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ORIGINAL ARTICLE

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

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Abstract

Sensitization of the respiratory tract is an important occupational health challenge, and understanding the mechanistic basis of this effect is necessary to support the development of toxicological tools to detect chemicals that may cause it. Here we use the adverse outcome pathway (AOP) framework to organize information that may better inform our understanding of sensitization of the respiratory tract, building on a previously published skin sensitization AOP, 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. These events mirror the skin sensitization AOP but with specific differences. For example, there is some evidence that respiratory sensitizers bind preferentially to lysine moieties, whereas skin sensitizers bind to both cysteine and lysine. 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. Knowledge gaps include the lack of understanding of which KE(s) drive the Th2 polarization. The construction of this AOP may provide insight into predictive tests that would in combination support the discrimination of respiratory-sensitizing from non- and skin-sensitizing chemicals, a clear regulatory need.

Keywords: AOP, *in silico*, *in vitro*, respiratory sensitization

Introduction

The adverse outcome pathway (AOP) has gained popularity as a framework for structuring information at different levels of biological complexity relevant to a particular adverse outcome (AO).[1] It is thought that constructing AOPs using available information on chemical response and response–response relationships along known pathways will allow the contextualization of results of predictive test methods across a diverse range of biological mechanisms.

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

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asthma and rhinitis on repeated exposure and is an important occupational health problem.[2] Due to the severity and irreversibility of the adverse effect, identifying chemical respiratory allergens is of considerable regulatory, industrial, and socioeconomic importance.[3] Efforts to outline a framework for assessment of potential respiratory-sensitizing chemicals are underway.[4] 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.[5]

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.[6–9] 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 article will consider information from models using skin and lung exposure (*in vivo*) and with dermal and respiratory cells and tissues (*in vitro/ex vivo*). However, since the AOP framework excludes chemical-specific events upstream of the molecular initiating event (MIE),[10] a detailed discussion of exposure or absorption/distribution/metabolism/excretion will not be undertaken.

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.

Here we propose an AOP for sensitization of the respiratory tract by covalent binding of low-molecular-weight organic chemicals to proteins, following the guidance provided by the Organization for Economic Cooperation and Development (OECD) in its AOP Wiki Handbook.[11] In doing so we outline, based on the available literature, the likely key events (KEs) and key event relationships (KERs) relevant to the eventual AO. The outlines of this pathway follow the already-published skin sensitization AOP.[12] 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[13]; 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. While not relied on to build the AOP, information from skin sensitization and protein respiratory allergy may be used when relevance can be established.

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.[14] A diagram of the AOP is illustrated in Figure 1, and a cartoon is provided in Figure 2. Details are described in this article and will be entered in the OECD AOP Wiki.

The Key Events

Molecular initiating event: covalent protein binding

The initial KE, or MIE, for induction of the pathway leading to sensitization of the respiratory tract is, as for skin sensitization, the formation of a covalent bond between a protein and a low-molecular-weight organic chemical (defined as chemicals consisting of only carbon, nitrogen, oxygen, sulfur, fluorine, chlorine, bromine, iodine, or hydrogen with a molecular mass less than 1000 g/mol). Classic chemical initiators of this pathway include some acrylates, diisocyanates,

