AOP 173 Response Document:

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1.0 Overall Comments and Revisions

1. Revise early section of the AOP (Background and early in overall AOP assessment) to discuss and cite literature on general lung fibrosis, role of epithelial type I versus type II cells and fibroblasts in the response to injury and also in promoting injury and inflammation. Consider specific references provided by reviewers in Appendix X, but also cite already included literature that is considered later in the AOP.

- Also expand the AO event with literature general references and discussion on human biology relevant to fibrosis

Response: The reviewers have agreed that the AOP173 document is already comprehensive and has covered the literature as extensively as possible. Thus, the suggestion to include additional references was reviewed and where possible and necessary, an attempt was made to include them. The description of AO was expanded to include human physiology and the cell types potentially known to be involved in the human lung fibrosis, adding relevant references. Additional background was also added in the main AOP page (the AOP background, and the overall assessment of the AOP sections). Reviewer suggested references have been cited where necessary and applicable.

However, we do not agree that the AOP should capture literature covering historical perspectives to current state-of-the-art. AOPs should not be viewed as review documents and it is not necessary to cite all published literature.

Revision: Text underlined in blue is a clickable hyperlink that directly links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot used for external review - Dec 2019 (pdf snapshot) was used to make the revisions.

AO Description (Event 1458):

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

excessive deposition of collagen by constitutively activated myofibroblasts during the wound healing response. This causes a pronounced decrease in the number of capillaries within the alveolar septa with asymmetric deposition of collagen and cells between part of the surface of a capillary and the nearby alveolar lining. In areas where capillaries are not present, the alveolar capillary membrane is occupied with collagen and cells.

Overall Assessment of AOP (AOP 173):

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

Background (Optional) (AOP 173):

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

Pulmonary fibrosis is a deadly lung pathology characterized by the destruction of native lung architecture, excessive collagen deposition, and extracellular matrix restructuring. Numerous respiratory diseases, such as pneumoconiosis, silicosis, asbestosis, bronchiolitis obliterans (‘popcorn lung’), and chronic beryllium disease have pulmonary fibrosis as a main or secondary symptom. In addition, pharmaceuticals and environmental contaminants such as bleomycin and arsenic, also induce the adverse outcome of pulmonary fibrosis through intravenous or oral exposure, without direct inhalation of the stressor. Idiopathic pulmonary fibrosis (IPF) is the most common type of lung fibrosis in humans and involves alveolar regions of the lung consisting of type 2 alveolar epithelial cells (AEC2s), type 1 cells (AEC1s) and mesenchymal cells. AEC1s are responsible for gas exchange and AEC2s synthesise surfactant. The AEC2s are capable of self-renewal and differentiate to AEC1s regularly during normal tissue maintenance (Barkauskas & Noble, 2014). In pro-fibrotic conditions, AEC2s fail to regenerate AEC1s lost by injury and do not respond normally to epithelial injury, undergoing hyperplasia. As a result, human patients suffering from IPF have dysregulated levels of surfactant proteins normally secreted by AEC2s (Barlo et al.,2009; Phelps et al., 2004). Furthermore,

immunohistochemical staining of human IPF lung slices shows AEC death as well as proliferation adjacent to fibrotic foci (Uhal et al., 1998). In animal models of bleomycin-induced lung fibrosis, abnormal AEC2s are incapable of protecting the basement membrane denuded by cell death and thus, result in fibrosis (Rock et al., 2011). AEC2s are hyperplastic and are located on top of the fibrotic lesions in the lung in human specimens (Katzenstein & Myers, 1998). The dysregulated proliferation of fibroblasts and myofibroblast differentiation leading to excessive ECM deposition in the fibrotic scar is thought to arise from disrupted cross talk between epithelial and mesenchymal cells (Barkauskas 2014). Targeted removal of AEC2s in mouse lungs results in fully manifested fibrosis (Sisson et al., 2010). Genetic studies have associated mutations in genes encoding surfactant proteins and the development of a familial type of lung fibrosis. These associations have been supported by experiments conducted in transgenic mice (reviewed in Barkauskas 2014). In certain infectious conditions, epithelial cell stress and dysfunction leading to inefficient repair capacity or transcriptional reprogramming of epithelial cells to secrete pro-fibrotic and pro-inflammatory factors can lead to lung fibrosis (Lawson et al., 2008; Lawson et al., 2011). Mesenchymal cells are the other main type of cell, which contribute to fibrosis development. Myofibroblasts exhibiting contractile properties of smooth muscle cells and expressing a-SMA and vimentin, are the types of mesenchymal cells that are most commonly associated with excessive collagen secretion in pro-fibrotic phenotypes (Todd et al., 2012). Myofibroblasts can arise mainly from differentiation of tissue resident fibroblasts, translocation of bone marrow derived fibrocytes into the lung, or from epithelial-to-mesenchymal transformation (EMT; a type of trans-differentiation) (Hung, 2020; Todd et al., 2012). These cells are critical to the normal process of wound healing, and are the main cells contributing to collagen deposition in both normal wear-and-tear repair processes and in disease promoting conditions. Following successful wound healing, myofibroblasts de-differentiate and disappear (Friedman, 2012). Myofibroblasts persistence is suggested to play a key role in progressive pulmonary fibrosis in humans. There is evidence for both EMT derived myofibroblasts and bone marrow derived fibrocytes in human pulmonary fibrotic conditions. Air epithelial biopsies from human patients suffering from bronchiolitis obliterans (BO) following lung transplant show significantly increased staining for mesenchymal markers (Vimentin and a-SMA), decreased staining for e-cadherin, and co-localization of epithelial and mesenchymal markers as compared to stable patients (Borthwick et al., 2009). With respect to bone marrow derived fibrocytes, these cells have been proposed as an indicator for poor prognosis in human IPF patients, and research has shown that the amount of fibrocytes in the human IPF lung correlates with the amount of fibroblastic foci (Andersson-Sjöland et al., 2008; Moeller et al., 2009). Additional cell types involved in fibrotic process include endothelial cells and immune cells such as macrophages, neutrophils, and T helper cells. Endothelial cells contribute to the fibrotic process through endothelial-to-mesenchymal transformation, as evidenced in bleomycin model systems in which endothelial cells in fibrotic conditions take on the characteristics of myofibroblasts (Kato et al., 2018). Macrophages present in the alveolar space as well as macrophages recruited to the lung during the fibrotic process also contribute to the inflammatory environment and potentiate the adverse outcome of pulmonary fibrosis. Direct interaction of fibrotic stressors, such as MWCNTs, silica, and asbestos, with the macrophage membrane can occur through scavenger receptors as well as through receptors such as MARCO (Li & Cao, 2018; Murphy et al., 2015). This can induce macrophage injury through frustrated or incomplete phagocytosis which leads to the production of alarmins such as IL-1b and ROS, and profibrotic mediators such as TNF-a, TGF-b, and PDGF (Dong & Ma, 2016; Li & Cao, 2018). The injured resident macrophages contribute to the initial acute phase inflammatory response during pulmonary fibrosis which recruits additional immune cells to the lung. Depending on the fibrotic stressor, different populations of immune cells can be initially recruited to the site of action. The recruitment of neutrophils into the lung space potentiates the inflammatory response and tissue damage. Furthermore, in conditions of acute lung injury, which can precede the development of

a fibrotic phenotype, neutrophilic recruitment to the lung through trans-epithelial migration can induce the formation of lesions in the epithelium and contribute to the loss of alveolar capillary membrane integrity (Zemans et al., 2009). Finally, T helper (Th) cells recruited to the lung potentiate the inflammatory environment, and through the induction of a Th2 response, stimulate the proliferation of fibroblasts and differentiation of myofibroblasts driving the development of a fibrotic phenotype (Shao et al., 2008; Wynn, 2004).

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

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

2. Consider using the 'omics' data including from the authors lab (if published before submission to the EAGMSTG to support the KERs and the quantitative understanding.

Response: The quantitative benchmark dose information from Labib et al., 2016 using transcriptomic pathway induction as a proxy for KE activation has been added to the "Quantitative Understanding" section of AOP 173, along with a concordance table.

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Quantitative Understanding (AOP 173):

The presented AOP is mostly qualitative and additional studies are needed to support the essentiality of the KEs and to build KERs. However, it is important to note that it is difficult to experimentally demonstrate the relevance of earlier KEs to the end outcome of fibrosis because of the redundancy in pathways involved. The mode or type of interactions between the resident cell membrane and a substance is dependent on the specific physical-chemical characteristics of the substance. Labib et al., 2016 (reproduced below) determined quantitatively the dose at which the events in AOP 173 are induced by CNTs, one of the pro-fibrotic stressor, using samples from three separate studies. Global transcriptomic analysis and benchmark dose (BMD) modelling was used to determine the dose at which the MIE, KE1, KE2, KE4, KE5, and KE6 are induced and compared these doses to the apical BMD derived for the AO of pulmonary fibrosis. From the results shown, it can be seen that the BMD intervals of transcriptional pathway induction associated with each KE largely overlap and are representative of the BMD of AO induction. These results highlight the complexity of a disease process, where KEs may also occur in parallel.

