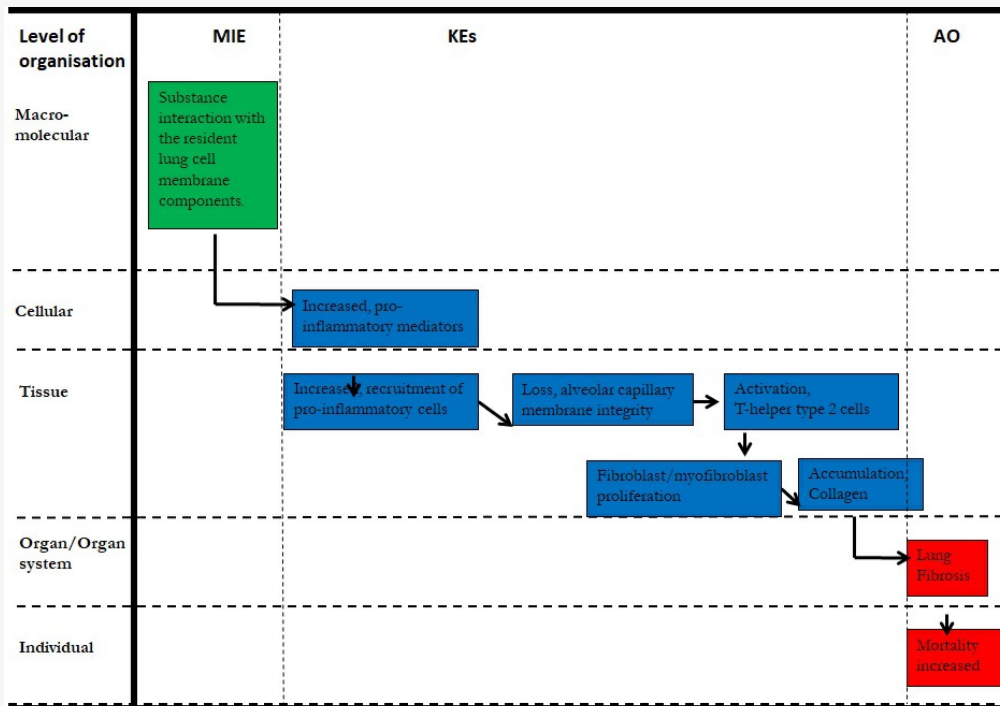## AOP ID and Title:

AOP 173: Substance interaction with the lung resident cell membrane components leading to lung fibrosis
Short Title: Substance interaction with the lung cell membrane leading to lung fibrosis

Graphical Representation

Authors

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Abstract

This AOP describes the qualitative linkages between interactions of substances (e.g. physical, chemical or, receptor-mediated) with the membrane components (e.g. receptors, lipids) of lung cells leading to fibrosis. This AOP represents a pro-fibrotic mechanism that involves a strong inflammatory component. It demonstrates the applicability of the AOP framework for nanotoxicology and describes a mechanism that is common to both chemical and nanomaterial-induced lung fibrosis. Lung fibrosis is a dysregulated or exaggerated tissue repair process. It denotes the presence of scar tissue in the localised alveolar capillary region of the lung where gas exchange occurs; it can be localised or more diffuse involving, bronchi and pleura. It involves the presence of sustained or repeated exposure to a stressor and intricate dynamics between several inflammatory and immune response cells, and the microenvironment of the alveolar-capillary region consisting of both immune and non-immune cells, and the lung interstitium. The interaction between the substance and components of the cellular membrane leading to release of danger signals/alarmins marks the first event, which is a molecular initiating event (MIE; Event 1495) in the process of tissue repair. As a consequence, a myriad of pro-inflammatory mediators are secreted (Key Event (KE) 1; Event 1496) that signal the recruitment of pro-inflammatory cells into the lungs (KE2; Event 1497). The MIE, KE1 and KE2 represent the same functional changes that are collectively known as inflammation. In the presence of continuous stimulus or persistent stressor, non-resolving inflammation and ensuing tissue injury, leads to the alveolar capillary membrane integrity loss (KE3; Event 1498) and activation of adaptive immune response, T Helper type 2 cell signalling (KE4; Event 1499), during which anti-inflammatory and pro-repair/fibrotic molecules are secreted. The repair and healing process stimulates fibroblast proliferation and myofibroblast differentiation (KE5; Event 1500), leading to synthesis and accumulation of extracellular matrix or collagen (KE6; Event 68). Excessive collagen deposition culminates in alveolar septa thickening, decrease in total lung volume, and lung fibrosis (Adverse Outcome (AO); Event 1458).

Lung fibrosis is frequently observed in miners and welders exposed to metal dusts, making this AOP relevant to occupational exposures. Other stressors include pharmacological products, fibres, chemicals, microorganisms or over expression of specific inflammatory mediators. Novel technology-enabled stressors, such as nanomaterials possess properties that promote fibrosis via this mechanism. Lung fibrosis occurs in humans and the key biological events involved are the same as the ones observed in experimental animals. Thus, this AOP is applicable to a broad group of substances of diverse properties and provides a detailed mechanistic account of the process of lung fibrosis across species.

Background

There is a high potential for inhalation exposure to toxicants in various occupational settings and polluted environments. Extensive investigation of pulmonary toxicity following inhalation of chemical and particulate stressors have demonstrated that these toxicants mount an exuberant inflammatory response early after exposure that, when unresolved, lays the foundation for later pathologies. Although inflammation is a normal immune reaction of the organism designed to effectively eliminate the invading threat, chronic and unresolved tissue inflammation is detrimental. Unresolved lung inflammation in humans plays a causative role in many debilitating and even lethal adverse health effects, such as decreased lung function, emphysema, fibrosis, and cancer. The various pathways, mechanisms, and biological processes associated with the pulmonary inflammatory process are well characterized in experimental animals and, to a great extent, in humans. Here, a mechanism underlying stressor-induced lung fibrosis that involves a pro-inflammatory component is described.

Pulmonary fibrosis is a chronic lung pathology, which when not treated, results in lethality. It is characterized by the excessive extracellular matrix deposition and restructuring. Numerous respiratory diseases, such as pneumoconiosis, silicosis, asbestosis, bronchiolitis obliterans ('popcorn lung'), and chronic beryllium disease have pulmonary fibrosis as a main or secondary symptom. In addition, exposure to pharmaceuticals and environmental contaminants such as bleomycin and arsenic via inhalation, oral or intravenous routes also induces the adverse outcome of pulmonary fibrosis. Idiopathic pulmonary fibrosis (IPF) is the most common type of lung fibrosis in humans and involves alveolar regions of the lung consisting of type 2 alveolar epithelial cells (AEC2s), type 1 cells (AEC1s) and mesenchymal cells. AEC1s are responsible for gas exchange and AEC2s synthesise surfactant. The AEC2s are capable of self-renewal and differentiate to AEC1s regularly during normal tissue maintenance (Barkauskas & Noble, 2014). In pro-fibrotic conditions, AEC2s fail to regenerate AEC1s lost by injury and do not respond normally to epithelial injury, undergoing hyperplasia. As a result, human patients suffering from IPF have dysregulated levels of surfactant proteins normally secreted by AEC2s (Barlo et al.,2009; Phelps et al., 2004). Genetic studies have associated mutations in genes encoding surfactant proteins and the development of a familial type of lung fibrosis. Furthermore, immunohistochemical staining of human IPF lung slices shows AEC death as well as proliferation adjacent to fibrotic foci (Uhal et al., 1998). AEC2s are hyperplastic and are located on top of the fibrotic lesions in the lung in human specimens (Katzenstein & Myers, 1998). In animal models of bleomycin-induced lung fibrosis, abnormal AEC2s are incapable of protecting the basement membrane destroyed by cell death, leading to aberrant repair and deposition of ECM, resulting in fibrosis (Rock et al., 2011). Targeted removal of AEC2s in mouse lungs results in full manifestion of the fibrotic disease (Sisson et al., 2010). In certain infectious conditions, epithelial cell stress and dysfunction leading to inefficient repair capacity or transcriptional reprogramming of epithelial cells to secrete pro-fibrotic and pro-inflammatory factors leads to lung fibrosis (Lawson et al., 2008; Lawson et al., 2011). Mesenchymal cells are the other main type of cell, which contribute to fibrosis development. The dysregulated proliferation of fibroblasts and myofibroblast differentiation leading to excessive ECM deposition in the fibrotic scar is the result of disrupted cross talk between epithelial and mesenchymal cells (Barkauskas 2014). Myofibroblasts exhibiting contractile properties of smooth muscle cells and expressing a-SMA and vimentin, are the types of mesenchymal cells that are most commonly associated with excessive collagen secretion in pro-fibrotic phenotypes (Todd et al., 2012). Myofibroblasts can arise mainly from differentiation of tissue resident fibroblasts, translocation of bone marrow derived fibrocytes into the lung, or from epithelial-to-mesenchymal transformation (EMT; a type of trans-differentiation) (Hung, 2020; Todd et al., 2012). These cells are critical to the normal process of wound healing, and are the main cells contributing to collagen deposition in both normal wear-and-tear repair processes and in disease promoting conditions. Following successful

Although this AOP is applicable to a broad group of chemicals of diverse properties, the AOP was specifically assembled keeping in mind, a novel class of engineered materials (nanomaterials) exhibiting sophisticated properties that have been shown to induce lung fibrosis via this mechanism. Thus, it demonstrates the applicability of the AOP framework to nanotoxicology.

Given the fundamental role of inflammation in organ homeostasis, well characterized AOPs targeting the pathological outcomes of unregulated inflammatory responses are important and will guide the development of appropriate assays to measure the key events that are predictive of inflammation-mediated chronic health impacts, and aid in screening a large array of inhalation toxicants that are inflammogenic, for their potential to induce lung diseases.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1495	Substance interaction with the lung resident cell membrane components	Interaction with the lung cell membrane
2	KE	1496	Increased, secretion of proinflammatory mediators	Increased proinflammatory mediators
3	KE	1497	Increased, recruitment of inflammatory cells	Recruitment of inflammatory cells
4	KE	1498	Loss of alveolar capillary membrane integrity	Loss of alveolar capillary membrane integrity
5	KE	1499	Increased, activation of T (T) helper (h) type 2 cells	Activation of Th2 cells
6	KE	1500	Increased, fibroblast proliferation and myofibroblast differentiation	Increased cellular proliferation and differentiation
8	KE	68	Accumulation, Collagen	Accumulation, Collagen
9	AO	1458	Pulmonary fibrosis	Pulmonary fibrosis

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components	adjacent	Increased, secretion of proinflammatory mediators	Moderate	Moderate
Increased, secretion of proinflammatory mediators	adjacent	Increased, recruitment of inflammatory cells	Moderate	Low
Increased, recruitment of inflammatory cells	adjacent	Loss of alveolar capillary membrane integrity	Moderate	Moderate
Loss of alveolar capillary membrane integrity	adjacent	Increased, activation of T (T) helper (h) type 2 cells	Moderate	Low
Increased, activation of T (T) helper (h) type 2 cells	adjacent	Increased, fibroblast proliferation and myofibroblast differentiation	High	Low
Increased, fibroblast proliferation and myofibroblast differentiation	adjacent	Accumulation, Collagen	High	High
Accumulation, Collagen	adjacent	Pulmonary fibrosis	High	Low

Stressors

Name	Evidence
Bleomycin	High
Carbon nanotubes, Multi-walled carbon nanotubes, single-walled carbon nanotubes, carbon nanofibres	High

Bleomycin

Bleomycin is a potent anti-tumour drug, routinely used for treating various types of human cancers (Umezawa et al., 1967; Adamson, 1976). Lung injury and lung fibrosis are the major adverse effects of this drug in humans (Hay J et al., 1991). Bleomycin is shown to induce lung fibrosis in experimental animals - in dogs (Fleischman et al., 1971), mice (Adamson IY and Bowden DH, 1974), hamsters (Snider GL et al., 1978) and is widely used as a model chemical to study the mechanisms of fibrosis in humans (reviewed in **Moeller** et al., **2008;** Gilhodes et al., 2017).

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Carbon nanotubes, Multi-walled carbon nanotubes, single-walled carbon nanotubes, carbon nanofibres

CNTs are high aspect ratio materials and cause lung fibrosis in experimental animals (Muller et al., 2005; Porter DW et al., 2010). In an intelligence bulletin published by NIOSH on 'Occupational exposure to carbon nanotubes and nanofibers', NIOSH reviewed 54 individual animal studies investigating the pulmonary toxicity induced by CNTs and reported that half of those studies consistently showed lung fibrosis (NIOSH bulletin, 2013). Multiwalled carbon nanotubes induce lung fibrosis in mice (Nikota et al., 2017; Rahman et al., 2017). However, the evidence is inconsistent and the occurrence of fibrotic pathology is influenced by the specific physical-chemical properties of CNTs (length, rigidity), their dispersion in exposure vehicle, and the mode of exposure.

1. Muller, J., Huaux, F., Moreau, N., Misson, P., Heilier, J., Delos, M., Arras, M., Fonseca, A., Nagy, J. and Lison, D. (2005). Respiratory toxicity of multi-wall carbon nanotubes. Toxicology and Applied Pharmacology, 207(3), pp.221-231.
2. NIOSH (2013). Occupational exposure to carbon nanotubes and nanofibers: current intelligence bulletin 65.
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inflammatory and pro-fibrotic responses in mice: Investigating the mechanisms of pulmonary carcinogenesis. Mutat Res. 2017 Nov;823:28-44.

Overall Assessment of the AOP

Pulmonary fibrosis is the thickening and scarring of lung tissue, caused by excessive deposition of collagen/extracellular matrix. The most common fibrotic disease of the lung in humans is IPF, a complex, progressive disease of unknown etiology with often poor prognosis. Pulmonary fibrosis in humans is also observed following exposure to pharmacological agents such as bleomycin, following inhalation of silica, asbestos, cigarette smoke, coal dust and following exposure to microbials and allergens. Regardless of the etiology, lung fibrosis in humans is characterised by the presence of inflammatory lesions, excessive extracellular matrix deposition, reduced lung volume and function. Mechanistically, using animals, it has been shown that key biological events that play a critical role in the onset and progression of the disease are similar in humans and animals. The main differences are limited to anatomical and physiological aspects of lung and its functions.

Some other considerations of relevance to this AOP:

This AOP represents a fibrotic mechanism that involves a strong inflammatory component. Exposure to pro-fibrotic stressors such as, bleomycin, silica, asbestos, CNTs, radiation or models of cytokine overexpression involve a profound inflammatory response. IPF in humans is more commonly observed in male subjects. A study in mice showed that male mice developed lung fibrosis more readily following exposure to bleomycin compared to female mice and that age is a risk factor, with aged male mice showing exuberant fibrosis (Redente et al., 2011). Scar formation is reduced in fetal wounds (Yates et al., 2012). Asbestosis and silicosis, (two types of fibrotic disease) are clinically manifested in aged humans. Thus, the AOP presented here is applicable to lung fibrosis observed in adults predominantly.

Different animal species have been used to study the pathology of fibrotic disease; with mice being the most common and rats the second most used. Australian sheep, horse, dogs, cats, donkeys, pigs and other animals have been studied to investigate different types of fibrosis. There are some limitations, however, in these animal systems with respect to modelling human pulmonary fibrosis. The most commonly used model, the bleomycin mouse model, presents a rapidly developing fibrotic phenotype which undergoes at least partial resolution following 28 days (Tashiro et al., 2017). Higher order organisms, like dogs, cats, and horses offer a chance to examine naturally occurring pulmonary fibrosis, with closer resemblance to human IPF in animals with a natural cough reflex (Williams & Roman, 2015). However, inherent limitations in these models, such as their outbred nature and lack of systematic characterization (Williams & Roman, 2015) make them poor candidates for routine fibrosis research. Regardless of the species or the type of fibrosis investigated, the key characteristic events that define the disease process are the same with few species-specific anatomical, physiological and histological differences. Thus, cross-species applicability for this AOP is strong.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	High

Sex/Gender and Age:

IPF in humans is more commonly observed in male subjects. Male mice develop lung fibrosis more readily following exposure to bleomycin compared to female mice and that age is a risk factor, with aged male mice showing exuberant fibrosis (Redente et al., 2011). Scar formation is reduced in fetal wounds (Yates et al., 2012). Asbestosis and silicosis, forms of fibrotic disease are clinically manifested in aged humans. Thus, the AOP presented here is applicable to lung fibrosis observed in adult males predominantly.

Taxonomy:

Different animal species have been used to study the pathology of fibrotic disease; with mice being the most common and rats the second most used. Australian sheep, horse, dogs, cats, donkeys, pigs and other animals have been studied to investigate different types of fibrosis. There are some limitations, however, in these animal systems with respect to modelling human pulmonary fibrosis. The most commonly used model, the bleomycin mouse model, presents a rapidly developing fibrotic phenotype which undergoes at least partial resolution following 28 days (Tashiro et al., 2017). Higher order organisms, like dogs, cats, and horses offer a chance to examine naturally occurring pulmonary fibrosis, with closer resemblance to human IPF in animals with a natural cough reflex

(Williams & Roman, 2015). However, inherent limitations in these models, such as their outbred nature and lack of systematic characterization (Williams & Roman, 2015) make them poor candidates for routine fibrosis research. Regardless of the species or the type of fibrosis investigated, the key characteristic events that define the disease process are the same with few species-specific anatomical, physiological and histological differences. Thus, cross-species applicability for this AOP is strong.

Types of Stressors:

Persistent and soluble stressors can induce fibrotic pathologies in humans (as well as in model animals) in concordance with the AOP presented. Asbestos exposure in humans has long been known to induce pulmonary fibrosis (asbestosis) due to chronic inflammation induced from persistent fibres deposited within the lung (Kamp and Weitzman 1997). Similarly, human exposure to silica leads to the development of silicosis in concordance with the AOP presented (Ding *et al.,* 2002). Furthermore, the soluble chemotherapeutic compound bleomycin has long been known to induce pulmonary fibrosis in humans (in line with this AOP) as a side effect of intravenous administration (Froudarakis *et al.,* 2013). In addition to these model stressors, exposure to various metals including uranium, arsenic, cadmium, and soluble copper can lead to fibrotic outcomes in humans (Assad et al., 2019). Occupational exposure to cobalt can induce interstitial lung disease in humans, which can progress to fibrotic outcomes (Traci et al., 2017). In male mice exposed via inhalation to cadmium oxide nanoparticles, increases in the pro-fibrotic and pro-inflammatory mediators IL-1b, TNF-a, and IFN-g were noted one day post exposure, with accompanying pulmonary inflammation (Blum et al., 2014). In another study , intratracheal instillation of cadmium chloride in mice induced peribronchiolar fibrosis through activation of myofibroblasts via SMAD signalling (Li et al., 2017). As with the aforementioned cadmium nanoparticles, murine animals exhibit pronounced acute inflammation and immune cell infiltration after pulmonary exposure to Copper oxide (CuO) nanoparticles (Gosens et al., 2016), which can progress to a fibrotic phenotype in some model systems after 28 days with marked increases of TGF-b detected in the BALF, activation of myofibroblasts, and pronounced deposition of extracellular matrix (Lai et al., 2018). In mice, intratracheal instillation of cobalt nanoparticles results in pronounced infiltration of neutrophils and macrophages into the alveolar and interstitial space, and increased amounts of CXCL1 in the BALF 1-7 days post exposure; pronounced pulmonary fibrosis was detected at 4 months post-exposure marked by increased collagen deposition and bronchiolization of the alveolar epithelium (Wan et al., 2017).

Essentiality of the Key Events

The essentiality of the MIE; Event 1495 was rated as moderate, due to the non-specific nature of the event, and the dynamic nature of the membrane interaction, especially with nanomaterials, which may adopt a molecular corona in biological environments that mediates cellular interactions.

The essentiality of KE1; Event 1496 and KE2; Event 1497 was rated as moderate, due to the redundant nature of the inflammatory response and the inherent challenges in abrogating this response without inducing another pathology in the model system.

For KE3; Event 1498, the essentiality was also listed as moderate, due to the fact that attenuation or abrogation of this response isn't practical, and as such the supporting evidence is indirect.

For KE4; Event 1499 and KE6; Event 68, the essentiality was rated as high due to the plethora of experimental evidence showing that modulation of these responses modifies the AO and downstream KEs. For additional information, please consult the Evidence Assessment Call Table below.

Weight of Evidence Summary

Concordance of Dose-Response Relationships:

The AOP presented here is qualitative. There is some evidence on dose-response relationships; however, dose-response relationships for each individual KE are not available. In Labib et al., 2016, Benchmark Dose (BMD) analysis of MWCNT-induced gene expression changes in lungs of mice and canonical pathways associated with each of the KEs identified in this AOP was conducted and the resulting BMD values were correlated with BMD values derived for the apical endpoints that measured histologically manifested fibrotic lesions in rodents. The study showed that low doses of MWCNTs induce early KEs of inflammation and immune response at the acute post-exposure timepoints, and histological manifestation of fibrosis required higher MWCNT doses and was only evident at the later timepoints. Similarly, in another study, the meta-analyses of transcriptomics data gathered from mouse lungs (over 2000 microarrays) exposed individually to a variety of pro-fibrotic agents showed that the gene expression profiles from the high dose MWCNT-exposed samples collected at sub-chronic timepoints were strongly associated with the Th2 response signalling observed in mouse fibrotic disease models compared to the low dose early timepoint MWCNT samples (Nikota et al., 2016). These studies showed temporal and dose-response relationships between KEs.

In another study, pharyngeal aspiration of 10, 20, 40, or 80 μg/mouse MWCNT induced lung fibrosis in a dose-dependent manner, which became apparent as early as 7 days post-exposure at 40 μg/mouse dose and persisted up to 56 days post-exposure (Porter et al., 2010). Pharyngeal aspiration of 10, 20, 40, or 80 μg/mouse MWCNTs induced significant alveolar septa thickness over time (1, 7, 28, and 56 days post-exposure) in 40 and 80 μg dose groups (Mercer et al., 2011). Similarly, inhalation of MWCNTs (10 mg/m3, 5h/day) for 2, 4, 8, or 12 days showed dose-dependent lung inflammation and lung injury with the development of lung fibrosis in mice (Porter et al., 2012). Lung inflammation and fibrosis was observed in mice intratracheally instilled with 162 μg/mouse MWCNTs at 28 days post-exposure (Nikota et al., 2017). The above studies involving CNTs showed elevated levels of pro-inflammatory mediators, pro-inflammatory cells and cytotoxicity in BALF.

Strength, Consistency, and Specificity of Association of Adverse Outcome and Initiating Event:

This AOP describes a non-specific MIE. Typically, in an experimental setting, the MIE itself is not assessed. Rather, the outcomes of MIE engagement or MIE trigger are assessed. Depending on the type of stressor and its physical-chemical property, the type of interactions between the stressor and the lung resident cells differ. High aspect ratio fibres such as asbestos and CNTs induce frustrated phagocytosis, acute cell injury (Boyles et al., 2015; Dörger et al., 2001; Brown et al., 2007; Kim et al., 2010; Poland et al., 2008), leading to inflammation, immune responses and fibrosis. Asbestos and silica crystals engage scavenger receptors present on the macrophages (Murthy et al., 2015), resulting in acute cell injury and inflammatory cascade, leading eventually to the AO. Bleomycin binds high affinity bleomycin binding sites present on rat alveolar macrophage surfaces, leading to macrophage activation (Denholm and Phan, 1990). Asbestos fibres also bind directly to cellular macromolecules including proteins and membrane lipids, which is influenced by their surface properties such as surface charge (reviewed in Agency for Toxic Substances and Disease Registry 2001). These studies demonstrate the types of interactions between cells and the pro-fibrotic stressors, which are often not measured in animal or cell culture experiments. Instead, the consequences or outcomes of triggering the MIE are measured, which are the release of Danger Associated Molecular Signals (DAMPs) or alarmins from cells.