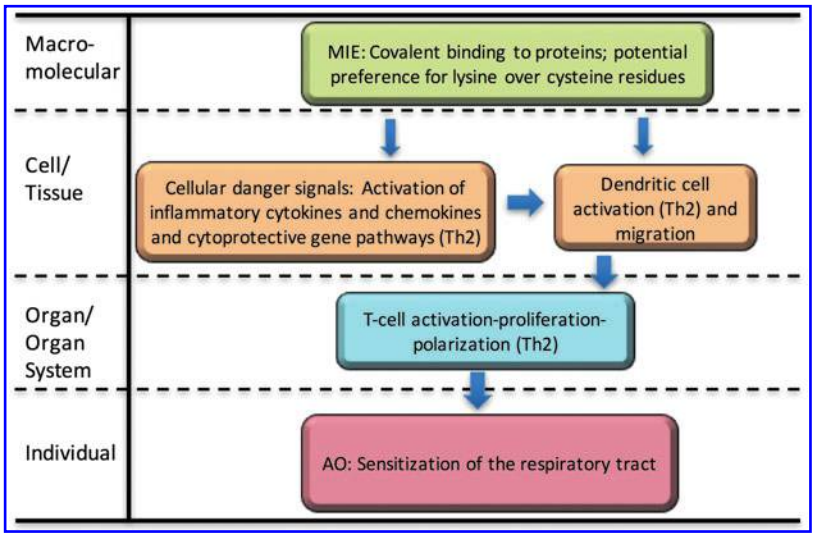

FIG. 1. A diagram of the proposed AOP. AOP, adverse outcome pathway.