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

Stressor	Species	Time Point	MIE[a] (1495)	KE1[a] (1496)	KE2[a] (1497)	KE3 (1498)	KE4[a] (1499)	KE5[a] (1500)	KE6[a] (1501)	AO (1458)
Mitsui 7 CNT	Mouse	24 Hr	4 – 9	3 - 7	9 – 13	N/A	5 – 11	10 – 21	9 – 13	N/A
Mitsui 7 CNT	Mouse	3 / 7 day	11 – 22	6 – 22	14 – 24	N/A	9 – 16	15 – 26	17 – 34	N/A
Mitsui 7 CNT	Mouse	28 day	No Effect	14 – 26	36 – 51	N/A	14 – 26	11 – 20	No Effect	N/A
Mitsui 7 CNT	Mouse	56 day	N/A	N/A	N/A	N/A	N/A	N/A	N/A	14 – 27[b]
NRCWE-026 CNT	Mouse	24 Hr	No effect	8 – 15	20 – 37	N/A	8 – 15	21 – 39	No Effect	N/A
NRCWE-026 CNT	Mouse	3 / 7 day	16 – 28	16 – 27	19 – 33	N/A	15 – 24	16 – 26	19 – 36	N/A
NRCWE-026 CNT	Mouse	28 day	No Effect	No Effect	No Effect	N/A	12 – 20	No Effect	No Effect	N/A
NM-401 CNT	Mouse	24 Hr	No Effect	3 – 20	8 – 22	N/A	8 – 22	13 – 22	18 – 29	N/A
NM-401 CNT	Mouse	3 / 7 day	11 - 17	12 - 19	12 - 20	N/A	7 - 20	14 - 22	13 – 21	N/A
NM-401 CNT	Mouse	28 day	20 - 37	17 - 28	No Effect	N/A	No Effect	13 - 21	18 – 31	N/A

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

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

3. Include additional evidence from human and also animal studies with persisting metal oxide dusts, asbestos, silica and cigarette smoke, that support particular KERs, including quantitative understanding in the inflammatory hub **and/or** the final AO, as appropriate. Additional references particularly on metallic oxide NMs provided by reviewers following the TC (Annex X/or further discussion).

Response: If a suggested study (Annex X provided by the reviewer) supported weight of evidence and if it is missing from the present document, it was added to the revised; however, we do not agree to conduct systematic literature review to build an exhaustive weight of evidence table. We do not agree that lung fibrosis is a primary adverse outcome for several of the metal oxides. Where applicable, additional studies related to animal and human research concerning metals and metal oxides, and silica have been added to various sections of the AOP to support KERs as well as for background information on the types of stressors involved.

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sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Domain of Applicability; Types of Stressors (AOP 173):

Types of Stressors:

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

KE1→2 (Empirical Evidence)

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

Dose-Response Evidence:

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

Ma, et al. (2016) studied inflammatory responses in male BALB/c mice exposed to multi-walled carbon nanotubes (MWCNT) administered intravenously at different doses (0.5-4 mg/kg) for 2 days. A dose-dependant relationship was found between the levels of the inflammatory

mediators IL-6 and TNF-α and the MWCNT dose. At the highest dose, 4 mg/Kg, white blood cells, lymphocytes, and neutrophils levels increased.

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

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

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

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

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

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

Temporal Evidence:

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

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

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

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

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

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

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

Rahman et al. 2017 evaluated whether different TiO_2 NPs induce lung inflammation. C57BL/6 mice were exposed to 18, 54, 162, or 486 μg/mouse of TiO_2 NPs via single intratracheal instillation. At 1-day post-exposure, gene expression analysis showed more changes in genes

associated with inflammation and fibrosis. Moreover, after 1- and 28-days post-exposure, an increase in cell counts in BALF was observed in a dose-dependant manner.

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

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

Kamata et al. (2011) studied the impact of carbon black nanoparticles on susceptible subjects with predisposing lung disease and the effects of nanoparticles on inflammation and fibrotic changes. To achieve this goal, female C57BL/6J mice were intratracheally administered with bleomycin 20 μg/mouse and carbon black nanoparticles 10 μg/mouse. Evaluations were performed post-exposure at different time points. An increase of IL-6 and CCL2 in BALF was observed at days 2 and 7. After 7- and 14-days, a recruitment of pro-inflammatory cells was observed. Oxidant injury (evaluated as nitrotyrosine expression) was observed after 7 days and 14 days. The levels of TGF-β_1 increased over time with the highest level at day 14. Finally, they observed an increase in lung collagen deposition, and a decrease in lung compliance at day 21.

KE2 → KE3 (Empirical Evidence):

Dose-Response Evidence:

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

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

Polyhexamethylene guanidine phosphate is used for the prevention of microorganism growth in humidifiers. To evaluate the inflammation response after the exposure to this chemical, three human lung cells (Calu-3, differentiated THP-1, and HMC-1 cells) were culture and exposed at

the air-liquid interface at 2.2, 4.4, 8.8, and 17.6 mg/ml for 1, 6 and 24 h. An increase in chemoattractant cytokine IL-8 release was observed in a concentration- and time-dependant manner. This response preceded the alveolar-capillary membrane integrity loss, which occurred at 24 h at the highest concentration. These changes were followed by reactive oxygen species (ROS) generation, an increase in the levels of MMP-2, TIMP-1, MMP-9, TIMP-2 mRNA expression, and the release of TNF-α, IL-6 and TGF-b1. Moreover, inflammatory cell infiltration, fibrosis, and the release of cytokines was observed in lung sections in Sprague-Dawley rats following a 3 week exposure (Kim et al., 2015).

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

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

Temporal Evidence:

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

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

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

Gautam et al. (1998) studied the effect of a chemotactic stimulant, fMLP, on polymorphonuclear cells resting on an endothelial cell monolayer in the upper compartment. Bovine aorta

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

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

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

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

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

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

Wan et al. (2017) studied the genotoxic effects of cobalt NPs and their capacity for causing oxidative stress and inflammation. Gpt delta transgenic mice were exposed to 50 μg/mouse of cobalt NPs by intratracheal instillation, and animals were sacrificed at days 1, 3, 7, and 28 post exposure, as well as 4 months post exposure. The levels of CXCL1/KC and neutrophils

increased at 1, 3, and 7 days post-exposure, but they decreased at day 28. LDH activity and protein content in BALF also increased, but their levels dropped after 28 days post-exposure. Four months after instillation, 8-OHdG levels were measured and were found in high levels. Moreover, histological changes were observed 7 days (infiltration of a large amount of neutrophils and macrophages in the alveolar space and septa, focal alveolar epithelial cell hyperplasia, and thickening of the alveolar wall) and 4 months (interstitial fibrosis, bronchiolization of the alveoli and collagen deposition in the alveolar septa) after the exposure.

KE3 → KE4 (Empirical Evidence):

Dose-Response Relationship:

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

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

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

KE4 → KE5 (Empirical Evidence):

Dose-Response Relationship:

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

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

determined that effector T cells purified from SiO_2-treated mice, in the absence of Treg cells, induced fibrosis by producing IL-4, suggesting that many T cell pathways lead to the fibroproliferative process.

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

KE5 → KE6 (Empirical Evidence):

Dose-Response Relationship:

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

Ma et al. (2017) studied the role of epithelial-mesenchymal transition (EMT) in cerium oxide (CeO_2) induced fibrosis. Male Sprague-Dawley rats were exposed to 0.15-7 mg/kg cerium oxide via intratracheal instillation and sacrificed at various times post-exposure. At 28 days post-exposure there was a dose-dependant increase in hydroxyproline content in lung tissue. Mice exposed to 3.5 mg/kg showed an increase in soluble collagen levels in BALF at day 3 and day 28 and an increase in α-SMA expression levels in lung tissue with a peak at day 1 post-exposure. From CeO_2 exposed rats (3.5 mg/kg), macrophages, fibroblast, and alveolar type II (ATII) cells were isolated. Macrophages produced TGF-β1 with peaks at day 3 and 10 post-exposure. Fibroblast proliferation decreased in a dose-dependant manner, and an increase in the levels of α-SMA in fibroblasts and ATII at day 28 post-exposure. They concluded that CeO_2 exposure affects fibroblast function and induces EMT in ATII cells.

