AOP ID and Title:

SNAPSHOT

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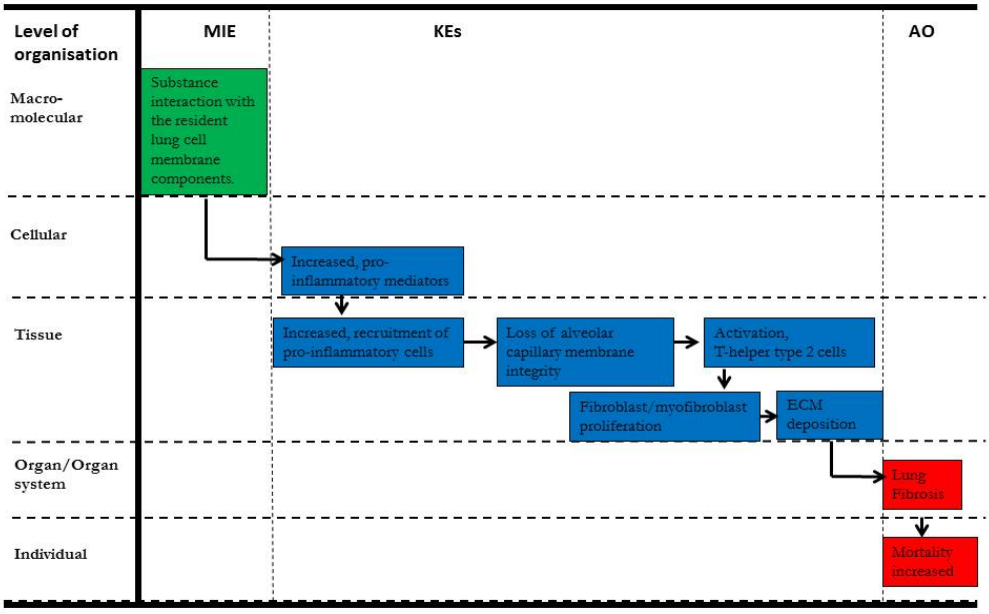
AOP 173: Substance interaction with the lung resident cell membrane components leading to lung fibrosis

Short Title: Substance interaction with the lung cell membrane leading to lung fibrosis

Graphical Representation



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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 an 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 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) in the process of tissue repair. As a consequence, a myriad of pro-inflammatory mediators are secreted (KE1) that signal the recruitment of pro-inflammatory cells into the lungs (KE2). 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) and activation of adaptive immune response, the T Helper type 2 cell signalling (KE4), during which anti-inflammatory and pro-repair/fibrotic molecules are secreted. The repair and healing process stimulates fibroblast proliferation and myofibroblast differentiation (KE5), leading to synthesis and deposition of extracellular matrix or collagen (KE6). Excessive collagen deposition culminates in alveolar septa thickening, decrease in total lung volume and lung fibrosis (Adverse Outcome).

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 foundation for the later pathology. 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. Recently, an AOP for stressor-induced pulmonary inflammation resulting in lung emphysema has been initiated and is currently under development. Here, a mechanism underlying stressor-induced lung fibrosis that involves a pro-inflammatory component is described.

Although this AOP is applicable to a broad group of chemicals of diverse properties, the AOP was specifcally 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 fundametal role of inflammation in organ homeostasis, well characterized AOPs targetting 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	Interaction with the lung resident cell membrane components (https://aopwiki.org/events/1495)	Interaction with the lung cell membrane
2	KE	1496	Increased, secretion of proinflammatory and profibrotic mediators (https://aopwiki.org/events/1496)	Increased proinflammatory mediators
3	KE	1497	Increased, recruitment of inflammatory cells (https://aopwiki.org/events/1497)	Recruitment of inflammatory cells
4	KE	1498	Loss of alveolar capillary membrane integrity (https://aopwiki.org/events/1498)	Loss of alveolar capillary membrane integrity
5	KE	1499	Increased, activation of T (T) helper (h) type 2 cells (https://aopwiki.org/events/1499)	Activation of Th2 cells

