AOP ID and Title:

SNAPSHOT

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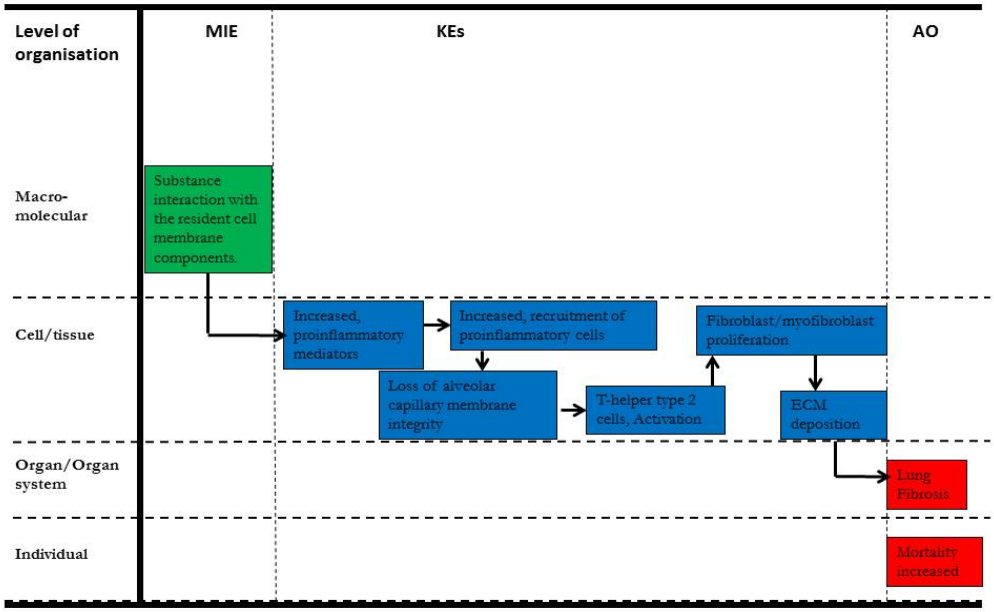
AOP 173: Increased substance interaction with the resident cell membrane components leading to lung fibrosis

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

Graphical Representation



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Abstract

This adverse outcome pathway (AOP) describes the linkages between the interaction of substances with the cellular membrane components and the lung fibrosis. Lung fibrosis is a dysregulated or an exaggerated tissue repair process. It denotes the presence of scar tissue in the alveolar capillary region of the lung where gas exchange occurs; it can be localised or more diffuse involving bronchi and pleura. The process involves intricate dynamics between several inflammatory and immune response cells, and the microenvironment of the alveolar-capillary membrane consisting of both immune and non-immune cells, and the lung interstitium, in the presence of sustained or repeated toxicant stimuli. Regardless of the type of stimulus, 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 and pro-fibrotic mediators are secreted (Key Event (KE) 1) 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, the purpose of which is to clear the invading pathogen or toxic substance. In the presence of continuous stimulus or persistent toxic substances, tissue injury ensues leading to the alveolar capillary membrane integrity loss (KE3) and activation of adaptive immune response. The purpose of the adaptive immune response is to resolve the inflammation and initiate healing process, involving activation of the T Helper type 2 cell signalling (KE4), during which anti-inflammatory and pro-repair/fibrotic molecules continue to be secreted. Once the healing process is initiated, fibroblast proliferation and myofibroblast differentiation is induced (KE5) leading to synthesis and deposition of extracellular matrix or collagen (KE6). Exaggerated collagen deposition leads to alveolar septa thickening, decrease in total lung volume, and lung fibrosis (Adverse Outcome). It is important to note that many of the individual KEs occur in parallel, early after exposure to fibrogenic stimuli and thus, it is difficult to establish key event relationships (KERs). The eventual clinical manifestation of the disease is influenced by the physical-chemical properties of the substance and duration of exposure.

Lung fibrosis can be induced by many substances, microorganisms or by over expression of specific inflammatory mediators such as cytokines and chemokines. This AOP is also applicable to materials such as nanomaterials that induce an inflammatory response as well as possess unique properties that allow for significant chronicity of the response, which takes place deep within the lung, beyond the airways and within the alveoli. Lung fibrosis occurs in humans and the key biological events involved are similar as the ones observed in animals. Thus, this AOP provides a detailed mechanistic account of the process of lung fibrosis across species.

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	Increased, interaction with the resident cell membrane components (https://aopwiki.org/events/1495)	Interaction with the 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	Increased, 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
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

Sequence	Type	Event ID	Title	Short name
8	AO	1458	Pulmonary fibrosis (https://aopwiki.org/events/1458)	Pulmonary fibrosis

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increased, interaction with the resident cell membrane components (https://aopwiki.org/relationships/1702)	adjacent	Increased, secretion of proinflammatory and profibrotic mediators	High	High
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	Increased, loss of alveolar capillary membrane integrity	High	High
Increased, 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	
Carbon nanotubes, Multi-walled carbon nanotubes, single-walled carbon nanotubes, carbon nanofibres	