Blaauboer et al. (2014) studied the expression of elastin, type V collagen, and tenascin C during the development of lung fibrosis and the effect of myofibroblast differentiation on this expression. Female C57Bl/6 mice were exposed to a single intratracheal instillation bleomycin 30 μl (1.25 U/ml in PBS). Seven days before sacrifice, mice received 35 μl D_2O/g via intraperitoneal injection to label new collagen. Mice were sacrificed 1, 3, 4 or 5 weeks post exposure. An increase in the level of α-SMA protein level in histological staining was

observed, with a peak after 2 weeks. Extracellular matrix proteins levels increased (histological staining). Elastin increased in a time-dependant manner with a peak after 4 weeks. Type V collagen and tenascin C increased after 1 week and decreased over time. They found that gene expression of elastin, type V collagen and tenascin C highly correlated to new collagen formation. Primary normal human lung fibroblast and human fetal lung fibroblast were exposed to different concentrations of TGF-β1. The expression of ACTA, COL1A1, ELN, COL5A1 and TNC increased in a dose-response relationship after 24 h. Fibroblast cultured in elastin coatings and stimulated with 10 ng/ml TGF-β1 for 48 h, showed an increase in the levels of ACTA2, COL1A1, and ELN. The *In vitro* study demonstrated that fibrotic changes in the composition of the extracellular matrix have a regulatory role during fibrosis development.

Judge et al. (2015) determined that the lactate dehydrogenase-A (LDHA) enzyme was upregulated in radiation and that lactate is required for radiation-induced myofibroblast differentiation. In lung biopsies obtained from patients who received thoracic radiation for cancer treatment, an overexpression of LDHA and α-SMA by immunostaining was seen, as well as the accumulation of collagen fibers. Mice C57BL/6mice were exposed to 5 Gy total body plus 10 Gy thoracic radiation. They found that LDHA overexpressed in lungs at 26 week, and LDHA mRNA increased over time at 16-26 weeks (post-radiation). Primary human lung fibroblasts were exposed to 3, and 7 Gy. At the highest dose 5 days post-radiation, an increase in the levels of LDHA protein expression, extracellular acidification, lactate levels in supernatants, α-SMA protein expression, soluble collagen I, Col1A1, and Col3A1 mRNA levels, and TGF-β1 bioactivity was seen. LDHA siRNA and an LDH inhibitor, inhibits radiation-induced myofibroblast differentiation.

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

Temporal Relationship:

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

Osterholzer et al. (2013) evaluated local inflammation and fibrosis after a targeted epithelial insult. Wild type (WT) C57BL/6 and transgenic mice expressing the diphtheria toxin receptor were intraperitoneally injected with diphtheria toxin (DT) once daily for 14 days at a dose of 100 μg/kg in 100 μl of PBS. Observations were evaluated at various days post DT initiation. At day 7 and 14, an accumulation of exudate macrophages and Ly-6C^{high} monocytes was observed. The immunophenotype of ExM and Ly-6C^{high} monocytes at day 14 showed an expression of arginase, iNOs, IL-13, TGF-β, CD45+, Col1+, and CCR4. Chemokine-receptor-2 deficient mice did not show an accumulation of inflammatory cells and fibrosis. Finally, at day 21, lung collagen deposition was evident, as measured by hydroxyproline content.

Fang et al. (2018) studied the endothelial-mesenchymal transition characterized by the loss of endothelial specific markers (Cdh5, PECAM1), the acquisition of the mesenchymal markers

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

Activated macrophages secrete TIMP1 into the ECM to inhibit matrix metalloproteinases and this could promote cell proliferation and inhibit fibroblast apoptosis through CD63/integrin β1 ERK signaling. Dong et al. (2017) characterized TiMP1 expression after multiwalled carbon nanotube (MWCNT) exposure. Male C57BL/6J WT and B6.129S4-Timp1tm1Pds/J (Timp1 KO) mice were administered with MWCNT at 40 μg per mouse by pharyngeal aspiration. Lungs were harvested at 1, 3, 7- and 14-days post-exposure. TIMP1 mRNA and protein levels increased in lung, BALF and serum at day 1, and then decreased over time. A similar behavior was observed for FN1, FSP, Ki67 and PCNA expression with a peak at 7 and 14 days post exposure. Collagen deposition was observed at 1 day post exposure with a peak at day 7. At day 7 they also observed an increase in the expression of Hsp47, vimentin, α-SMA, PDGFR-β, and genes involved in cell cycle regulation (Bub1b, Capg, Cenpa, Kif2c, Kif22, Mcm5, Plk1 and Tuba 6). TIMP1 KO mice displayed reduced responses. The formation of TIMP1/CD63/integrin β1 complex on the cell surface lead to an activation of the Erk1/3 pathway.

Hu et al. (2015) studied the effects of conditional mesenchymal-specific deletion of Notch1 on pulmonary fibrosis. A conditional knockout of Notch1 (CKO) in collagen I-expressing mesenchymal cells was generated (Notch1fl/fl, Col1α2-cre-ER(T)+/0). Col1α2-cre-ER(T)+/0 with WT Notch1 mice and CKO were given daily intra peritoneal injections of tamoxifen for 8 days to induce mesenchymal cell-specific expression of the Cre-ER(T) recombinase and the removal of the floxed Notch1 (Notch1 CKO Mice). Control mice and Notch1 CKO were injected endotracheally with 2 U/Kg Bleomycin. Mice were sacrificed after 7, 14, and 21 days. Jagged1 and Notch1 protein expression increased with a peak at 7- and 14-days post-exposure. After 14 days of the treatment, an increase in the levels of mRNA and protein of α-SMA and Col1 was seen, as well as an increase in the percentage of α-SMA+ lung fibroblasts. 28 days post-exposure there was an increase in the content of hydroxyproline in lungs. CKO mice showed a significant attenuation of collagen deposition and myofibroblast differentiation.

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

4. Clarify the role of ROS in the AOP173. How it connects to earlier events and its role in propagating the inflammation through positive feedback (feed forward) loop. Provide a schematic to clarify the point.

Response: Many xenobiotics including the stressors named in this AOP are capable of inducing ROS acutely after exposure, which serves as a signalling mechanism to alert the organism of the impending invasion and signal the inflammatory response. However, ROS is not an absolute requirement at this stage for initiating the inflammatory cascade but if present, can help build the acute inflammatory response. Thus, authors are of the opinion that ROS should only be described as an associative event in the presence of continuing inflammation, injury and exposure. However, considering the reviewers comments, the role of ROS in AOP 173 was clarified and included as an associative event potentiating KE 1-3, and KE5. All mention of associative events has been restricted to the AOP 173 page, to show how these events specifically potentiate the KEs of the AOP. Furthermore, a schematic has been developed which shows how the associative events influence the KEs in AOP 173.

Revision: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Evidence Assessment (AOP 173; Evidence Assessment Call Table):

Associative Event 2: Oxidative stress (Associative Events Schematic)

Oxidative stress is defined as an imbalance in the oxidant – antioxidant axis towards oxidants (hydrogen peroxide, superoxide anions, hydroxyl radicals) in a supraphysiological manner. Reactive oxygen species (ROS) serve as part of an important redox signalling system which helps cells adapt to their environment and tackle stress through modulation of transcriptional regulation. In the context of pulmonary fibrosis, ROS and oxidative stress potentiate the inflammatory response (KE1-2) and injury to the respiratory epithelium (KE3), and contribute to the differentiation and activation of myofibroblasts (KE5). The exact species of ROS, the specific cell types, and the perturbed oxidative stress related pathways may vary depending on the type of pulmonary fibrosis, and even among different human patients suffering from the same fibrosing disease (ex. IPF). Increased levels of ROS have been shown to activate TGF-b, and induce apoptosis of alveolar epithelial cells. Furthermore, oxidative stress induces secretion of pro-inflammatory mediators (mitochondrial DNA, Nalp3 inflammasome related molecules) from the injured epithelium as well as from resident immune cells like macrophages. This potentiates additional recruitment of immune cells to the site of injury, further compounding the inflammatory response, and inducing further production of ROS by effector cells like neutrophils. Clinical studies in IPF patients have consistently found higher levels of ROS biomarkers in the BALF, serum, as well as in exhaled condensate. Furthermore, increases in ROS and oxidative stress are associated with bronchiolitis obliterans, a fibrosing disease of the bronchioles instead of the alveolar tissue. While there is strong evidence for the involvement of ROS in the pathogenesis of pulmonary fibrosis, it acts to potentiate multiple KEs rather than acting as a KE or MIE in itself. Oxidative stress and increased ROS in this manner serve as both causative agents, and consequences of observed responses in a feedforward type mechanism.

Essentiality: Moderate.

Multiple studies, using knockdown and knockout mammalian models have shown that oxidative stress is involved in the development of pulmonary fibrosis. However, its essentiality in its pathogenesis is not conclusive, as antioxidant treatment offer no significant benefit in patients with IPF, the most common type of pulmonary fibrosis in humans. Furthermore, uncertainties remain concerning the exact molecular mechanisms underlying oxidative stress in the context of pulmonary fibrosis. (Checa & Aran, 2020; Cheresh et al., 2013; Dostert et al., 2008; Madill et al., 2009ab; Veith et al., 2019)

5. Clarify that the AOP is relevant to persistent stress(ors) associated with inhalation exposure and improve/clarify the discussion of how the evidence included in the AOP for other known types of exposure (bleomycin) and even unknown (e.g. environmental risks and ILF) links to the critical KE hub, the inflammation as a common and undelaying principle.