The alarmin HMGB1 is released from damaged or necrotic cells in cell culture models and in animals following exposure to asbestos and is involved in the inflammatory events elicited by asbestos (Yang et al, 2010), which plays a critical role in asbestosis. CNTs interact with HMGB1-RAGE, which is implicated in pro-inflammatory and genotoxic effects of CNTs (Hiraku et al., 2015). Mechanical stress and membrane damage following cellular uptake of long and stiff CNTs by lysosomes results in cell injury and consequent adverse effects (Zhu, et al., 2016). CNT-induced inflammatory response in vitro is mediated by IL-1, absence of which negatively impacts gap junctional intercellular communication (Arnoldussen et al., 2016). The levels of IL-1a are increased in BALF of mice immediately after exposure to MWCNT doses that induce fibrosis (Nikota et al., 2017).

Although there is enough empirical evidence to suggest the occurrence of the MIE; Event 1495 following exposure to pro-fibrogenic substances, there is incongruence in supporting its essentiality to the eventual AO. The inconsistency could be due to the fact that early defence mechanisms involving DAMPs is fundamental for the organism's survival, which may necessitate multifaceted signalling pathways. As a result, inhibition of a single biological pathway of the innate immune response may not be sufficient to completely abrogate the lung fibrotic response. For example, MWCNTs induce IL-1a secretion in BALF of mice (Nikota et al., 2017) and thus, IL-1a mediated signalling is involved in MWCNT induced lung inflammation and fibrosis (Rydman et al., 2015). Inhibition of IL-1a signalling alone does not alter the MWCNT-induced fibrotic response in mice (Nikota et al., 2017). This study further showed that simultaneous inhibition of both acute inflammatory events (KE1; Event 1496 and KE2; Event 1497) and Th2 –mediated signalling (KE4; Event 1499) is required to suppress lung fibrosis induced by MWCNTs (Nikota et al., 2017). Disengagement between innate immune responses (MIE; Event 1495, KE1; Event 1496 and KE2; Event 1497) and lung fibrosis is shown in mice following exposure to silica (Re et al., 2014). In this study, the role of innate immune responses in lung fibrosis were characterised in 11 separate knockout mouse models lacking individual members of the IL-1 family. The study supported the earlier hypothesis of Nikota et al., 2017 that inhibition of a single pathway may not be sufficient to attenuate the fibrotic response. On the contrary, IL-1a and IL-1R1 mediated signalling are shown to be involved in bleomycin-induced lung inflammation and fibrosis; inhibition of IL1-R1 signalling attenuated the bleomycin pathology (Gasse et al, 2007).

Biological Plausibility, Coherence, and Consistency of the Experimental Evidence:

As described above, there is significant evidence to support the occurrence of the MIE and individual KEs, and thus, evidence supporting the KEs involved in this AOP is strong. However, there is inconsistency in empirical evidence supporting the KERs. Again, this may be due to the redundancy in pathways involved in the early immune responses to injury and repair. Despite the incongruences, AOP presented is coherent and logical.

Alternative Mechanisms:

The AOP as presented is the most agreed upon sequence of biological events occurring in the process of lung fibrosis that involves robust inflammation following exposure to a variety of stressors of different physical-chemical properties. However, in a recent study, using ToxCast data, a different MIE that involves inhibition of PPARg resulting in lung fibrosis was proposed (Jeong et al., 2019). This alternate AOP for fibrosis placed activation of TGF-b1 upstream of inflammatory events (KE2; Event 1497, KE3; Event 1498), which is contrary to its perceived role in downstream events leading to fibroblast proliferation and differentiation, and ECM deposition. The stressors identified in this study were also different, suggesting the PPARg inhibition may be selective to a group of chemicals. The other alternative mechanisms may involve bypassing of the initial inflammatory KEs that directly trigger activation of fibroblast proliferation and differentiation leading to extracellular matrix deposition. For example, overexpression of TGF-b1 can promote excessive ECM deposition and fibrosis in rodents independent of inflammation (Hardie *et al.,* 2004)

Further mechanisms may involve the targeted inhibition of receptor tyrosine kinases by compounds like Gefitinib, Imatinib, and Sorafenib, as well as some monoclonal antibodies which affects receptors for growth factors like PDGF, EGF, and VEGF. This is thought to directly impair the regenerative capacity of lung epithelial cells (MIE; Event 1495 to KE3; Event 1497), resulting in an aberrant wound healing response (Li et al., 2018). Finally, one more alternative mechanism involves pulmonary fibrosis in the context of bronchiolitis obliterans. In this condition, the fibrotic phenotype is brochiolocentric and not alveolocentric – with the main insult involving the bronchiolar epithelium and an inability of the basal cells to replace lost bronchio epithelial cells. Stressors, such as soluble diacetyl used in popcorn flavouring and e-cigarette vape liquids, can cause bronchiolitis obliterans in humans. A recent human case study of a Canadian youth admitted to hospital with bronchiolitis obliterans following vaping flavoured liquid containing diacetyl, as well as tetrahydrocannabinol, shows septal thickening, type II pneumocyte hyperplasia, immune cell infiltration and myofibroblast proliferation & incorporation into pulmonary septa (Landmann et al., 2019). Pulmonary exposures in murine model systems indicate that diacetyl induces pronounced damage to the airway epithelium, and that repair processes result in a compositionally different epithelium (Reviewed in Brass & Palmer, 2017). In a study using rat models, inhalation of 200 ppm of diacetyl resulted in bronchiolar fibrosis, with chronic inflammation accompanying the fibrotic outcomes (Morgan et al., 2016).

Evidence Assessment Summary:

The MIE; Event 1495 and KE1; Event 1496 – KE2; Event 1497 occur in sequence, however most *in vivo* and *in vitro* experiments are not designed to measures these events separately. This is an area of focus for future pulmonary fibrosis research.

Support for Essentiality of KEs		
MIE; Event 1495: Interaction with the lung resident cell membrane components	Persistent fibres like CNTs and asbestos are known to induce frustrated or incomplete phagocytosis in resident lung cells following respiratory exposure. Particles such as silica, as well as asbestos fibres engage scavenger receptors on the surface of macrophages leading to activation and inflammation. The soluble pro-fibrotic compound bleomycin binds to as-of-yet uncharacterised sites on macrophages, leading to similar activation.	Essentiality: Moderate. While the specific receptors involved vary depending on the stressor, and there is evidence of compensation in the context of knockout models, over 20 years of research has shown that interaction between the fibrotic stressor and the resident lung cells is crucial for downstream responses. (Behzadi et al., 2017; Denholm & Phan 1990; Mossman & Churg 1998).
KE 1; Event 1496: Increased, secretion of proinflammatory mediators	Injured and activated resident lung cells release pro-inflammatory and fibrotic mediators, such as cytokines, chemokines, growth factors and reactive oxygen species, into the surrounding environment.	Essentiality: Moderate. It is accepted that one of the main mechanisms underlying pulmonary fibrosis involves a profound inflammatory component. This has been shown in animal models exposed to fibrotic stressors such as bleomycin, MWCNT, silica, and asbestos. The exact nature of the secreted mediators, and the essentiality of specific mediators requires further research. (Park & Im, 2019; Rahman et al., 2017; Rabolli et al., 2014).
KE 2; Event 1497: Increased, recruitment of inflammatory cells	Inflammatory cells migrate into the lung according to the pro-inflammatory stimuli released.	Essentiality: High. The migration of inflammatory immune cells relies upon secretion of chemotactic stimuli in response to a stressor. Knockout models have shown reduced recruitment of immune cells to the lung in response to fibrotic stressors such as bleomycin. However, compensation has been noted due to the redundant nature of these molecules. (Gasse et al., 2007; Girtsman et al., 2014; Rabolli et al., 2014)
KE 3; Event 1498: Loss of alveolar capillary membrane integrity	Significant alveolar damage from the inflammatory environment (including chronic inflammation and oxidative stress) results in the loss of alveolar capillary membrane (ACM) integrity.	Essentiality: Moderate. While it is generally recognized that damage to the alveolar capillary membrane is integral to the development of fibrosis, there evidence from knock-out models is lacking. Indirect evidence using bleomcyin has shown that animals deficient in Nrf2, and therefore presenting a weakened antioxidant response, have higher levels of ACM injury and more pronounced fibrosis as compared to Nrf2 competent mice. This was assessed by proxy, using LDH release into the BALF and the presence of pulmonary injury markers as a proxy for ACM injury.

		(Cho et al., 2004; Kikuchi et al., 2010)
KE 4; Event 1499: Increased, activation of T (T) helper (h) type 2 cells	T helper cells present in, and recruited to the lung environment commit to Th2 differentiation, which then release cytokines like IL-4, IL-5, and IL-13 and potentiate a Th2 driven response.	Essentiality: High. Induction of a Th2 response stimulates fibroblast proliferation & pulmonary fibrogenesis. Over expression of Th2 type cytokine IL-13 stimulates pulmonary fibrosis in the absence of external stressors. IL-13 can directly activate TGF-b1 and initiates fibroblast prolieration and differentiation in pulmonary fibrosis. In mice deficient in STAT6, MWCNT treatment reduced the Th2 response, and downstream fibroblast proliferation, and overall fibrotic response. There is some inconsistency however, as IL4 deficient mice had a lower fibrotic response compared to wild-type after bleomycin treatment, however with higher rate of mortality. This highlights that the timing of the Th2 response is important for the manifestation of fibrosis. (Lee et al., 2001; Huaux et al., 2003; Nikota et al., 2017; Sempowski et al., 1994; Zhu et al., 1999)
KE 5; Event 1500: Increased, fibroblast proliferation and myofibroblast differentiation	Fibroblasts originally present in the lung, and recruited to the lung, or which transdifferentiate from epithelial and endothelial cells proliferate and undergo differentiation into a collagen secreting myofibroblast phenotype which exprese smooth muscle actin. This is the main effector cell responsible for secretion of extracellular matrix components in pulmonary fibrosis, and represents a nexus KE.	Essentiality: High. The proliferation of fibroblasts and differentiation into myofibroblasts is integral to the development of pulmonary fibrosis. Inhibition or attenuation of fibroblast proliferation and differentiation using TGF-b antagonism attenuates fibrosis in bleomycin mice models. Targeted inhibition of the Wnt/b-catenin pathway inhibited myofibroblasts transition and reduced the overall fibrotic phenotype. (Cao et al., 2018; Chen et al., 2013; Kuhn & McDonald, 1991; Guan et al., 2016)
KE 6; Event 68: Accumulation, collagen	The balance between extra cellular matrix (ECM) synthesis and destruction is disrupted, with a sustained increase in the deposition of ECM bearing compositional differences as compared to the native matrix.	Essentiality: High. A sustained imbalance between ECM synthesis and destruction is a prerequisite for the development of pulmonary fibrosis, and as such this KE is essential to the AO. (Bateman et al., 1981; McKleroy et al., 2013)
AO; Event 1458: Pulmonary fibrosis	Destruction of lung architecture and alveolar capillaries due to increased and aberrant deposition of extra cellular matrix in the context of prolonged inflammation results in pulmonary fibrosis.	Essentiality: N/A. This is the adverse outcome of this AOP, and therefore, is essential.
Associative Event 1: Chronic Inflammation	In the presence of continuous stimulus (e.g., presence of biopersistent toxic fibres such as asbestos, MWCNTs) or following repeated stimulus (e.g., repeated exposure to silica or coal dust), the ensuing cell injury fuels the inflammatory mechanisms leading to accumulation of immune cells, prolonged inflammation and aggravated tissue damage. This sustained and perpetuated immunological response is termed as chronic inflammation. During this phase, active inflammation, tissue injury and destruction, and tissue repair processes proceed in tandem. Thus, the causative substance must contain unique physico-chemical properties that grant the material biopersistance in the pulmonary environment or the pulmonary system has to be repeatedly exposed to the same substance that perpetuates the tissue injury leading to loss of ACM. Although, increases in number of neutrophils are observed during chronic inflammation, mononuclear phagocytes (circulating monocytes, tissue macrophages) and lymphoid cells mark	Essentiality: Moderate. Knockdown and knockout models have shown that attenuation of the inflammatory response, attenuates the downstream fibrotic phenotype. Compensation from other inflammatory pathways makes complete abrogation of this response difficult. Furthermore, the essentiality of this associative event to fibrotic phenotypes like IPF is questionable, as treatment with

	this phase. The macrophages, components of mononuclear phagocyte system, are the predominant cells in chronic inflammation. Activated macrophages release a variety of cytokines, chemokines, growth factors, and reactive oxygen species that, which when uncontrolled, lead to extensive tissue injury. The other types of inflammatory cells involved in chronic inflammation include eosinophils in allergen induced lung fibrosis, lymphocytes and epithelial cells. Chronic inflammation exists to potentiate the KEs associated with inflammation and tissue injury, rather than acting as a separate KE itself.	anti-inflammatory agents like corticosteroids does not have substantial benefits for patients. (Strieter & Mehrad, 2009; Ueha et al., 2012; Wilson & Wynn, 2009)
Associative Event 2: Oxidative stress	Oxidative stress is defined as an imbalance in the oxidant – antioxidant axis towards oxidants (hydrogen peroxide, superoxide anions, hydroxyl radicals) in a supraphysiological manner. Reactive oxygen species (ROS) serve as part of an important redox signalling system which helps cells adapt to their environment and tackle stress through modulation of transcriptional regulation. In the context of pulmonary fibrosis, oxidative stress potentiates the inflammatory response (KE1-2) and injury to the respiratory epithelium (KE3), and contribute to the differentiation and activation of myofibroblasts (KE5). The exact species of ROS, the specific cell types, and the perturbed oxidative stress related pathways vary depending on the type of pulmonary fibrosis, and even among different human patients suffering from the same fibrosing disease (ex. IPF). Increased levels of ROS have been shown to activate TGF-b, and induce apoptosis of alveolar epithelial cells. Furthermore, oxidative stress induces secretion of pro-inflammatory mediators (mitochondrial DNA, Nalp3 inflammasome related molecules) from the injured epithelium as well as from resident immune cells like macrophages. This potentiates additional recruitment of immune cells to the site of injury, further compounding the inflammatory response, and inducing further production of ROS by effector cells like neutrophils. Clinical studies in IPF patients have consistently found higher levels of ROS biomarkers in the BALF, serum, as well as in exhaled condensate. Furthermore, increases in ROS and oxidative stress are associated with brionchiolitis obliterans, a fibrosing disease of the brionchioles instead of the alveolar tissue. While there is strong evidence for the involvement of ROS in the pathogenesis of pulmonary fibrosis, it acts to potentiate multiple KEs rather than acting as a key event itself. Oxidative stress is both causative and the consequence of observed responses in a feedforward type mechanism.	Essentiality: Moderate. Multiple studies, using knockdown and knockout mammalian models have shown that oxidative stress is involved in the development of pulmonary fibrosis. However, its essentiality in its pathogenesis is not conclusive, as antioxidant treatment offers no significant benefit in patients with IPF, the most common type of pulmonary fibrosis in humans. Furthermore, uncertainties remain concerning the exact molecular mechanisms underlying oxidative stress in the context of pulmonary fibrosis. (Checa & Aran, 2020; Cheresh et al., 2013; Dostert et al., 2008; Madill et al., 2009ab; Veith et al., 2019)
Associative Event 3: Macrophage polarization	Depending on the lung microenvironment (damaged cells, microbial products, activated lymphocytes), the precursor monocytes differentiate into distinct types of macrophages. Classically activated (M1) macrophages and alternatively activated (M2) macrophages are the important ones to consider in the context of this AOP. The M1 macrophages produce high levels of pro-inflammatory cytokines, mediate resistance to pathogens, induce generation of high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and T helper (Th) 1 type responses. M1 macrophages produce IL-1, IL-12, IL-23 and induce Th1 cell infiltration and activation. The M2 macrophages secrete anti-inflammatory mediators, by which they play a role in regulation of inflammation. The M2 polarisation is mediated by Th2 cytokines such as IL-4 and IL-13, which in turn, promotes M2 activation. M2 macrophages express immunosuppressive molecules such as IL-10, Arginase-1 and -2 (Arg-1, Arg-2), which suppress the induction of Th1 cells that produce the anti-fibrotic cytokine IFNg. The activity of M2 is associated with tissue remodelling, immune regulation, tumour promotion, tissue regeneration and effective phagocytic activity.	Essentiality: High. Inhibition of M2 polarization through genetic depletion of surface receptors such as MARCO, attenuates the fibrotic phenotype. Depletion of interstitial macrophages bearing the M2 phenotype has been shown to block radiation induced lung fibrosis. (He et al., 2013; Meziani et al., 2018; Murthy et al., 2015; Stahl et al., 2013)
Support for Biological Plausibility of KERs		
MIE --> KE1: Relationship 1702	Injury and activation resulting from the interaction of pro-fibrotic stressors with the membranes of resident lung cells results in the secretion of pro-inflammatory cytokines, chemokines, growth factors, and reactive oxygen species from the resident epithelial or immune cell.	Biological plausibility: High. There is a mechanistic relationship between the MIE and KE1 which has been evidenced in a number of both *in vitro* and *in vivo* model systems in response to stressors such as, asbestos, silica, bleomycin, carbon nanotubes, and metal oxide nanoparticles. (Behzadi et al., 2017; Denholm & Phan 1990; Mossman & Churg 1998)
KE1 --> KE2: Relationship 1703	The secreted pro-inflammatory and pro-fibrotic mediators induce chemotactic recruitment of immune cells to the lung, in a signal specific manner. Increases in the presence of macrophages, neutrophils, and eosinophils within pulmonary air spaces is commonly seen in the process of fibrosis, depending on the fibrotic stressor in question.	Biological plausibility: High. There are very well established functional relationships between the secreted signalling molecules and the chemotactic effects on pro-inflammatory and pro-fibrotic cells. (Harris, 1954; Petri & Sanz, 2018)
KE2 --> KE3:	Inflammatory cells recruited to the lung potentiate further injury to the alveolar	Biological plausibility: High.

Relationship 1704	capillary membrane through ROS production and direct damage, persistent inflammation, or an insufficient wound healing response. Type I alveolar cells are lost, type II cells exhibit enhanced proliferation, extracellular matrix changes are notable and alveoli collapse.	There is a mechanistic relationship between an increase in pro inflammatory cells and mediators, and damage to the ACM. (Bhalla et al., 2009; Ward, 2003; Zemans et al., 2009)
KE3 --> KE4; Relationship 1705	Continued loss of alveolar capillary membrane integrity, together with oxidative stress and chronic inflammation induce a Th2 response in the lung. T helper cells differentiate into Th2 cells in response to stimuli such as IL-6 and IL-4, which increase the secretion of IL-4 and IL-13. Increased Th2 cells in the lung polarize macrophages to the M2 phenotype which further suppresses Th1 cell differentiation.	Biological Plausibility: High. There is a mechanistic relationship between ACM injury (tissue damage), and the induction of a Th2 response (responsible for wound healing). (Gieseck et al., 2018; Wynn, 2004)
KE4 --> KE5; Relationship 1706	The increased population of Th2 cells and M2 polarized macrophages increases secretion of pro-fibrotic mediators, like TGF-b, IL-4, and IL-13 which activate lung resident fibroblasts, as well as fibroblasts and fibrocytes recruited to the lung, and potentiate endo/epithelial to mesenchymal transition. This induces their proliferation and differentiation into a contractile myofibroblast phenotype capable of extra cellular matrix synthesis and deposition.	Biological plausibility: High. There is a widely understood functional relationship between Th2 response related mediators, and their ability to induce proliferation and differentiation of fibroblasts. (Dong & Ma, 2018; Shao et al., 2008; Wynn, 2004; Wynn & Ramalingam, 2012)
KE5 --> KE6; Relationship 2625	Differentiated myofibroblasts represent the main effector cell responsible for the deposition of extracellular matrix during lung fibrosis. In the context of continuous stimuli and elevated levels of TGF-b, myofibroblasts are persistently activated and deposit excessive amounts of collagen in the lung.	Biological plausibility: High. There is an accepted mechanistic relationship between activated myofibroblasts, and the capacity to secrete collagen. (Hinz, 2016ab; Hu & Phan, 2013)
KE6 --> AO; Relationship 1629	Persistent myofibroblast activation and continued deposition of extracellular matrix causes destruction of alveolar structures and normal lung architecture. Reductions in lung function are noted, and pulmonary fibrosis develops.	Biological plausibility: High. By definition, pulmonary fibrosis is characterized by excessive deposition of extracellular matrix and destruction of native lung architecture. Thus, the plausibility of this association is undisputed. (Fukuda et al., 1985; Richeldi et al., 2017; Thannickal et al., 2004)
Empirical Support for KERs		
MIE --> KE1; Relationship 1702	Direct interaction with the membrane is not a typically assessed endpoint in fibrosis research, except when dealing with fibrous stressors. Specific receptors involved in the initial immune cell activation are not wholly understood, even for model fibrotic stressors such as bleomycin. Limited *in vitro* studies have shown toll like receptors are involved in silica and zinc nanoparticle macrophage recognition, which stimulates secretion of inflammatory factors. Similarly, bleomycin has been shown to bind to high affinity sites on the surface of macrophages, which stimulates secretion of growth factors and monocyte chemotactic molecules.	Empirical Support: Moderate. There are limited *in vitro* studies which show a temporal and dose-dependant relationship between these two events, using the upregulation of specific surface receptors as a proxy for direct membrane interaction. (Chan et al., 2018; Denholm & Phan 1990; Roy et al., 2014)
KE1 --> KE2; Relationship 1703	There are many studies which have shown empirically shown a relationship between secreted mediators and recruitment of immune cells to the lung. A paper by Chen et al., showed that increases in the levels of CXCL1, CXCL2, and CXCL5 in the lung preceded neutrophil recruitment following *in vivo* treatment with carbon nanoparticles. In an *in vitro* study, Schremmer *et al.,* exposed rat alveolar macrophages to nano silica and noted increases in CCL4, CXCL1, CXCL3, and TNF-a in the supernatant. This supernatant was able to induce chemotaxis in unexposed macrophages.	Empirical Support: Moderate. There are many studies which show temporal and dose-dependant recruitment of immune cells following increases in pro-inflammatory mediators. However, these mediators exhibit pleiotropy, and knockdown or knockout of a single pathway or mediator can result in compensation and recruitment of immune cells at a