Overall Assessment of the AOP

Overall assessment of AOP

Assessment of the Weight-of-Evidence supporting the AOP

Concordance of dose-response relationships

The pathway presented in this AOP is qualitative. There is some evidence on dose-response relationships; however, dose-response relationships for each individual KE are not available. Although there is empirical evidence to support the occurrence of each individual KE in the pathway of lung fibrosis, their essentiality to the final AO is not always experimentally investigated in the same studies or separately. For example, Gilhodes J-C (2017) showed that single intratracheal instillation of 0.25, 0.5, 0.75, and 1 mg/kg of bleomycin in mice induced alveolar thickening at 14 days post-exposure in a dose-dependent manner. However, this study did not investigate the occurrence of other KEs. *In vivo* exposure to different types of CNTs has been shown to induce all individual KEs involving inflammation and alveolar thickening in both mice and rats (Aiso et al., 2010; Dong et al., 2014; Lam et al., 2004; Mangum et al., 2006; Muller et al., 2005; Park et al., 2011; Porter et al., 2010, 2013; Shvedova et al., 2005). The inflammatory response following treatment with CNTs is typically characterised by the recruitment of inflammatory cells, and secretion of pro-inflammatory mediators; the lung fibrosis is characterised by the histopathological analysis for collagen deposition, fibrous lesions, and proliferation of fibroblasts. However, these studies did not evaluate the KERs.

The dose and the time-response of CNT-induced lung fibrosis is investigated in many studies. For example, pharyngeal aspiration of 10, 20, 40, or 80 µg/mouse MWCNT induced lung fibrosis in a dose-dependent manner which was apparent as early as 7 days post-exposure at 40 µg/mouse dose and persisted up to 56 days post-exposure (porter DW, 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 RR, 2011). Similarly, inhalation of MWCNTs (10mg/m3, 5h/day) for 2, 4, 8, or 12 days showed dose-dependent lung inflammation and lung injury with the development of lung fibrosis in mice (Porter DW, 2013). Lung inflammation and fibrosis was observed in mice intratracheally instilled with 162 µg/mouse MWCNTs at 28 days post-exposure Fibrotic changes in lungs persisted up to 1 year post-exposure to 40 or 80 µg/mouse MWCNTs. All of the studies involving CNTs showed elevated levels of pro-inflammatory mediators, pro-inflammatory cells and cytotoxicity in BALF.

Barbarin et al (2005) showed that silica particles induce early inflammatory KEs in both rats and mice; however, the magnitude of the inflammatory response was much severe in rats compared to mice. Regardless, the extent of fibrosis as measured by collagen deposition and alveolar thickening was the same between the two species. Again, a clear dose-response relationship between KEs and the end AOP was not established. Silica-induced lung injury involves all the KEs described in this AOP including the associative events of chronic inflammation and ROS generation (Hubbard AK, 2005). Although the fibrotic pathology induced by silica involves acute inflammatory phase and Th2 response, the role of these KEs in progression of the disease itself is not conclusive (Barbarin C, 2005; Brombacher MP, 2007).

Studies have shown that inhibition of inflammatory events early after exposure to bleomycin (Gasse P, 2007) or CNTs (Nikota J, 2017) attenuate fibrotic response in mice. These studies provide the necessary evidence supporting the essentiality of inflammatory KEs to the end AO.

There is enough empirical evidence to show the temporal associations between the individual KEs leading to the AO.

Strength, consistency, and specificity of association of adverse outcome and initiating event

Depending on the type of substance and its physical-chemical property, the type of interactions with resident cells differs. Asbestos fibres are shown to bind directly to cellular macromolecules including proteins and membrane lipids, which is influenced by their surface properties such as surface charge (reviewed in Hanley GD, 1995). The alarmin HMGB1 is released from damaged or nectrotic cells in cell culture models and in animals following exposure to asbestos and is suggested to be involved in the inflammatory events elicited by asbestos (Yang H, 2010). Interaction of CNTs with HMGB1-RAGE is implicated in pro-inflammatory and genotoxic effects of CNTs (Hiraku Y, 2016). Mechanical stress and membrane damage following cellular uptake of long and stiff CNTs by lysosomes is involved in cell injury and consequent adverse effects (Zhu W, 2016). CNT-induced inflammatory response in vitro is mediated by IL-1, absence of which negatively impacts gap junctional intercellular communication (Arnoldussen YJ, 2016). The levels of IL-1a are increased in BALF of mice immediately after exposure to MWCNTs (Nikota J, 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 final AO. The inconsistency could be due to the fact that early defence mechanisms involving DAMPs is fundamental for survival, which may necessitate redundancy in signalling pathways involved. 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 J, 2017) and thus, IL-1a mediated signalling is suggested to be involved in MWCNT-induced lung inflammation and fibrosis (Rydman EM, 2015). However, inhibition of IL-1a signalling alone did not alter the MWCNT-induced fibrotic response in mice (Nikota J, 2017). This study further showed that simultaneous inhibition of both acute inflammatory events and Th2 –mediated signalling may be required to suppress lung fibrosis induced by MWCNTs (Nikota J, 2017). Disengagement between innate immune responses including MIE, KE1 and KE2, and ultimate lung fibrosis has been shown in a mouse model following exposure to silica (Re SL, 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 suggested to be involved in bleomycin-induced lung inflammation and fibrosis; inhibition of IL1-R1 signalling attenuates the bleomycin pathology (Gasse P, 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 MIE and individual KEs, and thus evidence supporting the AOP is strong. Although, there is inconsistency in empirical evidence supporting the KERs, the AOP is established with the existing biological evidence. Thus, the 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 for any known substance. However, many may argue that innate immune response involving acute inflammation and associated KEs may not be necessary for lung fibrosis. This raises the question if the MIE should be a KE that involves lung injury or activation of cells that produce collagen.