Response: The relevance of AOP 173 to a range of stressors (both persistent and soluble) has been clarified in the "Domain of Applicability" section of the AOP.

Revision: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Domain of Applicability (AOP 173):

Types of Stressors:

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

6. Revise the WoE calls for KERs between early KEs, MIE-KE1-KE2-KE3, based on the discussion and the initial responses which point out limited evidence in relation to these early KERs. Suggestion was made to revise to Moderate.
    - Revisit the supporting evidence and provide the appropriate (wording) for the rationale based on the Users's handbook.
    - Transfer the relevant content from the external Table 1 to the relevant KER pages as much as possible.
    - For quantitative understanding specify the rationale using the guidance from the Handbook. Not necessarily downgrade needed but the rationale should be clearly stated in the KER pages.

Response:

The data previously present in Table 1 in the main AOP 173 page has been redistributed to weight of evidence tables for each KER. An evidence assessment call table has been prepared and integrated into the "Evidence Assessment" section in the main AOP 173 page. The WoE calls for the KERs, including the quantitative WoE calls, have been modified based on the guidance provided in the OECD handbook.

Revisions: Text in blue indicates clickable hyperlinks which lead to the WoE tables for each KER, as well as to the Evidence Assessment Call Table in the main AOP 173 page, which summarises the calls for the essentiality of the KEs, as well as the biological plausibility and empirical evidence calls for each KER.

MIE → KE1 WoE Table

KE1 → KE2 WoE Table

KE2 → KE3 WoE Table

KE3 → KE4 WoE Table

KE4 → KE5 WoE Table

KE5 → KE6 WoE Table

Evidence Assessment (AOP 173; Call table located at the bottom of the section)

7. Revisit the assessment/rationale of the essentiality of KEs according to the scoring suggested in the handbook. Even if evidence is scarce, indirect or points out to inconsistences, use it as a rationale to provide the score rather than omit it.

Response: The essentiality assessment of the KEs for AOP 173 were revised based on the scoring suggested in the OECD AOP handbook. The information has been presented in an evidence assessment summary table within the main AOP 173 page.

Revisions: See comment #6 (1.0 Overall Comments and revisions)

8. Include and reference to newer cellular (co-culture) models in the relevant KE (how it is measured).

a. reference better the section(s) dealing with limitations of certain models used in the study of lung fibrosis.

Response: New cell culture models have been added under the "How It Is Measured or Detected" section under the relevant KEs, with mention of the limitations involved. We are not sure if the AOP should discuss the limitations of certain models. Again, this is not a review article and we can only list the assays that are most commonly used and are readily available for the assessment of KEs.

Revisions: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

MIE: Interaction with the lung resident cell membrane components (How It Is Measured or Detected)

Cellular co-culture models of the pulmonary epithelium:

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

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

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

KE1: Increased, secretion of proinflammatory mediators (How it is measured or detected)

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

KE2: Increased, recruitment of inflammatory cells (How it is measured or detected)

In vivo, recruitment of pro-inflammatory cells is measured using BALF cellularity assay. The fluid lining the lung epithelium is lavaged (BALF) and its composition is assessed as marker of lung immune response to the toxic substances or pathogens. BALF is assessed quantitatively for types of infiltrating cells, levels and types of cytokines and chemokines. Thus, BALF assessment can aid in developing dose-response of a substance, to rank a substances' potency and to set up no effect level of exposure for the regulatory decision making. For NMs, in vivo BALF assessment is recommended as a mandatory test (discussed in ENV/JM/MONO(2012)40 and also in OECD inhalation TG for NMs). Temporal changes in the BALF composition can be prognostic of initiation and progression of lung immune disease (Cho et al., 2010).

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

KE3: Loss of alveolar capillary membrane integrity (How it is measured or detected):

Other:

The other methods include targeted RT-PCR or ELISA assays for tight junction proteins, cell adhesion molecules and inflammatory mediators such as IFNg, IL-10, and IL-13. Advanced *in*

vitro co-culture models, like the EpiAlveolar model system, and other similar systems present an intact capillary membrane that can be used to assess loss in the membrane integrity (via TEER) after exposure to pro-fibrotic stressors like crystalline silica and TGF-b (Barasova *et al.,* 2020, Kasper *et al.,* 2011).

KE5: Increased, fibroblast proliferation and myofibroblast differentiation (How it is measured or detected)

Advanced co-culture models (myofibroblast differentiation):

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

KE6: Increased, extracellular matrix deposition (How it is measured or detected)

Ex vivo and in vitro models of ECM deposition:

No models currently exist which allow for high-throughput in vitro assessment of ECM deposition. Using single, or co-cultures containing fibroblasts, the production of soluble ECM components can be assessed after exposure to a stressor of interest using either ELISA or qRT-PCR experiments as a proxy.

9. Expand the *Uncertainties, inconsistencies and data gap* section with the discussion about the knowledge gap with regard to the specific involvement/relevance of particular cell types and mediators for lung fibrosis and the on-going attempts to address them in terms of assay availability and development.

Response: The information concerning uncertainties, inconsistencies, and data gaps in the AOP has been expanded, and additional information has been presented in the “Quantitative understanding” section on the main AOP page.

Revisions: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers’ comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Quantitative Understanding (AOP 173):

The MIE of substance interaction with the lung cell membrane is broad and vague, reflecting the many interactions pro-fibrotic substances can have with the plasma membrane of cells. The presented AOP, while applicable to both soluble and persistent stressors, is specifically applicable to substances which induce fibrosis through immune responses. Nanomaterials are a group of such substances, which interact with organisms and cells via a dynamic biomolecular corona that is dependant on the biological microenvironment. While great strides have been

made in recent years to characterize and understand this corona and how it impacts cellular recognition, further research is needed in order to accurately describe the specific interactions necessary for the initiation of fibrosis pathogenesis. Indeed, this is also true for model soluble stressors such as bleomycin, for which cellular binding and uptake is incompletely understood.

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

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

1.2 From Further Discussions Section:

Reviewer 1: As it is a major objective of the AOP to provide alternative testing strategies for the large number of nanomaterials, available (in vivo) information on those related to lung fibrosis induction. A considerable number of commercialised nanomaterials are metals and metal oxides.

Review manager's note: In addition to the above, it is noted that the current draft AOP173 aims to be applicable to wide range of stresses as stated in the Abstract: Lung fibrosis is frequently observed in miners and welders exposed to metal dusts, making this AOP relevant to occupational exposures"; and in the Domain of applicability: "This AOP is applicable to occupational exposures as lung fibrosis is frequently observed in miners and welders exposed to metal dusts.". Therefore, the consideration of the additional evidence recommended by the reviewers, appears highly relevant for the AOP173.

Response: Additional references related to metal and metal oxide stressors (both *in vivo* and *in vitro*) have been added to the weight of evidence tables for each KER. Furthermore, specific references of interest have been elaborated upon in the "Evidence Supporting this KER" and "Quantitative Understanding of the Linkage" sections in the relevant KER wiki pages.

Revision: Please see revision and response related to Comment # 2; 1.0 Overall Comments and Revisions.

2.0 Individual Comments:

2.1 SCIENTIFIC QUALITY

Reviewer 1:

1. The cited scientific literature is comprehensive, adequate and useful to inform on the background and the events for the development of AOP 173. It is acknowledged that the literature cannot be exhaustively covered due to the complexity of the matter and processes involved.
2. Regarding CNTs as exemplary stressors, there are a number of publications/reviews addressing fibrosis and AOP development which should also be cited already on p.4 (e.g. Dong 2019, 2018, 2016, Duke 2017, Vietti 2016, 2016a).

Initial Response: We agree that there is much more available out there and could have been referenced. It is a possibility that some references may have been left out due to the exhaustive number of references already added to the text. We will go through the text and add additional references where necessary.

Revision: A number of the aforementioned references have been added to the stressor description in the AO page. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Pulmonary Fibrosis (Event 1458; Stressors)

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

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

1. Dong, J., & Ma, Q. (2016). Myofibroblasts and lung fibrosis induced by carbon nanotube exposure. Particle and fibre toxicology, 13(1), 60.

2. Duke, K. S., & Bonner, J. C. (2018). Mechanisms of carbon nanotube-induced pulmonary fibrosis: a physicochemical characteristic perspective. Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology, 10(3), e1498.

7. Vietti, G., Lison, D., & van den Brule, S. (2016). Mechanisms of lung fibrosis induced by carbon nanotubes: towards an Adverse Outcome Pathway (AOP). Particle and fibre toxicology, 13, 11.