		later time, as is seen in Nikota et al.,. 2017. (Chen et al., 2016; Nikota et al., 2017; Schremmer et al., 2016)
KE2 --> KE3; Relationship 1704	The chronic inflammatory environment and oxidative stress potentiated by an increase of immune cells in the lung is well known to precede significant alveolar damage. However, the variety of infiltrating leukocytes differs depending on the stressor in question. In a study with crystalline silica, Umbright *et al.,* were able to show that increases in pulmonary leukocytes at 3 weeks, preceded increases in total albumin (loss of ACM integrity) at 6 weeks. In another publication by Zeidler-Erdely *et al.*, mice exposed to stainless steel welding fumes had an increased amount of alveolar macrophages 1 day post exposure, while alveolar damage (as measured by total protein) was not evident until 4 days post exposure.	Empirical Support: Moderate. There is both temporal and dose-response evidence to suggest that an increased amount of pro-inflammatory immune cells potentiates alveolar capillary damage. However, few studies assessing these KEs include multiple concentrations and timepoints, and as such, these KEs are typically reported as occurring together (i.e. damage is detected along with an increase in cell abundance). (Umbright et al., 2017; Zeidler-Erdely et al., 2011)
KE3 --> KE4; Relationship 1705	Few studies have directly assessed the direct of ACM integrity loss on the induction of a Th2 response. In one publication, He *et al.,* showed that ROS induced by a specific superoxide dismutase induces M2 polarization in asbestosis, and inhibition of signalling by Jmjd3 reduces ROS, M2 polarization, and fibrosis. In another study using NRF2 knockout mice, a significant Th2 bias is observed following bleomycin treatment, with enhanced fibrosis noted. Discrepancies are present, for instance where many groups have found that TNF-a receptor 1 (TNF-R1) and TNF-R2 are associated with fibrosis, and even though TNF-a is a therapeutic target for IPF and asbestosis in humans, other groups have reported the opposite and that its exogenous delivery can reduce the fibrotic burden.	Empirical Support: Moderate. There is limited *in vitro* and *in vivo* evidence to support a direct relationship between these two KEs, with some inconsistencies with respect to the specific mediators in question. (Ortiz et al., 1998; Piguet, 1989; Redente et al., 2014)
KE4 --> KE5; Relationship 1706	Activation of a Th2 response is known to activate lung fibroblasts. Research by Hashimoto *et al.,* indicates that the Th2 cytokines IL-4 and IL-13 induces differentiation of human fibroblasts to myofibroblasts. Furthermore, IL-13 has been shown to directly activate TGF-b in vivo, and lead to pulmonary fibrosis.	Empirical Support: High. There is a plethora of dose and time response evidence which shows that Th2 cytokines induce the activation and proliferation of fibroblasts. (Hashimoto et al., 2001; Lee et al., 2001)
KE5 --> KE6; Relationship 2625	While it is difficult to show the accumulation and incorporation of extracellular matrix (ECM) *in vitro*, the levels of soluble collagen can be assessed. Many publications have reported secretion of soluble matrix components by activated myofibroblasts. For example, research by Li *et al.,* has shown that soluble cadmium can induce fibrosis in mice, and that *in vitro* treatment of fibroblasts with cadmium induces expression of alpha smooth muscle actin (hallmark of myofibroblasts), as well as soluble collagen.	Empirical Support: High. It is generally accepted knowledge that activated myofibroblasts are collagen secreting cells. (Blaauboer et al., 2014; Hinz, 2016a; Li et al., 2017)
KE6 --> AO; Relationship 1629	Pulmonary fibrosis results from excessive accumulation of collagen and extracellular matrix in the lungs, in the context of prolong inflammation, injury, and an aberrant healing response. Idiopathic pulmonary fibrosis is the most common form in humans, with a poor prognosis overall.	Empirical Support: High. Excessive ECM deposition is the defining characteristic of pulmonary fibrosis, and the evidence to support this relationship is unequivocal. (Meyer, 2017; Thannickal et al., 2004; Williamson et al., 2015; Zisman et al., 2005)

Quantitative Consideration

The presented AOP is mostly qualitative and additional studies are needed to support the essentiality of the KEs and to build KERs. However, it is important to note that it is difficult to experimentally demonstrate the relevance of earlier KEs to the end outcome of fibrosis because of the redundancy in pathways involved. The mode or type of interactions between the resident cell membrane and a substance is dependent on the specific physical-chemical characteristics of the substance. There has been an attempt to determine quantitatively the dose at which the events in AOP 173 are induced with respect to CNTs (Labib et al., 2016; reproduced below). In this manuscript, researchers applied global transcriptomic analysis and benchmark dose (BMD) modelling to determine the dose at which the MIE, KE1, KE2, KE4, KE5, and KE6 are induced using samples from three separate studies and compared the results to the apical BMD of the AO of pulmonary fibrosis. From the results shown, it can be seen that the BMD intervals of transcriptional pathway induction for each KE largely overlap but are representative of the BMD of AO induction. These results serve to highlight the parallel nature of the KEs in AOP 173, with many of the events occurring concurrently in addition to occurring sequentially.

Quantitative concordance table for AOP 173 KERs. Data is reproduced from Labib et al., 2016 (Figure 4., Additional file 4: Table S3). CNT: carbon nanotube. N/A: Not assessed

Stressor	Species	Time Point	MIE[a] (1495)	KE1[a] (1496)	KE2[a] (1497)	KE3 (1498)	KE4[a] (1499)	KE5[a] (1500)	KE6[a] (68)	AO (1458)
Mitsui 7 CNT	Mouse	24 Hr	4 – 9	3 - 7	9 – 13	N/A	5 – 11	10 – 21	9 – 13	N/A
Mitsui 7 CNT	Mouse	3 / 7 day	11 – 22	6 – 22	14 – 24	N/A	9 – 16	15 – 26	17 – 34	N/A
Mitsui 7 CNT	Mouse	28 day	No Effect	14 – 26	36 – 51	N/A	14 – 26	11 – 20	No Effect	N/A
Mitsui 7 CNT	Mouse	56 day	N/A	N/A	N/A	N/A	N/A	N/A	N/A	14 – 27[b]
NRCWE-026 CNT	Mouse	24 Hr	No effect	8 – 15	20 – 37	N/A	8 – 15	21 – 39	No Effect	N/A
NRCWE-026 CNT	Mouse	3 / 7 day	16 – 28	16 – 27	19 – 33	N/A	15 – 24	16 – 26	19 – 36	N/A
NRCWE-026 CNT	Mouse	28 day	No Effect	No Effect	No Effect	N/A	12 – 20	No Effect	No Effect	N/A
NM-401 CNT	Mouse	24 Hr	No Effect	3 – 20	8 – 22	N/A	8 – 22	13 – 22	18 – 29	N/A
NM-401 CNT	Mouse	3 / 7 day	11 - 17	12 - 19	12 - 20	N/A	7 - 20	14 - 22	13 – 21	N/A
NM-401 CNT	Mouse	28 day	20 - 37	17 - 28	No Effect	N/A	No Effect	13 - 21	18 – 31	N/A

[a]: Benchmark dose (BMD) (Benchmark dose low (BMDL) à BMD) intervals in µg / lung based on transcriptional pathway induction.

[b]: BMDL – BMD interval in µg / lung based on alveolar thickness.

The MIE of substance interaction with the lung cell membrane is intentionally kept broad and vague, to reflect the many interactions pro-fibrotic substances can have with the plasma membrane of cells. The presented AOP, while applicable to both soluble and persistent stressors, is specifically applicable to substances which induce fibrosis through immune responses. Nanomaterials are a group of such substances, which interact with organisms and cells via a dynamic biomolecular corona that is dependant on the biological microenvironment. While great strides have been made in recent years to characterize and understand this corona and how it impacts cellular recognition, further research is needed in order to accurately describe the specific interactions necessary for the initiation of fibrosis pathogenesis. Indeed, this is also true for model soluble stressors such as bleomycin, for which cellular binding and uptake is incompletely understood.

The specific mediators involved in the first KE (KE1; Event 1496), and the threshold necessary for progression to subsequent KEs is incompletely understood. Knockout models have shown that ablation of alarmins, such as IL-1, changes the initial trajectory of pulmonary fibrosis, however, compensation from other pathways makes it difficult to determine its essentiality to the end pathogenesis.

The role of reactive oxygen species (ROS) and oxidative stress in potentiating pulmonary fibrosis is also ambiguous. Many pro-fibrotic substances induce the formation of ROS and subsequent oxidative stress, as do many non-fibrotic stressors. While it is hard

to deny that ROS and oxidative stress serve an important role in fibrosis (by increasing cellular injury, potentiating an environment of chronic inflammation & damage, and activation of pro-fibrotic factors like TGF-b, a causal relationship between the two has not been established. Furthermore, anti-oxidant treatment in IPF patients have been largely unsuccessful, indicating a lack of knowledge of the specific redox mechanisms involved. Recent research has indicated a potential role of specific redox mechanisms, such as mitochondrial ROS and NOX derived ROS, however further research is needed to elucidate their role in potentiating pulmonary fibrosis. The development of newer fibrosis model systems which better capitulate the human condition will assist in clarifying this aspect.

Considerations for Potential Applications of the AOP (optional)

This AOP is applicable to occupational exposures as lung fibrosis is frequently observed in miners and welders exposed to metal dusts.

Pulmonary fibrosis is a progressive debilitating disease with no cure. A number of environmental and occupational agents, such as cigarette smoke, agriculture or farming, wood dust, metal dust, stone and sand dust, play a causative role in the development of lung fibrosis. More recently, laboratory experiments in animals have shown that exposure to nanomaterials, novel technology-enabled materials of sophisticated properties induce lung fibrosis. Fibrosis also develops in other organs (skin, liver, kidney, heart and pancreas) and the underlying mechanisms are similar. Thus, this AOP is applicable to screening of a broad group of suspected inhalation toxicants and allows the development of in silico and in vitro testing strategies for chemicals suspected to cause inhalation toxicity. Indeed, recent efforts aimed at collating all AOPs with potential relevance to NM risk assessment has led to the production of an AOP network which identified shared KEs of relevance to multiple AOs (Halappanavar et al., 2020). From this list, KE1 and KE2 from this AOP are among the most commonly shared between the various AOPs in the network. Shared KEs such as these can be prioritized for *in vitro* bio-assay development and tier-1 testing strategies. In a recent review, AOP 173 was used as a case study to define a testing strategy consisting of a slew of targeted bio assay alternatives that can be used to screen for the *in vivo* occurrence of a number of the contained KEs (Halappanavar et al., 2021). These recent efforts serve to highlight the utility of AOP 173 in guiding the development of rapid screening strategies as well as research recommendations spanning across multiple AOPs with shared events.

This AOP is also currently being used by the various European Union nano research consortia to inform the design and development of relevant in vitro and in silico models for screening, prioritising, and assessing the potential of nanomaterials to cause inhalation hazard. Specifically, this AOP has recently informed the development of a Nano Quantitative Structure Activity Relationship (NanoQSAR) model of CNT induced pulmonary inflammation, which found that the transcriptional response is associated with the aspect ratio of the nano fibres (Jagiello et al., 2021). Furthermore, this AOP can also inform the creation of biomarkers for fibrosis, such as the preliminary biomarker PFS17, which was produced using global transcriptional datasets from mice exposed to CNTs (Rahman et al., 2020). Although in a preliminary stage, this signature composed of 17 genes can be used to assess the response of the MIE (Event 1495), KE1 (Event 1496), KE2 (Event 1497), KE4 (Event 1499), and KE5 (Event 1500), based on the differential expression of key bioinformatics-informed transcripts.

Given the fact that a number of pharmacological agents and allergens cause fibrosis via a similar mechanism; the mechanistic representation of the lung fibrotic process in an AOP format, clearly identifying the individual KEs potentially involved in the disease process, enables visualisation of the possible avenues for therapeutic interference in humans.

Confidence in the AOP

Mechanistically, there is enough evidence to support the occurrence of each individual KE in the process of lung fibrosis as described. There is also enough evidence to support each KERs. However, as mentioned earlier, the early KEs constitute an organism's defence system and thus exhibit high heterogeneity in the signalling pathways and biological networks involved. Therefore, the results of the essentiality experiments may show incongruence based on the individual protein, gene or a pathway selected for intervention.

How well characterised is the AOP?

The adverse outcome is established and there is some quantitative data for some stressors.

How well are the initiating and other key events causally linked to the outcome?

The occurrence of each individual KE in the process leading to lung fibrosis is well accepted and established. However, individual studies mainly focus on a single KE and its relationship with the end AO. Quantitative data to support individual KERs is scarce.

What are the limitations in the evidence in support of the AOP?

As described earlier, attempts have been made to establish an in vitro model to predict the occurrence of fibrosis. However, the model has not been validated for screening the potential fibrogenic substances; the model has been used to identify drug targets that can effectively inhibit the progression to fibrosis (Chen et al., 2009). This is mainly due to the inability to accurately capture the responses induced by different cell types involved, and the intricate dynamics between the cell types, biological pathways and the biomolecules involved. Studies conducted to date have mainly focussed on the adverse outcome.

Is the AOP specific to certain tissues, life stages/age classes?

Fibrosis is a disease that affects several organ systems in an organism including lung, liver, heart, kidney, skin, and eye. The hallmark events preceding the end AO are similar to the one described here for lung fibrosis and involve similar cell types and

biomolecules. Thus, the AOP can be extended to represent fibrosis in other organs. The AOP is mainly applicable to adults as evidence to support applicability to different life stages is lacking. Lung fibrosis is thought to be a disease of male subjects. The early inflammatory KEs represented in this AOP constitute functional changes that describe inflammation in general. Several diseases are known to be mediated by inflammation and thus, early KEs in this AOP can be extended to any study investigating inflammation mediated adverse outcomes.

Are the initiating and key events expected to be conserved across taxa?

The events and pathways captured in this AOP are suggested to be conserved across different species and the process itself is influenced by the physical-chemical properties of the toxic substance.

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Appendix 1

List of MIEs in this AOP

Event: 1495: Substance interaction with the lung resident cell membrane components

Short Name: Interaction with the lung cell membrane

Key Event Component

Process	Object	Action
pattern recognition receptor signaling pathway		increased
toll-like receptor signaling pathway	Toll-like receptor	increased
toll-like receptor 4 signaling pathway	Toll-like receptor 4	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the lung resident cell membrane components leading to lung fibrosis	MolecularInitiatingEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

As stated earlier, there are many different ways by which pro-fibrotic stressors can interact with the components of cell membrane and often involve multiple interactions at the same time. Few studies investigate the exact interaction between the stressor and the cellular membrane components. Asbestos and silica crystals engage scavenger receptors present on the macrophages (Murthy et al., 2015). Bleomycin binds high affinity bleomycin binding sites present on rat alveolar macrophage surfaces, leading to macrophage activation (Denholm and Phan, 1990). However, the consequences of such interactions such as, the release of PRR agonists DAMPs (alarmins) from dying or injured cells, increased gene or protein synthesis downstream of receptor binding or in the case of NMs, their cellular uptake, are measured routinely as indicative of occurrence of such interactions (Nel et al., 2009; Cheng et al., 2013). Because of the phys-chem properties such as surface charge, NMs and asbestos like materials can bind to cellular macromolecules and cell surface/membrane components, which in turn, facilitate their uptake and intracellular sequestration by the cells (NIOSH, 2011a; Pascolo et al., 2013). Several DAMPs that can be effectively measured in biological samples and cultured cells include High Mobility Group Binding 1 (HMGB1) protein, Heat Shock proteins (HSPs), uric acid, annexins, and S100 proteins (Bianchi, 2007). Of all DAMPs, interleukin (IL)-1α is the most commonly measured alarmin. IL-1α is the principal pro-inflammatory moiety and is a designated 'alarmin' in the cell that alerts the host to injury or damage (Di Paolo and Shayakhmetov, 2016). It is shown that administration of necrotic cells to mice results in neutrophilic inflammation that was entirely mediated by IL-1α released from the dying or necrosed cells and consequent activation of IL-1 Receptor 1 (IL-1R1) signalling (**Suwara** et al., 2014). IL-1α is released following exposure to MWCNTs (Nikota et al., 2017) and silica (Rabolli et al., 2014). Although IL1-b is not a designated alarmin, its secretion following exposure to stressors is routinely assessed and is linked to initiation of cell or tissue injury.

Other high aspect ratio fibres such as asbestos and CNTs induce frustrated phagocytosis and acute cell injury (Boyles et al., 2015; Dörger et al., 2001; Brown et al., 2007; Kim et al., 2010; Poland et al., 2008), leading to DAMP release (Nikota et al, 2017), inflammation and immune responses.

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence

Human, mouse, rat.

Although the expression of DAMPs following exposure to pro-fibrotic substances is not assessed across species, it is known that alarmins are released after trauma or injury, and their release is important for initiating the inflammatory response in all species including humans. The immediate acute inflammatory response involving DAMP signalling is also observed in human IPF; however, anti-inflammatory drugs have proven ineffective for treating IPF. Danger signalling axis including uric acid, ATP and IL-33/ST2 has been proven to promote lung fibrosis in animals.

Key Event Description

The human lung consists of approximately 40 different resident cell types that play different roles during homeostasis, injury, repair and disease states (Franks et al., 2008). Of these, resident airway epithelial cells, alveolar/interstitial macrophages and dendritic cells are well characterised for their ability to sense the danger upon interaction with harmful substances and relay the message to mount the necessary immune/inflammatory response. The resident macrophages are present in all tissues, and in a steady state, macrophages contribute to epithelial integrity, survey the tissue for invading pathogens or chemicals and maintain an immunosuppressive environment. Their main function is to clear the incoming irritants and microbes. They are named differently based on the tissue type and their specific functions (Kierdorf et al., 2015).

Substance interactions:

The chemicals or pathogens interact with cellular membrane to gain access to the organisms' interior. A predominant interaction mechanism involves the recognition of innate immune response agonists by pattern recognition receptors (PRRs) present on resident cells such as epithelial and alveolar macrophages. PRRs are also present on other immune and parenchymal cells. PRRs can be activated by two classes of ligands. Pathogen Associated Molecular Patterns (PAMPs) are microbial molecules derived from invading pathogens. PAMPs will not be discussed further as pathogens are not the focus for the AOP presented here. The other class of ligands are called Danger Associated Molecular Patterns (DAMPs) that include cellular fragments, nucleic acids, small molecules, proteins and even cytokines released from injured or dying cells. Most fibrogenic stressors discussed in this AOP act via DAMPs-driven PRR activation. High aspect ratio (HAR) materials such as asbestos or carbon nanotubes (CNTs) pierce the cellular membrane of epithelial cells or resident macrophages resulting in cell injury or non-programmed cellular death. Alveolar macrophages trying to engulf High Aspect Ratio (HARs) fibres that are long and stiff undergo frustrated phagocytosis because of their inability to engulf the piercing fibres and subsequently lead to cell injury (Mossman and Churg, 1998; Donaldson K et al., 2010). The cellular debris from injured or dying cell then serves as ligands for PRRs (Nakayama, 2018), leading to cell activation. In case of pro-fibrotic insoluble particles such as silica, coal dust and nanomaterials (NMs), the particle adsorbed opsonins such as, immunoglobulins, complement proteins, or serum proteins act as ligands to the receptors on the macrophage cell surface (Behzadi et al., 2017). The tissue response to these materials resembles that observed following foreign body invasion in lungs.

Toll-like receptors (TLRs) are highly conserved PRRs that are associated with fibrogenic stressors (Desai et al., 2018). Inhibition of TLR-4 is protective against bleomycin-induced fibrosis (Li et al., 2015). However, the exact role and mechanisms by which TLRs mediate lung fibrosis are yet to be uncovered and some studies have shown TLRs to be protective against lung fibrosis (Desai et al., 2018). Asbestos and silica crystals are suggested to engage scavenger receptors present on the macrophages. Mice deficient in class A scavenger receptor MARCO are shown to induce reduced fibrogenic response following chrysotile asbestos exposure; although, the direct binding of MARCO by asbestos is not investigated in the study (Murthy et al., 2015). In case of soluble substances such as bleomycin, paraquat (Dinis-Oliveira et al., 2008) (N, Ndimethyl-4, 4′-bipyridinium dichloride) and other soluble fibrogenic chemicals, direct damage of lung epithelial cells and resulting cellular debris or secreted cytokines (DAMPs) serve as triggers for downstream cascading pro-inflammatory events, tissue injury and fibrosis. Engagement of PRRs and consequent cell activation is observed in various organisms including flies and mammals (Matzinger, 2002).

How it is Measured or Detected

Detection of Danger Associated Molecular Patterns (DAMPs) or homeostasis-altering molecular processes (HAMPs):

Cellular interaction with substances or particles can be measured by assessing the release of DAMPs from stressed, injured or dying cells - indicative of binding of PRRs on the cell surface. Release of DAMPs is reflective of substance interaction with resident cells and their activation, a key step in the process of inflammation.

The release of DAMPs can be measured by the techniques listed in the published literature (Suwara et al., 2014; Nikota et al., 2017; Rabolli et al., 2014).

Targeted enzyme-linked immunosorbent assays (ELISA) (routinely used and recommended):

ELISA assays – permit quantitative measurement of antigens in biological samples. For example, in a cytokine ELISA (sandwich ELISA), an antibody (capture antibody) specific to a cytokine is immobilised on microtitre wells (96-well, 386-well, etc.). Experimental samples or samples containing a known amount of the specific recombinant cytokine are then reacted with the immobilised antibody. Following removal of unbound antibody by thorough washing, plates are reacted with the secondary antibody (detection antibody) that is conjugated to an enzyme such as horseradish peroxidase, which when bound, will form a sandwich with

the capture antibody and the cytokine (Amsen and De Visser, 2009). The secondary antibody can be conjugated to biotin, which is then detected by addition of streptavidin linked to horseradish peroxidase. A chromogenic substrate can also be added, which is the most commonly used method. Chromogenic substrate is chemically converted by the enzyme coupled to the detection antibody, resulting in colour change. The amount of colour detected is directly proportional to the amount of cytokine in the sample that is bound to the capture antibody. The results are read using a spectrophotometer and compared to the levels of cytokine in control samples where cytokine is not expected to be secreted or to the samples containing known recombinant cytokine levels.

IL-1a and IL-1b is activated or secreted into the cytosol following stimulus. Targeted ELISA can be used to quantify IL-1a or IL1b that is released in the culture supernatant of the cells exposed to toxicants, in bronchoalveolar lavage fluid and serum of exposed animals. The assay is also applicable to human serum, cerebrospinal fluid, and peritoneal fluids.

Similarly, other alarmins can also be quantified by ELISA. Westernblot is another method that can be used to quantify the release of various alarmins using specific antibodies. qRT-PCR or ELISA assays can also be used to quantify expression of genes or proteins that are regulated by the receptor binding – e.g. downstream of TLR binding.

Frustrated phagocytosis and cellular uptake of NMs:

In vitro, interaction of NMs with the cellular membrane is investigated by assessing their uptake by lysosomes (Varela et al., 2012). Immunohistochemistry methods targeting lysosome specific proteins are regularly employed for this purpose. In co-localisation experiments, lysosomal marker LAMP1 antibody is used to detect particle co-localisation with lysosomes. A combination of Cytoviva hyperspectral microscope and immunolocalisation (Decan et al., 2016) or confocal microscopy to visualise co-localisation of fluorescence labelled nanoparticles with lysosomal markers have been used. Frustrated phagocytosis is assessed using microscopic techniques (Donaldson et al., 2010).