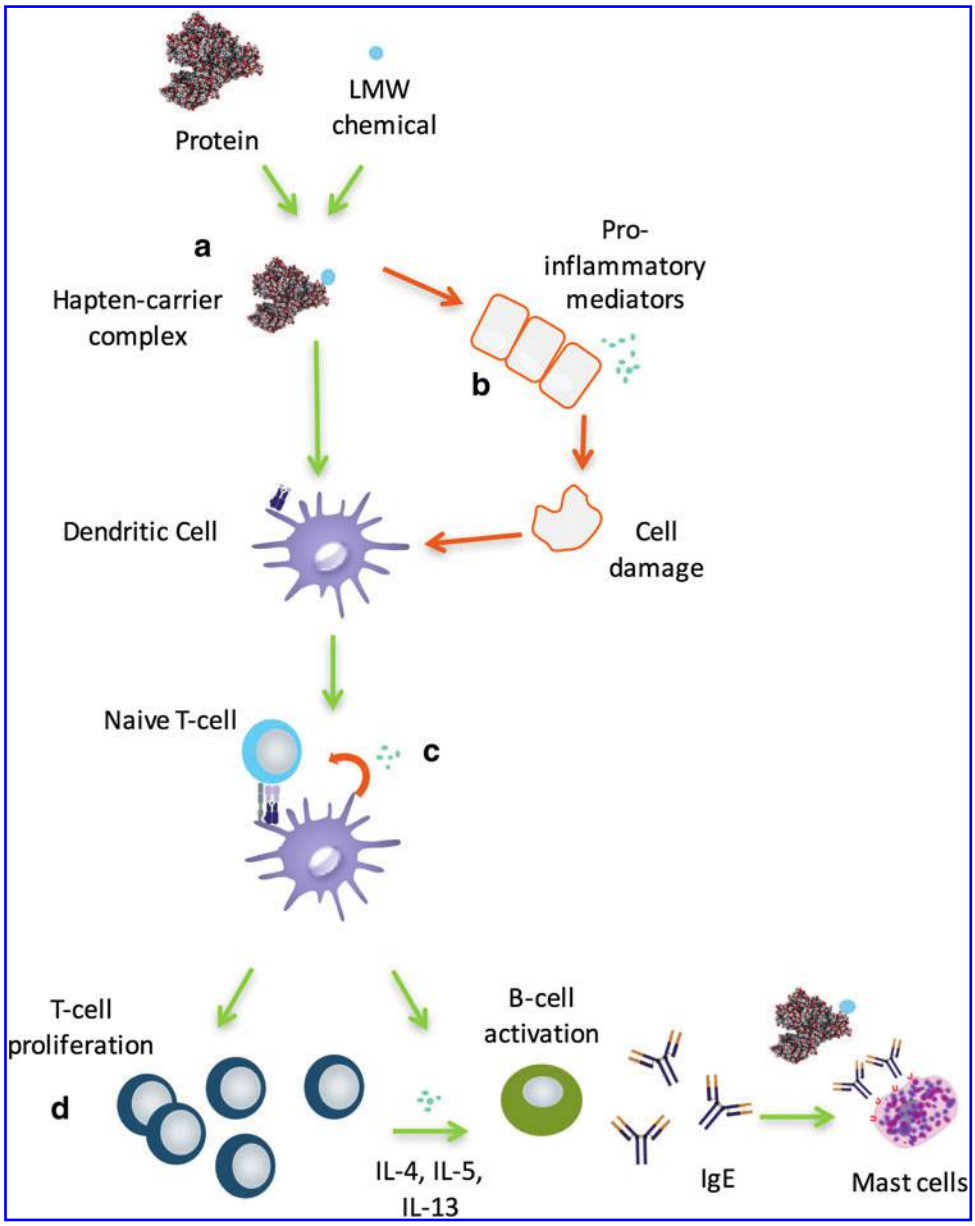

FIG. 2. The proposed AOP for respiratory sensitization includes covalent binding of low-molecular-weight chemicals to lung or skin proteins **(a)**, the activation of inflammatory cytokines and chemokines and cytoprotective gene pathways typical of a Th2 immune response **(b)**, activation and migration of Th2-skewed dendritic cells to lymph nodes **(c)**, T cell activation, proliferation, and polarization **(d)** leading to sensitization of the respiratory tract. Th2, T helper 2.

and acid anhydrides. Clinical observation and experimental *in vivo* studies lead to a somewhat unexpected conclusion that protein binding—or haptenation—leading to respiratory sensitization can occur via chemical exposure to either the skin or the lung.[15–17]

There is extensive evidence in the literature for haptenation being the MIE for respiratory sensitization.[18–23] In general, haptenation can be divided into five types of chemistry, so-called mechanistic domains. These being acylation, aliphatic nucleophilic substitution (S_N1/S_N2), aromatic nucleophilic substitution (S_NAr), Michael addition, and Schiff base formation.[20] There has been much research showing that grouping chemicals into one of these mechanistic domains (which are based around the chemistry of the MIE) is the key first step in the nonanimal prediction of toxicity for both skin and respiratory sensitization.[24–28] These studies add further weight to the importance of the haptenation event for the nonanimal prediction of respiratory sensitization.

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[31–34]; 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.[35]

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, for example, trimellitic anhydride (TMA) and fluorescein isothiocyanate (FITC).[34]

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).[25] 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.[25,36–40] A detailed mechanistic chemistry analysis suggested that a reactivity threshold existed that could be passed either by a chemical's intrinsic reactivity alone or by a combination of reactivity and the ability to form multiple covalent bonds with proteins leading to crosslinking.[25] This existence of such a threshold is in keeping with skin sensitization studies that have consistently demonstrated that the rate of covalent bond formation plays a key role in determining potency in the local lymph node assay (LLNA).[26,41,42] The mechanistic rationale for this observation can be explained in terms of the balance between the rate a chemical is cleared from a biological system versus the rate it reacts with a protein. The faster the rate of covalent bond formation, the faster the haptenation occurs and the greater the degree of sensitization (either skin or respiratory). This importance of the rate of chemical reactivity can also be considered as modeling the effective dose that an individual receives of a given chemical; the faster the rate, the higher the dose, the greater the degree of sensitization.

KE2: activation of cellular danger signals (inflammatory cytokines and chemokines and cytoprotective gene pathways)

The innate immune system plays a crucial role in the initiation of adaptive immune responses.[43,44] It is a first-line of defense against invading microbial pathogens and is activated via a range of pattern recognition receptors (PRRs) that recognize conserved patterns present on pathogens, that is, the toll-like receptors (TLRs) and the nucleotide-binding domain leucine-rich repeat containing receptor (NLR) family. These PRRs can be activated by endogenous danger-associated molecular patterns (DAMPs), released under oxidative stress and cell damage and include components of the extracellular matrix generated after tissue injury, for example, hyaluronic acid fragments, intracellular proteins such as heat shock proteins and nonprotein DAMPs such as uric acid crystals.[45–47] NLR protein-3 (NLRP3) is a PRR that belongs to the NLR family, a group of intracellular receptors activated by mitochondrial oxidative stress, for example, by adenosine triphosphate and uric acid.[48] On activation, TLR and NLRP3 activate innate immunity signaling pathways leading to the release of proinflammatory cytokines and chemokines. In recent years, increasing attention has been paid to the role of the innate immune system in asthma. The sentinel role of the innate immune systems includes the activation of pathways by pathogen-associated molecular patterns and DAMPs. By this, KEs during sensitization such as activation and migration of DCs are set into motion.[49] Proinflammatory molecules are also known to induce the expression of surface molecules on immune cells such as antigen-presenting cells (APCs), which are greatly involved in the induction of adaptive immune responses. Thus, whether an immune response or tolerance response is induced in APCs depends not only on the presence of antigenic properties of a substance but also on danger signals.