3. Further literature may be included which discusses relevant (nano-)material characteristics for cellular interaction (MIE) and inflammatory response induction

(e.g. ROS generation by metallic contaminants of CNTs) and cellular consequences of interaction or uptake, respectively (e.g. apoptosis, immobilisation/chemoattraction, autophagy/ER-stress).

Response: Substantial new information concerning metal and metal oxide nanomaterials has been added to substantiate the various KERs as well as in the AOP background and relevance sections. Please see comment #2; 1.0 Overall Comments and Revisions for a detailed overview of *in vivo* evidence related to these compounds.

We agree that ROS generation can be viewed as a consequence of triggered MIE and can be used to measure the MIE. This information can be used under the measurement section as an endpoint of consideration. However, the suggested literature related to metallic contaminants of nanomaterials is not necessary. As such, in our experience, even the most inert nanomaterials induce ROS and it is not detrimental. However, it is important to note that AOPs are stressor agnostic and MIE/KEs have to be described independent of stressors inducing them. Thus, the AOP describes the most commonly accepted mechanism of fibrosis. Some evidence is provided to support the occurrence of MIE/KEs but specific details are avoided. Some other points to consider: the target stressors for this AOP are not limited to nanomaterials and the AOP should not be viewed as a review of nanomaterial literature or to specify nanomaterial property mediated differences. Where necessary, this point is already emphasised in the text. Moreover, at this point in time, there is incongruence in the literature available in the context of specific properties or characteristics-dependent responses. AOP 173 focuses on the most commonly accepted mechanism of fibrosis and is applicable to a wide variety of stressors but not necessarily to every single nanomaterial present out there.

4. Some lack of congruency has been found upon cursory checking citations in the text and the corresponding reference lists. For instance, KE Description of event 1497 (p. 19) cites Zuo, 2002 and Beamer, 2013 (2012?), both missing in the reference list on the same page. It is recommended to carefully check for missing or incorrect citations in all reference lists.

Response: All KE & KER pages, as well as the main AOP page has been checked for consistency, and referencing has been updated where necessary

5. The topic is well covered considering the current scientific knowledge with regard to providing evidence for KER, also addressing critical issues and knowledge gaps. Altogether, the scientific quality is high and up to date. However, the somewhat indiscriminative treatment of soluble and particulate (fibrous) stressors CNTs (exemplified by bleomycin and CNTs, respectively) would need more critical

appraisal, e.g. in terms of target cells, types of cell injury and mediators involved in inflammation and fibrosis.

Initial Response: Information has been added on a nano QSAR model for CNT toxicity built using AOP 173, a pro-fibrotic biomarker (PFS17) designed using this AOP as well as data from model soluble and particulate stressors. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

We agree that the target cells, type of cell injury and mediators involved could all differ. The consensus from the literature is that the acute injury mediated by pro-fibrotic stressors mainly involves alveolar macrophages and epithelial cells. Chronic or adaptive phase of the disease mainly involves macrophages and fibroblasts. In allergen induced fibrosis, eosinophils are involved in addition to the others. Similarly, types of pro-inflammatory mediators secreted acutely vary. In some cases, same mediators may be secreted but to a different magnitude. Also, there is no consensus on how many mediators are sufficient to make a 'positive' call. This was one of the topics for discussion at the 2017 expert workshop on inflammation. It was agreed that in most cases, what is measured for inflammation depends on individual experiences/expertise and available resources in a given laboratory. We agree that more thought has to be given to this aspect; however, this is something we are working on in our own laboratory. We are using meta-analysis approaches to identify clusters of genes that can be used for predicting occurrence of inflammation and even the AO.

Considerations for Potential Applications of the AOP (AOP 173):

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

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

Reviewer 2.

6. Overall, the scientific quality of the AOP is strong. There is evidence for each of the KEs in the pathway that these can be related to the AO pulmonary fibrosis. In addition, the authors provide substantial evidence from literature, including data interpretation and discussion of uncertainties and inconsistencies. Especially the evidence for the later KEs and the AO is strong. A limitation of the AOP is that there is some incongruence in the essentiality of the MIE and some KE for the eventual AO. The authors do describe this in the document, but they could be more clear. MIE substance interaction, KE1 increased pro-inflammatory mediators and KE2 increased recruitment of pro-inflammatory cells are all part of an inflammation response. Inflammation does not necessarily lead to pulmonary fibrosis. Therefore, the authors need to be more clear on which type of substance interactions with membrane components can be linked to pulmonary fibrosis, which pro-inflammatory mediators can be linked to fibrosis and which pro-inflammatory cells can be linked to fibrosis. Now, it is not specific enough to understand the mechanism as inflammation could also lead to pulmonary emphysema, cancer or decreased lung function.

Response: We agree with the comment and have discussed extensively the specified incongruence. The AOP 173 applies to those stressors that mediate their effects via immune and inflammatory reactions. The argument that inflammation is not necessary for eventual AO comes from the transgenic studies that lack a single pro-inflammatory mediator and inactivation of a specific pro-inflammatory pathway. However, as noted in many places in the AOP 173 text, these early responses to stressor exposure serve as defence mechanisms. As a result, they exhibit high level of redundancy and pleiotropy, which is absolutely needed for the organism's survival. As pointed out in one of our studies, unless we are able to completely abrogate the acute as well as adaptive immune responses, one cannot stop the fibrotic disease. Fibrosis is a progressive disease and involves signalling and cross talk between several cell types, mediators and molecules. Its occurrence is highly dependent on the material properties (persistence), level of injury (repeated exposure) and the temporal microenvironment. At each stage, there is a feedback signalling that either propagates the AO response or inhibits it from further progress. AOP173 describes a path forward towards the AO that has overcome the inhibitory loops. Again, there are 'classical' pro-fibrotic markers that have been routinely used in the literature; however, in our experience, they don't work always. The only way to specify a set of genes/proteins with confidence is to derive a set by meta-data analysis. In our own laboratory, we have conducted meta-analysis of high content data and have come up with a 17-gene signature that is promising. Although further validation of the 17-gene set is necessary, for now we can confidently say that these genes are induced both by bleomycin and CNTs, and the results can be correlated to histopathological findings. In fibrosis, the acute inflammatory phase will also secrete pro-fibrotic markers. Please note that we are not referring to diagnostic markers of fibrosis that are present in the fibrotic lesion. We are referring to markers that promote the process of fibrosis and can be used to predict its occurrence. Similarly, in emphysema, we see several metalloproteinase secreted during the acute phase that

we don't see following pro-fibrotic stressors. Halappanavar's group is developing a parallel AOP for lung emphysema and it is clear that acute inflammatory phase can be discriminatory. In cancer, it is a different story. Fibrosis can precede cancer growth if material properties and exposure scenarios are conducive. There is a lot of debate about this and in our opinion, we are no way closer to solving this debate.

Finally harmonising the inflammatory key events – we have now revised the higher order title to 'Altered expression of pro-inflammatory mediators' removing the reference to fibrosis from the title.

At present, there are about 5-6 AOPs of relevance to nanomaterials. The MIE in each of these is the same – nano-bio interaction. However, each AOP describes this interaction with subtle differences. For example, frustrated phagocytosis is the MIE in the AOP for lung cancer. Interaction with surfactants is the MIE in the AOP for acute lung toxicity. However, in all these AOPs, MIE itself is not measured, rather the consequence/s of such interaction. Frustrated phagocytosis is one type of interaction that can lead to lung fibrosis. The same material that induces frustrated phagocytosis can also interact with surfactants and inhibit their activity, thus inducing acute inhalation toxicity. So the point is that the MIE in this AOP does not follow the prescribed norms and it is essential for it to be that way. Especially for nanomaterials, this interaction can vary from material to material and more than one type of interaction can occur at the same time. Even for bleomycin, higher doses of bleomycin can interact with DNA and induce DNA damage but the low doses can bind to specific receptors and initiate fibrosis via inflammation.

Revision: Additional data related to the uncertainty surrounding the MIE has been added to the main AOP 173 page. Please see Comment # 9; 1.0 Overall Comments and Revisions for an overview of the changes made.

7. More specifically, the MIE is still a bit vague: interaction of substances (physical, chemical or receptor-mediated) with membrane components (receptors, lipids) leading to danger signals. The MIE itself is usually not measured and thus not specified. The danger signals are identified as alarmins that initiate an immune response. Alarmins can induce a cascade leading to fibrosis, however, alarmins can also induce other diseases such as cancer. Therefore, the MIE seems not specific for the AO. In addition, it is not clear which cell types are involved here (macrophages? Or epithelial cells? Both are mentioned) and whether there should be a specific pro-inflammatory response to start the cascade leading to pulmonary fibrosis.