Sequence	Type	Event ID	Title	Short name
6	KE	1500	Increased, fibroblast proliferation and myofibroblast differentiation (https://aopwiki.org/events/1500)	Increased cellular proliferation and differentiation
7	KE	1501	Increased, extracellular matrix deposition (https://aopwiki.org/events/1501)	Increased extracellular matrix deposition
8	AO	1458	Pulmonary fibrosis (https://aopwiki.org/events/1458)	Pulmonary fibrosis

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Interaction with the lung resident cell membrane components (https://aopwiki.org/relationships/1702)	adjacent	Increased, secretion of proinflammatory and profibrotic mediators	High	Not Specified
Increased, secretion of proinflammatory and profibrotic mediators (https://aopwiki.org/relationships/1703)	adjacent	Increased, recruitment of inflammatory cells	High	High
Increased, recruitment of inflammatory cells (https://aopwiki.org/relationships/1704)	adjacent	Loss of alveolar capillary membrane integrity	High	High
Loss of alveolar capillary membrane integrity (https://aopwiki.org/relationships/1705)	adjacent	Increased, activation of T (T) helper (h) type 2 cells	High	Moderate
Increased, activation of T (T) helper (h) type 2 cells (https://aopwiki.org/relationships/1706)	adjacent	Increased, fibroblast proliferation and myofibroblast differentiation	High	High
Increased, fibroblast proliferation and myofibroblast differentiation (https://aopwiki.org/relationships/1707)	adjacent	Increased, extracellular matrix deposition	High	High
Increased, extracellular matrix deposition (https://aopwiki.org/relationships/1708)	adjacent	Pulmonary fibrosis	High	High

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** (file:///D:/pubmed/?term=Moeller%20A[Author]&cauthor=true&cauthor_uid=17936056) et al., **2008;** (file:///D:/entrez/eutils/elink.fcgi?dbfrom=pubmed&retmode=ref&cmd=prlinks&id=17936056) 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.

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Overall Assessment of the AOP

Overall assessment

Ideopathic pulmonary fibrosis (IPF) is a complex, progressive disease of unknown etiology that is most commonly observed in humans. Lung 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 microbials and allergen exposure. 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 functions. Some other considerations of relevance to this AOP:

This AOP represents fibrotic mechanism that involves a strong inflammatory component. Exposure to pro-fibrotic stressors such as, bleomycin, silica, asbestos, CNTs, radiation or models of overexpression of cytokines 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, Hebda and Wells, 2012). Asbestosis and silicosis, 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, cats, donkeys, pigs and other animals have been studied to investigate different types of fibrosis. 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.

Assessment of the Weight-of-Evidence supporting the AOP

Concordance of dose and time-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 fibrosis 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 (over 2000 microarrays) mouse lungs exposed individually mouse 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 (10mg/m^3, 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., 2013). 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 J-E 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 Hanley, 1995). 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 alarmins from cells.

The alarmin HMGB1 is released from damaged or nectrotic 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., 2016). 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 MIE 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 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 and KE2) and Th2 –mediated signalling (KE4) is required to suppress lung fibrosis induced by MWCNTs (Nikota et al., 2017). Disengagement between innate immune responses including MIE, KE1 and KE2, and ultimate lung fibrosis is shown in a 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 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, the alarmin 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). Thus, the results supporting the KERs are not consistent.

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 that may be described

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). The alternate AOP for fibrosis placed activation of TGFb1 upstream of inflammatory events (KE2, KE3), which is contrary to its perceived role in downstream events leading to fibroblast proliferation and differentiation, and extracellular matrix 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 TGFb1 can promote excessive ECM deposition and fibrosis in rodents independent of inflammation.