KER: protein binding (MIE) leads to activation of cellular danger signals (KE2)

Since different cells express PRRs, including epithelial cells, endothelial cells, macrophages, fibroblasts, and DCs, it is likely that different cell types are involved. Since skin exposure can result in respiratory sensitization, both tissue types should be considered as cellular sources.

Hur et al.[50] found that toluene diisocyanate (TDI)-human serum albumin (HSA) conjugates increased reactive oxygen species (ROS) production in A549 cells. Exposure of reconstituted three-dimensional (3D) human airway epithelia (MulcilAir™) to respiratory sensitizers TMA and 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-α (GRO-α)/C-X-C motif (CX)CL1, and regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5.[51] Similarly, typical respiratory sensitizers caused an elevation of proinflammatory cytokines IL-1α (TMA) and tumor necrosis factor (TNF)-α (glutaraldehyde) in precision-cut lung slices.[52]

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-α, IL-1 receptor, and cytokine signaling pathways were upregulated by HDI and TMA.[53] NLRP3 has been demonstrated to be important in respiratory sensitization by proteins,[54] 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.[55] Conflicting reports as to whether IL-18 is associated with a Th1 or Th2 immune response hamper interpretation of this result.

Finally, peripheral blood mononuclear cells (PBMCs) *in vitro* take up HDI-HSA conjugates resulting in morphologic changes, increased expression of genes associated with antigen processing, and increased expression of proinflammatory chemokine macrophage migration inhibitory factor (MIF) and MCP-1 and PRRs that bind chitin, known to be associated with asthma in humans. Similar morphological changes were found after *in vivo* human exposure to controlled amounts of HDI vapour.[56] Curiously, TMA-HSA conjugates did not induce MIF, MCP-1, or chitinase-1 production in PBMCs.

Besides activation of PRRs, there are other ways to activate signaling pathways involved in innate immune responses. Some of these pathways, including the nuclear factor erythroid-2 (Nrf2)-Kelch-like ECH-associated protein-1 (Keap1)-antioxidant response element (Nrf2-Keap1) and NF-κB, are sensitive to the redox balance.[57,58] Respiratory sensitizers, being protein reactive, are known to have an impact on thiol-redox homeostasis and GSH metabolism. HDI, for example, upregulates genes related to thiol-redox homeostasis and cytoprotective pathways in human airway epithelial cells[59] and macrophages.[60] Conjugation of respiratory sensitizers to GSH leads to depletion of GSH[61,62] and to a diminished antioxidant defence, making the cells more vulnerable to toxicity induced by respiratory sensitizers,[58,63] resulting in activation of cytoprotective pathways, such as the Nrf2-Keap1 pathway.

Under physiological conditions, the transcription factor (Nrf2) is bound to the sensory protein Keap1. In response to either oxidative stress[64] or if there is a covalent interaction between the cysteine residues on Keap1 and electrophiles, Keap1 dissociates from Nrf2. Subsequently, Nrf2 accumulates in the nucleus and triggers transcriptional activation of cytoprotective genes.[65] 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.[66–68] 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,[25] *in chemico* studies show that cysteine binding occurs as well.[21,69] 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[70]); however, actual Nrf2 and heme oxygenase-1 proteins accumulated only minimally in the cells.

Finally, the canonical phosphatase and tensin homolog (PTEN)-signaling pathway might be relevant for respiratory sensitization.[71] This pathway regulates cell survival signaling pathways and plays a protective role in the pathogenesis of asthma.[72] 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-κB activation.[73] 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[68] but another diisocyanate, HDI, did.