Cellular co-culture models of the pulmonary epithelium:

Complex co-culture systems, such as those containing epithelial cells and immune cells, better model the environment of the lung epithelium and can be used to study the interaction of potentially pro-fibrotic fibres and particles with resident lung cells. This type of model has been used, alongside electron microscopy, to study lung cell interactions with CNTs following 24 Hr *in vitro* exposure (Clift *et al.,* 2014). More recently, the EpiAlveolar model, which contains primary human alveolar epithelial cells, endothelial cells, as well as fibroblasts was assessed for its ability to predict fibrosis induced by CNTs (Barasova *et al.,* 2020). Using laser scanning, fluorescence, and enhanced darkfield microscopy, CNT interaction with the resident cells of the model was shown, and this interaction induced the formation of holes in the epithelial model (Barasova *et al.,* 2020). While new co-culture models are a better recapitulation of the native lung environment as compared to traditional mono-cultures, the increased complexity necessitates enhanced expertise in tissue culture techniques, and can make them less practical as compared to submerged mono culture methods.

Ex vivo model of the lung – Precision Cut Lung Slices:

Even closer to the *in vivo* condition than co-culture models, precision cut lung slice (PCLS) techniques capture the native lung architecture, cell-cell communication and cellularity of the lung. Advancement in culturing and cryopreservation techniques has increased accessibility and use of PCLS for longer term studies (Bai *et al.*, 2016, Neuhaus *et al.*, 2017). These slices can be cultured *ex vivo* for up to a week with minimal reduction in viability, and the technique has recently been assessed for its applicability to assess nanomaterial induced fibrosis *ex vivo* (Rahman *et al.,* 2020). Using MWCNT and darkfield microscopy, interaction between the nanofibers and the lung epithelium could be determined. The main downside of this technique is the animal requirement, which precludes their use in a first-pass screening context for the MIE.

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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 lung resident cell membrane components leading to lung fibrosis	KeyEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
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 of Low Molecular Weight Organic Chemicals to Proteins leads to Sensitisation (Sensitization) of the Respiratory Tract	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

Biological Context

Level of Biological Organization

Cellular
Level of Biological Organization

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 GM-CSF are involved in the adaptive immune responses. The pro-inflammatory cytokines include IL-1 family (IL-1a, IL-1b, IL-1ra, IL-18, IL-36a, IL-36b, IL-36g, IL-36Ra, IL-37), IL-6 family, TNF family, IL-17, and IFNg (Turner et al., 2014). While IL-4 and IL-5 are considered T helper (Th) cell type 2 response, IFNg 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, TNFa, IFNg 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 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 ROS (reactive oxygen species). Exposure to crystalline silica induces release of inflammatory cytokines (TNFa, IL-1, IL-6), transcription factors (NF-kb, AP-1) and kinase signalling pathways in mice that contain NFkB luciferase reporter (Hubbard et al., 2002). Boyles et al., 2015 found that lung responses to long MWCNTs included high expression levels of pro-inflammatory mediators MCP-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 NMs 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.

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.

ELISA assays – 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-a, IL-1b, and the pro-fibrotic TGF-b using ELISA assays (Barasova *et al.,* 2020). A similar, but less complicated co-culture model of immortalized human alveolar epithelial cells and IPF patient derived fibroblasts was used to assess pro-fibrotic signalling, and noted enhanced secretion of PDGF and bFGF (basic fibroblast growth factor), 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.

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Event: 1497: Increased, recruitment of inflammatory cells

Short Name: Recruitment of inflammatory cells

Key Event Component

Process	Object	Action
inflammatory response	inflammatory cell	increased
macrophage activation	macrophage	increased
neutrophil activation	neutrophil	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the lung resident cell membrane components leading to lung fibrosis	KeyEvent
Aop:303 - Frustrated phagocytosis-induced lung cancer	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	

AOP ID and Name		Event Type
[Acute Respiratory Distress Syndrome (ARDS)](#)		Key Event
[Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer](#)		Key Event

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	[NCBI](#)
mouse	Mus musculus	High	[NCBI](#)
rats	Rattus norvegicus	High	[NCBI](#)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	High

Human, mouse, rat

Key Event Description

Pro-inflammatory cells originate in bone marrow and are recruited to the site of infection or injury via circulation following specific pro-inflammatory mediator (cytokine and chemokine) signalling. Pro-inflammatory cells are recruited to lungs to clear the invading pathogen or the toxic substance. Monocytes (dendritic cells, macrophages, and neutrophils) are subsets of circulating white blood cells that are involved in the immune responses to pathogen or toxicant stimuli. They are derived from the bone marrow. They can differentiate into different macrophage types and dendritic cells. They can be categorised based on their size, the type of cell surface receptors and their ability to differentiate following external or internal stimulus such as increased expression of cytokines. Monocytes participate in tissue healing, clearance of toxic substance or pathogens, and in the initiation of adaptive immunity. Recruited monocytes can also influence pathogenesis (Ingersoll et al., 2011). Sensing or recognition of pathogens and harmful substances results in the recruitment of monocytes to lungs (Shi and Pamer, 2011). Activated immune cells secrete a variety of pro-inflammatory mediators, the purpose of which is to propagate the immune signalling and response, which when not controlled, leads to chronic inflammation, cell death and tissue injury. Thus, Event 1496 and Event 1497 act in a positive feedback loop mechanism and propagate the proinflammatory environment.

***Literature* evidence for its perturbation:**

Macrophages accumulate in bronchoalveolar fluid (BALF) post-exposure to bleomycin (Phan et al., 1980; Smith et al., 1995). NM-induced inflammation is predominantly neutrophilic (Shvedova et al., 2005; Rahman L et al., 2016; Rahman et al., 2017; Poulsen et al., 2015). An increased number of neutrophils (Reynolds et al., 1977) is observed in the BALF of patients with idiopathic pulmonary fibrosis. Eosinophils are a type of white blood cells and a type of granulocytes (contain granules and enzymes) that are recruited following exposure to allergens, during allergic reactions such as asthma or during fibrosis (Reynolds et al., 1977). MWCNTs induce increased eosinophil count in lungs (Købler C et al., 2015). MWCNTs act as allergens and induce lung infiltration of eosinophils and cause airway hypersensitivity (Beamer et al., 2013).

It is important to note that the stressor-induced Event 1495, Event 1496, and Event 1497 are part of the functional changes that we collectively consider as inflammation, and together, they mark the initiation of acute inflammatory phase. Event 1495 and Event 1496 occur at the cellular level. Event 1497 occurs at the tissue level.

How it is Measured or Detected

In vivo, recruitment of pro-inflammatory cells is measured using BALF cellularity assay. The fluid lining the lung epithelium is lavaged (BALF) and its composition is assessed as marker of lung immune response to the toxic substances or pathogens. BALF is assessed quantitatively for types of infiltrating cells, levels and types of cytokines and chemokines. Thus, BALF assessment can aid in developing dose-response of a substance, to rank a substances' potency and to set up no effect level of exposure for the

regulatory decision making. For NMs, in vivo BALF assessment is recommended as a mandatory test (discussed in ENV/JM/MONO(2012)40 and also in OECD inhalation TG for NMs). Temporal changes in the BALF composition can be prognostic of initiation and progression of lung immune disease (Cho et al., 2010).

In vitro, it is difficult to assess the recruitment of pro-inflammatory cells. Thus, a suit of pro-inflammatory mediators specific to cell types are assessed using the same techniques mentioned above (qRT-PCR, ELISA, immunohistochemistry) in cell culture models, as indicative of recruitment of cells into the lungs. Alternatively, the use of precision cut lung slices can allow for limited assessment of recruitment of tissue resident inflammatory cells, based on the repertoire of cells remaining in the specific slice following harvesting. This method was used to show that there is a histological increase in inflammatory foci following treatment with bleomycin and MWCNTs (Rahman et al., 2020). Finally, more complicated microfluidic lung-on-a-chip devices can be used to assess the migration of select immune cells and fibroblasts toward a simulated epithelium following treatment with a pro-fibrotic compound (He et al., 2017). However, this method is limited to two cell types, and it lacks the reservoirs of immune cells present in the body in vivo.

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Event: 1498: Loss of alveolar capillary membrane integrity

Short Name: Loss of alveolar capillary membrane integrity

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the lung resident cell membrane components leading to lung fibrosis	KeyEvent
Aop:302 - Lung surfactant function inhibition leading to decreased lung function	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

lung

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	Not Specified	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Male	High
Female	Not Specified

Key Event Description

The alveolar-capillary membrane (ACM) is the gas exchange surface of the lungs that is only ~0.3μm thick and is the largest surface area within the lung that separates the interior of the body from the environment. It is comprised of the microvascular endothelium, interstitium, and alveolar epithelium. As a consequence of its anatomical position, and the large surface area, it is the first point of contact for any inhaled pathogen, particles or toxic substances. Thus, ACM is subjected to injury constantly and rapidly repaired following the external insults without formation of fibrosis or scar tissue. The extent of ACM injury or how rapidly its integrity is restored is a pivotal determinant of whether the lung restores its normal functioning following an injury or is replaced by fibrotic lesion or scar tissue (Fukuda et al., 1987; Shwarz et al., 2001). Significant loss of endothelium and epithelium of the ACM results in loss of the barrier and membrane integrity. Increased membrane permeability leading to efflux of protein-rich fluid into the peribronchovascular interstitium and the distal airspaces of the lung, disruption of normal fluid transport via downregulated Na channels or malfunctioning Na+/K+ATPase pumps, loss of surfactant production, increased expression of epithelial or endothelial cell markers such as intercellular adhesion molecule-1 (ICAM-1) or decreased expression of surfactant D are few of the markers of decreasing lung compliance arising from the lost integrity of ACM (Johnson and Matthay, 2010).

***Literature* evidence for its perturbation:**

Bleomycin exposure causes alveolar barrier dysfunction (Miyoshi et al., 2013). Cigarette smoke impairs tight junction proteins and leads to altered permeability of the epithelial barrier (Schamberger et al., 2014). Exposure to bleomycin destroys the structural architecture of tight junctions, increases permeability, epithelial death and loss of specialised repair proteins such as claudins. Thoracic radiation and bleomycin induced lung injury results in decreased expression of E-cadherin and Aquaporin-5 expression (Almeida et al., 2013; Gabazza et al., 2004).

Repeated exposure to or biopersistent toxic substances, pathogens or lung irritants initiate non-resolving inflammation and ACM injury (Costabel et al., 2012). Chronic inflammation mediated by overexpression of cytokines such as IL-1 (Kolb et al., 2001), TNFa (Sime et al., 1998), T-helper type 2 cytokine IL-13 or exposure to specific proteinases initiate ACM injury, leading to significant loss of the epithelium and endothelium of the ACM resulting loss of barrier integrity. In patients diagnosed with idiopathic pulmonary fibrosis (IPF), both type 1 pneumocyte & endothelial cell injury with ACM barrier loss is observed.

Bleomycin and silica exposure generate persistent inflammation and lung damage (Chua et al., 2005; Thrall and Scaliso, 1995). Exposure to SWCNTs induces persistent inflammation, granuloma formation and diffuse intestinal fibrosis in mice after pharyngeal aspiration (Shvedova *et al.,* 2005). MWCNTs act as allergens and induce lung infiltration of eosinophils and cause airway hypersensitivity (Beamer *et al.,* 2013). Inhaled particles induce chronic inflammation (Hamilton et al., 2008; Thakur et al., 2008; Ernst *et al.,* 2002). Increased numbers of alveolar macrophages, neutrophils and eosinophils are observed in the BALF of patients suffering from IPF and chronic inflammation is associated with decreased survival (Parra *et al.*, 2007; Schwartz *et al.,* 1991; Yasuoka *et al.,* 1985).

The BALF of patients diagnosed with interstitial diseases contains increased levels of 8-isoprostane (Psathakis *et al.*, 2006) and carbonyl-modified proteins (Lenz et al., 1996), markers of oxidative modification of lipids and proteins. In vivo, increased ROS levels in rodents (Ghio *et al.*, 1998) and enzymatic production of nitric oxide in rat alveolar macrophages is observed after asbestos exposure (Quinlan *et al.*, 1998). Some nanoparticles induce oxidative stress that contributes to cellular toxicity (Shi *et al.*, 2012). NADPH oxidase derived ROS is a critical determinant of the pulmonary response to SWCNTs in mice (Shvedova *et al.*, 2008). Oxidative lipidomics analysis of the lungs of CNT-exposed mice showed, phospholipid oxidation (Tyurina *et al.*, 2011). ROS synthesis is suggested to be important for inflammmosome activation involving NLR-related protein 3 complex, activated caspase-1 and IL-1b, which is observed following exposure to a variety of pro-inflammatory stimuli including, asbestos and crystalline silica (Cassel *et al.*, 2008; Dostert *et al.*, 2008) and long needle-like CNTs. In the case of asbestos, frustrated phagocytosis triggered ROS synthesis leads to inflammmosome activation, which is associated with asbestos induced pathology (Dostert *et al.*, 2008).

How it is Measured or Detected

Proteinosis, BAL fluid protein content:

Compromised ACM barrier integrity in vivo can be measured by measuring total protein or total albumin content in the BAL fluid derived from experimental animals exposed to lung toxicants or in human patients suffering from lung fibrosis. In addition to albumin, the total urea in BAL fluid is also a good indicator of the ACM integrity loss (Schmekel et al., 1992).

Cell type considerations:

ACM loss is a tissue level event. In vitro, assays with human cells are desired; however, the use of cells derived from experimental animals including alveolar macrophages, dendritic cells, epithelial cells, and neutrophils are routinely used. Primary cells are preferred over immortalised cell types that are in culture for a long period of time. In vitro, studies often assess the altered expression of pro-inflammatory mediators, increased ROS synthesis or oxidative stress and cytotoxicity events, an interplay between these three biological events occurring following exposure to stressors, is suggested to induce cell injury, which is reflective of tissue injury or loss of ACM (Halappanavar *et al.,* 2019) in vivo.

Cytotoxicity assessment:

Cellular viability or cytotoxicity assays are the most commonly used endpoints to assess the leaky or compromised cell membrane. The most commonly employed method is the trypan blue exclusion assay – a dye exclusion assay where cells with intact membrane do not permit entry of the dye into cells and thus remain clear, whereas the dye diffuses into cells with damaged membrane turning them to blue colour. Other high throughput assays that use fluorescent DNA stains such as ethidium bromide or propidium iodide can also be used and cells that have incorporated the dye can be scored using flow cytometry.

LDH release assay is a very sensitive cytotoxicity assay that measures the amount of LDH released in the media following membrane injury. The assay is based on measuring the reduction of NAD and conversion of a tetrazolium dye that is measured at a wavelength of 490 nm.

The Calcein AM assay depends on the hydrolysis of calcein AM (a non-fluorescent hydrophobic compound that permeates live cells by simple diffusion) by non-specific intracellular esterases resulting in production of calcein, a hydrophilic and strongly fluorescent compound that is readily released into the cell culture media by the damaged cells.

Although the above mentioned assays work for almost all chemicals, insoluble substances such as NMs can confound the assay by inhibiting the enzyme activity or interfering with the absorbance reading. Thus, care must be taken to include appropriate controls in the assays.

Transepithelial/transendothelial electrical resistance (TEER):

TEER is an accepted quantitative technique that measures the integrity of tight junctions in cell culture models of endothelial and epithelial cell monolayers. They are based on measuring ohmic resistance or measuring impedance across a wide range of frequencies.

Other:

The other methods include targeted RT-PCR or ELISA assays for tight junction proteins, cell adhesion molecules and inflammatory mediators such as IFNg, IL-10, and IL-13. Advanced *in vitro* co-culture models, like the EpiAlveolar model system, and other similar systems present an intact capillary membrane that can be used to assess loss in the membrane integrity (via TEER) after exposure to pro-fibrotic stressors like crystalline silica and TGF-b (Barasova *et al.,* 2020, Kasper *et al.,* 2011).

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Event: 1499: Increased, activation of T (T) helper (h) type 2 cells

Short Name: Activation of Th2 cells

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the lung resident cell membrane components leading to lung fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Key Event Description

Naïve CD4+ T cells differentiate into four types of Th cells – Th1, Th2, Th17 and inducible regulatory T cells following exposure to infectious agents. The differentiation process begins when antigen presenting cells (APCs) come in contact with toxic substances and is mainly driven by cytokines that make up the microenvironment. For example, increased concentrations of IL-12 secreted by APCs in the environment may be biased towards Th1 type and increased IL-6 or IL-4 in the environment may commit to Th2 type differentiation. Th1 cytokines IFNg and IL-12 induce inflammation, aid in clearance of toxic substances, induce tissue damage and control the fibrotic responses. IFNg has suppressive effects on the production of extracellular matrix proteins including collagen and fibronectin. The Th2 response suppresses Th1 mediated response, which results in decreased Th1 cell-mediated tissue damage but at the same time contributing to the persistence of toxic substances leading to perpetuation of tissue damage, triggering uncontrolled healing response. The major sources of Th2 cytokines are Th2 cells themselves; however, mast cells, macrophages, epithelial cells and activated fibroblasts have shown to produce IL-4, IL-13 and IL-10 upon appropriate stimulation. Th2 cytokines IL-4 and IL-13 regulate wound healing.

Literature evidence for its perturbation in the context of pulmonary fibrosis:

For fibroplasia or fibrosis, the type of CD4+ T cell response that develops is crucial. Studies conducted in mice that do not express Th2 cytokines IL-4, IL-5 and IL-13 show complete attenuation of fibrosis despite the highly active Th1 response. Th2 cytokines IL-4 and IL-13 are elevated in fibrotic lungs; IL-13 activates TGFb1 and initiates fibroblast proliferation and differentiation in lung fibrosis (Lee *et al.,* 2001). Overexpression of IL-13 induces sub-epithelial airway fibrosis in mice in the absence of any other external pro-inflammatory or pro-fibrotic stimulus (Zhu *et al.,* 1999). Both MWCNTs and SWCNTs induce elevated expression of IL-4 and IL-13 in BALF of mouse lungs (Park *et al.,* 2011), and increased levels of IL-25 and IL-33 in BALF and mouse lungs exposed to MWCNTs (Dong and Ma, 2016). In a rare human study, increased levels IL-4 and IL-5 were observed in the sputum of humans exposed to MWCNTs at an occupational setting (Fatkhutdinova *et al.,* 2016). Overexpression of IL-10 increases IL-4 and IL-13 production and lung fibrosis following exposure to silica (Barbarin *et al.,* 2005). Alveolar macrophages from asbestosis patients (a form of lung fibrosis) exhibit M2 phenotype (He et al., 2013). Ex vivo culture of alveolar macrophages obtained from BALF of patients suffering from IPF with collagen type I showed enhanced levels of M2 macrophage markers CCL-18, CCL-2 and CD204 (Stahl *et al.,* 2013). Th2 response associated expression of IL-33 cytokine enhances polarisation of M2 macrophages and inducing M2-mediated expression of IL-13 and TGFb1 in mice (Dong *et al.,* 2014). Cigarette smoke induces expression of genes associated with M2 sub-phenotypes, which is further enhanced in smokers presenting with COPD (Shaykhiev *et al.,* 2009).

How it is Measured or Detected

Targeted enzyme-linked immunosorbent assays (ELISA) or real-time quantitative polymerase chain reaction (qRT-PCR) (routinely used and recommended):

The ELISA and qRT-PCR are routinely used to assess the levels of protein and mRNA of several Th1 and Th2 cytokines including IL-4, IL-5, IL- 13, IL-10, IL-12, IFNg. In addition, the levels of transforming growth factor b (TGFb) is also assessed, expression of which is increased following induction of IL-13 synthesis. The other genes of relevance to Th2 response and eventual pro-fibrotic response include Arg-1 and Arg-2. BALF supernatant collected from lungs of animals exposed to toxic substances or human patients is used. Tissue homogenates or cell pellets can also be used. Expression of these genes and proteins can be assessed in in vitro cell cultures exposed to pro-fibrotic stimulus. Apart from assaying single protein or gene at a time, cytokine bead arrays or cytokine PCR arrays can be used to detect a whole panel of Th1 and/or Th2 cytokines using a multiplex method. This method is quantitative and especially advantageous when the sample amount available for testing is scarce. The details of ELISA and qRT-PCR are described under Event 1495. The details of BALF sample collection is described under Event 1497.

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Event: 1500: Increased, fibroblast proliferation and myofibroblast differentiation

Short Name: Increased cellular proliferation and differentiation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the lung resident cell membrane components leading to lung fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Male	High
Female	Not Specified

Key Event Description

Fibroblasts are non-hematopoietic, non-epithelial and non-endothelial cells. In steady state conditions, they are distributed throughout the mesenchyme. During the wound healing process, fibroblasts are rapidly recruited from mesenchymal cells or in case of exaggerated repair, and they can also be derived from fibrocytes in the bone marrow. They are not terminally differentiated. They synthesise structural proteins (fibrous collagen, elastin), adhesive proteins (laminin and fibronectins) and ground substance (glycosaminoglycans – hyaluronan and glycoproteins) proteins of the ECM that provide structural support to tissue architecture and function. Fibroblasts play an important role in ECM maintenance and turnover, wound healing, inflammation and angiogenesis.

They provide structural integrity to the newly formed wound. Fibroblasts with a-smooth muscle actin expression are called myofibroblasts. It is thought that differentiating fibroblasts residing in the lung are the primary source of myofibroblast (CD45 Col I α-SMA) cells (Hashimoto et al., 2001; Serini and Gabbiani, 1999). Myofibroblasts can also originate from epithelialmesenchymal transition (Kim et al., 2006). The other sources of fibroblasts include fibrocytes that likely originate in the bone marrow and migrate to the site of injury upon cytokine signaling. Fibrocytes are capable of differentiating into fibroblasts or myofibroblasts, and comprise less than 1% of the circulating pool of leukocytes and express chemokines CCR2, CXCR4 and CCR7 in addition to a characteristic pattern of biomarkers, including collagen I and III, CD34, CD43 and CD45 (Bucala *et al.*, 1994; Chesney et al., 1998; Abe et al., 2001). In bleomycin induced lung fibrosis model, human CD34 CD45 collagen I CXCR4 cells (fibrocytes) are shown to migrate to the lungs in response to both bleomycin and CXCL12 (which is the only chemokine known to bind to CXCR4) (Phillips et al., 2004). Myofibroblasts exhibit features of both fibroblasts and smooth muscle cells. The myofibroblasts synthesise and deposit ECM components that eventually replace the provisional ECM. Because of their contractile properties, they play a major role in contraction and closure of the wound tissue. Apart from secreting ECM components, myofibroblasts also secrete proteolytic enzymes such as metalloproteinases and their inhibitors tissue inhibitor of metalloproteinases, which play a role in the final phase of the wound healing which is scar formation phase or tissue remodelling.