Uncertainties, inconsistencies and data gap

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.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Unspecific	High

This AOP applies to the following:

Stressors

1. Stressors that persist in the lung environment for a long duration of time causing chronic injury (silica, coal dust). Repeated exposure to stressors such as bleomycin, cigarette smoke, other pharamcological drugs that cause chronic lung injury.

2. The long and rigid fibres or high aspect ratio fibres (asbestos, CNTs).

3. Stressors that indcue a strong inflammatory component (bleomycin, silica, silica dust, etc).

4. Stressors exhibiting unique physical-chemical properties including shape (fibres, particles), crystal structure (crystalline silica), etc.

Sex/Gender and age

Ideopathic pulmonary fibrosis (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, cats, donkeys, pigs and other animals have been studied to investigate different types of fibrosis. 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.

Other applications

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

Essentiality of the Key Events

Essentiality of the Key Events (key event relationships)

Please refer to Table-1.

Although the MIE, KE1, and KE2 occur in sequence and are described as separate KEs, the animal or cell culture experiments are generally not designed to measure these events separately. As a result, there is not enough empirical support to build individual KERs. Thus, in the KER description below, the following KERs will be considered together.

MIE – KE1: Substance interaction with the resident lung cell membrane components leads to increased pro-inflammatory mediators

KE1- KE2: Increased pro-inflammatory mediators leads to increased recruitment of pro-inflammatory cells

KER 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. The secreted mediators, in turn, signal the recruitment of neutrophils, which are the first cell types to be recruited in acute inflammatory conditions. Other types of cells including macrophages, eosinophils, lymphocytes are recruited in a signal-specific manner. Neutrophil influx in sterile inflammation is driven mainly by IL-1a (Rider et al., 2011). IL-1 mediated signalling regulates neutrophil influx (Horning et al., 2008). IL-1 signalling also mediates neutrophil influx in other tissues and organs including liver and peritoneum. Recruitment of leukocytes induces critical cytokines associated with the Th2 immune response, including TNF-α, IL-1β, and IL-13.

Weight of Evidence

Both empirical evidence and biological plausibility are strong. 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 (Horning 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; Re 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).

Uncertainties or inconsistencies

Attenuation or complete abrogation of KE1 and KE2 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.

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.

KE2 – KE3

Increased recruitment of pro-inflammatory cells leads to loss of alveolar capillary membrane integrity

KER 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 (Grandel, 1998).

Role of ROS synthesis and chronic inflammation in the loss of ACM integrity

In general, chronic or persistent inflammation occurs after prolonged acute inflammation (Soehnlein et al., 2017), which leads to ACM integrity loss. Neutrophils are the dominant cell population during acute inflammation. Clearance of neutrophils from the inflammatory site triggers resolution of inflammation and consequently, the tissue repair process (Nathan, 2002). Failure to trigger neutrophil deathand continued secretion of damaging enzymes by the neutrophils contributes to the propagation of inflammatory response and cell injury.

In the presence of persisting or repeated tissue injury, the macrophages induce a large amount of pro-inflammatory cytokines that can prolong the lifespan of neutrophils resulting in prolongation of the acute inflammatory phase. The pro-inflammatory cytokines such as IL-1b, TNF-α, and others secreted by macrophages are shown to delay neutrophil death. The other factor that can delay neutrophil death is ROS, synthesised by neutrophils, which can activate specific membrane receptors that inhibit neutrophil apoptosis. Humans suffering from sepsis exhibiting neutropenia (deficiency of neutrophils) have fewer macrophages in their BALF compared to the healthy human population. Excessive production of ROS leads to inflammation, pulmonary injury and subsequently, fibrosis in experimental bleomycin models (Chaudhary, Schnapp and Park, 2006). ROS released by neutrophils via the multicomponent enzyme nicotinamide adenine dinucleotide phosphate oxidase (NAPDH) is a known contributor to tissue injury and mediator of both lung and liver fibrosis. ROS can activate TGF-β directly or indirectly via proteases, and TGF-β itself further induces ROS production through NADPH oxidase catalytic subunit NOX4 (Caielli, Banchereau and Pascual, 2012; Koli et al., 2008).