In conclusion, evidence mostly from *in vitro* studies show that respiratory sensitizers are able, via protein-chemical conjugates, to generate cellular danger signals, including induction of oxidative stress and proinflammatory cytokines and chemokines. A link between oxidative stress and the initiation of signal transduction pathways involved in inflammation and allergy has been shown.[74] In the skin, for example, oxidative stress may lead to activation of signal transduction pathways such as NF-κB and p38 MAPK, which leads to the release of cytokines and chemokines. These inflammatory signals drive the maturation and activation of DCs and promote the adaptive immune response.[75] Some evidence for this process by respiratory sensitizers is discussed in the section on KE3: DC activation and migration.

KE3: DC activation and migration

DCs are referred to as a bridge between innate and adaptive immunity, and their maturation is an essential event in the sensitization of an organism.[76] DC activation results in mature cells with a changed phenotype and function.[77] The most prominent changes include antigen-presenting capacity, enhanced levels of major histocompatibility complex (MHC) (Class II) and costimulatory molecules such as cluster of differentiation (CD)54, CD80, and CD86, and receptors that are essential for migration. These modified DCs carry the hapten ''message'' to the lymph where they are presented to T helper cells for further action.

As with skin sensitization, the available *in vitro* and *in vivo* evidence indicates that chemical-hapten conjugates cause cellular ''danger signals,'' and also act directly on naive DCs to trigger subsequent sensitization. The relationships among KEs 1, 2, and 3 may provide insight into the mechanistic events underlying immune response skewing toward a Th2, and therefore respiratory sensitization, phenotype.

KER: protein binding (MIE) leads to DC activation and migration (KE3)

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.[70] 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.[78] 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-α 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.[79] Finally, TMA induced increased production of IL-10 when incubated with precision cut lung slices (PCLS) for 24 hours.[52]

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.[80] 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. Also, 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.[81]

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. 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.[82–84] 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.[79,81]

KER: activation of cellular danger signals (KE2) leads to DC activation and migration (KE3)

The presence of cellular danger signals at the local exposure site plays a crucial role in the induction and amplification of lung immune responses associated with respiratory sensitization. Silva et al.[85] 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.

KE4: activation/proliferation/polarization of T cells

In both respiratory and dermal sensitization, activated T cells proliferate in the DLN, resulting in a primed population of lymphocytes. Both respiratory and skin sensitizers lead to T cell proliferation *in vivo*.[86] However, as mentioned, there is likely a difference in skewing of T cell proliferation, cytokine release, and subsequent response[3,87] between skin and respiratory sensitizers. Data on such cytokine profiles have mainly been derived from animals exposed via the skin or respiratory tract to well-characterized respiratory sensitizers, including TMA,[88] FITC, PA, and TDI.[89,90] Recent human data have emerged that concurs with this pattern of response. For example, Newell et al.[91] have reported that weaker skin sensitization occurs in individuals with atopic dermatitis, who already have a systemic Th2 bias. In addition the cells studied have been either peripheral blood or tonsil derived, and therefore, it is currently unknown if results would be different for cells derived from the skin or respiratory tract. Ouyang et al.[92] found increased methylation of the IFNg gene promoter in workers who had developed asthma to diisocyanates, suggesting downregulation of this Th1 cell marker. In addition it has been reported that atopic status can affect the stimulatory capacity and production of cytokines by DC, which in turn affect T cell stimulation and subsequent polarization of the immune response.[79]

KER: DC activation and migration (KE3) lead to activation/proliferation/polarization of T cells (KE4)

Once activated DCs have migrated to DLN, they interact with T cells, presenting MHC-associated immunogen for recognition. It is known that the efficiency with which, and pathway by which, an antigen is processed and presented by a DC can influence the subsequent immune response raised against it. 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.[34] found increased expression of type 2 cytokines in mouse lymph node cells after topical exposure to TMA and FITC. T cell activation, proliferation, and polarization not only occur in response to antigen recognition but also additional cytokine signals from the DC and other cells such as the airway epithelia.[51]