Uncertainties, inconsistencies and data gap

As mentioned earlier, the AOP is based on the existing knowledge and describes the sequence of events that are shown to occur following exposure to many fibrogenic substances. However, it is mostly qualitative and additional studies are needed to support the essentiality of the KEs.

As described above, 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. Regardless of the type of interactions or the substance characteristics, the end outcome of lung fibrosis follows the same sequence of events.

Essentiality of the Key Events (key event relationships)

Weight of evidence summary

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: Increased, substance interaction with the resident cell membrane component leads

to increased pro-inflammatory mediators

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

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, IL-1 is a pleotropic cytokine and impacts nearly every cell in the body. Members of the IL-1 signalling pathway are evolutionarily conserved across many species including mammals, insects, plants, and yeast. Certain members of the IL-1 signalling pathway seem to have been present in the unicellular ancestral organisms of both plants and animals, suggesting that IL-1 represents an ancient defence system that provided protection against pathogen invasion. In higher vertebrates, IL-1 signalling plays a vital role in defence against infection and injury (O'Neill LAJ and Greene C, 1998). IL-1a is an alarmin. When released from the damaged cells, IL-1a stimulates host-derived alarmin/IL-1a synthesis, thus perpetuating and sustaining the response. The stressor can also trigger the IL-1R1 signalling without the initial cell death. In such cases, the external stressors trigger translocation of intracellular pro-IL-1a onto the plasma membrane, where IL-1a appears as membrane bound molecule ready to bind IL-1R1 on the neighbouring nonhematopoietic cells or resident macrophages, activating the IL-1R1 cascade. Binding of IL-1a to IL-1R1 can release NF-κb resulting in its translocation to nucleus and transactivation of pro-inflammatory genes including cytokines, growth factors and acute phase genes, and increased 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 CJ, 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, peritoneum. Other types of cells including macrophages, eosinophils, lymphocytes are also recruited in a signal-specific manner. Recruitment of leukocytes (neutrophils mainly) 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 V, 2008; Girtsman TA, 2014; Gasse P, 2007; Nikota J, 2017; Suwara MI, 2013; Rabolli V, 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 J, 2017), cigarette smoke (Halappanavar S, 2013), silica crystals (Rabolli V, 2014; Re SL, 2014) and bleomycin (Gasse P, 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 J, 2017). The inconsistency could be due to the fact that early defence mechanisms involving DAMPs is fundamental for survival, which may necessitate redundancy in signalling pathways involved. 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.

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 that greater concentrations or doses induce higher release of IL-1a and concomitant IL-1R1 signalling, resulting in a higher neutrophil influx in lungs. However, these studies demonstrate strong temporal relationships between the individual KEs.

KE2 – KE3

Increased, recruitment of proinflammatory cells leads to loss of alveolar capillary membrane integrity

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 C, 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 (Robert M, 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 (Robert M, 2009). In rodents treated with bleomycin, the damaged ACM resembles that seen in the fibrotic human lung (Grandel NR, 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 O, 2017), which leads to ACM integrity loss. Neutrophils are the dominant cell population during acute inflammation and communicate with other immune cell types such as platelets, monocytes, dendritic cells, and different types of lymphocytes via the mediators they synthesise and secrete (Nathan C, 2002). However, the exact mechanisms by which acute neutrophilic inflammation leads to chronic inflammation vary and depend on the type of tissue injury or properties of the toxic substance. Some of the common links between neutrophils and chronic inflammation include: 1) neutrophils deposit a variety of granule proteins on the endothelium, some of which act as chemotactic for monocytes, 2) it is shown that neutrophils also secrete alarmins such as S1008, S1009, and high-mobility group box 1, which drive myeloid cell recruitment and induce recruitment of pro-inflammatory macrophages, 3) prolonged life span of neutrophils - normally, death of neutrophils marks the beginning of resolution phase of the acute inflammation, during which dead neutrophils are cleared by macrophages and normal homeostasis is established, and 4) neutrophils committed to apoptosis also activate signals that degrade pro-inflammatory mediators, and thus deplete and dampen the inflammatory stimulus. Clearance of neutrophils from the inflammatory site triggers resolution of inflammation and consequently, the tissue repair process (Nathan C, 2002).

In the presence of persisting or repeated tissue injury, these 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. It has been shown that in 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 can lead to inflammation, pulmonary injury and subsequently, fibrosis in experimental bleomycin models (Chaudhary NI, 2006). ROS released by neutrophils via the multicomponent enzyme nicotinamide adenine dinucleotide phosphate oxidase 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 S 2012; Koli K, 2008).

Exposure to high doses of insoluble materials such as ENMs 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 2004), which in turn, is dependent on activation of NF-κB and ROS synthesis (Shi et al 1998; Cassel et al 2008; Kawasaki 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 W, 2001). Several studies have reported that anti-oxidant treatment attenuates the bleomycin-induced oxidative burden and subsequent pulmonary fibrosis (Wang, HD, 2002; Serrano-Mollar A, 2003; Punithavathi, D, 2000).

Uncertainties or inconsistencies

Although there is enough evidence to suggest a role for persistent inflammation 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, the 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 SN, 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 (Bin Ma, 2016).