Weight of evidence

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).

Uncertainties or 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).

KE3-KE4

Loss of alveolar capillary membrane integrity leads to activation of Th2 type cell signalling

KER 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 Jet 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 subepethelial 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).

Weight of evidence

Studies establishing this KER are very scarce and data is not available to establish the quantitative dose- or time- response relationships.

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 (Kold, 2001). TNFα and IL1β are the therapeutic targets in IPF and asbestosis (Zhang et al., 1993). Overexpression of TNFa induces spontaneous fibrosis in mouse lungs (Miyaki 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).

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).

KE4-KE5

Activation of Th2 type cell signalling leads to fibroblast proliferation and myofibroblast differentiation

KER 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., 2006). These cytokines are suggested to initiate polarisation of macrophages to 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).

Weight of evidence

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 (Redington et al., 1997; Kurosaka et al., 1998). 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).

Uncertainties or 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, favours 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 the AOP can function in parallel in a positive feedback loop, perpetuating and magnifying the response at each stage. The resulting microenvironment may contain 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.

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.

KE5 – KE6

Fibroblast proliferation and myofibroblast differentiation leads to ECM/collagen deposition

KER 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 (Satoshi et al., 2012; Wallace et al., 2007).

Weight of evidence, Uncertainties or inconsistencies, Quantitative understanding of the linkage

Mice infused subcutaneously with bleomycin showed pronounced lung fibrosis, characterised by the elevated levels of TGFβ1 and collagen genes (Hoyt et al., 1988). Radiation induced lung fibrosis was shown to precede high levels of TGFβ1 expression (Eunhee et al., 1996). Mice lacking TGFβ-receptor II showed resistance to bleomycin-induced 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). 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.

KE5 – KE6

Excessive ECM/collagen deposition leads to alveolar septa thickness (fibrosis)

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.

Quantitative considerations

Since the adverse outcome of lung fibrosis involves multiple cell types, cell - cell interactions and cell–biomolecule interactions, it is difficult to recapitulate the entire process in one model. Therefore an integrated approach, such as one consisting of cell systems that assess individual KEs and quantitative relationships between the KEs, is needed to predict the AO in humans.

Quantitative Consideration

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Considerations for Potential Applications of the AOP (optional)

Considerations for potential applications of the AOP

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 nanomaterial, 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. Especially, in the field of nanotoxicology, considering the vast number of nanomaterials and their property variants that require testing, the AOP will allow rapid screening and identification of potentially pro-fibrogenic materials. This AOP is 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.

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 organisms' 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 C, 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: Interaction with the lung resident cell membrane components (https://aopwiki.org/events/1495)

Short Name: Interaction with the lung cell membrane

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 (https://aopwiki.org/aops/173)	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Evidence for MIE perturbation

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 (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High

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

How this MIE works

Background

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 K 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 to 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, N-dimethyl-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

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 (Nelson et al., 2016; 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).

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List of Key Events in the AOP

Event: 1496: Increased, secretion of proinflammatory and profibrotic mediators (https://aopwiki.org/events/1496)

Short Name: Increased proinflammatory mediators

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 (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rats	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

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 lietrature is avaiable 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

How this KE works

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).

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 fibrogenic 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., 2010; Husain et al., 2015). 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 proinflammatory mediators for investigation varies based on the expertise of the lab, cell type studied and availability of the specific antibodies.

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 flourophore 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., 2015). 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 et al., 2009).

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Event: 1497: Increased, recruitment of inflammatory cells (https://aopwiki.org/events/1497)

Short Name: Recruitment of inflammatory 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 (https://aopwiki.org/aops/173)	KeyEvent
Aop:303 - Frustrated phagocytosis-induced lung cancer (https://aopwiki.org/aops/303)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Key Event Description

Increased, recruitment of pro-inflammatory cells

How it works

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 MA et al., 2011). Sensing or recognition of pathogens and harmful substances results in the recruitment of monocytes to lungs (Shi C and Pamer EG, 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, KE1 and KE2 act in a positive feedback loop mechanism and propagate the pro-inflammatory environment. All pro-fibrotic agents induce leukocyte infiltration in a dose and time-dependent manner.