Literature **evidence for its perturbation** *in the context of pulmonary fibrosis:*

IPF is characterised by progressive fibroblast and myofibroblast proliferation and excessive deposition of extracellular matrix (Kuhn and McDonald., 1991). High levels of a-SMA protein and increased number of a-SMA positive cells were observed in mouse lungs treated with MWCNTs as early as day 1 post-exposure (Dong et al., 2015). Fibrotic lesions observed in mice treated with asbestos show proliferating fibroblasts and collagen deposition. The same study also demonstrated that BALF supernatant derived from asbestos exposed lungs was sufficient to stimulate fibroblast proliferation in vitro (Lemaire et al., 1986). Fibrotic foci developed in rat lungs following exposure to bleomycin show a-SMA expressing myofibroblasts (Vyalov et al., 1993). Several in vitro studies have shown fibroblast proliferation following CNT treatment (Wang et al., 2010a; Wang et al., 2010b; Hussain et al., 2014).

How it is Measured or Detected

Immunohistochemistry (routinely used and recommended):

Proliferation of fibroblasts and activation of myofibroblasts is normally detected using individual antibodies against vimentin, procollagen 1 and alpha-smooth muscle actin, specific markers of fibroblasts and myofibroblasts (Zhang, 1994). It is recommended to use more than one marker to confirm the activation of fibroblasts. The species-specific antibodies for all the markers are commercially available and the technique works in both in vitro and in vivo models as well as in human specimens. Immunohistochemistry is performed using immunoperoxidase technique. Formalin fixed and paraffin embedded lung sections are sliced in 3-5μm thin slices and reacted with diluted H O for 10 min to block the endogenous peroxidase activity. The slices are then incubated with appropriate dilutions of primary antibody against the individual markers followed by incubation with the secondary antibody that is biotinylated. The slices are incubated for additional 30 minutes for avidin-biotin amplification and reacted with substrate 3'3' diaminobenzidine before visualising the cells under the light microscope. Although only semiquantitative, morphometric analysis of the lung slices can be conducted to quantify the total number of cells expressing the markers against the control lung sections where expression of specific markers is expected to be low or nil. For the morphometric analysis, using ocular grids, images of 20-25 non-overlapping squares (0.25 mm) from 2-3 random lung section are taken under 20x magnification. Minimum of three animals per treatment group are assessed. Some researchers include only those cells that are positive for both procollagen I and alpha smooth muscle markers.

The limitation of the technique is that the antibodies have to be of high quality and specific. Background noise due to non-specific reactions can yield false-positive results.

In vitro, expression of type-1 collagen, Thy-1, cyclooxygenase-2 and vaeolin-1 are used as markers of homogeneous population of fibroblasts. Increased expression of TGF-b and a-smooth muscle actin is used as markers of differentiated myofibroblasts. Transcription factor Smad3 is the other marker measured in vitro to assess the fibroblast proliferation and differentiation. Several in vitro studies using lung epithelial cells (e.g. A549 cells) have shown that asbestos induces markers of epithelial-mesenchymal transition (Tamminen et al., 2012), which is mediated by the activation of TGF-β-p-Smad2 (Kim et al., 2006).

Hydrogels:

Hydrogels are water-swollen crosslinked polymer networks. They are used to mimic the original extracellular matrix (ECM). Hydrogels consist of collagen, fibrin, hyaluronic acid or synthetic materials such as polyacrylamide enriched with ECM proteins, etc. Hydrogels can be prepared to express inherent biological signals, mechanical properties (e.g., modulus) and biochemical properties (e.g., proteins) of the ECM. Fibroblasts are usually cultured in fibrin and type-1 collagen that represent the matrix of the wound healing. Thus, the well-constructed hydrogel can be used to assess cell proliferation, activation and matrix synthesis as reflective of fibroblast activation. For naturally derived hydrogen scaffolds, cells derived directly from animal or human tissues can be used (Smithmyer et al., 2014).

Fibroblast proliferation assay:

Several primary and immortalised fibroblast types can be used for the assay. Proliferation assays such as water-soluble tetrazolium salts (WST)-1 and propidium iodide (PI) staining of cells have been used to show dose-dependent increase in MWCNT-induced increase in fibroblast proliferation that is in alignment with in vivo mouse fibrogenic response (Vietti et al., 2013; Azad et al., 2013) to the same material.

Advanced co-culture models (myofibroblast differentiation):

Co-culture models that mimic the alveolar capillary membrane (such as those listed for Event 1496 & Event 1498) can be used to assess myofibroblast differentiation in response to pro-fibrotic stressors using immunofluorescent staining for a-SMA. More complex in vitro microfluidic lung-on-a-chip models (such as the one listed for Event 1497) can be used to assess myofibroblast differentiation in the same stead. These provide a more realistic exposure model as opposed to a submerged monoculture of fibroblasts, however they require a higher degree of technical skill and advanced fabrication which may not be suitable for all labs.

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Event: 68: Accumulation, Collagen

Short Name: Accumulation, Collagen

Key Event Component

Process	Object	Action
collagen biosynthetic process	collagen	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:38 - Protein Alkylation leading to Liver Fibrosis	KeyEvent
Aop:241 - Latent Transforming Growth Factor beta1 activation leads to pulmonary fibrosis	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:382 - Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis	KeyEvent
Aop:173 - Substance interaction with the lung resident cell membrane components leading to lung fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term
connective tissue

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Humans: Bataller and Brenner, 2005; Decaris et al., 2015.

Mice: Dalton et al., 2009; Leung et al., 2008; Nan et al., 2013.

Rats: Hamdy and El-Demerdash, 2012; Li, Li et al., 2012; Natajaran et al., 2006; Luckey and Petersen, 2001.

Key Event Description

Collagen is mostly found in fibrous tissues such as tendons, ligaments and skin. It is also abundant in corneas, cartilage, bones, blood vessels, the gut, intervertebral discs, and the dentin in teeth. In muscle tissue, it serves as a major component of the endomysium. Collagen is the main structural protein in the extracellular space in the various connective tissues, making up from 25% to 35% of the whole-body protein content. In normal tissues, collagen provides strength, integrity, and structure. When tissues are disrupted following injury, collagen is needed to repair the defect. If too much collagen is deposited, normal anatomical structure is lost, function is compromised, and fibrosis results.

The fibroblast is the most common collagen producing cell.Collagen-producing cells may also arise from the process of transition of differentiated epithelial cells into mesenchymal cells (EMT). This has been observed e.g. during renal fibrosis (transformation of tubular epithelial cells into fibroblasts) and in liver injury (transdifferentiation of hepatocytes and cholangiocytes into fibroblasts) (Henderson and Iredale, 2007)·

There are close to 20 different types of collagen found with the predominant form being type I collagen. This fibrillar form of collagen represents over 90 percent of our total collagen and is composed of three very long protein chains which are wrapped around each other to form a triple helical structure called a collagen monomer. Collagen is produced initially as a larger precursor molecule called procollagen. As the procollagen is secreted from the cell, procollagen proteinases remove the extension peptides from the ends of the molecule. The processed molecule is referred to as collagen and is involved in fiber formation. In the extracellular spaces the triple helical collagen molecules line up and begin to form fibrils and then fibers. Formation of stable crosslinks within and between the molecules is promoted by the enzyme lysyl oxidase and gives the collagen fibers tremendous strength (Diegelmann,2001)· The overall amount of collagen deposited by fibroblasts is a regulated balance between collagen synthesis and collagen catabolism. Disturbance of this balance leads to changes in the amount and composition of collagen. Changes in the composition of the extracellular matrix initiate positive feedback pathways that increase collagen production.

Normally, collagen in connective tissues has a slow turn over; degradating enzymes are collagenases, belonging to the family of matrix metalloproteinases (MMPs).Other cells that can synthesize and release collagenase are macrophages, neutrophils, osteoclasts, and tumor cells (Di Lullo et al., 2001; Prockop and Kivirikko, 1995; Miller and Gay, 1987;Kivirikko and Risteli, 1976).

How it is Measured or Detected

Determination of the amount of collagen produced in vitro can be done in a variety of ways ranging from simple colorimetric assays to elaborate chromatographic procedures using radioactive and non-radioactive material. What most of these procedures have in common is the need to destroy the cell layer to obtain solubilized collagen from the pericellular matrix. Rishikof et al describe several methods to assess the in vitro production of type I collagen: Western immunoblotting of intact alpha1(I) collagen using antibodies directed to alpha1(I) collagen amino and carboxyl propeptides, the measurement of alpha1(I) collagen mRNA levels using real-time polymerase chain reaction, and methods to determine the transcriptional regulation of alpha1(I) collagen using a nuclear run-on assay (Rishikof et al., (2005) .

Histological staining with stains such as Masson Trichrome, Picro-sirius red are used to identify the tissue/cellular distribution of collagen, which can be quantified using morphometric analysis both in vivo and in vitro. The assays are routinely used and are quantitative.

Sircol Collagen Assay for collagen quantification:

The Serius dye has been used for many decades to detect collagen in histology samples. The Serius Red F3BA selectively binds to collagen and the signal can be read at 540 nm (Chen & Raghunath, 2009; Nikota et al., 2017).

Hydroxyproline assay:

Hydroxyproline is a non-proteinogenic amino acid formed by the prolyl-4-hydroxylase. Hydroxyproline is only found in collagen and thus, it serves as a direct measure of the amount of collagen present in cells or tissues. Colorimetric methods are readily available and have been extensively used to quantify collagen using this assay (Chen & Raghunath, 2009; Nikota et al., 2017).

Ex vivo precision cut tissue slices

Precision cut tissue slices mimic the whole organ response and allow histological assessment, an endpoint of interest in regulatory decision making. While this technique uses animals, the number of animals required to conduct a dose-response study can be reduced to 1/4th of what will be used in whole animal exposure studies (Rahman et al., 2020).

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List of Adverse Outcomes in this AOP

Event: 1458: Pulmonary fibrosis

Short Name: Pulmonary fibrosis

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:241 - Latent Transforming Growth Factor beta1 activation leads to pulmonary fibrosis	AdverseOutcome
Aop:173 - Substance interaction with the lung resident cell membrane components leading to lung fibrosis	AdverseOutcome
Aop:347 - Toll-like receptor 4 activation and peroxisome proliferator-activated receptor gamma inactivation leading to pulmonary fibrosis	AdverseOutcome

Stressors

Name
Bleomycin

Carbon nanotubes, Multi-walled carbon nanotubes, single-walled carbon nanotubes, carbon nanofibres
Name

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

lung

Evidence for Perturbation by Stressor

Bleomycin

Bleomycin is a potent anti-tumour drug, routinely used for treating various types of human cancers (Umezawa H et al., 1967; Adamson IY, 1976). Lung injury and lung fibrosis are the major adverse effects of this drug in humans (Hay J et al., 1991). Bleomycin is shown to induce lung fibrosis in animals – such as dogs (Fleischman RW et al., 1971), mice (Adamson IY and Bowden DH, 1974), and hamsters (Snider GL et al., 1978) and is widely used as a model to study the mechanisms of fibrosis (reviewed in **Moeller** A et al., **2008;** Gilhodes J-C et al., 2017).

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Carbon nanotubes, Multi-walled carbon nanotubes, single-walled carbon nanotubes, carbon nanofibres

Carbon nanotubes (CNTs) are allotropes of carbon, are made of rolled up sheet of graphene (single-walled carbon nanotubes) and are tubular in shape. A multi-walled carbon nanotube (MWCNT) is a multi-layered concentric cylinder of graphene sheets stacked one inside the other (N. Saifuddin et al., 2013). CNTs exhibit a combination of unique mechanical, thermal, and electronic properties and are highly desired commercially. They are light weight but their tensile strength is 50 times higher than that of steel, and they are stable chemically as well as in the environment. Consequently, they are produced in massive amounts and are increasingly incorporated in several industrial products.

CNTs are high aspect ratio materials and are shown to cause lung fibrosis in animals (Muller J et al., 2005; Porter DW et al., 2010; Dong and Ma 2016; Vietti, et al., 2016). In an intelligence bulletin published by NIOSH on 'Occupational exposure to carbon nanotubes and nanofibers', NIOSH reviewed 54 individual animal studies investigating the pulmonary toxicity induced by CNTs and reported that half of those studies consistently showed lung fibrosis (NIOSH bulletin, 2013). However, the evidence is inconsistent and the occurrence of fibrotic pathology is influenced by the specific physical-chemical properties of CNTs (i.e. length, rigidity), their dispersion in exposure vehicle, and the mode of exposure (Duke and Bonner 2018).

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Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Description

Pulmonary fibrosis is broadly defined as the thickening or scarring of lung tissue, due to excessive deposition of extracellular matrix. In the normal human lung, the nasopharynx and the conducting airways are mainly covered by epithelium composed of ciliated, mucous secreting cells in direct contact with the basement membrane with submucosal glands containing goblet, duct, and serous cells also contributing to the fluid balance and mucous production (Koval & Sidhaye, 2017). Within this epithelium, basal cells are found which are stimulated to proliferate and differentiate in response to injury (Koval & Sidhaye, 2017). Further down the lung, in the terminal bronchiole region, the epithelium does not contain submucosal glands, but instead contains club cells which produce pulmonary surfactant and can differentiate into bronchiolar or alveolar epithelial cells. Finally, in the terminal airspaces, the epithelium is made up entirely of type I and type II alveolar epithelial cells. In between the two adjacent alveoli are two layers of alveolar epithelium resting on basement membrane, which consists of interstitial space, pulmonary capillaries, elastin and collagen fibres. Thus, the alveolar capillary membrane, where gas exchange takes place, is made up of the alveolar epithelium and alveolar endothelium (Gracey et al, 1968). In pulmonary fibrosis, damage to the pulmonary epithelium results in excessive deposition of collagen by constitutively activated myofibroblasts during the wound healing response. This causes a pronounced decrease in the number of capillaries within the alveolar septa with asymmetric deposition of collagen and cells between part of the surface of a capillary and the nearby alveolar lining. In areas where capillaries are not present, the alveolar capillary membrane is occupied with collagen and cells.

How it is Measured or Detected

In vivo, histopathological analysis is used for assessing fibrotic lung disease. Morphometric analysis of the diseased area versus total lung area is used to quantitatively stage the fibrotic disease. Although, some inconsistencies can be introduced during the analysis due to the experience of the individual scoring the disease, the histological stain, etc., a numerical scale with grades from 0 to 8, originally developed by Ashcroft et al., 1988 is assigned to indicate the amount of fibrotic tissue in histological samples. This scale is applied to diagnose lung fibrosis in both human and animal samples. Modifications to this scoring system were proposed (Hubner et al., 2008), which enables morphological distinctions thus enabling a better grading of the disease. Using the modified scoring system, bleomycin induced lung fibrosis in rats was scored as follows: Grade 0 – normal lung, Grade 1 – isolated alveolar septa with gentle fibrotic changes, Grade 2 – knot like formation in fibrotic areas in alveolar septa, Grade 3 – contiguous fibrotic walls of alveolar septa, Grade 4 – single fibrotic masses, Grade 5 – confluent fibrotic masses, Grade 6 – large contiguous fibrotic masses, Grade 7 – air bubbles and Grade 8 – fibrotic obliteration. Further morphometric analysis can be conducted to quantify the total disease area (Nikota et al., 2017).

Lungs are formalin fixed and paraffin embedded such that an entire cross section of lung can be presented on a slide. The entire cross section is captured in a series of images using wide field light microscope. Areas of alveolar epithelium thickening and consolidated air space are identified. ImageJ software (freely available) is used to trace the total area (green line) and the diseased area (red line) imaged and quantified. The diseased area is equal to disease area/total area (Nikota et al., 2017).

In vitro, there is no single assay that can measure the alveolar thickness. However, a combination of assays spanning various KEs described above provide a measure of the extent of fibrogenesis potential of tested substances. qRT-PCR and ELISA assays measuring increased collagen, TGFβ1 and various pro-inflammatory mediators are used as sensitive markers of potential of substances to induce the adverse outcome of lung fibrosis.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1702: Interaction with the lung cell membrane leads to Increased proinflammatory mediators

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis	adjacent	Moderate	Moderate
Interaction with lung resident cell membrane components leads to lung cancer	adjacent	Moderate	Moderate

Key Event Relationship Description

Innate immune response is the first line of defence in any organism against invading infectious pathogens and toxic substances. It involves tissue triggered startle response to cellular stress and is described by a complex set of interactions between the toxic stimuli, soluble macromolecules and cells (reviewed in Nathan, 2002). The process culminates in a functional change defined as inflammation, purpose of which is to resolve infection and promote healing. In lungs, the interaction of toxic substances with resident cells results in cellular stress, death or necrosis leading to release of intracellular components such as alarmins (DAMPs, IL-1a, HMGB1). Released alarmins (danger sensors) bind cell surface receptors such as Interleukin 1 Receptor 1 (IL-1R1), Toll Like Receptors (TLRs) or others leading to activation of innate immune response signalling.

For example, binding of IL-1a to IL-1R1 can release Nuclear Factor (NF)-κb resulting in its translocation to nucleus and transactivation of pro-inflammatory genes including cytokines, growth factors and acute phase genes. The signalling also stimulates secretion of a variety of pro-inflammatory mediators. Overexpression of IL-1a in cells induces increased secretion of pro-inflammatory mediators. Products of necrotic cells are shown to stimulate the immune system in an IL-1R1-dependent manner (Chen et al., 2007).

The secreted alarmins activate resident cells pre-stationed in the tissues such as mast cells or macrophages leading to propagation of the already initiated immune response by releasing more eicosanoids, cytokines, chemokines and other pro-inflammatory mediators. Thus, secreted mediators signal the recruitment of neutrophils, which are the first cell types to be recruited in acute inflammatory conditions. Neutrophil influx in sterile inflammation is driven mainly by IL-1a (Rider P, 2011). IL-1 mediated signalling regulates neutrophil influx in silica-induced acute lung inflammation (Horning V, 2008). IL1 signalling also mediates neutrophil influx in other tissues and organs including liver and peritoneum. Other types of cells including macrophages, eosinophils, lymphocytes are also recruited in a signal-specific manner. Recruitment of leukocytesinduces critical cytokines associated with the Th2 immune response, including TNF-α, IL-1β, and IL-13.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this relationship is high. There is a mechanistic relationship between the MIE (Event 1495) and KE1 (Event 1496) which has been evidenced in a number of both in vitro and in vivo model systems in response to stressors such as, asbestos, silica, bleomycin, carbon nanotubes, and metal oxide nanoparticles (Behzadi *et al.,* 2017; Denholm & Phan 1990; Mossman & Churg 1998).

Increased expression of IL-1a or IL-1b following lung exposure to MWCNTs, bleomycin, micro silica particles, silica crystals, and polyhexamethyleneguanidine phosphate has been shown to be associated with neutrophil influx in rodents (Hornung et al., 2008; Girtsman et al., 2014; Gasse et al., 2007; Nikota et al., 2017; Suwara et al., 2013; Rabolli et al., 2014). Inhibition of IL-1 function by knocking out the expression of IL-1R1 using IL-1R1 KO mice or via treatment with IL-1a or IL-1b neutralising antibodies results in complete abrogation of lung neutrophilic influx following exposure to MWCNTs (Nikota et al, 2017), cigarette smoke (Halappanavar et al., 2013), silica crystals (Rabolli et al., 2014) and bleomycin (Gasse et al., 2017). In transgenic mice lacking IL1R1, Myd88 signalling or the IL-33 receptor St2, early inflammatory responses are suppressed following silica or bleomycin treatment (Dong, et al., 2014; Gasse et al., 2017).

Empirical Evidence

Empirical support for this KER is moderate. There are limited *in vitro* studies, which show a temporal and dose-dependent relationship between these two events, using the upregulation of specific surface receptors as a proxy for direct membrane interaction (Chan et al., 2018; Denholm & Phan, 1990; Roy et al., 2014). There are also studies that provide general support for the idea that an interaction with the lung resident cell membrane components leads to increased, secretion of pro-inflammatory and pro-fibrotic mediators (Table 1).

Dose-Response Evidence:

There are a few studies which provide evidence for a dose-response relationship in this KER. An *in vitro* study demonstrated a concentration-response relationship, in which silica exposure induced increases in pro-inflammatory cytokines through scavenger receptors in cultured bone marrow-derived murine mast cells. Cells were exposed to 6.25, 12.5, 25 or 50 mg/cm^2 SiO$_2$ for 24 h. Scavenger receptor MSR2 expression increased over time at 50 mg/cm^2 and in a concentration dependant relationship. Moreover, TNF-a, IL-13 and MCP-1 increased in a concentration-dependent manner (Brown et al., 2007). This provides indications that at higher concentrations of the stressor, the interaction with the lung resident cell membrane components (Event 1495) leads to an increased secretion of pro-inflammatory mediators (Event 1496).

Temporal Evidence:

In vitro and *in vivo* studies have demonstrated temporal concordance of the KEs.

Toll-like receptor 4 signal pathway was evaluated in differentiated macrophages exposed to silica at 2.5 mg/cm^2. After 16 and 24 h, the mRNA expression level of TLR4 increased. Moreover, the protein expression level of TLR-4 and related MyD88/TIRAP pathway increased at 24 h. Release of IL-1b, IL-6, IL-10, and TNF-a was induced by silica exposure at 24 h. Pre-treatment with TAK-242, an inhibitor of TLR4 signaling, suppressed the release of the cytokines (Chan et al., 2018).

Macrophages exposed to ZnO nanoparticles at 2.5 mg/mL for 24 h increased the expression level of TLR6 and MyD88, TRAF, and IRAK. At 24 h, they also observed an increase in the mRNA and protein levels of the pro-inflammatory cytokines IL-1b, IL-6, and TNF-a. These results demonstrated that ZnO nanoparticles induced pro-inflammatory mediators by TLR stimulation and MAPKs activation (Roy et al., 2014).

The pro-inflammatory IL-1b induced granulocyte migration and can be produced as a result of cellular detection of PAMPs. Mice exposed to 2.5 mg/mouse of silica by instillation showed an increase of mRNA expression of pro-IL-1b in BALF at 6, 12, and 24 h post-exposure in a time-dependent manner. At early time points (1 h, 3 h, 6 h), there was an increase in the release of an alarmin (IL-1a) which indicates that the alarmin was released due to cell damage leading to cytokine production and an inflammatory reaction. Moreover, at 24 h, the levels of mature IL-1b and neutrophil accumulation in BALF increased. Neutralization or deletion of IL-1a reduced the observed responses (Rabolli et al., 2014).

Epithelial damage can lead to the release of alarmins. In this stead, conditioned media from primary human bronchial epithelial cells (PBECs) exposed to thapsigargin was able to induce a pro-inflammatory response in primary human lung fibroblasts. PBECs were exposed to thapsigargin 20 mM for 2 h. After that, the cell culture medium was replaced, and cells were incubated for 24 h. At this time, the medium was recovered and used to culture lung fibroblast for 5 h. This conditioned media from epithelial cells damage contains the alarmin IL-1a, which induced increased gene expression of IL-6, IL-8, MCP-1, and GMC-CSF in fibroblasts. These responses were reduced with anti-IL-1a treatment (Suwara et al., 2014).

Heijink et al. 2015 conducted a similar strategy to identify the relationship between DAMPs and pro-inflammatory mediator release after exposure to cigarette smoke (CS). Neutrophils treated with CS bubbled for 1 min, released high levels of HMGB1 as a consequence of necrotic cell death. The cell-free supernatant, which contains HMGB1, was used to culture human bronchial epithelial cells, and after 24 h it promoted the production of CXCL 8 by lung epithelial cells. Pharmacological inhibitors, such as 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC) and RAGE antagonist peptide (RAP), reduced the effect of CXC8L release.

