Z., AZOURY, M. E., GULDEMANN, J., GIMÉNEZ-ARNAU, E., LEPOITTEVIN, J. P., KERDINE-RÖMER, S. & PALLARDY, M. 2013. 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,* 133**,** 259-74.

NAYAK, A. P., HETTICK, J. M., SIEGEL, P. D., ANDERSON, S. E., LONG, C. M., GREEN, B. J. & BEEZHOLD, D. H. 2014. Toluene diisocyanate (TDI) disposition and co-localization of immune cells in hair follicles. *Toxicol Sci,* 140**,** 327-37.

OUWEHAND, K., SANTEGOETS, S. J., BRUYNZEEL, D. P., SCHEPER, R. J., DE GRUIJL, T. D. & GIBBS, S. 2008. CXCL12 is essential for migration of activated Langerhans cells from epidermis to dermis. *Eur J Immunol,* 38**,** 3050-9.

OUWEHAND, K., SPIEKSTRA, S. W., WAAIJMAN, T., BREETVELD, M., SCHEPER, R. J., DE GRUIJL, T. D. & GIBBS, S. 2012. CCL5 and CCL20 mediate immigration of Langerhans cells into the epidermis of full thickness human skin equivalents. *Eur J Cell Biol,* 91**,** 765-73.

OUWEHAND, K., SPIEKSTRA, S. W., WAAIJMAN, T., SCHEPER, R. J., DE GRUIJL, T. D. & GIBBS, S. 2011. Technical advance: Langerhans cells derived from a human cell line in a full-thickness skin equivalent undergo allergen-induced maturation and migration. *J Leukoc Biol,* 90**,** 1027-33.

TOEBAK, M. J., MOED, H., VON BLOMBERG, M. B., BRUYNZEEL, D. P., GIBBS, S., SCHEPER, R. J. & RUSTEMEYER, T. 2006. Intrinsic characteristics of contact and respiratory allergens influence production of polarizing cytokines by dendritic cells. *Contact Dermatitis,* 55**,** 238-45.

Relationship: 2651: Increased proinflammatory mediators leads to Activation, Dendritic Cells

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	adjacent	Low	Low

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	High

Sex Applicability

Sex	Evidence
Unspecific	High

Dendritic cell activation is not limited to a specific sex or age.

Key Event Relationship Description

The presence of cellular danger signals at the local exposure site leads to the induction and amplification of immune responses in dendritic cells.

Evidence Supporting this KER

Biological Plausibility

DCs express receptors for, and respond to, constitutive and inflammatory chemokines and other chemoattractants, such as platelet-activating factor and formyl peptides. 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., 2011)

Empirical Evidence

(Silva et al., 2014) found that Hexamethylene diisocyanate increased ROS by inhibiting superoxide dismutase (SOD1) in THP-1 (human monocytic) cells. Increased ROS also led to extracellular signal-related kinase (ERK) signaling pathway phosphorylation and the transcription of cytoprotective and maturation pathways (HMOX1 and CD83). (Silva et al., 2014) also found that coincubation with the antioxidant *N*-acetyl cysteine and SOD decreased ERK phosphorylation in hexamethylene diisocyanate-treated THP-1 cells.

References

OUWEHAND K, SPIEKSTRA SW, WAAJIMAN T, SCHEPER RJ, DE GRUJIL TD, GIBBS S. 2011. Technical advance: Langerhans cells derived from a human cell line in a full-thickness skin equivalent undergo allergen-induced maturation and migration. J Leukoc Biol. 290(5):1027-33.

SILVA, A., NUNES, C., MARTINS, J., DINIS, T. C., LOPES, C., NEVES, B. & CRUZ, T. 2014. Respiratory sensitizer hexamethylene diisocyanate inhibits SOD 1 and induces ERK-dependent detoxifying and maturation pathways in dendritic-like cells. *Free Radic Biol Med,* 72**,** 238-46.

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.