Evidence for its perturbation

Macrophages acuumulate in bronchoalveolar fluid (BALF) post-exposure to fibrogenic bleomycin (Phan, 1980; Smith, 1995). NM-induced inflammation is predominantly neutrophilic (Shevedova, 2005; Rahman L, 2017; Rahman, 2017; Poulsen 2015). Increased number of pro-inflammatory cells (Zuo, 2002), neutrophils (Reynolds 1997) is observed in the BALF of IPF patients. 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, 1997). MWCNTs induce increased eosinophil count in lungs (Køobler C, 2015). MWCNTs act as allergens and induce lung infiltration of eosinophils and cause airway hypersensitivity (Beamer, 2013).

It is important to note that the stressor-induced MIE, KE1 and KE2 are part of the functional changes that we collectively consider as inflammation, and together, they mark the initiation of acute inflammatory phase. MIE and KE1 occur at the cellular level. KE2 occurs at the tissue level.

How it is Measured or Detected

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 (BALF) is lavaged 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. Details of in vitro methods are described under KE2.

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Event: 1498: Loss of alveolar capillary membrane integrity (https://aopwiki.org/events/1498)

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 (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	Not Specified	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Male	High
Female	Not Specified

Key Event Description

Loss of alveolar capillary membrane integrity

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 (Fakuda 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).

KE3 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 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, which when uncontrolled, lead to extensive tissue injury, cellular death and necrosis, the other characteristics of chronic inflammation, leading to ACM loss. The other types of inflammatory cells found in chronic inflammation include eosinophils in allergen induced lung fibrosis, lymphocytes and epithelial cells.

KE3 associative event 2 - Oxidative stress

Superoxide anion (O2-) and the hydroxyl radical (OH) are the common ROS found in the biological systems that are unstable due to unpaired electrons. As such, all tissues including lung have efficient antioxidant system to counteract the ROS induced oxidation. The antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase act directly to inactivate ROS and associated reactions. In addition, phase II detoxifying enzymes, including glutathione-S-transferase, NADPH quinone oxidoreductase and glutamate-cysteine ligase catalytic act as indirect antioxidant enzymes. However, when the balance between oxidant and antioxidants is tipped towards oxidants, oxidative stress occurs. ROS, when interacted with biomolecules, initiate their oxidation. During the process, proteins, DNA and lipids are oxidized. Oxidative stress modulates the cellular signalling processes and contributes to oxidative stress-induced tissue injury. It also plays a role in the tissue injury caused by inflammatory processes in lungs. The infiltrating neutrophils and macrophages generate superoxide anion which is converted to hydrogen peroxide by superoxide dismutase enzyme. OH is formed by a secondary reaction in the presence of Fe2+. ROS can also be produced by NADPH oxidase present in phagocytes. The other enzyme that contributes to ROS synthesis during inflammatory processes is the myeloperoxidase from neutrophils. In a self-perpetuating loop, inflammatory cells generate oxidative stress leading to increased airspace epithelial permeability, increased cell death and increased expression of pro-inflammatory genes, all of which lead to secretion of inflammatory cytokines/chemokines leading to prolonged and chronic inflammation.

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 pro-fibrotic drug bleomycin destroys structural architecture of the 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).

Epithelium and basement membrane injury is a prerequisite for the development of fibrotic lesion (Brody et al., 1981). 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, significant loss of the epithelium and endothelium of the ACM resulting in loss of the barrier. In patients diagnosed with idiopathic pulmonary fibrosis, both type 1 pneumocyte and endothelial cell injury with the ACM barrier loss is observed.