Response: Additional details concerning the uncertainties of the MIE as well as changes to the role of ROS in AOP 173 can be found in the comments above. Please see Comment # 3 and Comment # 9; 1.0 Overall Comments and Revisions for details concerning the exact changes made. An addition in the "Considerations for Potential Applications of the AOP" has been made, highlighting the recent successes concerning

the creation of an AOP network for NM induced lung toxicity, and its use in guiding the creation of alternative testing strategies (below).

However, it is important to note that MIE in this AOP is non-specific and should be left as that. We would argue that MIEs that lead to defence mechanisms such as inflammation or healing process will be redundant in most cases. There is another AOP for lung fibrosis that describes specific binding and inhibition of PPAR receptor leading to fibrosis. In this AOP, inflammation plays a role of an associative event and not a KE. From our own work, we have seen that carbon nanotubes that induce frustrated phagocytosis also potentially interact with selectins, receptors required for agranulocyte diapedesis. The binding is assumed to result in inhibition of selectin-mediated signalling and impede diapedesis (Jagiello et al., 2021). In the context of ROS, in our own work we have observed that even most inert nanomaterials induce increase in ROS. For now, the evidence is scarce and inconsistent.

Revision: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Considerations for Potential Applications of the AOP (AOP 173)

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

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

8. The inflammation response plays a critical role in the AOP. Inflammation can be reversible, as is also explained in the section on biological plausibility, coherence and consistency. This leads to inconsistent results. The AOP could reflect the feedback loops.

Revision: The associative events in AOP 173 have been summarized in a general schematic, present in the main AOP 173 page. The schematic can be accessed through this hyperlink.

At the moment, there is not enough information in the literature to recommend a threshold below which inflammation is reversible and above which inflammation progresses to AO. For this, we will need to agree on a set of inflammatory markers that will be assessed commonly and then define thresholds. We are working on it. This is work in progress. For now, we know that if exposure persists, inflammation persists and AO ensues. However, we would like to bring up the example of expression of SAA3 following exposure to all types of nanomaterials and other pro-fibrotic stressors that involve inflammation. SAA3 expression in mouse lungs is directly proportional to the extent of neutrophil influx and potential potency of stressors (in this example, nanomaterials) to induce pathology. Further work is needed to validate if this is an appropriate marker. A lot of work is being taken up by Dr. Vogel's group, who is an author on this AOP. Regarding reversibility - The AOP is supposed to describe a mechanism that leads to AO and not inhibitory loops that inhibit the disease process. Granted that all disease processes have two trajectories but the AOP describes the trajectory that surpasses the inhibitory loop to the final AO. Therefore, if we start adding all feedback loops as suggested, it will not be anymore an AOP, it will be a MOA.

9. Finally, there is an overall lack of quantitative information on the KERs. The authors explain that many studies only tested a single dose and that therefore quantitative information is missing. This is an important point as quantification of KERs would help to increase the understanding of the AOP. Especially for the KEs related to inflammation, quantitative information could help to understand at which doses inflammation can progress into fibrosis and at which doses the inflammation is reversible. Maybe for a future project, it would be feasible to collect available quantitative data on each of the KERs and start modelling the data to find quantitative KERs.

Response: Additional quantitative information relevant to the KERs was added where possible.

Revision: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Quantitative Understanding (AOP 173):

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

Quantitative concordance table for AOP 173 KERs. Data is reproduced from Labib et al., 2016 (Figure 4., Additional file 4: Table S3). [a]: benchmark dose (BMD) (Benchmark dose low (BMDL) à BMD) intervals in µg / lung based on transcriptional pathway induction. [b]: BMDL – BMD interval in µg / lung based on alveolar thickness. CNT: carbon nanotube. N/A: Not assessed

Stressor	Species	Time Point	MIE[a] (1495)	KE1[a] (1496)	KE2[a] (1497)	KE3 (1498)	KE4[a] (1499)	KE5[a] (1500)	KE6[a] (1501)	AO (1458)
Mitsui 7 CNT	Mouse	24 Hr	4 – 9	3 - 7	9 – 13	N/A	5 – 11	10 – 21	9 – 13	N/A
Mitsui 7 CNT	Mouse	3 / 7 day	11 – 22	6 – 22	14 – 24	N/A	9 – 16	15 – 26	17 – 34	N/A
Mitsui 7 CNT	Mouse	28 day	No Effect	14 – 26	36 – 51	N/A	14 – 26	11 – 20	No Effect	N/A
Mitsui 7 CNT	Mouse	56 day	N/A	N/A	N/A	N/A	N/A	N/A	N/A	14 – 27[b]
NRCWE-026 CNT	Mouse	24 Hr	No effect	8 – 15	20 – 37	N/A	8 – 15	21 – 39	No Effect	N/A
NRCWE-026 CNT	Mouse	3 / 7 day	16 – 28	16 – 27	19 – 33	N/A	15 – 24	16 – 26	19 – 36	N/A
NRCWE-026 CNT	Mouse	28 day	No Effect	No Effect	No Effect	N/A	12 – 20	No Effect	No Effect	N/A
NM-401 CNT	Mouse	24 Hr	No Effect	3 – 20	8 – 22	N/A	8 – 22	13 – 22	18 – 29	N/A
NM-401 CNT	Mouse	3 / 7 day	11 - 17	12 - 19	12 - 20	N/A	7 - 20	14 - 22	13 – 21	N/A
NM-401 CNT	Mouse	28 day	20 - 37	17 - 28	No Effect	N/A	No Effect	13 - 21	18 – 31	N/A

MIE→KE1 (Quantitative Understanding of the Linkage)

Response-response Relationship

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

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

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

Time-scale

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

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

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

KE2→KE3 (Quantitative Understanding):

Time-scale

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

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

Reviewer 3:

10. Lung fibrosis as an adverse outcome (AO) is a chronic and progressive disease that leads to scarred alveolar tissue. Occupational and environmental factors as well as medication can induce lung fibrosis, but rare cases also occur without a known cause, which are summarized as idiopathic pulmonary fibrosis (IPF). The sequence of effects includes: the interaction of the substance / chemical / drug / material with the outer cell membrane, i.e. molecular initiating event (MIE), pro-inflammatory mediator release (KE1), recruitment of inflammatory cells into the lung tissue (KE2), alveolar capillary membrane integrity loss (KE3), activation of adaptive immune response by T Helper type 2 cell signalling accompanied by anti-inflammatory and pro-repair/fibrotic mediator release (KE4), fibroblast proliferation and myofibroblast differentiation (KE5), which finally leads to the synthesis and deposition extracellular matrix deposition (KE6). All these events culminate in a thickening of the alveolar septa, a decrease in lung volume and lung fibrosis (AO). It is a comprehensive work on this AOP with a careful revision based on the comments of the internal reviewers. It provides clear guidance for in vitro and in vivo work to coordinate and conduct specific experiments to assess the potential of a chemical / substance / nanomaterial to induce fibrosis.

Initial Response: No Response Needed

11. In the overall assessment of the AOP, the different animal species used for this research area are listed. It is found that the key characteristic events for fibrosis are more or less the same, with some minor differences such as inter-species variation in the respiratory system. It is recommended to add some references that summarize the limitations of the different models, e.g.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5532376
https://err.ersjournals.com/content/28/153/190029
https://onlinelibrary.wiley.com/doi/full/10.1002/path.4658

Response: Two of the suggested readings were incorporated into the main AOP page, describing some of the limitations in using different animal models

Revision: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Overall Assessment of the AOP (AOP 173):

Some other considerations of relevance to this AOP:

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

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

2.2 WEIGHT OF EVIDENCE:

Reviewer 1:

1. Regarding the WoE for the KERs, comments are provided for each KER described, also addressing uncertainties, inconsistencies and challenges in terms of quantification study and inconsistencies are identified for several studies. The KER table on p.3 provides “high” evidence scoring for all KERs. The described KERs (MIE-K1E, KE1-KE2) emphasize the decisive role of the alarmin IL-1a/ NFk B signaling pathway in triggering KE2. However, the example of MWCNT-macrophage interaction shows that more signaling pathways may be involved (Vietti, 2016, Li, 2018), and IL-1a/inflammasome activation appears to be predominantly induced by fibre-like MWCNTs exposure resulting in macrophage necrosis (Palomäki, 2011). Likewise, Il-1R1 knock-out models which provide strong evidence for the relevance for IL-1 signaling in PMN recruitment also tested fibre-like but no other types of MWCNTs (Nikota, 2017). Because of the narrow evidence base in relation to its suggested comprehensive applicability, downgrading the WoE evidence from “high” to “moderate” for the first KER is recommended.

Response: Please see response to Comment # 6; 1.0 Overall Comments and Revisions.

2. Regarding the essentiality assessment of all KE’s in section 4, it would be helpful to organize a separate Excel sheet. The table provided, termed “weight of evidence”, which lists key studies for different stressors and the KEs they address. It is not clear this table is intended to be used as an equivalent for Table 5 in the OECD Handbook. In any case, it is recommended to use the format of the Handbook for appraising the quality of the available evidence (direct, indirect, no or contradictory evidence), considering specificities of stressors.