References

1. ↑ 1.0 1.1 Antonopoulos C, Cumberbatch M, Mee JB, Dearman RJ, Wei XQ, Liew FY, Kimber I, Groves RW. 2008. IL-18 is a key proximal mediator of contact hypersensitivity and allergen induced Langerhans cell migration in murine epidermis. J. Leukoc. Biol. 83: 361-367.
2. ↑ 2.0 2.1 Ouwehand K, Santegoets SJAM, Bruynzeel DP, Scheper RJ, de Gruijl TD, Gibbs S. 2008. CXCL12 is essential for migration of activated Langerhans cells for epidermis to dermis. Eur. J. Immunol. 38: 3050-3059.
3. ↑ Martin SF, Esser PR, Schmucker S, Dietz L, Naisbitt DJ, Park BK, Vocanson M, Nicolas JF, Keller M, Pichler WJ, Peiser M, Luch A, Wanner R, Maggi E, Cavani A, Rustemeyer T, Richter A, Thierse HJ, Sallusto F. 2010. T-cell recognition of chemical, protein allergens and drugs; toward the development of *in vitro* assays. Cell. Mol. Life Sci. 67: 4171-4184.
4. ↑ 4.0 4.1 Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF 2009. Effector and regulatory mechanisms in allergic contact dermatitis. Allergy 64: 1699-1714.
5. ↑ Api AM, Basketter DA, Cadby PA, Cano MF, Ellis G, Gerberick GF, Griem P, McNamee PM, Ryan CA and Safford B. 2008. Dermal sensitisation quantitative risk assessment (QRA) for fragrance ingredients. Reg. Toxicol. Pharmcol. 52: 3-23.
6. ↑ Zou T, Caton AJ, Koretzky GA, Kambayashi T. 2010. Dendritic cells induce regulatory T cell proliferation through antigen-dependent and –independent interactions. J. Immunol. 185:2790-2799.

TAYLOR, L. W., FRENCH, J. E., ROBBINS, Z. G., BOYER, J. C. & NYLANDER-FRENCH, L. A. 2020. Influence of Genetic Variance on Biomarker Levels After Occupational Exposure to 1,6-Hexamethylene Diisocyanate Monomer and 1,6-Hexamethylene Diisocyanate Isocyanurate. Front Genet, 11, 836.

Relationship: 1701: Activation/Proliferation, T-cells leads to Increase, Allergic Respiratory Hypersensitivity Response

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

In brief, once antigen has been processed and presented by DCs and Th2 cells activated (KEs 398 and 272), the differentiation and clonal expansion of Th2 cells lead to production of Th2 cytokines that induce immunoglobulin (Ig) class switching to production of antigen-specific allergic antibody (IgE) by B cells and clonal expansion of naive and memory B cell populations. (Dearman et al., 2003) These antibodies are then found throughout the body, in circulation and/or bound to Fce receptors on cells such as mast cells and basophils in tissues, including the respiratory tract. On subsequent re-exposure, antigen can crosslink IgE bound to the surface of the aforementioned cells and induce degranulation, releasing various mediators that lead to the clinical symptoms of asthma and rhinitis.

Evidence Supporting this KER

Empirical Evidence

Type 2 innate lymphoid (GATA3+/CD3-) cells were found in bronchial biopsies of patients exhibiting TDI-induced asthma. (Blomme et al., 2020).

Antihapten antibodies have been found in mice treated epicutaneously with skin and respiratory sensitizers, although they produce qualitatively different immune responses, likely reflecting the different cytokine milieus (Th1 or Th2) produced by the activated T cells in each case. While IgG1 production occurred in response to both groups of chemicals, the skin sensitizers DNCB and oxazalone preferentially drove production of IgG2a, while the respiratory sensitizers TMA and PA preferentially drove production of IgG2b. In addition, only the respiratory sensitizers were associated with an increase in serum IgE. (Dearman and Kimber, 1992, Dearman and Kimber, 1991)

Uncertainties and Inconsistencies

There is still remaining uncertainty regarding the role of IgE in chemical respiratory allergy, because specific IgE has not been demonstrated in all subjects sensitized to chemicals. (Kimber et al., 2014b, Kimber et al., 2014a, Quirce, 2014)

IgE production can occur both in the germinal centers of lymph nodes and locally in the airway mucosa, with the latter reported to be linked to nasal polyps associated with chronic rhinosinusitis and in response to inhaled protein allergens. (Baba et al., 2014, Hoddeson et al., 2010) The extent of germinal center involvement or local IgE production in respiratory sensitizers is currently unknown.