AO: sensitization of the respiratory tract

The development of chemical respiratory allergy, defined here as *an immune-mediated hypersensitivity reaction to an exogenous low-molecular-weight chemical resulting in symptoms such as asthma and rhinitis,* is a two-step process. The first step (induction or sensitization) is when exposure (which may be via various routes) leads to immunological priming and sensitization of the respiratory tract. The second step occurs when a sensitized person has subsequent exposures of the respiratory tract to the same substance, resulting in elicitation of clinical symptoms such as rhinitis and asthma, with the latter characterized by breathlessness and wheezing, airflow obstruction, bronchoconstriction, and tightness of the chest.[52] Reactions can be acutely life threatening or lead to chronic occupational asthma.[3] There is also often a long lag phase (in some cases many months) between the two steps described.

KER: activation/proliferation/polarization of T cells (KE4) lead to sensitization of the respiratory tract (AO)

In brief, once antigen has been processed and presented by DCs and Th2 cells activated (KEs 3 and 4), 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.[93] These antibodies are then found throughout the body, in circulation and/or bound to Fcε 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.

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

authors have published recently on this issue and therefore it will be not be discussed in detail here.[13,94,95]

Class switching to IgE is most commonly triggered when Th2 or T follicular helper cells provide costimulatory signals to antigen-stimulated B cells via CD40L-CD40 binding and secreted cytokines (mainly IL-4 and IL-13), which turn on the epsilon germ line transcript.[96] The immediate precursor of an IgE-switched B cell can be either a ''naive'' non-switched B cell or mature IgG switched B cell, with the routes to IgE described as direct (IgM to IgE) and indirect (IgM via IgG to IgE) pathways, respectively. Davies et al.[97] reviewed the available data on this phenomenon and argued that, in contrast to the conclusion of Xiong et al.[96] based on mouse data, it was likely that atopic IgE responses are characterized by a low IgG/IgE ratio, low B cell memory, and modest affinity maturation, which fits more with a direct pathway. Most recently, however, work published by Looney et al.[98] and Ramadani et al.[99] indicates that the indirect pathway is the predominant mechanism of IgE production in humans. It is noted, however, that this phenomenon has not been investigated specifically with respect to chemical respiratory allergy and the data available are based primarily on responses to protein allergens and parasite antigens. In addition, the cells studied have been either peripheral blood or tonsil derived, and therefore, it is currently unknown if results would be different for cells derived from the skin or respiratory tract.

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.[87,100] To date, analysis of hapten-specific antibody subclasses in humans sensitized to skin and respiratory sensitizers does not appear to have been undertaken.

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.[101,102] 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.[103] A role for airway and alveolar epithelial cells in antigen presentation and induction and maintenance of adaptive responses is also becoming increasingly recognized.[104]

Most recently, data have been published on the cross talk between DCs and different immune cells, including B cells, showing that activated human B cells can regulate DC maturation and function. Maddur et al.[105] report that DCs receiving signals from BCR-activated B cells selectively induce Th2 polarization via the OX-40 ligand and that the Th2 cells induced via this phenomenon are capable of stimulating B cells to produce IgE, although the levels were low and variable and further work with more donors is required. Again the role of this phenomenon specifically related to respiratory sensitizers is currently unexplored.

Measuring KEs

Available methods to measure the KEs outlined in this article are discussed here. Currently, no validated test methods are available for discriminating respiratory and skin sensitizers, although some work with very small data sets has been done to develop both *in vitro* and *in vivo* models. North et al.[4] outline advantages and disadvantages of a broader range of potential test methods, which may contribute to a weight-of-evidence (WoE) assessment of respiratory sensitization.