HMGB1 and HSP-70 can be released by damaged hepatocytes. In a study, mice were treated with acetaminophen 350 mg/Kg for 3 and 6 h. At these time points, the liver perfusate was obtained and an increase in HSP-70 and HMGB1 protein levels was observed. RAW 264.7 cells treated with the liver perfusate exhibited increased mRNA expression levels of MCP-1 and IL-1b (Brittany et al. 2010).

Female mice were intratracheally administered with bleomycin at 5 mg/kg to represent idiopathic pulmonary fibrosis. IL-33, a molecule that can act as a DAMP, increased in lungs after 3 and 7 days of treatment. In serum, at 7-, 14- and 28-days post-exposure, IL-4 and IL-13 increased. It was concluded that IL-33/ST2 signaling pathway is involved in pulmonary fibrosis by bleomycin (Xu et al., 2016).

Uncertainties and Inconsistencies

Attenuation or complete abrogation of KE1 (Event 1496) and KE2 (Event 1497) following inflammogenic stimuli is observed in rodents lacking functional IL-1R1 or other cell surface receptors that engage innate immune response upon stimulation. However, following exposure to MWCNTs, it has been shown that absence of IL-1R1 signalling is compensated for eventually and neutrophil influx is observed at a later post-exposure time point (Nikota et al., 2017). In another study, acute neutrophilic inflammation induced by MWCNT was suppressed at 24 hr in mice deficient in IL1R1 signalling; however, these mice showed exacerbated neutrophilic influx and fibrotic response at 28 days post-exposure (Girtsman et al., 2014). The early defence mechanisms involving DAMPs is fundamental for survival, which may necessitate activation of compensatory signaling pathways. As a result, inhibition of a single biological pathway mediated by an individual cell surface receptor may not be sufficient to completely abrogate the lung inflammatory response. Forced suppression of pro-inflammatory and immune responses early after exposure to substances that cannot be effectively cleared from lungs, may enhance the injury and initiate other pathways leading to exacerbated response.

Quantitative Understanding of the Linkage

A majority of the in vivo studies are conducted with only one dose and thus, it is difficult to derive quantitative dose-response relationships based on the existing data. However, it is clear from the studies referenced above that greater concentrations or doses of pro-fibrotic substances results in higher release of alarmins, and consequently, higher pro- inflammatory signalling. The above studies also demonstrate strong temporal relationships between the individual KEs.

Response-response relationship

One study has demonstrated a response-response relationship for this KER.

Human intervertebral disc cells were treated with 0, 0.5, 1, or 2 mg/ml of recombinant HMGB1 for 24 h. Protein levels were determined in cell medium supernatant by ELISA. HMGB1 stimulates the expression of IL-6 and MMP-1 in a response-response relationship. A strong correlation was observed by Spearman's rank correlation coefficient between HMGB1 treatment and IL-6 or MMP-1 levels (Shah et al., 2018).

Other reports have studied both KEs, but they do not indicate if the response-response relationship was linear or not (coefficient or correlation is not shown) (Fukuda et al. 2017; Kim et al., 2020, Piazza et al., 2013; Yang et al., 2012; Chakraborty et al., 2017).

Time-scale

Some studies have described how long after a change in the MIE (Event 1495; interaction substance and components), KE1 (Event 1496;pro-inflammatory mediators are secreted) is impacted (Table 3.).

Table 3. Time-scale related studies relevant to the MIE (Event 1495) - KE1 (Event 1496) relationship.

Reference	In vitro/in vivo/population study	Design	MIE (Event 1495) Timepoint	KE1 (Event 1496) Timepoint
Xu et al., 2016	In vivo	40 Female Kunming strain mice Bleomycin was intratracheally administered 5 mg/Kg. Days post-exposure	IL-33 3, 7 days	IL-4, IL-13 7, 14, and 28 days
Roy et al., 2014	In vitro	Primary mice macrophages exposed to 2.5 mg/ml ZnO for 24 hrs.	Increased TLR6 expression 0.5, 3, 6, 12, and 24 h	Increased IL-6, TNF-a 24 h
Rabollli et al., 2014	In vivo	Female C57BL/6 mice	Increased the release of IL-1 a	Increased mRNA expression of

		Exposed to silica 2.5 mg/mouse by instillation	1, 3, and 6 h	pro IL-1b 6, 12, and 24 h

Known Feedforward/Feedback loops influencing this KER

Pancreatic cancer cells stimulated with S100A8 and S100A9 released pro-inflammatory cytokines IL-8, TNF-a, and FGF. Cancer cell-derived conditioned media and the individual cytokines (TNF-a and TGF-b) induced the protein expression of S100A8 and S100A9 in HL-60 monocytic cell line and primary human monocytes (Nedjadi et al. 2018).

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Relationship: 1703: Increased proinflammatory mediators leads to Recruitment of inflammatory cells

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis	adjacent	Moderate	Low
Decreased fibrinolysis and activated bradykinin system leading to hyperinflammation	adjacent		
Frustrated phagocytosis leads to malignant mesothelioma	adjacent	High	Not Specified
Interaction with lung resident cell membrane components leads to lung cancer	adjacent	Moderate	Low

Key Event Relationship Description

Pro-inflammatory mediators are the chemical and biological molecules that initiate and regulate inflammatory reactions. They are secreted following inflammation or exposure to an inflammogen. Commonly measured pro-inflammatory mediators include IL-1 family cytokines, IL-4, IL-5, IL-6, TNFa, IFNg. (https://aopwiki.org/events/1496)

 Proinflammatory mediator increase is caused when there's increased inflammation. This can be found in many ways, including bradykinin system activation or hypofibrinolysis (Koller, https://doi.org/10.1161/ATVBAHA.119.313536).With more proinflammatory mediators, this causes increased signaling from proinflammatory cytokines, which promotes leukocyte recruitment, which will differentiate into proinflammatory cells ((Villenueve et al, https://doi.org/10.1093/toxsci/kfy047)). Increased proinflammatory mediators means this process happens more, which means increase recruitment of inflammatory cells.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this KER is high. There are very well established functional relationships between the secreted signalling molecules and the chemotactic effects on pro-inflammatory cells (Harris, 1954; Petri & Sanz 2018).

Increased proinflammatory mediators means more proinflammatory cytokines, chemokines, vasoactive amines, and lipid mediators (Villenueve et al, https://doi.org/10.1093/toxsci/kfy047). Increased Signaling from these Cytokines and Chemokines promote leukocyte recruitment to areas of infection, including monocytes and neutrophil (Leick et al, doi: 10.1007/s00441-014-1809-9). The

leukocytes will differentiate into mature proinflammatory cells, in response to mediators they encounter in the local tissue microenvironment (Villenueve et al, https://doi.org/10.1093/toxsci/kfy047). With higher levels of leukocytes from increased proinflammatory mediators, it causes an increase in proinflammatory cells (Libby, https://doi.org/10.1093/cvr/cvv188).

Empirical Evidence

The empirical support for this KER is moderate. There are many studies which show temporal and dose-dependent recruitment of immune cells following increases in pro-inflammatory mediators. However, these mediators exhibit pleiotropy, and knockdown or knockout of a single pathway or mediator can result in compensation and recruitment of immune cells at a later time, as is seen in Nikota et al.,. 2017. (Chen et al., 2016; Nikota et al., 2017; Schremmer et al., 2014) (Additional studies available in Table 1.).

Dose-Response Evidence:

Many studies provide dose-response evidence of this KER. For example, *in vitro* and *in vivo* studies testing stressors at different doses/concentrations have demonstrated a dose-response relationship; at the higher dose of the stressor, the pro-inflammatory mediators increased, leading to an increase of pro-inflammatory cell recruitment.

Ma, et al. (2016) studied inflammatory responses in male BALB/c mice exposed to multi-walled carbon nanotubes (MWCNT) administered intravenously at different doses (0.5-4 mg/kg) for 2 days. A dose-dependant relationship was found between the levels of the inflammatory mediators IL-6 and TNF-a and the MWCNT dose. At the highest dose, 4 mg/Kg, white blood cells, lymphocytes, and neutrophils levels increased.

Porter et al. (2020) have demonstrated that MWCNT caused dose-dependent and time-dependent pulmonary inflammation in male C57BL/6J mice. Animals received a single dose of 2.5, 10, or 40 mg/mouse. At 40 mg/mouse, IL-1b and IL-18 increased at one day post-exposure. Moreover, polymorphonuclear leukocyte increased on day 1, and after 7 days the number of inflammatory cells was higher.

Zinc oxide nanoparticles (NPs) can induce metal fume fever and acute inflammation. Female C57BL/6J mice were intratracheally instilled once at 11, 33, and 100 mg/kg with coated ZnO NPs. Inflammatory responses were evaluated after 1, 3, and 28 days of exposure. An increase in serum amyloid A3 mRNA in lung tissue was observed at 33 and 100 mg/kg. Neutrophils accumulated in BAL fluid after 28 days of exposure in a dose-dependant manner (Hadrup et al., 2019).

Polyhexamethyleneguanidine phosphate (PHMG-P) is used as a disinfectant. PHMG-P at 0.3, 0.9, and 1.5 mg/kg was instilled into the lungs of mice. At 7- and 14-days post-exposure an increase in the levels of pro-inflammatory markers (IL-1b, IL-6 and CXCL1) and an increase in mRNA levels of MCP1, MMP2, and MMP12 was seen. Moreover, on day 7, neutrophils were recruited to the inflamed site. These changes were observed in a dose-response manner (Song et al., 2014).

Bourdon et al. 2012 evaluated the toxicity of carbon black nanoparticles (CBNPs) in mouse lung and liver. C57BL/6 mice were exposed to Printex 90 CBNPs with 0.018, 0.054, or 0.162 mg, and after 1, 3, and 28 days of the single instillation, BAL fluid was analyzed. Polymorphonuclear cell counts in BAL increased in a dose-dependant manner with the strongest recruitment 1- and 3-days post-exposure and remained elevated at day 28. CBNP also increased the expression of Saa3 mRNA levels in lung tissue on days 1, 3, and 28 in a dose-dependant manner. Although this response decreased over time, the expression of Saa3 mRNA increased at all time points, which indicates a persistent acute phase response.

A study evaluated the mechanisms of toxicity after exposure to PM2.5 in a tri-culture system: A549 cells and THP-1 differentiated macrophages in the apical chamber; meanwhile, EA.hy926 endothelial cells were cultured in the basolateral chamber. The system was exposed to PM2.5 at three different concentrations 20, 60, and 180 mg/ml for 24 h. An increase in the pro-inflammatory mediators IL-6, IL-8, and TNF-a was observed, as well an increase in mRNA expression of MMP9, ICAM-1, and CAV-1. These genes are involved in the movement and recruitment of leukocytes in sites of inflammation. Changes were observed in a concentration-dependant manner (Wang et al., 2019).

In another study female C57BL/6 mice were exposed to 18, 54, or 162 mg of MWCNT/mouse via single intratracheal instillation. An increased gene expression of Cxcl1, IL-6, Mt2, Saa1, and Saa2 was observed in a dose-dependent manner at 24 h post-exposure. Moreover, an increase in the recruitment of pro-inflammatory cells was observed in a dose-dependent manner (Poulsen et al., 2013).

Temporal Evidence:

There is significant evidence of the temporal relationship between the two KES. *In vitro* and *in vivo* studies have demonstrated that pro-inflammatory mediators (Event 1496) increased prior to the recruitment of pro-inflammatory cells (Event 1497).

Female C57BL/6J mice were exposed to carbon nanoparticles at 20 mg/mouse via intratracheal instillation. An increase in the levels of cytokines CXCL1, CXCL2, and CXCL5 at 3 h post-exposure was observed, with peaks after 12 and 18 h post-exposure. These pro-inflammatory mediators preceded neutrophil recruitment (12 and 24 h post-exposure) (Chen et al., 2016). Alveolar macrophages (AM) were isolated from lungs 3 to 12 h after CNP instillation, but they did not show a pro-inflammatory response. The authors suggest that AM are not involved in the initiation of the inflammatory response. Meanwhile, ATII cells induced the highest CXCL levels and acute neutrophilic inflammation.

Nickel oxide NPs intratracheally instilled at one single dose 200 cm^2/rat into female Wistar rats induced an increase of pro-inflammatory cytokines in BALF, at 24 and 74 h for CINC-3 and eotaxin, respectively. At 24 h and 48 h, neutrophils were observed, and after 72 h, the levels of neutrophils, eosinophils, and macrophages increased (Lee et al., 2016).

Porter et al. (2002) have shown pulmonary inflammation in rats exposed to crystalline silica aerosol at a concentration of 15 mg/m^3 (6h/day, 5 days/week) for 116 days. Lung disease was linked to TNF-a and IL-10 production in a timely response (10-116 days). The number of polymorphonuclear cells in the BALF increased progressively from day 41 - 116.

One study has demonstrated a dose-response and temporal relationship for these two KEs (Patowary et al., 2020). Female Wistar rats were exposed to oleoresin capsicum (OC) sprays at 2, 6, and 10%, and after 1, 3, and 24 h post-exposure, blood cell and BALF cytokines were evaluated. The pro-inflammatory cytokine TNF-a increased in a dose-dependant manner, and polymorphonuclear cells increased in a time-dependant manner.

Schremmer et al. (2014) have reported the time course of chemotaxis *in vitro* in response to the challenge of biopersistent particles and their relation to inflammatory mediators. NR8383 rat alveolar macrophages were challenged with different types of particles for 1, 4, and 16 h. The cell supernatants obtained from different time points were used to evaluate the chemotaxis of unexposed NR8383 macrophages. They found that nanosized silica at 16 mg/cm^2 induced an elevated transcription of CCL4, CXCL1, CXCL3, and TNF-a in a time-dependant manner. The pro-inflammatory cytokines present in the supernatants induced chemotaxis of unexposed macrophages at 4 and 16 h post-exposure.

Husain et al. (2015) found increased expression of genes related to chemotactic recruitment of pro-inflammatory cells at 3 h and 1 day after exposure to 162 mg/mouse carbon black nanoparticles in female C57BL/6 mice. They observed an increase in the gene expression of pro-inflammatory mediators at day 1 (Cxcl2, Ccl2), day 3 (IL-17, IL-33), day 14 (Cd2), and day 42 (Cxcl) post-exposure. The KE2 (Event 1497) increased over time with the maximum levels of neutrophils, macrophages, eosinophils, and lymphocytes at 4- and 5-days post-exposure. This response suggests chronic inflammation occurs because of an incomplete resolution of acute inflammation.

Rahman et al. 2017 evaluated whether different TiO$_2$ NPs induce lung inflammation. C57BL/6 mice were exposed to 18, 54, 162, or 486 mg/mouse of TiO$_2$ NPs via single intratracheal instillation. At 1-day post-exposure, gene expression analysis showed more changes in genes associated with inflammation and fibrosis. Moreover, after 1- and 28-days post-exposure, an increase in cell counts in BALF was observed in a dose-dependant manner.

Ho et al., 2013 evaluated the inflammatory response in mice exposed to coated quantum dots (QD705-PEG, QD705-COOH) at 12 or 60 mg/mouse. At 2-, 17- and 90-days post-exposure, an increase in the level of TNF-a, IL-1b, IL-6, CXCL1, CCL2, CCL1, CCL17, and CXCL13 mRNA levels in lungs was observed and the amount of polymorphonuclear cells in BALF increased in a dose-dependent manner at day 7 post-exposure. The inflammatory response increased on days 2 and 17, but on day 90 decreased. QD705-COOH induced granulomas persistently presented from 2 to 90 days.

Morimoto et al. 2010 examined the different kinds of cytokines related to lung inflammation by nickel oxide exposure. Rats were intratracheally exposed to 0.33 mg/Kg and 0.66 mg/kg nickel oxide NPs and were sacrificed at day 3, after 1 week, 1, 3, and 6 months post exposure. Infiltration of alveolar macrophages in lung tissue and BALF was observed from day 3 to 3 months post exposure, with higher levels after 1 and 3 months. Before the recruitment of inflammatory cells, an increase in the level of pro-inflammatory cells as MCP-1 and IL-1b in BALF was observed. Nickel oxide nanoparticles induced a persistent inflammatory effect.

Kamata et al. (2011) studied the impact of carbon black nanoparticles on susceptible subjects with predisposing lung disease and the effects of nanoparticles on inflammation and fibrotic changes. To achieve this goal, female C57BL/6J mice were intratracheally administered with bleomycin 20 mg/mouse and carbon black nanoparticles 10 mg/mouse. Evaluations were performed post-exposure at different time points. An increase of IL-6 and CCL2 in BALF was observed at days 2 and 7. After 7- and 14-days, a recruitment of pro-inflammatory cells was observed. Oxidant injury (evaluated as nitrotyrosine expression) was observed after 7 days and 14 days. The levels of TGF-b$_1$ increased over time with the highest level at day 14. Finally, they observed an increase in lung collagen deposition, and a decrease in lung compliance at day 21.

Uncertainties and Inconsistencies

Attenuation or complete abrogation of KE1 (Event 1496) and KE2 (Event 1497) following inflammogenic stimuli is observed in rodents lacking functional IL-1R1 or other cell surface receptors that engage innate immune response upon stimulation. However, following exposure to MWCNTs, it has been shown that absence of IL-1R1 signalling is compensated for eventually and neutrophil influx is observed at a later post-exposure time point (Nikota et al., 2017). In another study, acute neutrophilic inflammation induced by MWCNT was suppressed at 24 hr in mice deficient in IL1R1 signalling; however, these mice showed exacerbated neutrophilic influx and fibrotic response at 28 days post-exposure (Girtsman et al., 2014). The early defence mechanisms involving DAMPs is fundamental for survival, which may necessitate activation of compensatory signalling pathways. As a result, inhibition of a single biological pathway mediated by an individual cell surface receptor may not be sufficient to completely abrogate the lung inflammatory response. Forced suppression of pro-inflammatory and immune responses early after exposure to substances that cannot be effectively cleared from lungs, may enhance the injury and initiate other pathways leading to exacerbated response.

Most of the studies evaluate one dose at different time points or one-time point at different concentrations. Moreover, some studies have demonstrated that a stressor can lead to the recruitment of pro-inflammatory cells, but the presence of pro-inflammatory mediators was not determined (Westphal et al., 2015).

Recruitment of pro-inflammatory cells is a key event that is complicated to replicate *in vitro* conditions as cell migration is induced by cooperative chemotactic mediators (Gouwy et al., 2015) which are produced and released from different cells. Therefore, more kinetics studies in co-culture techniques are needed to fill this gap.

Quantitative Understanding of the Linkage

A majority of the in vivo studies are conducted with only one dose and thus, it is difficult to derive quantitative dose-response relationships based on the existing data. However, it is clear from the studies referenced above that greater concentrations or doses of pro-fibrotic substances results in higher release of alarmins, and consequently, higher pro- inflammatory signalling. The above studies also demonstrate strong temporal relationships between the individual KEs.

Known Feedforward/Feedback loops influencing this KER

Activated pro-inflammatory cells secrete pro-inflammatory mediators, and those mediators' goal is to cause signalling and response, which can lead to chronic inflammation (https://aopwiki.org/events/1497). Chronic inflammation means proinflammatory mediators increase and increased recruitment of inflammatory cells acts in a positive feedback loop, which continues a pro-inflammatory environment.

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Relationship: 1704: Recruitment of inflammatory cells leads to Loss of alveolar capillary membrane integrity

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis	adjacent	Moderate	Moderate

Key Event Relationship Description

Acute lung injury followed by normal repair of the ACM results in rapid resolution of the tissue injury and restoration of tissue integrity and function. The irreversible loss of alveolar membrane integrity occurs when 1) acute inflammation is not able to get rid of the toxic substance or invading pathogen (this happens following exposure to a toxic substance that is persistent or when the host is repeatedly exposed to the substance over a long period of time, 2) acute inflammation, originally incited to protect the host from external stimuli and to maintain normal homeostasis, by itself damages the host, resulting in tissue injury, and 3) the host fails to initiate a resolution response, which is essential to override the self-perpetuating inflammation response (Nathan, 2002). Loss of type-1 epithelial cells and endothelial cells, the collapse of alveolar structures and fusion of basement membranes, and persistent proliferation of type II alveolar epithelial cells on a damaged ECM, mark this phase (Strieter and Mehrad, 2009). The lung tissues

from patients diagnosed with idiopathic pulmonary fibrosis show ultrastructural damage to the ACM with type-1 pneumocyte and endothelial cell injury (Strieter and Mehrad, 2009). In rodents treated with bleomycin, the damaged ACM resembles that seen in the fibrotic human lung (Grande *et al.,* 1998).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this KER is high. There is a mechanistic relationship between an increase in pro-inflammatory cells and mediators, and damage to the ACM (Bhalla et al., 2009; Ward 2003; Zemans et al., 2009).

Exposure to high doses of insoluble nanomaterials can impair the macrophage-mediated clearance process, initiating chronicity of inflammation characterized by cytokine release, ROS synthesis and the tissue damage cascade (Palecanda and Kobzik, 2001) and subsequently leading to tissue injury. For example, exposure to crystalline silica generates oxidative stress, increased release of pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6), activation of transcription factors (e.g. NF-κB, AP-1), and other cell signalling pathways including MAP and ERK kinase (Hubbard et al., 2001; Hubbard et al., 2002; Fubini and Hubbard 2003). In silicosis, TNF-α is suggested to play a critical role in the observed pathogenicity (Castranova et al., 2004), which in turn, is dependent on activation of NF-κB and ROS synthesis (Shi et al.,1998; Cassel et al.,2008; Kawasaki et al., 2015). It has been proposed that IPF is a disorder of elevated oxidative stress, with the existence of an oxidant-antioxidant imbalance in distal alveolar air spaces (MacNee, 2001). Several studies have reported that anti-oxidant treatment attenuates the bleomycin-induced oxidative burden and subsequent pulmonary fibrosis (Wang et al., 2002; Serrano-Mollar et al., 2003; Punithavathi, et al., 2000).

Mice deficient in Nalp3 showed reduced inflammation, lower cytokine production and dampened fibrotic response following exposure to asbestos or silica (Dostert et al., 2008). SWCNT exposure induces alveolar macrophage activation, enhanced oxidative stress, increased and persistent expression of pro-inflammatory mediators associated with chronic inflammation and severe granuloma formation in mice (Chou et al., 2008). Bleomycin treatment induces increased lung weight, epithelial cell death, inflammation, increased hydroxyproline content, collagen accumulation and fibrotic lesions in mice, all of which were elevated in mice deficient in Nrf2 (Cho et al., 2004). MWCNT-induced fibrotic response is the result of interplay between oxidative stress and inflammation, which determines the severity of the fibrotic pathology. Mice lacking Nrf2 (the nuclear factor erythroid 2-related factor 2), that is associated with mounting anti-oxidant defense against oxidative stress, exhibit exuberant fibrotic responses to MWCNT (Dong and Ma, 2016).

Empirical Evidence

The empirical support for this KER is moderate. There is both temporal and dose-response evidence to suggest that an increased amount of pro-inflammatiory immune cells potentiates alveolar capillary damage. However, few studies assessing these KEs include multiple concentrations and timepoints, and as such, these KEs are typically reported as occurring together (i.e. damage is detected along with an increase in cell abundance) (Umbright et al., 2017; Zeidler-Erdley et al., 2011; Additional references available in Table 1).