While there is considerable evidence that DCs are likely the most efficient APC for stimulating naive T cells, there is evidence that IgE at the surface of allergen-specific IgE-positive B cells and other APCs, such as alveolar macrophages, may also facilitate antigen presentation. (Zhong et al., 1997) A role for airway and alveolar epithelial cells in antigen presentation and induction and maintenance of adaptive responses is also becoming increasingly recognized. (Hasenberg et al., 2013)

Recent characterizations of the role of IL-21 in mouse models of protein allergy show that IL-21 promotes IgG1 in B cells when IL-4 is in low supply. This was supported by the finding that human ex-vivo naïve B cells from tonsils increased IgG1 but not IgE, suggesting that IgG1 may be associated with skin sensitization. (Gong et al., 2019) IL-21 was found to suppress the IgE response through IL-21R - STAT3 signaling in murine B cells. (Yang et al., 2020) However, these studies focused on murine models of allergy including dust mite and peanut, as well as a 4-hydroxy-3-nigrophenylacetyl-modified globulin, so the relevance of these mechanisms to low molecular weight chemical allergy is not clear.

Quantitative Understanding of the Linkage

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
miR-155		Type 2 innate lymphoid cells have been shown to be dependent on miR-155 to induce airway hyperresponse and IgE elevation in mice. Using a murine miR-155 KO model, mice who did not express miR-155 did not display these effects following dermal senstization to toluene diisocyanate, in contrast to control mice.	Blomme et al., 2020)

References

BABA, S., KONDO, K., TOMA-HIRANO, M., KANAYA, K., SUZUKAWA, K., USHIO, M., SUZUKAWA, M., OHTA, K. & YAMASOBA, T. 2014. Local increase in IgE and class switch recombination to IgE in nasal polyps in chronic rhinosinusitis. *Clin Exp Allergy,* 44**,** 701-12.

BLOMME, E. E., PROVOOST, S., BAZZAN, E., VAN EECKHOUTTE, H. P., ROFFEL, M. P., POLLARIS, L., BONTINCK, A., BONATO, M., VANDENBROUCKE, L., VERHAMME, F., JOOS, G. F., COSIO, M. G., VANOIRBEEK, J. A. J., BRUSSELLE, G. G., SAETTA, M. & MAES, T. 2020. Innate lymphoid cells in isocyanate-induced asthma: role of microRNA-155. Eur Respir J, 56.

COLLINS, J. J., ANTEAU, S., CONNER, P. R., CASSIDY, L. D., DONEY, B., WANG, M. L., KURTH, L., CARSON, M., MOLENAAR, D., REDLICH, C. A. & STOREY, E. 2017. Incidence of Occupational Asthma and Exposure to Toluene Diisocyanate in the United States Toluene Diisocyanate Production Industry. Journal of occupational and environmental medicine, 59 Suppl 12, S22-S27.

DEARMAN, R. J. & KIMBER, I. 1991. Differential stimulation of immune function by respiratory and contact chemical allergens. *Immunology,* 72**,** 563-70.

DEARMAN, R. J. & KIMBER, I. 1992. Divergent immune responses to respiratory and contact chemical allergens: antibody elicited by phthalic anhydride and oxazolone. *Clin Exp Allergy,* 22**,** 241-50.

DEARMAN, R. J., STONE, S., CADDICK, H. T., BASKETTER, D. A. & KIMBER, I. 2003. Evaluation of protein allergenic potential in mice: dose-response analyses. *Clin Exp Allergy,* 33**,** 1586-94.

GONG, F., ZHENG, T. & ZHOU, P. 2019. T Follicular Helper Cell Subsets and the Associated Cytokine IL-21 in the Pathogenesis and Therapy of Asthma. Front Immunol, 10, 2918.

HASENBERG, M., STEGEMANN-KONISZEWSKI, S. & GUNZER, M. 2013. Cellular immune reactions in the lung. *Immunol Rev,* 251**,** 189-214.

HODDESON, E. K., PRATT, E., HARVEY, R. J. & WISE, S. K. 2010. Local and systemic IgE in the evaluation and treatment of allergy. *Otolaryngol Clin North Am,* 43**,** 503-20, viii.