Bleomycin and silica exposure generate persistent inflammation and lung damage (Thrall, 1995; Chau, 2005). Exposure to SWCNTs induces persistent inflammation, granuloma and diffuse intestinal fibrosis in mice after pharyngeal aspiration (Shevedova, 2005). MWCNTs act as allergens and induce lung infiltration of eosinophils and cause airway hypersensitivity (Beamer, 2013). Inhaled particles induce chronic inflammation (Hamilton, 2008; Thakur, 2008; Ernst, 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, 2007; Schwartz 1991; Yasuoka, 1985).

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

How it is Measured or Detected

Proteinosis, BAL fluid protein content

The compromised ACM 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).

Cel 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, 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.

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.

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Event: 1499: Increased, activation of T (T) helper (h) type 2 cells (https://aopwiki.org/events/1499)

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 (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Key Event Description

How does this KE work

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.

KE4 associative event - Macrophage polarisation

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. During chronic inflammation, the phenotype of infiltrating macrophages is suggested to resemble that of M2, which is suggested to play a role in lung fibrosis.

Evidence for its perturbation

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, 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, 1999). Both MWCNTs and SWCNTs induce elevated expression of IL-4 and IL-13 in BALF of mouse lungs (Park, 2011), and increased levels of IL-25 and IL-33 in BALF and mouse lungs exposed to MWCNTs (Dong, 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, 2016). Overexpression of IL-10 increases IL-4 and IL-13 production and lung fibrosis following exposure to silica (Barbarin, 2005). Alveolar macrophages from asbestosis patients (a form of lung fibrosis) exhibit M2 phenotype (Chao 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, 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, 2014). Cigarette smoke induces expression of genes associated with M2 sub-phenotypes, which is further enhanced in smokers presenting with COPD (Shaykhiev, 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 MIE. The details of BALF sample collection is described under KE2.

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Event: 1500: Increased, fibroblast proliferation and myofibroblast differentiation (https://aopwiki.org/events/1500)

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 (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

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 epithelial-mesenchymal 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, 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) (Phillip 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.

Evidence for its perturbation

IPF is characterised by progressive fibroblast and myofibroblast proliferation and excessive deposition of extracellular matrix (Kuhn et al., 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., 2010; Wang et al., 2010; 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 (Kai, 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_2O_2 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 semi-quantitative, 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^2) 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.

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Event: 1501: Increased, extracellular matrix deposition (https://aopwiki.org/events/1501)

Short Name: Increased extracellular matrix deposition

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 (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Key Event Description

ECM is a macromolecular structure that provides physical support to tissues and is essential for organ function. The composition of ECM is tissue specific and consists mainly of fibrous proteins, glycoproteins, and proteoglycans. The ECM in lung is compartmentalised to basement membrane and the interstitial space. Fibroblasts found in the interstitial space are the main sources of ECM in lung (White, 2015). Altered composition of ECM is observed in several lung diseases of inflammatory origin in humans including Chronic Obstructive Pulmonary Disease, asthma and idiopathic lung fibrosis. The composition and architecture of the ECM determines 1) the open sites of attachment that are available to cells, 2) the mechanical properties of the ECM and 3) the mechanical loading (breathing) experienced by the cells. Thus, changes in the ECM composition during the exaggerated wound healing process determines if an organism commits to fibrotic process or completes the wound healing (Blaubouer et al., 2014).