KE3-KE4

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

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 and pro-inflammatory and fibrotic mediators including GATA-3, IL-13 and Arg-1 are increased in lung-irradiation induced fibrosis (Wynn TA, 2004; Brush J, 2007; Han G, 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, shows that Th2 response associated genes are upregulated in fibrotic lungs (Nikota J, 2016). Exposure of mice lacking STAT6 transcription factor to fibrosis causing doses of MWCNTs resulted in abrogated expression of Th2 genes and reduced lung fibrosis (Nikota J, 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 MS and Wynn TA, 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 E, 2005). Chemokines associated with the Th2 response in airway epithelial cells include CCL1, CCL17, CCL20, and CCL22 (Lekkerkerker N, 2012).

Weight of evidence studies establishing the KER are very scarce and data is not available to establish the quantitative dose- or time- response relationships.

KE4-KE5

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

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 TGF-β, 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 TGFb1 in macrophages and its absence results in reduced TGFb1 expression and decrease in collagen deposition (Fichtner-Feigl S, 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 L and Wynn TA, 2011).

Weight of evidence

C57BL/6 mice exposed to MWCNTs for seven days showed altered expression of a wide variety of Th2 cytokines including IL-4 and IL-13 (Dong J and Ma Q, 2016). The study implicated IL-4 and IL-13 signalling as well as activation of transcription factors STAT6 and GATA3 are necessary for MWCNT-induced lung fibrosis in rodents. In agreement with this study, in a meta-analysis, comparison of gene expression profiles of lungs of mice exposed to different types of nanomaterials to the lung transcriptomics data from mouse models of lung inflammation and lung fibrosis showed significant similarities between Th2-cytokine model and MWCNT-induced lung fibrosis model (Nikota J, 2016). Moreover, exposure of STAT6 transgenic mice to MWCNTs suppressed the expression of Th2-mediated gene expression and the overall fibrotic response to MWCNTs (Nikota J, 2017).

Uncertainties or inconsistencies

Since a vast number of pro-inflammatory mediators with redundant functions are involved in these processes, targeting a single molecule or a pathway is not sufficient enough to impact the KE or the eventual adverse outcome.

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

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-1b and TGF-β, 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 IA, 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 TGF-β, 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 U, 2012; Wallace WA, 2007).

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

There is some evidence to show that inhibition of fibroblast proliferation and differentiation by counteracting the activity of TGF-β attenuates bleomycin-induced lung fibrosis (Chen Y-L, 2013; Guan R, 2016). Several studies have shown that inhibition of TGF-β involved both 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.

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 redundancy in the signalling pathways involved. Therefore, the results of the essentiality experiments may show incongruence based on the individual protein, gene or a pathway selected for inhibition.

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 lacking.

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 of other organs. Moreover, 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 this some of the KEs in this AOP can be extended to any study investigating inflammation mediated adverse outcomes.

Are the initiating and key evets 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.

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

Quantitative Consideration

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.

Considerations for Potential Applications of the AOP (optional)

Considerations for potential applications of the AOP

Efforts are ongoing to develop a predictive *in vitro* model to assess the fibrogenic potential of substances. Additional work is needed to optimise these cell systems to mimic the *in vivo* conditions. 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, design and development of relevant *in vitro* models for screening, prioritising, and assessing the chemicals' potential to induce fibrosis. This in turn will facilitate uptake of data derived from alternative toxicity testing methods for the purposes of regulatory decision making. The assessment of the extent of perturbation of the MIE, KE1, and KE2 can help rank the fibrogenic capacity of a substance, all of which are influenced by the physical-chemical properties of a substance.

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Appendix 1

List of MIEs in this AOP

Event: 1495: Increased, interaction with the resident cell membrane components (https://aopwiki.org/events/1495)

Short Name: Interaction with the cell membrane

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Increased substance interaction with the 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

Not many studies investigate the exact mode of interaction between the toxic substance and the cellular membrane components. Rather, the consequences of such interaction such as, the release of intracellular contents (alarmins), increases in genes or protein synthesis downstream of receptor binding or cellular uptake are measured as indicative of occurrence of such interactions (Nel AE et al., 2009; Cheng L-C et al., 2013).

Because of the physical –chemical properties such as surface charge, ENMs 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 2011 a; Pascolo L et al., 2013). Intratracheally instilled crystalline silica interacts with lung epithelial cells and alveolar macrophages and induces pro-inflammatory cascade via NF-κb signaling pathway (Hubbard AK, 2002).

Exposure to MWCNTs results in increased IL-1a protein levels in the bronchoalveolar lavage fluid (BALF) of lungs in mice (Nikota J et al., 2017) and lung epithelial cells exposed to silica show lysosomal accumulation of silica in a size-dependent manner (Decan N et al., 2016).

Key Event Description

Background

The human lung with the surface area of ~100 m^2 is ventilated by 10-20,000 litres of air per day. Thus, it is the largest organ of the human body that is exposed to the environment contaminated with pathogens, organic and inorganic materials. The gas exchange between blood and the inhaled air takes place across a membrane that is only 1-2 µm thick, making the lung an easy target for foreign invasion and toxic chemicals in the environment. To combat the constant attack by the pathogens and chemicals, the lung is equipped with several defence mechanisms; a layer of epithelial cells acting as physical barrier to limit entry of pathogens and specialised cells of the immune system with defence functions (reviewed in **Weitnauer M** (file://ncr-a_hecsbu4s/pubmed/?term=Weitnauer%20M[Author]&cauthor=true&cauthor_uid=26627458) et al., 2016). The human lung consists of approximately 40 different resident cell types that play different roles during homeostasis, injury, repair, and disease states (**Franks TJ** (file://ncr-a_hecsbu4s/pubmed/?term=Franks%20TJ[Author]&cauthor=true&cauthor_uid=18757314) et al., 2008). Of these cells, resident airway epithelial cells, alveolar 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.