KIMBER, I., DEARMAN, R. J. & BASKETTER, D. A. 2014a. Diisocyanates, occupational asthma and IgE antibody: implications for hazard characterization. *J Appl Toxicol,* 34**,** 1073-7.

KIMBER, I., DEARMAN, R. J., BASKETTER, D. A. & BOVERHOF, D. R. 2014b. Chemical respiratory allergy: reverse engineering an adverse outcome pathway. *Toxicology,* 318**,** 32-9.

QUIRCE, S. 2014. IgE antibodies in occupational asthma: are they causative or an associated phenomenon? *Curr Opin Allergy Clin Immunol,* 14**,** 100-5.

YANG, Z., WU, C. M., TARG, S. & ALLEN, C. D. C. 2020. IL-21 is a broad negative regulator of IgE class switch recombination in mouse and human B cells. J Exp Med, 217.

ZHONG, G., REIS E SOUSA, C. & GERMAIN, R. N. 1997. Antigen-unspecific B cells and lymphoid dendritic cells both show extensive surface expression of processed antigen-major histocompatibility complex class II complexes after soluble protein exposure in vivo or in vitro. *J Exp Med,* 186**,** 673-82.

List of Non Adjacent Key Event Relationships

Relationship: 2650: Covalent Binding, Protein leads to Increase, Allergic Respiratory Hypersensitivity Response

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	non-adjacent	High	Low

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	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Consistent epidemiologic evidence shows that allergic respiratory hypersensitivity is caused by exposure to electophilic low molecular weight chemicals, which are too small to activate the immune system without first generating hapten-protein conjugates.

Quantitative Understanding of the Linkage

Frequency of exposures to toluene diisocyanate exceeding 3 ppb in the time-weighted average (8 hrs, TWA-8) without respiratory protection were found to be associated with incidence. In this study, TWA-8 values above 3 ppb were indicative of peak exposure events, i.e. spills. (Plehiers et al., 2020a and 2020b) This is consistent with a prior report by (Collins et al., 2017) which found a significant link between peak exposure and asthma incidence.

Response-response relationship

Mounting evidence supports a threshhold relationship between hapten exposure and airway hypersensitivity. While average exposures to toluene diisocyanate have decreased significantly from the 1970s and 1980s, asthma incidence rates have stayed the same. A pair of studies (Plehiers et al., 2020a and 2020b) found that gross cumulative exposure does not correlate with asthma incidence. Instead, frequency of unprotected exposurse over a certain threshold was positively associated with incidence. A recent review highlighted the evidence that sensitization is threshold-based, but noted practical difficulties in defininig accurate numerical threshold exposure values (Cochrane, SA et al., 2015).

Time-scale

The rapid onset of symptoms (within 1 hour and often within minutes of chemical exposure) of respiratory allergic symptoms in sensitized individuals is indicative of an antibody-mediated (type I hypersensitivity) mechanism.

References

Cochrane SA, Arts JHE, Ehnes C, et al. 2015. Thresholds in chemical respiratory sensitisation. *Toxicology*. 333:179-194.

Kimber I, Basketter DA, Gerberick GF, Ryan CA, Dearman RJ. 2011. Chemical allergy: translating biology into hazard characterization. *Toxicol Sci*. 120 Suppl 1:S238-S268.

Kimber I, Poole A, Basketter DA. 2018. Skin and respiratory chemical allergy: confluence and divergence in a hybrid adverse outcome pathway. *Toxicol Res (Camb)*. 2018;7(4):586-605.

PLEHIERS, P. M., CHAPPELLE, A. H. & SPENCE, M. W. 2020a. Practical learnings from an epidemiology study on TDI-related occupational asthma: Part I-Cumulative exposure is not a good indicator of risk. Toxicol Ind Health, 36, 876-884.
PLEHIERS, P. M., CHAPPELLE, A. H. & SPENCE, M. W. 2020b. Practical learnings from an epidemiology study on TDI-related occupational asthma: Part II-Exposure without respiratory protection to TWA-8 values indicative of peak events is a good indicator of risk. Toxicol Ind Health, 36, 885-891.