Response: Please see response to Comment # 6; 1.0 Overall Comments and Revisions.

3. Weight of evidence for involvement of specific stressors in the different KEs is provided from the scientific literature, most often for bleomycin, CNT, asbestos, silica, as well as IPF. However, the evidence is rather anecdotal and it is questionable if reported changes of the expression or secretion of cytokines (varying with type of stressor) are sufficient to predict the sequential occurrence of KEs such as inflammatory cell recruitment or loss of capillary membrane integrity. Moreover, as dose-response concordance is missing it is difficult to substantiate hypothesized KERs with respective data. In this context it is surprising that the BMDs related to

specific KEs derived from toxicogenomics analysis by the AOP developers has not been considered yet (Labib, 2016) for a concordance table, as suggested in the OECD Handbook.

Response: Please see response to Comment # 2; 1.0. Please see overall responses to comments.

The reason we hesitate deriving conclusions based on what is available is the heterogenous nature of info on inflammation. As mentioned above, increased expression of SAA3 is directly correlative of neutrophil influx in mouse lungs. There are other cytokines and chomokines which are also highly expressed in the same study and not all correlate with neutrophil influx. Thus, it is important to define or agree on which cytokines and chemokines we consider as relevant to inflammation and then build such concordance relations. This is something we are doing at the moment.

Reviewer 2:

4. There are a number of studies supporting the AOP leading to pulmonary fibrosis. From these studies, there are some inconsistencies that are pointed out in the WoE table. There are some inconsistencies that seem to indicate that the correlation between inflammation and pulmonary fibrosis is not that clear. For example, in knock-out mice that lack specific genes (for a receptor for example), the inflammation response is not changed, while the fibrosis is decreased or even absent. In addition, in mice lacking IL1R1 signalling, the inflammation response is first suppressed, while later on the fibrosis is exacerbated. This makes clear that the inflammation response is complex and that the correlation between inflammation and fibrosis is still not completely clear.

Response: Absolutely. Please see the responses to earlier comments.

The essentiality and empirical evidence scores for each KER were adjusted to take into account the inconsistencies present. Please see response to Comment # 6; 1.0 Overall Comments and responses.

5. Another observation from the WoE table is that the number of substances that lead to pulmonary fibrosis seem limited. The WoE is mainly based on Bleomycin, some carbon nanotubes (depending on the characteristics), asbestos and silica. For CNTs, asbestos and silica, it is known that these could also induce cancer. Therefore, it is important to better understand when the inflammation that is induced progresses into fibrosis or into cancer or when it is still able to resolve

Response: Most endorsed AOPs are built on one or two stressors. AOP173 provides evidence from several of stressors. Role of inflammation in cancer is yet to be defined. Identifying the specific markers that allow differentiation between inflammation leading

to cancer vs inflammation leading to lung fibrosis is a research program in itself. Thus, we will not attempt to address this here. Please see other responses above.

Reviewer 3:

6. Yes, the evidence and qualitative understanding is high for most KEs, as there is many human and animal data that clearly underline the described KEs and KERs. Only for the integrity of alveolar capillary membrane, the KER is difficult to prove with animal data, and more data must be generated by in vitro model to support KE3 with quantitative data.

Response: We agree that it is difficult to prove.

7. Weight of evidence support for dose and time-response relationship focuses on data for carbon nanotubes. But a lot of human data exists for occupational exposure, such as inhalation of asbestos / silica and for cigarette smoking. Is it possible to make a similar estimation from these data as for CNTs?

Response: There are numerous studies included throughout the AOP documents which refer to asbestos and silica exposures to support various KERs and as weight of evidence. A few references relating to fibrosis in humans as a result of asbestos and silica exposure has been added to the "Types of Stressors" section in the main AOP 173 document.

Revision: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents revisions made to the AOP sections, addressing the reviewers' comments. The Snapshot for external review - Dec 2019 pdf snapshot was used to make the revisions.

Domain of Applicability; Types of Stressors (AOP 173):

Types of Stressors:

Persistent and soluble stressors can induce fibrotic pathologies in humans (as well as in model animals) in concordance with the AOP presented. Asbestos exposure in humans has long been known to induce pulmonary fibrosis (asbestosis) due to chronic inflammation induced from persistent fibres deposited within the lung (Kamp and Weitzman 1997). Similarly, human exposure to silica leads to the development of silicosis in concordance with the AOP presented (Ding *et al.,* 2002). Furthermore, the soluble chemotherapeutic compound bleomycin has long been known to induce pulmonary fibrosis in humans (in line with this AOP) as a side effect of intravenous administration (Froudarakis *et al.,* 2013). In addition to these model stressors, exposure to various metals including uranium, arsenic, cadmium, and soluble copper can lead to fibrotic outcomes in humans (Assad et al., 2019). In male mice exposed via inhalation to cadmium oxide nanoparticles, increases in the pro-fibrotic and pro-inflammatory mediators IL-1b, TNF-a, and IFN-g were noted one day post exposure, with accompanying pulmonary inflammation (Blum et al., 2014). In another study utilizing cadmium chloride, intratracheal instillation in mice induced peribronchiolar fibrosis through activation of myofibroblasts via

SMAD signalling (Li et al., 2017). As with the aforementioned cadmium nanoparticles, murine animals exhibit pronounced acute inflammation and immune cell infiltration after pulmonary exposure to CuO nanoparticles (Gosens et al., 2016), which can progress to a fibrotic phenotype in some model systems after 28 days with marked increases of TGF-b detected in the BALF, activation of myofibroblasts, and pronounced deposition of extracellular matrix (Lai et al., 2018). Occupational exposure to cobalt can induce interstitial lung disease in humans, which can progress to fibrotic outcomes (Traci et al., 2017). In mice, intratracheal instillation of cobalt nanoparticles results in pronounced infiltration of neutrophils and macrophages into the alveolar and interstitial space, and increased amounts of CXCL1 in the BALF 1-7 days post exposure; pronounced pulmonary fibrosis was detected at 4 months post exposure marked by increased collagen deposition and bronchiolization of the alveolar epithelium (Wan et al., 2017).

2.3 ADDITIONAL OBSERVATIONS

Reviewer 1:

1. This AOP is quite ambitious as it spans a large distance from a stressor's portal of entry to fibrosis, thereby linking highly complex processes and mechanisms such as acute and chronic inflammation with fibrotic effects, considering respective cells, signalling pathways, and soluble mediators involved in culminating in adverse fibrotic scarring. The developers of the AOP emphasize its generic features and events, irrespective of the nature of the stressor (soluble chemical, [nano]particle, fibre, biological pathogen, unknown stressor). Converging evidence is provided for both, soluble (exemplified by bleomycin) and a particulate stressors (exemplified by CNT). The rationale for AOP project reads as if its development is primarily motivated by an alternative strategy for nanomaterial testing and assessment. Evidence for nanomaterials and solid materials predominates the database. Bleomycin and ILF data is included primarily because these models provide well established mechanistic information, thus supporting the universal character of the AOP. The later events are indeed independent of the type of stressor, as long as the stress persists. However, the type of stressor matters in terms of the MIE and upstream inflammatory events. For instance, there are profound differences in solid and soluble stressors with regard to toxic properties, compartmentalisation cellular interaction. These should be pointed out more clearly.

Response: MIE and KEs should be described independent of stressors. We have clearly stated that this AOP is applicable to any pro-fibrotic stressor that initiates immune and inflammation response. Although MIE and the first two KEs seem generic, the magnitude of their activation is dictated by the property of materials. Thus, the injury and consequent DAMP release following interaction of 'toxic' materials with lung cells will be many fold higher compared to the response observed following exposure to inert materials. From the 75 different nanomaterials that we have studied, we can clearly see a gradient of inflammatory response that can be associated to eventual pathological potential of each material. This is all work in progress. It is also important to note that metal oxides activate proteinases, which are not observed following CNT exposure. In

addition, the magnitude (how many, how much) of proteinase response again varies based on how toxic a material is. Some pro-emphysematic stressors can also induce fibrosis. Again, for the diseases that are mediated by immune and inflammation response, there is a thin line that differentiates the final outcome and it is all dictated by the material properties and changing microenvironment. It has to be acknowledged that stressors that induce a robust inflammatory and immune response can induce multiple AOs.

Response: Additional information relating to metal and metal oxides has been added to AOP 173, in both the main document and a number of the connected KER pages. Please see Comment #3; Section 1.0 Overall Comments and Revisions.

2. Measurements for KEs on the cellular and partially on the tissue level heavily rely on PCR and ELISA methods to investigate the expression and secretion of soluble mediators. Because pro-inflammatory mediators can be induced depending on stressors and because mediators are usually multifunctional, it can become difficult to agree on a set of mediators predictive for fibrogenic inflammation. To circumvent this issue, a large set of mediators may be screened, e.g. by using array and –omics techniques. However, such an approach is still facing a high degree of uncertainty, reproducibility issues and random in interpretation of results. Likewise, primary target cells differ depending on the type of stressor. Thus, agreeing on relevant cells for in vitro assays can be challenging.