Receptor-mediated interactions

The chemicals or pathogens interact with cellular membrane to gain access to the organisms' interior. This interaction between the pathogens/substances and lung cells can occur via binding of the pathogens or pathogen associated molecular patterns (PAMPs, microbial molecules) to the receptors present on the surfaces of the resident cells. For example, the airway epithelium and the mucosal layer form a physical barrier and contribute to the first line of defence. The resident epithelial cells, by default, under the normal homeostatic conditions, are adjusted to the local microbial burden and therefore, are less responsive to microbial stimulation. The resident epithelial cell surfaces express receptors that recognise and sense the presence of pathogens through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) resulting in epithelial cell activation. PRRs are also present on other resident cells including alveolar macrophages. PRRs can be activated by interaction with debris from dying cells (cellular fragments) as a result of interaction with toxic substances. Engagement of PRRs and consequent cell activation is observed in various organisms including flies and mammals (Matzinger, 2002).

Opsonisation driven interactions

In case of non-pathogens such as insoluble particles or engineered nanomaterials (ENMs), interaction with cellular membrane can occur via the process of opsonisation. For example, the surfaces of ENMs can be adsorbed with opsonins (protein corona) such as immunoglobulins, complement proteins, or serum proteins that are then recognised by the phagocytes (macrophages). Opsonised ENMs attach to the cellular membrane of phagocytes via ligand-receptor interactions. Some of the receptors on the cell surface that are engaged by the opsonins include Fc receptors and complement protein receptors (reviewed in Behzadi S et al., 2017). The endocytosis processes such as phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and micropinocytosis define the interactions between cell membrane and the particles (airborne or ENMs).

Physical or mechanical interactions

The other type of interaction can involve physical damage to cells. High aspect ratio (HAR) materials such as asbestos or CNTs can pierce the cellular membrane of epithelial cells or resident macrophages resulting in cell injury or non-programmed cellular death. The resident macrophages are present in all tissues, and in a steady state, 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. Resident macrophages in the lung, bone, brain, liver, spleen, skin, and in the intestine are known as alveolar macrophages, osteoclasts, microglia, Kupffer cells, splenic macrophages, Langerhans cells and intestinal macrophages, respectively (Kierdorf K et al., 2015). It has been shown that alveolar macrophages trying to engulf HARs including asbestos and CNTs that are long and stiff undergo frustrated phagocytosis because of their inability to engulf the piercing fibres and subsequently lead to cell injury (Mossman BT et al., 1998; Donaldson K et al., 2010).

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 or activation of TLR receptors - indicative of binding of PAMPs to 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. DAMPs, also called as alarmins are endogenous molecules that are released by stressed cells. A few of the putative alarmins that can be effectively measured in biological samples including cultured cells are High Mobility Group Binding 1 (HMGB1) protein, Heat Shock proteins (HSPs), uric acid, annexins, S100 proteins and IL-1α (Bianchi ME, 2007).

Of all, IL-1a is the most commonly measured alarmin. IL-1a is the principal pro-inflammatory moiety and is a designated 'alarmin' in the cell that

alerts the host to injury or damage (Di Paolo NC, 2016). It is shown that administration of necrotic cells to mice results in neutrophilic inflammation that was entirely mediated by IL-1a released from the dying or necrosed cells and consequent activation of IL-1 Receptor 1 (IL-1R1) signalling (Suwaraa MI et al., 2014). IL-1a is released following exposure to MWCNTs (Nikota J et al., 2017) and silica (Rabolli V et al., 2014). IL-1a can be cleaved from its precursor form to the active form; however, it is not a prerequisite since both precursor and cleaved forms of IL-1a are active that bind and activate IL-1R1 signaling.

The release of DAMPs can be measured by the techniques listed below.

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 D et al., 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 is activated or secreted into the cytosol following stimulus. Targeted ELISA for IL-1a can be used to quantify IL-1a 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.

Measuring cellular uptake of ENMs

Lysosomal localisation – In vitro, interaction of ENMs with the cellular membrane can be investigated by assessing their uptake by the lysosomes (Varela JA 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 N et al., 2016) or confocal microscopy to visualise co-localisation of fluorescence labelled nanoparticles with lysosomal markers have been used.

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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 - Increased substance interaction with the resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Key Event Description

Proinflammatory mediators are the chemical and biological molecules that initiate and regulate inflammatory reactions. Cell-derived proinflammatory mediators include cytokines, chemokines, and growth factors. Blood derived proinflammatory 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 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. Some of the proinflammatory 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 MD 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 proinflammatory mediators are secreted during innate or adaptive immune responses across various species (Mestas J et al., 2004). However, IL-1 family cytokines, IL-4, IL-5, IL-6, TNFa, IFNg are the commonly measured mediators in experimental animal models and in humans. Proinflammatory mediators are secreted following exposure to an inflammogen in a gender/sex or developmental stage independent manner.