Dose-Response Evidence:

There are some studies that provide dose-response evidence of this KER. For example, *in vitro* and *in vivo* studies testing stressors at different doses/concentrations have demonstrated a dose-response relationship; at the higher dose/concentration of the stressor, the recruitment of pro-inflammatory cells increased leading to loss of alveolar-capillary membrane integrity.

Microvascular endothelial and the human lung adenocarcinoma cell lines in co-culture were exposed to 6-600 mg/ml silica nanoparticles (NPs). After 4 h of exposure and 20 h recovery, siCAM-1 (intracellular adhesion molecule 1), IL-6, and IL-8 increased in a concentration-dependant manner. These cytokines increased the recruitment and regulation of neutrophils. The study suggests that there is crosstalk of both cell types in co-culture that leads to basolateral cytokine secretion. Moreover, TEER decreased in a concentration-dependant manner after 4 h exposure, and changes in the assembly of cell-cell junctions were observed (Kasper J et al. 2011).

Polyhexamethylene guanidine phosphate is used for the prevention of microorganism growth in humidifiers. To evaluate the inflammation response after the exposure to this chemical, three human lung cells (Calu-3, differentiated THP-1, and HMC-1 cells) were culture and exposed at the air-liquid interface at 2.2, 4.4, 8.8, and 17.6 mg/ml for 1, 6 and 24 h. An increase in chemoattractant cytokine IL-8 release was observed in a concentration- and time-dependant manner. This response preceded the alveolar-capillary membrane integrity loss, which occurred at 24 h at the highest concentration. These changes were followed by reactive oxygen species (ROS) generation, an increase in the levels of MMP-2, TIMP-1, MMP-9, TIMP-2 mRNA expression, and the release of TNF-a, IL-6 and TGF-b1. Moreover, inflammatory cell infiltration, fibrosis, and the release of cytokines was observed in lung sections in Sprague-Dawley rats following a 3 week exposure (Kim et al., 2015).

Arras et al. (2001) studied the effect of IL-9 on the development of lung fibrosis after crystalline silica particle (DQ12) exposure. Transgenic Tg5 mice expressing high levels of IL-9 and wild type (WT) FVB mice were exposed to DQ12 particles by intratracheal instillation at 1 or 5 mg, and mice were sacrificed 2, 4, and 6 months after instillation. Recruitment of pro-inflammatory cells and an increase in the level of LDH and proteins were observed in BALF after 2 and 4 months at the highest exposure (5 mg). Hydroxyproline content in the lung increased over time with the highest levels seen 4 months after instillation. Moreover, IL-4 increased in a dose-dependent manner after 2 months of exposure. In contrast, IFN-g decreased after 2 months and 4 months after the highest exposure dose. In Tg5 mice, fibrosis was less severe than in WT mice. Moreover, intraperitoneal injection of IL-9 in C57BL/6 reduced the amplitude of silica-induced lung fibrosis associated with a recruitment of B lymphocytes in the lung

parenchyma.

Morimoto et al. (2015) studied the inflammatory response of cerium oxide NPs in the acute and chronic phases. Male F344 rats were exposed once to 0.2 mg or 1 mg of NPs. Total cell counts in BALF increased at 1 mg after one week, and decreased after 1 and 3 months. Only neutrophils increased at the lowest dose at 3 days and 1 week after exposure and dropped after 1, 3, and 6 months. LDH activity in BALF increased after 3 days of exposure and decreased over time (1 week to 3 months). In the chronic phase, the total cell counts increased 3-days post-exposure, with no effects after 1 and 3 months. LDH increased after 3 days of administration and decreased in a time-dependant manner. The effects were more severe at the highest dose.

Temporal Evidence:

There is strong evidence of a temporal relationship between the two KES. *In vitro* and *in vivo* studies have demonstrated that the recruitment of pro-inflammatory cells increased prior to loss of alveolar-capillary membrane integrity.

Mice exposed to aerosolized multiwalled carbon nanotubed (MWCNT) at 10mg/m^3 (5 h per day for 2, 4, 8 or 12 days) developed pulmonary inflammation 1-day post-exposure. There was an increase in the recruitment of polymorphonuclear cells, neutrophil chemoattractant, albumin concentration, and LDH activity in the whole lung fluid in a time-dependant manner (Porter et al., 2013).

Research has showb that crystalline silica induces pulmonary toxicity. Rats exposed to this stressor at 15 mg/m^3, 6 h/day, 5 days/week for 3, 6, and 12 weeks, showed an accumulation of macrophages and neutrophils in BALF. Moreover, an increase in BALF LDH activity and albumin content was also observed (Umbright et al., 2017).

Gautam et al. (1998) studied the effect of a chemotactic stimulant, fMLP, on polymorphonuclear cells resting on an endothelial cell monolayer in the upper compartment. Bovine aorta endothelial cells exposed to 10^{-7} M fMLP in the upper compartment induced adhesion of polymorphonuclear (PMN) cells followed by a decrease in transendothelial electrical resistance and an increase in protein permeability for 10-50 min in a time-dependant manner. The findings indicate that PMN are activated by fMLP and adhere to the endothelium which induces an increase in cytosolic free Ca^{2+}. This lead to a decrease in electrical resistance, leading to a structural rearrangement of endothelial cells which impairing their barrier integrity.

Paraquat is a herbicide that induces pulmonary toxicity. Chronic exposure to Paraquat results in inflammation, damage to alveolar epithelial cells, and fibrosis. In sheep exposed to Paraquat at 5 mg/Kg (intramuscular), there was an increase in the number of granulocytes after 1, 2, and 3 weeks in a dose-dependent manner. After 3 weeks of paraquat administration alveolar wall thickening was observed, and the concentration of lung malondialdehyde increased as an indicator of lipid peroxidation. These results indicate that the recruitment of pro-inflammatory cells precedes alveolar damage (Shinozaki et al., 1992).

Exposure to nickel NPs can induce oxidative stress and lung inflammation. In a dose-response study, mice were intratracheally instilled with 0, 10, 20, 50, and 100 mg per mouse of nickel NPs and sacrificed at day 3 post-exposure. There was an increase in the neutrophil count in BALF, LDH activity, and total protein in BALF in a dose-dependent manner; the highest response was observed at 50 mg per mouse.

In a time-response study, mice were intratracheally instilled with 50 mg per mouse of nickel NPs and sacrificed at days 1, 3, 7, 14, 28, and 42 post-instillation. The recruitment of neutrophils increased at day 1 and 3 post-exposure. The levels of TBARS and 8-OHdG increased after 3 days of instillation. LDH activity and total protein in BALF increased at day 3 and 7 post-exposure. These responses decreased after 14 days post-exposure. However, at 42-day post-exposure, an increase in the level of hydroxyproline content was observed in lung tissues exposed to NPs (Mo et al., 2019).

Park et al. (2009) used BALF indicators as a tool for evaluating radiation-induced lung damage. Sprague-Dawley rats received 20 Gy of radiation to the right lung. At 3, 7, 14, 28, and 56 days after radiation, rats were sacrificed. Total cells in the BALF increased at 14 and 28 days, with the highest levels at day 56. Meanwhile, total protein in BALF increased after 7 days of radiation and peaked at 28 days post-radiation. The levels of TGF-b increase after 56 days of radiation.

Sapoznikov et al. (2019) studied the role of neutrophils in the early disruption of the alveolar-capillary barrier in a ricin-induced ARDS mouse model. Female CD-1 mice were administered intranasally with crude ricin (50 ml; 7mg/Kg diluted in PBS), and after 3, 6, 24, 48, and 72 h animals were sacrificed. The neutrophil count increased at 24, 48, and 72 h post-exposure. Alveolar capillary membrane integrity loss was evaluated as Evans blue dye extravasation and the protein expression of VE-cadherin claudin 18, claudin 5, connexin 43, and occludin. After 6 h post-exposure, alveolar permeability increased in a time-dependent manner. From 3 h post-exposure, the decrease of junction proteins was evident. Animals treated with anti-ricin antibody, anti-Ly6G (neutrophil depletion), and marismat (MMP inhibition) showed less severity in alveolar membrane integrity loss.

Wan et al. (2017) studied the genotoxic effects of cobalt NPs (Nano-Co) and their capacity for causing oxidative stress and inflammation. Gpt delta transgenic mice were exposed to 50 μg/mouse of Nano-Co by intratracheal instillation, and animals were sacrificed at days 1, 3, 7, and 28 post exposure, as well as 4 months post exposure. The levels of CXCL1/KC and neutrophils increased at 1, 3, and 7 days post-exposure, but they decreased at day 28. LDH activity and protein content in BALF also increased, but their levels dropped after 28 days post-exposure. Four months after instillation, 8-OHdG levels were measured and were found in high levels. Moreover, histological changes were observed 7 days (infiltration of a large amount of neutrophils and macrophages in the alveolar space and septa, focal alveolar epithelial cell hyperplasia, and thickening of the alveolar wall) and 4 months (interstitial fibrosis, bronchiolization of the alveoli and collagen deposition in the alveolar septa) after the exposure.

Uncertainties and Inconsistencies

Although there is enough evidence to suggest a role for persistent inflammation and oxidative stress in ACM integrity loss, a direct

relationship is hard to establish as studies involving inhibition of early pro-inflammatory cellular influx alter other immune cell types, thereby altering the end outcome.

Quantitative Understanding of the Linkage

In the context of lung fibrosis, data supporting quantitative dose-response relationships between the individual KEs is scarce. A majority of the mechanistic studies investigating the role of inflammation in lung fibrosis report acute neutrophilic inflammation and how altering neutrophil influx acutely after exposure to a toxic substance alters the end fibrotic outcome. However, these studies do not characterise the impact on immediate downstream KEs including the loss of ACM integrity or chronic inflammation in the absence of acute neutrophilia. Few studies have shown such concordance. For example, in mice exposed to different doses of bleomycin, total number of cells in BALF increased in a dose-dependent manner with predominant neutrophil phenotype at 7 days post-exposure and macrophage dominance at 24 days post-exposure (Kim et al., 2010). Other studies have shown that upon onset of chronic inflammation, secondary stimuli such as persisting toxic substance can make the injured tissue highly sensitive to acute inflammatory stimuli and may in turn fuel the ongoing chronic inflammation and affect the disease process (Ma et al., 2016).

Time-scale

One publication examined the timescale of KE induction with relation to this KER, in the context of AOP 173. Mo et al., 2019 found that KE2 (Event 1497) (1 and 3 days post-exposure) precedes KE3 (Event 1498) (3 and 7 days post-exposure) in mice exposed to 50 µg per mouse of nickel nanoparticles by intratracheal instillation.

Reference	In vitro/in vivo/population study	Design	KE1 (Event 1496)	KE2 (Event 1497)	KE3 (Event 1498)	KE6 (Event 1501)
Mo Y et al., 2019	In vivo	Mice C57BL/6, 50 mg per mouse intratracheal instillation	CXCL1/KC 1- and 3-days post-exposure	Neutrophil content 1 and 3 days Post-exposure	LDH activity, oxidative stress protein content 3- and 7-days post-exposure	Hydroxyproline content 42 days post-exposure

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Relationship: 1705: Loss of alveolar capillary membrane integrity leads to Activation of Th2 cells

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis	adjacent	Moderate	Low

Key Event Relationship Description

During the tissue injury-mediated immune response, naïve CD4+ Th cells differentiate into two major functional subsets: Th1 and Th2 type. Both Th1 and Th2 secrete distinct cytokines that promote proliferation and differentiation of their respective T cell population and inhibit proliferation and differentiation of the opposing subset. Th2 cytokines including pro-inflammatory and fibrotic mediators such as GATA-3, IL-13 and Arg-1 are increased in lung-irradiation induced fibrosis (Wynn, 2004; Brush et al., 2007; Han et al., 2011). Th2 immune response is implicated in allergen-mediated lung fibrosis. Meta-analysis of gene expression data collected from lungs of mice exposed to various fibrogenic substances including MWCNTs, showed that the expression and function of Th2 response associated genes and pathways are altered in fibrotic lungs (Nikota et al., 2016). Exposure of mice lacking STAT6 transcription factor to MWCNTs resulted in abrogated expression of Th2 genes and reduced lung fibrosis (Nikota et al., 2017). IL-4, the archetypal Th2 cytokine is a pro-fibrotic cytokine and is elevated in IPF and lung fibrosis. Overexpression of pro-fibrotic Th2 cytokine IL-13 results in sub-epithelial fibrosis with eosinophilic inflammation (Wilson and Wynn, 2009). In silica-induced pulmonary fibrosis in mice, T regulatory lymphocytes are recruited to the lungs where they increase expression of platelet-derived growth factor (PDGF) and TGF-β (Maggi et al., 2005). Chemokines associated with the Th2 response in airway epithelial cells include CCL1, CCL17, CCL20, and CCL22 (Lekkerkerker et al., 2012).

Evidence Supporting this KER

Biological Plausibility

The biological plasubility of this KER is high as there is a mechanistic relationship between ACM injury (tissue damage), and the induction of a Th2 response (responsible for wound healing) (Gieseck et al., 2018; Wynn, 2004).

Empirical Evidence

The empirical support for this KER is moderate. There is limited in vitro and in vivo evidence to support a direct relationship between these two KEs, with some inconsistencies with respect to the specific mediators in question (Ortiz et al., 1998; Piguet, 1989; Redente et al., 2014; Additional references can be found in Table 1).

In mice lacking both TNFα receptor 1 (TNF-R1) and receptor 2 (TNF-R2) or in wild type mice treated with anti-TNFα, bleomycin-induced lung fibrosis is attenuated (Ortiz, 1998; Piguet, 1989). Persistent activation of TNF-α and IL1-β results in elevated secretion of pro-inflammatory cytokines that are tissue damaging. Over expression of IL-1β induces acute lung injury and lung fibrosis in mice (Kolb, 2001). TNFα and IL1β are the therapeutic targets in IPF and asbestosis (Zhang et al., 1994). Overexpression of TNFa induces spontaneous fibrosis in mouse lungs (Miyazaki et al., 1995). In cases of infestation with parasitic worm helminths, chronic injury activates a large immune response, resulting in secretion of pro-inflammatory mediators that can inflict cell and tissue damage. Effective treatment involves control of immune-response mediated damage (reviewed in Jackson et al., 2009).

Dose-Response Relationship:

There are some *in vivo* studies that provide dose-response evidence of this KER.

Li et al. (2017) studied the immunotoxic effects of the lung after arsenic exposure in an acute and a subchronic phase. Female C57BL/6 mice were exposed to 2.5, 5, and 10 mg/Kg NaAsO2 via a single oral intragastric administration. After 24 h, BALF and lung tissue were collected. The induction of KE3 (Event 1498; ACM injury) was observed as an increase in the total protein levels in BALF, MDA content, and Nrf2 protein expression in the lung. The induction of KE4 (Event 1499; Th2 activation) was determined as an increase in the levels of Gata3, IL-4, Foxp3, and IL-10 levels, as well as a decrease in the levels of T-bet, Ifn-γ, Ror- γ t, and IL-23. All these changes were observed in a dose-dependant manner. After a subchronic exposure (100 mg/mL NaAsO2 administered freely in drinking water for 2 months), an increase in the levels of NF-κB, p-38, p-JNK, p-ERK mRNA and an increase in the levels of IL-4, IL-23, IL-10, Ifn- γ, IL-1β was observed.

Chang et al. (2017) studied the role of NF-κB activation and Th1/Th2 imbalance in pulmonary inflammation induced by NiO nanoparticles. Male Wistar rats were exposed to 0.015, 0.06, and 0.24 mg/Kg by intratracheal instillation twice a week for 6 weeks. At the highest dose, an increase of nitrative stress in rat lung tissue was observed. TNF-a, IL-2, IL-10, CINC increased in a dose-response manner. Activation of the NF-kB signalling pathway (NF-kB, IKK-α and NIK) also increased in a dose-response manner. An increase in the levels of GATA3 and a decrease in T-bet was observed, indicating a Th1/T2 imbalance after exposure to NiO

nanoparticles. NiO enhances the nitrative stress and inflammatory response in lung tissue, related to NF-kB and Th1/Th2 imbalance.

Temporal Evidence:

In vivo studies have demonstrated that the loss of alveolar capillary membrane integrity precedes the activation of T helper (Th) type 2 cells.

It has been described that oxidant and antioxidant Th1/Th2 balances are essential in the inflammatory response and fibrosis. To evaluate whether transcription factor Nrf2 is a mediator, wild type (WT) C57BL/6 mice, and Nrf2-deficient mice were administered with 5 mg/kg bleomycin intratracheally, and the inflammatory response, antioxidant response, and fibrosis were evaluated at different time points post-exposure. The loss of alveolar membrane integrity was observed at days 1 and 3, as well as a decrease in the induction of antioxidant genes. On day 7, an increase in the level of Th2 cytokine production (IL-4 and IL-13) was observed. The expression of GATA-3, a transcription factor that regulates the differentiation of Th1/Th2 cells, was also increased at this time point. These responses were more intense in Nrf2-deficient mice. Moreover, bleomycin administration increased the recruitment of cells at day 1 and 3, and fibrosis were observed after 28 days of treatment (Kikuchi et al., 2010).

Haczku et al. (2006) observed in mice sensitized and challenged with Aspergillus fumigatus extract (intraperitoneal injection at day 0 and day 14, intranasal challenge at day 24) an increase in the expression of SP-D mRNA and protein levels in lungs in a time-dependant manner after allergen challenge. Total BALF protein increased over time at 12 and 24 h; after that, protein content decreased. IL-4, IL-5, and IL-13 increased at 12 h and 24 h, but decreased at later time points. They found that allergen exposure increased the expression of SP-D and IL-4/IL-13 in a time-dependent manner.

Venosa et al. (2016) evaluated the presence of M1 and M2 macrophages in lung after the exposure to nitrogen mustard. Rats were exposed to nitrogen mustard at 0.125 mg/kg, intratracheally, and the inflammatory response was evaluated 1, 3, 7- and 28-days post-exposure. A decrease in the levels of resident macrophages and an accumulation of infiltrating M1 and M2 macrophages was observed. M1 macrophages were prominent after 1- and 3-days of exposure; meanwhile, M2 macrophages were more prominent after 28 days of exposure. M1 and M2 genes were also upregulated. These events were in a time-dependant manner. M2 macrophages in early time points phagocytized cellular debris and counterbalanced M1 cell activation. At later time points, they promoted matrix deposition, tissue remodeling, and fibrosis.

Caldwell et al. (2009) studied the inflammatory response after exotoxin pyocyanin (PCN) exposure in WT and FVBN mice. Mice were exposed to PCN 10 or 25 µg intranasally inoculated chronically into the lungs three times a week for the intervals of 3, 6, and 12 weeks. At 25 µg, after 6 and 12 weeks, alveolar airspace destruction was observed (KE3 (Event 1498)). The KE4 event (Event 1499) decreased resident macrophages and increased CDT cells/ml in BALF, changed in a time-dependant manner with maximum levels after 12 weeks. STAT6 mediated the induction of Th2 cytokines by PCN.

Uncertainties and Inconsistencies

Exogenous delivery of TNFα to mouse lungs with established fibrosis, reduced the fibrotic burden. Exogenous treatment with TNFα slowed the M2 macrophage polarisation. TNFα deficient mice showed prolonged pro-fibrotic response and M2 polarisation following bleomycin treatment (Redente et al., 2014).

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Relationship: 1706: Activation of Th2 cells leads to Increased cellular proliferation and differentiation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis	adjacent	High	Low

Key Event Relationship Description

The wound healing process involves an inflammatory phase, during which the damage tissue/wound is provisionally filled with ECM. This phase is characterised by secretion of cytokines/chemokines, growth factors and recruitment of inflammatory cells, fibroblasts and endothelial cells. The activated Th1/Th2 response and increased pool of specific cytokines and growth factors such as IL-1b, IL-6, IL-13, and TGFβ, induce fibroblast proliferation. Th2 cells can directly stimulate fibroblasts to synthesise collagen with IL-1 and IL-13. Th2 cytokines IL-13 and IL-4, known to mediate the fibrosis process induce phenotypic transition of human fibroblasts (Hashimoto S, 2001). IL-13 is shown to inhibit MMP-mediated matrix degradation resulting in excessive collagen deposition by downregulating the synthesis and expression of matrix degrading MMPs. IL-13 is also suggested to induce TGFβ1 in macrophages and its absence results in reduced TGFβ1 expression and decrease in collagen deposition (Fichtner-Feigl et al., 2005). These cytokines are suggested to initiate polarisation of macrophages to the alternative M2 phenotype. Th2 cells that synthesise IL-4 and IL-13 induce synthesis of Arg-1 in M2 macrophages. The Arg-1 pathway stimulates synthesis of proline for collagen synthesis required for fibrosis (Barron and Wynn, 2011).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility for this KER is high. There is a widely understood functional relationship between Th2 response related mediators, and their ability to induce proliferation and differentiation of fibroblasts (Shao et al., 2008; Wynn, 2012; Wynn, 2004).

Empirical Evidence

The empirical support for this KER is high. There is a plethora of dose and time response evidence, which show that Th2 cytokines

induce the activation and proliferation of fibroblasts (Hashimoto et al., 2001; Lee et al., 2001; additional references can be found in Table 1).

A majority of the weight of evidence studies assess collagen synthesis as a proxy to fibroblast proliferation and myofibroblast differentiation. A few studies have shown that Th2 cytokine IL-4 stimulates fibroblast proliferation (Sempowski et al., 1994) and production of ECM components (Postlethwaite et al., 1992). In human studies, the progression of idiopathic pulmonary fibrosis is also associated with a sustained IL-4 production (Wallace and Howie, 1999; Ando et al., 1999). Th2 cytokines induce expression and activity of TGFb1, levels of which are elevated in BALF of patients suffering from lung interstitial diseases, is a potent inducer of myofibroblast differentiation and collagen synthesis (Kurosaka et al., 1998; Redington et al., 1997). Exposure of STAT6 deficient mice to MWCNTs, suppressed acute lung inflammation, expression of Th2-mediated gene expression, reduced vimentin positive cells (marker of fibroblasts), levels of collagen synthesis and reduced the overall fibrotic response to MWCNTs (Nikota et al., 2017). Mice deficient in IL-33r (St2, Th2 response cytokine) or mice treated with anti-IL33 antibody, showed reduced lung inflammation, reduced collagen production and fibrotic pathology induced by bleomycin. IL-33 deficient mice treated with bleomycin showed reduced levels of IL-1 and other pro-inflammatory cytokines. Mice administered exogenously with mature IL-33 enhanced bleomycin-induced lung inflammation, collagen synthesis and fibrotic lesions (Dong et al., 2014).

Dose-Response Relationship:

In vivo and *in vitro* studies have demonstrated a dose-response relationship, at the higher dose of the stressor, T helper (Th) type 2 cells leads to increased, fibroblast proliferation, and myofibroblast differentiation.