MIE: protein binding

Covalent binding can be detected using *in chemico* depletion assays[32,69,106,107] and is an OECD test guideline[108]; both respiratory and skin sensitizers are detected.[109] The rate of covalent binding can also be measured.[41] 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.[35]

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.[29–31,34,110]

In silico structural alerts can also be used to identify respiratory sensitizers[24,111] and build computational profilers.[112]

KE2: cellular danger signals (activation of inflammatory cytokines and chemokines and cytoprotective gene pathways)

It is not fully understood which cell types are the most important sources for the endogenous danger signals involved in sensitization of the respiratory tract. Relevant cell types representing cellular sources for danger signals are probably alveolar and bronchial epithelial cells, keratinocytes, macrophages, DCs, natural killer cells, endothelial cells, and nerve fiber endings.[113] In particular, macrophages are able to respond with high levels of, for example, cytokines and ROS after stimulation of PRRs. Human cell lines representative of the cells mentioned above might be used for the measurements of danger signal induction. A limitation of the use of submerged cell lines is that certain respiratory sensitizers hydrolyze in an aqueous environment, which may lead to negative results.[114] Air/liquid exposure in 3D skin or airway models might provide a more robust model although this has not been explored in great detail.

There are no predictive markers for cellular danger or proinflammatory responses described for respiratory sensitizers yet. The studies performed up until now did not result in any proteins, genes, or molecular pathways that are consistently regulated by a broad range of respiratory sensitizers or genes[51,52,68]; however, only a few chemicals have been tested. Cytokine production can be measured by ELISA or Bio-Plex systems either in the supernatants or intracellular matrix. Cell systems that can be used include also complex models such as the 3D epithelial cell models, that is, MucilAir™[51] and PCLS.[52]

Activation of innate immune response can also be assessed using commercial immunoassays for signal transduction

pathways, that is, p38 MAPK, JNK 1/2, and ERK 1/2. Other possible detection methods, focusing on ROS production or the induction of cytoprotective pathways, might be used as well to assess the ability of chemicals to generate endogenous danger signals (DAMPs). For ROS production, commercial assays are available that can be applied. The induction of Nrf2-KEAP1 can be assessed using the Keratinosens®[66,67] or LuSens[115] assays[116] and by measuring gene expression of Nrf2-dependent genes by quantitative polymerase chain reaction (qPCR), that is, HMOX,[70] although the utility of this pathway for respiratory sensitizers is unclear. The BEAS-2B cell line, coupled with microarray analysis, reveals the PTEN pathway as potentially useful.[71] The predictivity of these assays has not been studied with a large number of respiratory sensitizers.

KE3: DC activation and migration

Studying the molecular mechanisms behind DC activation and maturation is impeded by the fact that primary DCs constitute a heterogeneous and minute population of cells among many functionally specialized DC subpopulations. To circumvent this issue, various human myeloid cell lines (e.g., THP-1, U937, KG-1, and MUTZ-3) have been used both for acquiring mechanistic understanding and for development of predictive tests; this work is more advanced for skin sensitizer assessment.[70,117–119]

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.[120] Preliminary data on the performance of the GARD for assessing chemical respiratory sensitizers using transcriptional readouts of a genomic biomarker signature indicated 80% accuracy.[121]

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.[122,123] However, only limited data are available substantiating its performance on chemical respiratory sensitizers.[109] 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.[124] While some respiratory sensitizers have been assessed, it is unclear whether this event will provide discrimination between skin and respiratory sensitizers.[117]

KE4: activation/proliferation/polarization of T cells

In mice, induction of respiratory immune response, measured by lymphocyte maturation and proliferation in local lymph nodes, can be detected using an LLNA protocol[125] with subsequent cytokine fingerprinting or IgE testing[126] although inconsistencies prevent wider adoption.[3,127]

In humans, T cell proliferation and DC and T cell cytokine profiles produced in response to chemical respiratory stimuli have been measured *in vitro*.[79,128]

AO: sensitization of the respiratory tract

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.[129] 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. 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.[94,95] Immune assays such as ELISA or ImmunoCAP are also used to investigate allergen-specific antibody isotype profiles.[130]

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.[97]

Discussion

Evaluation of the AOP

We have outlined the available evidence supporting an AOP for sensitization of the respiratory tract. This AOP follows the one already outlined for skin sensitization,[12] with the aim of highlighting evidence from known respiratory sensitizers and opportunities for discrimination between skin and respiratory sensitizers. According to the available evidence, the domain of applicability of the AOP includes mammals of either sex; ultimately, a regulatory interest in protecting workers and consumers requires an emphasis on confirmatory evidence and experimental models reflecting the human situation.