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. 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 AK, 2002). Long and thin CNTs (>5 μm) can elicit asbestos-like pathogenicity through the continual release of pro-inflammatory cytokines and ROS (Poland CA, 2008). Lung responses to long MWCNTs included high expression levels of MCP-1, TGF-β1, and TNF-α (Boyles MSP, 2015). In vitro, CNTs have been shown to induce increased secretion of cytokines and chemokines (He X, 2011; Herano S, 2010).

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.

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 proinflammatory mediators in several species including mouse, rat and humans, are readily available commercially.

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 D et al., 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.

Both ELISA and qRT-PCR assays 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 M 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 proinflammatory mediators and its downstream effectors in any given tissue (Costa PM 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 D 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 - Increased substance interaction with the resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Key Event Description

Proinflammatory cells originate in bone marrow and are recruited to the site of infection or injury via circulation following specific proinflammatory mediator (cytokine and chemokine) signalling. Proinflammatory 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).

Dendritic cells are antigen-presenting cells (APCs) and they stimulate naïve T-cell proliferation. They are found in the airway epithelium, the alveolar septa, pulmonary capillaries and airway spaces. APCs identify, ingest and process an antigen and present the antigen to resident T-cells in the lymph node initiating the immune response (Kopf M et al., 2015).

Macrophages are the components of mononuclear phagocyte system; they phagocytose pathogens, nanoparticles and other cellular debris. Mononuclear phagocytes originate in bone marrow as blood monocytes, which migrate to different tissues where they differentiate into tissue macrophages. At least three types of macrophages exist in lung: bronchial macrophages, interstitial macrophages and alveolar macrophages. They are found in the air space of the alveoli, parenchymal space (interstitial space) between adjacent alveoli. Lung macrophages can be characterised by their expression of surface markers. Activated macrophages release a variety of cytokines, chemokines, and growth factors (Kopf M et al., 2015).

Neutrophils are a type of polymorphonuclear leukocytes that are among the first to be recruited to an injured or inflamed site and play a major role in acute inflammation. They are the fundamental effectors of the host immune system and their basic role is to clear the infection or exogenous agents causing tissue injury. Neutrophils are continuously generated in the bone marrow and can be found in spleen, liver and lung. The lung holds a large reservoir of mature, terminally differentiated neutrophils, which are patrolling within the lung vasculature during steady state conditions (Kolaczkowska E and Kubes P, 2013). Neutrophil recruitment is initiated by the changes in the endothelium surface following stimulation by alarmin, DAMPs or inflammatory mediators released from resident leukocytes when they come in contact with pathogens or injurious external stimuli.

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 allergen induced fibrosis.

Activated immune cells secrete a variety of proinflammatory mediators, the purpose of which is to propagate the immune signalling and response, which when not controlled, lead to chronic inflammation, extensive cellular death and tissue injury.

It is important to note that the stressor-induced MIE, KE1 and KE2 are part of the functional changes that we collectively considered as inflammation, and together, they mark the initiation of acute inflammation phase.

How it is Measured or Detected

In vivo, recruitment of proinflammatory cells is measured using bronchoalveolar lavage fluid (BALF) cellularity assay.

The airway epithelium is the largest surface that is targeted by inhaled substances including ENMs. The fluid lining the epithelium (BAL fluid) is lavaged and its composition is assessed as marker of lung immune response to the toxic substances or pathogens. BAL is assessed quantitatively for types of infiltrating cells, levels and types of cytokines and chemokines. Thus, BAL fluid 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 regulatory purposes. For ENMs, in vivo BAL assessment is recommended as a mandatory test (discussed in ENV/JM/MONO(2012)40 and also in OECD inhalation TG for ENMs). Temporal changes in the BAL composition can be prognostic of initiation and progression of lung immune disease (Cho W-S et al., 2010).

In vitro, it is difficult to assess the recruitment of proinflammatory cells. Thus, a suit of proinflammatory 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.

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Event: 1498: Increased, 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 - Increased substance interaction with the resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

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. The cellular composition of ACM includes the type 1 alveolar epithelial cells, the capillary endothelial cells, and the endothelial and epithelial basement membranes. As a consequence of its anatomical position, and the large surface area, it is the first point of contact for any inhaled pathogens, particles or toxic substances. Thus, ACM is subjected to injury 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. Repeated exposure to or biopersistent toxic substances, pathogens or lung irritants leading to non-resolving inflammation contribute to the ACM injury. Chronic inflammation mediated by overexpression of cytokines such as IL-1, TGFb, T-helper type 2 cytokine IL-13 or exposure to specific proteinases initiate ACM injury leading to significant loss of the epithelium and endothelium of the ACM resulting 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. In addition, in normal conditions, the epithelial barrier consisting of alveolar epithelial type 1 and type 2 cells establish close contacts with the neighbouring cells via cell-cell adhesions through the intercellular junction complexes such as tight junctions, adherens junction and desmosomes (Vareille M et al., 2011; Kulkarni T et al., 2016). The various proteins found at the epithelial cell surface play a role in maintaining the cellular adhesions and intercellular junction complexes, which are critical for maintaining the integrity of alveolar epithelium. Exposure to pro-fibrotic drug bleomycin destroys structural architecture of the tight junctions leading to increased permeability, epithelial death and loss of specialised proteins such as caludins required for repair and restoration of alveolar epithelial barrier. Thoracic radiation and bleomycin-induced lung injury result in decreased expression of E-cadherin and Aquaporin-5 expression – proteins involved in adherens and in transgenic mice lacking aquaporin-5, increased lung fibrosis is observed. 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 ER et al., 2010).