Lo Re et al. (2011) evaluated the role of Treg cells in a mouse model of lung fibrosis induced by silica particles. SiO_2 particles administered 2.5 mg per mouse by pharyngeal instillation induced an increase in the levels of $CD4^+Foxp3^+$ regulatory T lymphocytes in lungs after 3 and 15 days of administration. Treg cells, purified from Foxp3-GFP transgenic mice administered with SiO_2, stimulated lung fibroblast proliferation *in vitro* by producing PDGF-B and TGF-b in a dose-dependant manner. Moreover, these results indicated that the activation of Th2 response (KE4; Event 1499) was needed to activate fibroblast proliferation (KE5; Event 1500). They determined that effector T cells purified from SiO_2-treated mice, in the absence of Treg cells, induced fibrosis by producing IL-4, suggesting that many T cell pathways lead to the fibroproliferative process.

Liu et al. (2011) investigated the role of FIZZ2 in pulmonary fibrosis in a rodent bleomycin model and the potential role of FIZZ2 in human fibrotic lung disease. FIZZ2 has been found in pulmonary fibrosis after 14 days of exposure to bleomycin in mice (2U and 10 U/Kg BLM) and in lung tissue from patients with IPF and nonspecific interstitial pneumonia. The expression was localized mainly to airway epithelial cells and alveolar epithelial cells, and to a lesser extent in alveolar macrophages and smooth muscle and endothelial cells. Alveolar epithelial cells were isolated from rats and humans, and they were exposed to 10 ng/ml rIL-4, rIL-13, rIL-17 and INF-g. After 4 h, rIL-4 and rIL-13, induced FIZZ2 mRNA expression in rat lungs. After 8 h, rIL-13 increased FIZZ2 mRNA expression in rat lungs, and rIL-4 and IL-13 induced an expression of FIZZ2 MRNA in human lungs. These results indicate that FIZZ2 mRNA expression is driven by Th2-type cytokines. Mouse lung fibroblasts (MFLs) were isolated and treated with recombinant mouse FIZZ2 at different concentrations. Collagen I deposition was observed at 10 and 25 ng/ml, and a-smooth muscle actin was induced at 25, 50, and 200 ng/ml; meanwhile, cell proliferation was observed at 10, 25, and 50 ng/ml. These results suggested that FIZZ2 had direct profibrogenic activity. Furthermore, FIZZ2 acts as a chemoattractant for bone marrow cells, especially BM-derived CD11[C+] dendritic cells. In knockout mice treated with bleomycin, a decrease in the FIZZ2 expression was seen, and the adverse effects produced by FIZZ2 decreased. The authors concluded that FIZZ2 is a Th2-associated multifunctional mediator which plays a role in fibroblast proliferation mediated via STAT6 signaling.

Temporal Relationship:

In vitro and in vivo studies have demonstrated that Th2 cells are activated prior to fibroblast proliferation and myofibroblast differentiation.

Dong et al. (2016) demonstrated that multiwalled carbon nanotubes (MWCNT) activated Th2 immune responses. Male C57BL/6J mice were administered with 40 mg/mouse carbon nanotubes by pharyngeal aspiration. On days 1, 3, and 7 post-exposure, an increase in the expression of Th2 cytokines (IL-4 and IL-3), as well as an induction of STAT6 and GATA-3 was seen. At day 7, the presence of collagen I fibers was evident.

In another study, male Wistar rats were administered with 5 mg/kg bleomycin by intratracheal instillation. The inflammatory response was evaluated after 7, 14, and 28 days of exposure. Bleomycin increased hydroxyproline levels, total cell counts, and the expression of NFkB p65 in lung tissue. Collagen type I increased in a time-dependant manner. At day 7, the Th1 response was suppressed, based on a decrease of IFN-g and an increase of IL-4 levels. Mice treated with hydrogen sulfide showed less intense effects than mice treated with bleomycin (Cao et al., 2014).

Yin et al. (2013) studied the role of IL-33 in cutaneous wound healing. Male BALB/c mice were injured on the dorsal skin and administered with murine recombinant IL-33 (1.0 mg/mouse). After 5 days of injury, alternatively activated macrophages (AAM) accumulation and mRNA expression of AAM-associated genes increased (fibronectin and collagen). Collagen deposition increased in a time-dependant manner, and the percentage of wound closure and re-epithelization increased over 14 days. This study indicates that macrophage polarization, which is associated with KE4 (Event 1499) preceded the KE5 (Event 1500: fibroblast proliferation). IL-33 is essential for homeostasis and wound healing.

Wynes et al. (2004) evaluated the proliferative response associated with Th2 activation. Insulin-like growth factor-I (IGF-I) is a fibroblast growth and survival factor that has been implicated in the pathogenesis of idiopathic pulmonary fibrosis. The authors observed that mouse bone marrow-derived macrophages from C3H/HeJ mice treated with IL-4 2 ng/ml for 26 h released

IGF-I. CCL39 myofibroblasts, cultured with conditioned media from IL-4-treated macrophages, consumed IGF-1 and avoided apoptosis (caspase-3 activity reduced and pro-survival kinases Akt and ERK were activated). The survival effect was lost when IGF was immunodepleted from macrophage-condition media with IGF-I-specific antibodies. These results indicate that a Th2 response conditions macrophages to release mediators which induce persistence of fibroblasts in a fibrotic setting.

Meziani et al. (2018) studied the role of large doses of radiation in promoting M2 macrophage polarization. In patients with thoracic malignancies and preoperative radiotherapy between 25 and 60 Gy, an infiltration of CD163+ macrophages was found in fibrotic areas. The pulmonary infiltration was characterized during radiation-induced lung fibrosis in a murine model. Female C57BL/6J mice were locally irradiated at the thorax with a dose rate of 1.08 Gy min^{-1}. A single dose of 16 Gy was locally administered to the whole thorax. Infiltrating macrophages (IMs) and alveolar macrophages (AMs) were isolated post-irradiation. They observed the number of Icam1+ IMs transiently increased at day 6, and an increase number of CD206+ IMs at week 20 post-irradiation. At this time, Th1 cytokines decreased, and TIMP-1 increased. Moreover, IMs express high levels of Arg-1. IMs were co-cultured with normal fibroblasts and increased the expression of a-SMA and TGF-b1. They found that IMs isolated from normal mouse lungs, activated *in vitro* with IL-13 and IL-4 for 24 h, were able to increase a-SMA levels. After 20 weeks, irradiation induced collagen deposition and an increase in the expression of TGF-b1, PAI-1, and Smad2/3 phosphorylation in lung tissue. Depletion of tissue IMs by anti-CSF1R after thoracic irradiation, blocks the observed effects.

Gibbons et al. (2011) studied the role of circulating monocytes and lung macrophages in the pathogenesis of lung fibrosis and the importance of alternatively activated macrophages and Ly6Chi monocyte phenotype. Female C57Bl/6 mice were given 0.05 U bleomycin or 1X10^8 pfu AdTGF-b intratracheally. Bleomycin induced early fibrosis at day 18, progressive fibrosis at day 32, or resolving fibrosis at day 56. Bleomycin increased the expression of a-SMA and Col1A1 at day 25. At this time point, they also found an increase in the level of arginase activity and Ym1, and at day 32 an increase in the number of cells per field (markers of alternatively activated macrophages). Macrophages isolated from patients with idiopathic pulmonary fibrosis showed CD163, a human marker of alternative macrophages. AdTGF-b induced an increase in BALF TGF-b at day 5, and collagen deposition at day 14. The administration of liposomal clodronate intratracheally (100 ml) at 10, 21-23 after the exposure to bleomycin or AdTGF-b decreased fibrosis, collagen deposition and alternatively activated macrophages. The depletion of circulating monocytes reduced fibrosis and alternatively activated macrophages. It was found that Ly6Chi inflammatory monocytes were the direct precursor of the alternatively activated lung macrophages.

Uncertainties and Inconsistencies

Due to multifarious functions of several cytokines involved in the process of inflammation and repair, the timing of when a pathway is intervened in an experiment is important in the assessment of the KER studies. For example, exposure to pro-fibrotic bleomycin stimulates IL-4 production during the acute inflammatory phase, which is suggested to limit the recruitment of T lymphocytes and production of damaging cytokines such as TNFα, IFNγ, and nitric oxide, playing a tissue protective role. However, production of IL-4 during the chronic phase of tissue repair and healing, favors fibrosis manifestation. Treatment of IL4 -/- mice with low doses of bleomycin induced fewer fibrotic lesions compared to IL-4 +/+ mice. However, treatment of high doses of bleomycin induced more lethality in IL-4 -/- mice compared to the wild type mice (Huaux et al., 2003). Moreover, the KEs represented in AOP 173 can function in parallel in a positive feedback loop, perpetuating and magnifying the response at each stage. The resulting microenvironment may contain the same molecules in different proportions exhibiting different functions. Thus, the complexity of the process and the functional heterogeneity of the molecular players involved, makes it nearly impossible to establish KERs using a targeted deletion of one single gene or a pathway in a study, which is how most of the studies are designed.

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Relationship: 2652: Increased cellular proliferation and differentiation leads to Accumulation, Collagen

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis	adjacent	High	High

Key Event Relationship Description

When activated, fibroblasts migrate to the site of tissue injury and build a provisional ECM, which is then used as a scaffold for tissue regeneration. Activated fibroblasts in turn produce IL-13, IL-6, IL-1β and TGFβ, propagating the response. In the second phase, which is the proliferative phase, angiogenesis is stimulated to provide vascular perfusion to the wound. During this phase more fibroblasts are proliferated and they acquire a-smooth muscle actin expression and become myofibroblasts. Thus, myofibroblasts exhibit features of both fibroblasts and smooth muscle cells. The myofibroblasts synthesise and deposit ECM components that eventually replace the provisional ECM. Because of their contractile properties, they play a major role in contraction and closure of the wound tissue (Darby et al., 2014). Apart from secreting ECM components, myofibroblasts also secrete proteolytic enzymes such as metalloproteinases and their inhibitors tissue inhibitor of metalloproteinases, which play a role in the final phase of the wound healing which is scar formation phase or tissue remodelling.

During this final phase, new synthesis of ECM is suppressed to allow remodelling. The wound is resolved with the secretion of procollagen type 1 and elastin, and infiltrated cells including inflammatory cells, fibroblasts and myofibroblasts are efficiently removed by cellular apoptosis. However, in the presence of continuous stimulus resulting in excessive tissue damage, uncontrolled healing process is initiated involving exaggerated expression of pro-fibrotic cytokines and growth factors such as TGFβ, excessive proliferation of fibroblasts and myofibroblasts, increased synthesis and deposition of ECM components, inhibition of reepithelialisation, all of which lead to replacement of the normal architecture of the alveoli and fibrosis (Ueha et al., 2012; Wallace et al., 2006).

Evidence Supporting this KER

Biological Plausibility

The biological plausibility of this KER is high. There is an accepted mechanistic relationship between activated myofibroblasts, and the capacity to secrete collagen (Hinz, 2016a; Hinz, 2016b; Hu & Phan, 2013).

Empirical Evidence

The empirical evidence to support this KER is high. It is generally accepted knowledge that activated myofibroblasts are collagen secreting cells (Blaauboer et al., 2014; Hinz, 2016a; Li et al., 2017; For additional references see Table 1).

Mice infused subcutaneously with bleomycin showed pronounced lung fibrosis, characterised by elevated levels of TGFβ1 and collagen genes (Hoyt and Lazo, 1988). Radiation induced lung fibrosis was shown to precede high levels of TGFβ1 expression (Yi et al., 1996). Mice lacking TGFβ-receptor II showed resistance to bleomycininduced lung fibrosis (Li et al., 2011). Inhibition of fibroblast proliferation and differentiation by counteracting the activity of TGF-β attenuates bleomycin-induced lung fibrosis (Chen et al., 2013; Guan et al., 2016). Adenoviral vector-mediated gene transfer based transient overexpression of TGFb1 in lungs of mice induced progressive lung fibrosis (Bonniaud et al., 2004). Targeted inhibition of Wnt/b-catenin signalling by a small molecule drug inhibited the mesenchymal-myofibroblast transition and repressed matrix gene expression leading to attenuated lung fibrosis (Cao et al., 2018).

Dose-Response Relationship:

There are a number of *in vitro* and *in vivo* studies that indicate a dose-response relationship in this KER. At a higher dose of the stressor, an increased in fibroblast proliferation and myofibroblast differentiation leads to increases in extracellular matrix deposition.

Ma et al. (2017) studied the role of epithelial-mesenchymal transition (EMT) in cerium oxide (CeO_2) induced fibrosis. Male Sprague-Dawley rats were exposed to 0.15-7 mg/kg cerium oxide via intratracheal instillation and sacrificed at various times post-exposure. At 28 days post-exposure there was a dose-dependant increase in hydroxyproline content in lung tissue. Mice exposed to 3.5 mg/kg showed an increase in soluble collagen levels in BALF at day 3 and day 28 and an increase in α-SMA expression levels in lung tissue with a peak at day 1 post-exposure. From CeO_2-exposed rats (3.5 mg/kg), macrophages, fibroblast, and alveolar type II (ATII) cells were isolated. Macrophages produced TGF-b1 with peaks at day 3 and 10 post-exposure. Fibroblast proliferation decreased in a dose-dependant manner, and an increase in the levels of a-SMA in fibroblasts and ATII at day 28 post-exposure. They concluded that CeO_2 exposure affects fibroblast function and induces EMT in ATII cells.

Blaauboer et al. (2014) studied the expression of elastin, type V collagen, and tenascin C during the development of lung fibrosis and the effect of myofibroblast differentiation on this expression. Female C57Bl/6 mice were exposed to a single intratracheal instillation bleomycin 30 ml (1.25 U/ml in PBS). Seven days before sacrifice, mice received 35 ml D_2O/g via intraperitoneal injection to label new collagen. Mice were sacrificed 1, 3, 4 or 5 weeks post exposure. An increase in the level of a-SMA protein level in histological staining was observed, with a peak after 2 weeks. Extracellular matrix proteins levels increased (histological staining). Elastin increased in a time-dependant manner with a peak after 4 weeks. Type V collagen and tenascin C increased after 1 week and decreased over time. They found that gene expression of elastin, type V collagen and tenascin C highly correlated to new collagen formation. Primary normal human lung fibroblast and human fetal lung fibroblast were exposed to different concentrations of TGF-b1. The expression of ACTA, COL1A1, ELN, COL5A1 and TNC increased in a dose-response relationship after 24 h. Fibroblast cultured in elastin coatings and stimulated with 10 ng/ml TGF-b1 for 48 h, showed an increase in the levels of ACTA2, COL1A1, and ELN. The *In vitro* study demonstrated that fibrotic changes in the composition of the extracellular matrix have a regulatory role during fibrosis development.

Judge et al. (2015) determined that the lactate dehydrogenase-A (LDHA) enzyme was upregulated in radiation and that lactate is required for radiation-induced myofibroblast differentiation. In lung biopsies obtained from patients who received thoracic radiation for cancer treatment, an overexpression of LDHA and a-SMA by immunostaining was seen, as well as the accumulation of collagen fibers. Mice C57BL/6mice were exposed to 5 Gy total body plus 10 Gy thoracic radiation. They found that LDHA overexpressed in lungs at 26 week, and LDHA mRNA increased over time at 16-26 weeks (post-radiation). Primary human lung fibroblasts were exposed to 3, and 7 Gy. At the highest dose 5 days post-radiation, an increase in the levels of LDHA protein expression, extracellular acidification, lactate levels in supernatants, a-SMA protein expression, soluble collagen I, Col1A1, and Col3A1 mRNA levels, and TGF-b1 bioactivity was seen. LDHA siRNA and an LDH inhibitor, inhibits radiation-induced myofibroblast differentiation.

Lia et al. (2018) studied whether copper oxide nanoparticles (CuO NPs) could induce epithelial cell injury, pulmonary inflammation, and fibrosis in C57BL/6 mice. Animals were nasally instilled with 1, 2.5, 5, and 10 mg/kg of CuO NPs, and responses were evaluated at 7, 14, and 28 days post-exposure. In a dose-dependent manner, authors found increased mRNA levels of proinflammatory genes such as CCL-2, CCL-3, IL-4, IL-10, IFN-α, and TGF-β1 in lung tissue. Cell apoptosis was also increased in a dose-dependant manner at 1, 2.5, and 5 mg/Kg. Also, the increase of TGF-B1 in BALF at day 14 and the increase of α-SMA at day 28 in lung tissue followed a dose-response relation ship at 2.5 and 5 mg/Kg. After 28 days of exposure, there was an increase in collagen-I and hydroxyproline content at 2.5 and 5 mg/Kg.

Temporal Relationship:

In vitro and *in vivo* studies highlight the temporal relationship between the two KEs in this KER.

Osterholzer et al. (2013) evaluated local inflammation and fibrosis after a targeted epithelial insult. Wild type (WT) C57BL/6 and transgenic mice expressing the diphtheria toxin receptor were intraperitoneally injected with diphtheria toxin (DT) once daily for 14 days at a dose of 100 mg/kg in 100 ml of PBS. Observations were evaluated at various days post DT initiation. At day 7 and 14, an accumulation of exudate macrophages and Ly-6C^{high} monocytes was observed. The immunophenotype of ExM and Ly-

$6C^{high}$ monocytes at day 14 showed an expression of arginase, iNOs, IL-13, TGF-b, CD45+, Col1+, and CCR4. Chemokine-receptor-2 deficient mice did not show an accumulation of inflammatory cells and fibrosis. Finally, at day 21, lung collagen deposition was evident, as measured by hydroxyproline content.

Fang et al. (2018) studied the endothelial-mesenchymal transition characterized by the loss of endothelial specific markers (Cdh5, PECAM1), the acquisition of the mesenchymal markers (Col1A1, Acta 2), and the expression of a-SMA and Collagen I and III. Stock TEK-GFP 287 Sato/JNiu Tie2-GFP mice were administered with 0.5 g/Kg SiO_2 instilled intratracheally in one dose. After 28 days of treatment GFP were localized with a-SMA/Acta2 and the amount of Sirius red (collagen I and III) increased. Mouse microvascular lung cells (MML1) were exposed to 50 mg/cm^2 for 0, 6, 12, 24, and 48 h. An increase in the level of mesenchymal markers, a decrease in the level of endothelial markers, and an increase in cell proliferation and migration were observed after 12 h in a time-dependant manner. The exposure to SiO_2 increased the expression of circHECTD1 (a circular RNA which regulates the SiO_2-induced endothelial mesenchymal transition) after 1, 3 and 24 h of exposure, and decrease the expression of HECTD1 12, 24 and 48 h post-exposure.

Activated macrophages secrete TIMP1 into the ECM to inhibit matrix metalloproteinases and this could promote cell proliferation and inhibit fibroblast apoptosis through CD63/integrin b1 ERK signaling. Dong et al. (2017) characterized TiMP1 expression after multiwalled carbon nanotube (MWCNT) exposure. Male C57BL/6J WT and B6.129S4-Timp1tm1Pds/J (Timp1 KO) mice were administered with MWCNT at 40 mg per mouse by pharyngeal aspiration. Lungs were harvested at 1, 3, 7- and 14-days post-exposure. TIMP1 mRNA and protein levels increased in lung, BALF and serum at day 1, and then decreased over time. A similar behavior was observed for FN1, FSP, Ki67 and PCNA expression with a peak at 7 and 14 days post exposure. Collagen deposition was observed at 1 day post exposure with a peak at day 7. At day 7 they also observed an increase in the expression of Hsp47, vimentin, a-SMA, PDGFR-b, and genes involved in cell cycle regulation (Bub1b, Capg, Cenpa, Kif2c, Kif22, Mcm5, Plk1 and Tuba 6). TIMP1 KO mice displayed reduced responses. The formation of TIMP1/CD63/integrin b1 complex on the cell surface lead to an activation of the Erk1/3 pathway.

Hu et al. (2015) studied the effects of conditional mesenchymal-specific deletion of Notch1 on pulmonary fibrosis. A conditional knockout of Notch1 (CKO) in collagen I-expressing mesenchymal cells was generated (Notch1fl/fl, Col1a2-cre-ER(T)+/0). Col1a2-cre-ER(T)+/0 with WT Notch1 mice and CKO were given daily intra peritoneal injections of tamoxifen for 8 days to induce mesenchymal cell-specific expression of the Cre-ER(T) recombinase and the removal of the floxed Notch1 (Notch1 CKO Mice). Control mice and Notch1 CKO were injected endotracheally with 2 U/Kg Bleomycin. Mice were sacrificed after 7, 14, and 21 days. Jagged1 and Notch1 protein expression increased with a peak at 7- and 14-days post-exposure. After 14 days of the treatment, an increase in the levels of mRNA and protein of a-SMA and Col1 was seen, as well as an increase in the percentage of a-SMA+ lung fibroblasts. 28 days post-exposure there was an increase in the content of hydroxyproline in lungs. CKO mice showed a significant attenuation of collagen deposition and myofibroblast differentiation.

Li et al. (2017) evaluated whether low-dose cadmium exposure induces peribronchiolar fibrosis through site-specific phosphorylation of vimentin. C57BL/6 mice were exposed to 0.009 or 0.018 mg/kg cadmium chloride ($CdCl_2$) via non-surgical intratracheal instillation in saline every other day for eight weeks. On weeks 1, 2, 4, and 8, mice were sacrificed, and lungs were removed for histology. At week 4, the expression of α-SMA, collagen-I, and picro-sirius red increased. Also, subepithelial thickness and airway resistance increased at this time point. Collagen content was also raised in a time-dependant manner. In a parallel experiment, primary human fibroblasts were incubated with $CdCl_2$ at 5, 10, and 20 μM for 3 h and then allowed to recover for 3, 24, 48, and 72 h. α-SMA protein expression and soluble collagen increased in a dose-dependent manner; meanwhile, α-SMA, fibronectin, and collagen-I increased in a time-dependant manner. These results demonstrated that cadmium induces myofibroblast differentiation and extracellular matrix deposition around small airways.

Uncertainties and Inconsistencies

Several studies have shown that inhibition of TGF-β involved in fibroblast activation and collagen deposition results in attenuated fibrotic response in lungs; however, results are inconsistent. More studies are required to support the quantitative KER.

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Relationship: 1629: Accumulation, Collagen leads to Pulmonary fibrosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Latent Transforming Growth Factor beta1 activation leads to pulmonary fibrosis	adjacent	High	
Substance interaction with the lung resident cell membrane components leading to lung fibrosis	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adult	High

Humans (Zisman 2005, Meyer 2017), rats (Williamson 2015), mice (Williamson 2015).

Key Event Relationship Description

Fibrosis by definition is the end result of a healing process. It involves a series of lung remodelling and reorganisation events leading to permanent alteration in the lung architecture and a fixed scar tissue or fibrotic lesion (Wallace WA, 2007). Excessive deposition of ECM or collagen is the hallmark of this disease and there is ample evidence to support this KER (Fukuda 1985, Meyer 2017, Richeldi 2017, Thannickal 2004, Zisman 2005).

Evidence Supporting this KER

Biological Plausibility

By definition, pulmonary fibrosis is characterized by excessive deposition of extracellular matrix and destruction of native lung architecture (Fukuda 1985, Richeldi 2017, Thannickal 2004). Thus, the plausibility of this association is undisputed.

Empirical Evidence

Excessive ECM deposition is the defining characteristic of pulmonary fibrosis, and the evidence to support this relationship is unequivocal. (Meyer 2017, Thannickal 2004, Zisman 2005).

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