Efforts to discern the mechanisms involved in respiratory sensitization, including this one, are motivated by the need for test methods and strategies to identify respiratory sensitizers. While some do exist, none has reached the level of regulatory acceptance necessary to be implemented globally or regularly relied on. The utility of an AOP approach is that all available and relevant information can be gathered and transparently assessed according to recognized criteria. This assessment subsequently informs potential regulatory applications, which may include support for grouping and read across of chemicals, identification of relevant and biologically plausible test methods, support for the development of Integrated Approaches to Testing and Assessment (IATA), identification or characterization of hazard, or quantitative risk assessment.[131]

The potential regulatory applicability of any particular AOP is informed by the degree of confidence in the biological plausibility of each of the KERs, the degree of confidence in the essentiality of the KEs, and the empirical support for each of the KERs and the overall AOP.[11]

Biological plausibility of the KERs. Each of the hypothesized KERs is supported by evidence from studies with at least one, and sometimes a few, known respiratory sensitizer. 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.''

Support for essentiality of each KE. Strong evidence exists for the essential nature of protein binding,[18–23] cellular danger signals,[85] DC activation,[79] and T cell activation in the continuation of the subsequent KE, as shown by experiments that block important features of each KE. 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.[79,91,92]

Empirical support for KERs and overall AOP. The literature data available for this AOP specifically with respiratory sensitizers are comparatively low; most studies use one or a few classical chemicals. There are also remaining uncertainties related to how respiratory sensitizers direct the immune response toward a Th2 phenotype, how APCs present haptens to effector T cells, and why responses home to the respiratory tract even with dermal exposure. However, when taken in the context of what is known about sensitization in general and the consistency of the available evidence, the WoE assessment for the KEs and the AOP overall is judged to be ''moderate.''

Quantitative understanding of each KER. Quantitative information on the relationships between KEs is low. Nayak et al.[80] provides a detailed temporal and dose–response analysis of TDI-induced protein binding, colocalization of immune messenger cells, and migration to DLNs. However, the quantitative relationship between one KE and another is not known. For example, it is not known the level at which intercellular ''danger signals'' (KE2) approach a threshold beyond which DC activation (KE3) is certain.

Other pathways/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,[132] but it does not cause a Th2 cytokine response in mice.[133] 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.[134] and Wisnewski et al.,[56] 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.[135] 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).[136] 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.

Future research needs

A common nonregulatory use for an AOP is to focus future research by highlighting areas where lack of understanding is impeding progress. That is, an AOP identifies gaps in knowledge that should be filled to increase the regulatory utility of that pathway and make advances in predicting the AO, with the recognition that not all gaps in knowledge can or must be filled.

From the evidence presented here, it is clear that our understanding of the development of respiratory sensitization is growing. However, 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,[25,137,138] 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.

Regulatory applicability

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.

Conclusion

The AOP provides a starting point for the development of methods and strategies that will fit into an IATA for the assessment of potential respiratory sensitizers. The available evidence highlights events that may be targeted in the development of discriminatory test methods; likely candidates are selective protein binding assays and DC-based Th2-related assays (e.g., cytokine release).[13]

As with other AOPs, identification of the endpoints for some of the proposed key steps has relied on event-based cell and tissue culture models. The predictive validity of these disease models for the human outcome is unclear and needs to be confirmed by an assessment of their relevance, reproducibility, and reliability.

Currently, MIE-based computational profilers have been developed. Given the strong link between the MIE and the rest of the AOP, these tools provide evidence as to the potential for a low-molecular-weight organic chemical to cause respiratory sensitization. However, although protein binding is necessary, it is not sufficient to the continuation of the pathway. The assessment of additional respiratory sensitizers in emerging test methods linked to the other KEs in this AOP is urgently needed.

Author Disclosure Statement

No competing financial interests exist.

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