Alveolar capillary membrane integrity loss is also associated with other events such as chronic inflammation and Reactive Oxygen Species (ROS) synthesis. Chronic inflammation and ROS synthesis are described below as associative events to ACM loss.

Chronic inflammation as an associative event in the loss of ACM

Chronic inflammation, by definition is associated with tissue injury (Wallace WA et al., 2007). In the presence of continuous stimulus (e.g., presence of biopersistent toxic fibres such as asbestos, multi walled carbon nanotubes) or following repeated stimulus (e.g., repeated exposure to silica or coal dust), the ensuing tissue 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. The chronicity of the inflammatory process occurs after prolonged acute inflammation and is a crucial component of the lung fibrotic response. During the chronic inflammatory 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.

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. Macrophages are the key inflammatory cells linking inflammation with repair and fibrosis. 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. The other types of inflammatory cells found in chronic inflammation include eosinophils in allergen induced lung fibrosis, lymphocytes and epithelial cells.

Oxidative stress as an associative event in the ACM loss

Oxidative stress is an important event that influences the extent of lung injury (reviewed in MacNee W, 2001). Superoxide anion (O2-) and the hydroxyl radical (OH) are the common ROS found in the biological systems that are unstable due to unpaired electrons. ROS, when interacted with biomolecules, initiate their oxidation. 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. 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 proinflammatory genes, all of which lead to secretion of inflammatory cytokines/chemokines leading to prolonged and chronic inflammation. Nrf2, a member of the cap'n'collar basic leucine zipper transcription factors is suggested to play an important role in orchestrating the antioxidant defence against ROS via antioxidant response element. Nrf2 is shown to be protective against pulmonary inflammation and fibrosis induced by oxidants (Kikuchi N et al., 2010).

Human idiopathic pulmonary fibrosis is suggested to result from increased ROS synthesis and imbalances in oxidant/antioxidant levels in distal alveolar space. Antioxidant treatment of mice attenuates bleomycin-induced oxidative stress and subsequent lung fibrosis (Wang HD, 2002). Several ENMs have been shown to induce oxidative stress (a state of redox disequilibrium), an event associated with their in vivo toxicity (Xia T, 2008). Bleomycin induced lung pathology involves oxidative stress. In mice treated with antioxidants, bleomycin-induced inflammation and pathology is attenuated (Kelly C, 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 B et al., 1992).

The physical-chemical properties of chemicals, their intended applications and exposure levels must be considered as important factors influencing the extent of lung injury. Although not quantitative, in case of ENMs, the physical presence of ENMs (biopersistence) must be confirmed in lungs following exposure, using Transmission Electron Microscopy or Cytoviva Nanoscale Hyperspectral Microscope. High aspect ratio materials including ENMs injure the ACM in rodent models.

Cell type considerations

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.

Cellular damage

Upon interaction with toxic substances, phagocytic cells internalize toxic substances leading to respiratory burst and release of ROS. A fluorimetric assay that relies on the intracellular oxidation of 5- and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy H2DCFDA) (Molecular Probes) has been used to detect ROS release in cells in vitro (Decan et al., 2016); however, it is important to note that the results are not specific to the types of radicals detected. In addition, lipid peroxidation, protein oxidation, and protein carbonylation can be measured as indicative of oxidative stress using proteomics techniques (Riebeling et al., 2001). Measurement of intracellular glutathione levels using the ThiolTracker™ Violet assay (Decan et al., 2016) or glutathionylation of proteins is also used. Oxidative stress can also be measured by assessing the relevant genes and proteins associated with antioxidant pathways (Riebeling et al., 2001). Other biomolecule modifications such as nitrosylation, reflective of oxidative stress, can be measured by measuring nitrosylated tissue proteins, or increase in NO production, and

nitrate/nitrite ratio in BAL. In addition to tissue analysis, acellular glutathione levels, antioxidants and NO production in BAL supernatant can be used to assess ROS synthesis. These methods have been routinely used to measure ROS release following exposures to several toxic substances including ENMs.

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.

Lactate dehydrogenase (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 ENMs can confound the assays by inhibiting the enzyme activity or interfering with the absorbance reading. Thus, care must be taken to include appropriate controls in the assay.

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 qRT-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 - Increased substance interaction with the 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

The loss of ACM involving epithelial cell injury engages the adaptive immune system resulting in the activation of CD4+ T cells. 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 (Kidd P, 2003). As described above, 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 (Lukacs NW. et al., 2001). 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 have shown complete attenuation of fibrosis despite the highly active Th1 response, clearly demonstrating the link between Th2 response and fibrosis. 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. In animal models of fibrosis, overexpression of IFNg negatively regulates fibrotic process. On the other hand, Th2 cytokines IL-4 and IL-13 regulate wound healing and contribute to the development and extension of fibrosis. IL-4 and IL-13 are suggested to stimulate the production of extracellular matrix proteins by activated fibroblasts; overexpression of IL-4 or IL-13 in fibroblasts increases ECM deposition. 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. Neutrophils recruitment during acute inflammation initiates Th2 immune response and secretion of Th2 type cytokines and chemokines (Lekkerkerker N, 2014). The members of the FIZZ (resistin-like molecules, RELM) proteins is induced in lung airway and epithelial cells following exposure to fibrogenic bleomycin (Liu T, 2004; Liu T, 2014). The expression of FIZZ is shown to be mediated by Th2 signalling and is involved in recruitment of inflammatory cells to lungs (Nair 2003; Munitz 2008; Angelini 2010; Madala 2012).