In lung fibrosis, an exaggerated amount of ECM is distributed in the alveolar parenchyma in a non-heterogenous manner, leading to lower spirometry readings implying occlusion of alveolar regions and reduced gas exchange. Collagen 1 and Collagen III are suggested to be the main components of the ECM in the thickened alveolar septa in fibrosis with other constituents such as fibronectin, elastin and tenacin C (Zhang et al.,

1994; Hinz, 2006; Kuhn and McDonald, 1991; Crabb et al., 2006; Bensadoun, 1996; Klingberg et al., 2013; McKleroy et al., 2013). It is suggested that ECM composition dramatically changes during the fibrotic process. The early fibrotic process is characterised by collagen III deposition and collagen 1 predominates the later stages of the fibrosis. Excessive collagen production by myofibroblasts is necessary for the development of fibrosis (scarred tissue), with established areas of scar formation containing almost exclusively Type I collagen (Bateman et al., 1981; McKleroy et al., 2013; Zhang et al., 1994). Studies have demonstrated that while total collagen increases in IPF, there is also a shift toward the less elastic type I collagen, which contributes to the stiffness of the scar tissue within the lung (Nimni, 1983; Rozin et al., 2005; McKleroy, Lee and Atabai, 2013).

The fibrotic ECM contains characteristic accumulation of fibroblasts and myofibroblasts, which are the major contributors of ECM synthesised. The proliferation of fibroblasts and their differentiation into myofibroblasts is, in turn, guided by the composition and structure of the ECM. For example, Studies have demonstrated that cytokines secreted in response to inflammation are capable of activating fibroblasts, and that these changes could cause alterations in the fibroblasts that lead to excessive proliferation and ECM deposition (Sivakumar, 2012; Wynn, T.A., 2011).

How it is Measured or Detected

How it is measured

The qRT-PCR, ELISA, and immunohistochemistry are routinely used to assess the levels of protein and mRNA levels. The various genes and proteins that are assessed include, collagen I, collagen III, elastin and tenacin C. 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 and 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 and Raghunath, 2009; Nikota et al., 2017).

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List of Adverse Outcomes in this AOP

Event: 1458: Pulmonary fibrosis (https://aopwiki.org/events/1458)

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 (https://aopwiki.org/aops/241)	AdverseOutcome
Aop:173 - Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	AdverseOutcome

Stressors

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

Biological Context

Level of Biological Organization
Organ

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** (file:///\\NCR-A_HECSBU4S\pubmed\%3fterm=Moeller%20A%5bAuthor%5d&cauthor=true&cauthor_uid=17936056) A et al., **2008;** (file:///\\NCR-A_HECSBU4S\entrez\eutils\elink.fcgi%3fdbfrom=pubmed&retmode=ref&cmd=prlinks&id=17936056) 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). 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.

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Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Description

Lung/pulmonary fibrosis – thickened alveolar septa

Fibrosis or scarring is a fixed end result of damage in a tissue not capable of regeneration with no possibility of restoring the original tissue architecture. In normal lung, an individual alveolus spans about 0.2mm in diameter and there are 300 million alveoli in an adult male lung. 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, 1968). In fibrotic disease however, a pronounced decrease in the number of capillaries within the alveolar septa is found 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

How it is measured

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, 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 (https://aopwiki.org/relationships/1702)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	**adjacent**	**High**	**Not Specified**

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 C, 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.

Relationship: 1703: Increased proinflammatory mediators leads to Recruitment of inflammatory cells (https://aopwiki.org/relationships/1703)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	**adjacent**	**High**	**High**

Relationship: 1704: Recruitment of inflammatory cells leads to Loss of alveolar capillary membrane integrity (https://aopwiki.org/relationships/1704)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	**adjacent**	**High**	**High**

Relationship: 1705: Loss of alveolar capillary membrane integrity leads to Activation of Th2 cells (https://aopwiki.org/relationships/1705)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	**adjacent**	**High**	**Moderate**

Relationship: 1706: Activation of Th2 cells leads to Increased cellular proliferation and differentiation (https://aopwiki.org/relationships/1706)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	**adjacent**	**High**	**High**

Relationship: 1707: Increased cellular proliferation and differentiation leads to Increased extracellular matrix deposition (https://aopwiki.org/relationships/1707)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	**adjacent**	**High**	**High**

Relationship: 1708: Increased extracellular matrix deposition leads to Pulmonary fibrosis (https://aopwiki.org/relationships/1708)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	**adjacent**	**High**	**High**