Macrophage polarisation as an associative event in the activation of Th2 cells

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 induce T helper (Th) 1 type responses. M1 macrophages produce IL-1, IL-12, IL-23 and induce Th1 cell infiltration and activation. M1 macrophages are associated with antigen presentation, microbiocidal and antitumour activities. 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-1 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 (Martinez FO and Gordon S, 2014). 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.

Pharyngeal aspiration of MWCNT for 7 days in mice induced lung fibrosis via activation of Th2 cells and Th2- mediated immune response, and included increased expression of Th2 cytokines such as IL-4, IL-13 and other genes. In addition, activation of Th2-mediated signaling pathway involving STAT6 transcription factor was also observed (Dong J and Ma Q, 2016). Meta-analysis of gene expression data derived from lungs of mice exposed to MWCNTs showed that the MWCNT-induced gene expression profiles are similar to the expression profiles induced in Th2 signalling-mediated lung fibrosis (Nikota J, 2016). In another study, mice deficient in STAT6 transcription factor showed attenuated lung fibrosis following exposure MWCNT, that was accompanied by reduced expression of Th2 cytokines and chemokines (Nikota J, 2017). Polarisation to Th2 and M2 phenotypes is observed in bleomycin-induced lung fibrosis (Li D, 2014).

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 FIZZ-1, 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 - Increased substance interaction with the resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Key Event Description

Fibroblasts are non-hematopoietic, non-epithelial and non-endothelial cells. In steady state conditions, they are distributed throughout the mesenchyme (Phan SH, 2008). 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. Fibroblasts are the

workhorse of the connective tissue that joins and holds other tissues together in the body. 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. In the context of lung fibrosis, fibroblasts play an important role in ECM maintenance and turnover, wound healing, inflammation and angiogenesis (Kendall RT, 2014). 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 et al 1994; Chesney et al 1998; Abe et al 2001). In bleomycin induced lung fibrosis model, human $CD34^+$ $CD45^+$ collagen I $CXCR4^+$ cells (fibrocytes) are shown to migrate to the lungs in response to both bleomycin and CXCL12 (CXCL12 binds 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[SH1] . 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.

Epithelial-mesenchymal transition is a process by which epithelial cells lose their original phenotype, acquire fibroblast-like properties, and display reduced cell adhesion (which is crucial for the detachment of epithelial cells prior to migration to the site of injury) and increased motility (Lekkerkerker et al 2012). During this process, epithelial cells also lose typical markers such as E-cadherin and zona occludens-1 (ZO-1) and acquire mesenchymal markers such as fibroblast-specific protein-1, vimentin and α-SMA (Grunert et al 2003; Zeisberg and Neilson 2009).

[SH1]Darby Clinical, Cosmetic and Investigational Dermatology 2014:7 301–311

How it is Measured or Detected

Immunohistochemistry (routinely used and recommended)

Proliferation of fibroblasts and activation of myofibroblasts is normally detected using individual antibodies against vimentin, procollagen 1 and alpha-smooth muscle actin, specific markers of fibroblasts and myofibroblasts (Zhang 1994). It is recommended to use more than one marker to confirm the activation of fibroblasts. The species-specific antibodies for all the markers are commercially available and the technique works in both in vitro and in vivo models as well as in human specimens. Immunohistochemistry is performed using immunoperoxidase technique. Formalin fixed and paraffin embedded lung sections are sliced in 3-5µm thin slices and reacted with diluted H_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 (Megan 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 to the same material (Vietti 2013; Azad 2013)

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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 - Increased substance interaction with the 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. 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 (reviewed in White 2015).

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. 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, 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 (Blaauboer ME, 2014). 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, P., 2012; Wynn, T.A., 2011).

In lung fibrosis, an exaggerated amount of ECM is distributed in the alveolar parenchyma in a non-heterogenous manner, occluding alveolar regions leading to 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, K., 1994; Hinz, B., 2006; Kuhn, C., 1991; Crabb, R.A., 2006; Bensadoun, E.S., 1996; Klingberg, F., 2013; McKleroy, W., 2013). Excessive collagen production by myofibroblasts forms the basis of scar formation containing almost exclusively Type I collagen (Bateman, ED, 1981; McKleroy, W., 2013; Zhang, K., 1994). Several types of collagen exist, with differences based on their tissue localisation and function. However, type I collagen is the most abundant throughout the body, as well as in lung scar tissue. 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, M.E.1983; Rozin, G.F., 2005; McKleroy, W., 2013).

How it is Measured or Detected

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 (Clarice ZC, 2009).

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 (Clarice ZC, 2009).

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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 - Increased substance interaction with the 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 DR, 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

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 R-H, 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 as shown in (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 J, 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 cell membrane leads to Increased proinflammatory mediators (https://aopwiki.org/relationships/1702)

AOPs Referencing Relationship

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

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
Increased substance interaction with the 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
Increased substance interaction with the 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
Increased substance interaction with the 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
Increased substance interaction with the 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
Increased substance interaction with the 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
Increased substance interaction with the resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173)	**adjacent**	**High**	**High**