Response: The publication concerning the 17 gene signature (PFS17) has been published, and the AOP page was adjusted to include its mention. Please see Comment # 5; Reviewer #1; Section 2.1 Scientific quality.

3. MIE 1495: "Interaction with the lung resident cell membrane components" is very vague terminology (perturbation, chemical interaction, receptor-binding, etc.?). Lack of precision is likely due to the broad range of stressors the AOP is developed for but should be avoided. In fact, depending on the sort of stressor, it is reasonable to assume that there is more than one MIE. For the time being it is recommended to do without a MIE and use the earliest known KE instead, in accordance with the OECD Handbook.

Initial Response: For nanomaterials, it is accepted that nano-bio interaction is a must for any response to occur, without which there is no AOP. Thus, even though vague, the MIE is important to have. As such, vagueness describes the non-specificity of such interaction. This brings us back to the same points discussed above – one stressor can initiate multiple interactions. The MIE in AOP 173 is unconventional and may be it is important that this is acknowledged.

4. The uncertainty deteriorates the usefulness of this AOP for regulatory purposes. It is therefore recommended to adapt and limit the AOP to specific types or class of stressors. For instance, limiting it to CNTs, inflammation-promoting interaction would clarify on primary target cells (resident AM) and interactions (phagocytosis, piercing). In this case, oxidative stress (due to substance-related or cell-dependent ROS generation) would act as an initiating or upstream event in terms of inflammation compared to its current role defined as KE associative event No.3 in the context of loss of ACM integrity only. Accordingly, both cellular and acellular ROS would need to be measured.

Response: We do not agree to limit it to one class of materials as the KEs identified are broadly applicable to fibrosis process induced by several different stressors. The associative events in AOP 173 were removed from the KE pages and included only in the AOP page, to show how they potentiate KEs specifically related to the AOP. Furthermore, the associative event of oxidative stress has been modified. Please see Comment # 4; 1.0 Overall responses to Comments.

5. KE 2 (1497): Increased, recruitment of inflammatory cells. Authors admitted that measurement in vitro is difficult, thus referring to the detection of proinflammatory cytokines as indicators of cellular recruitment. Alternatively, the appropriateness of co-culture transwell chemotaxis assays and differential expression of integrins/selectins could be checked (e.g. see Zemans RL, Colgan SP, Downey GP. Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury. Am J Respir Cell Mol Biol. 2009;40(5):519–535. doi:10.1165/rcmb.2008-0348TR).

Response: Advanced co-culture systems and *ex vivo* methodologies have been added to the assessment section of a number of KERs. Furthermore, the publication referencing PFS17 has been published and the main AOP page was updated to mention this preliminary biomarker. Please see Comment # 8; 1.0 Overall Comments and revisions & Comment # 5; Reviewer # 1; Section 2.1 Scientific Quality.

6. Potential applications: This topic is insufficiently addressed. It should be elaborated more in detail what this AOP is aiming for and how it can be applied to inform risk assessment (including screening, grouping). Does it aim at replacing animal testing, such as repeat dose inhalation testing? If so, this would have an impact on sampling and measurement of tissue-level KEs in vivo (e.g. histopathology analysis, BALF analysis, use of primary cells, etc.).

Response: The paper describing a nano QSAR model produced using AOP 173 has been published and the main AOP page has been modified to include its mention. As well, recent efforts at creating an AOP network (of which AOP 173 was a major

component) have been summarised, highlighting how this AOP can guide the development of testing strategies and research focus. Please see Comment # 5; Reviewer # 1; Section 2.1 Scientific Quality and Comment #11; Reviewer #3; Section 2.1 Scientific Quality.

7. Altogether, AOP 173 is certainly a project that deserves to be followed-up, provided that there is an agreement on its applicability but also on its limitations. Refinement and strengthening of the MIE/KEs and KERs appears to be most effectively achieved when focusing on a specific class of stressors (even if redundancy of stressors is deemed a common feature of AOPs). This would also help reducing the complexity of this AOP, specifying the involved cellular interactions, pathways and molecules, and fostering the integration of its linear approach into respective AOP networks that highlight further stressors and KEs.

Response: We do not agree that intervention strategies suggested will help reduce the complexity. Instead, Three additional alternative mechanisms were added to the “alternative mechanisms” section in the AOP.

Revision: Text underlined in blue is a clickable hyperlink that links to the relevant section on the AOP wiki. Text in red represents a modification or addition to the material specifically relating to the reviewer comment, based on the Snapshot for external review - Dec 2019 pdf snapshot.

Evidence Assessment; Alternative Mechanisms (AOP 173):

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

Further mechanisms may involve the targeted inhibition of receptor tyrosine kinases by compounds like Gefitinib, Imatinib, and Sorafenib, as well as some monoclonal antibodies which affects receptors for growth factors like PDGF, EGF, and VEGF. This is thought to directly impair the regenerative capacity of lung epithelial cells (MIE; Event 1495 to KE3; Event 1497), resulting in an aberrant wound healing response (Li et al., 2018). Finally, one more alternative mechanism involves pulmonary fibrosis in the context of bronchiolitis obliterans. In this

condition, the fibrotic phenotype is brochiolocentric and not alveolocentric – with the main insult involving the bronchiolar epithelium and an inability of the basal cells to replace lost bronchio epithelial cells. Stressors, such as soluble diacetyl used in popcorn flavouring and e-cigarette vape liquids, can cause bronchiolitis obliterans in humans. A recent human case study of a Canadian youth admitted to hospital with bronchiolitis obliterans following vaping flavoured liquid containing diacetyl, as well as tetrahydrocannabinol, shows septal thickening, type II pneumocyte hyperplasia, immune cell infiltration and myofibroblast proliferation & incorporation into pulmonary septa (Landmann et al., 2019). Pulmonary exposures in murine model systems indicate that diacetyl induces pronounced damage to the airway epithelium, and that repair processes result in a compositionally different epithelium (Reviewed in Brass & Palmer, 2017). In a study using rat models, inhalation of 200ppm of diacetyl resulted in bronchiolar fibrosis, with chronic inflammation accompanying the fibrotic outcomes (Morgan et al., 2016).

Reviewer 2:

8. The authors put a lot of effort in describing the AOP, there is a lot of information available in the document that is very helpful for understanding the mechanism behind pulmonary fibrosis. Such an AOP can be very valuable for developing in vitro methods that can be used for screening for the potential of substances/particles to induce pulmonary fibrosis. In my opinion, the main issue of this AOP are the MIE and the earlier KEs. There is uncertainty whether these are essential for the AO and they seem not specific for the AO. The MIE and early KEs can be grouped as the inflammation response. Pulmonary inflammation can be resolved, or it can lead to many different outcomes. Recent insight show that mechanisms related to chronic inflammation, fibrosis and cancer are all interrelated. This is the case for CNTs, asbestos and silica, which are used in the WoE for the AOP. More information on the complex interplay between these mechanisms is needed to refine the AOP and to be able to use it. A suggestion forward is to start building networks of AOPs, as many share the same KEs. By gathering available data on each of the KEs and the AO, as is done in the current AOP 173 but then including cancer and chronic inflammation, one could unravel if there are specific pro-inflammatory mediators or specific inflammatory cells involved in the different AOs. In addition, one could start to model the KERs. Probably, by unravelling the quantitative KERs, the correlation between each of the KEs and the AO becomes more clear.

Response: A section has been added, highlighting how a network of AOPs was used to derive a set of shared KEs to guide future bio assay development. Please see Comment #11; Reviewer #3; Section 2.1 Scientific Quality.

Reviewer 3:

9. The AOP is applicable to a broad group of chemicals / substances / drugs / materials and can also be applied to engineered nanomaterials. For carbon nanotubes a lot of data is provided. In this area there is an ongoing and intensive discussion about dose-response relationship. This is not covered in the current AOP concept, where

only the MIE is relevant, but not the delivered dose / concentration. The quantitative understanding of this class of materials should be considered as relevant information.

Response: A study published recently from our lab has been used to construct a quantitative concordance table for KE induction based on transcriptomic data from CNTs. Please see Comment # 2; 1.0 Overall Comments and Revisions.

10. Currently, oxidative stress response is not covered as the first KE, but the oxidative stress paradigm for nanomaterials is an accepted fact. Since this endpoint, in addition with inflammatory mediators, would facilitate cross-species comparison, this should be reconsidered and assays to cover this point should be expanded.

Response: Oxidative stress is another higher order KE that involves multiple hub KEs. Acute oxidative stress can play a signalling role and can be reversed. Similar to feedback loops involved in inflammation, multiple feedback loops are involved in oxidative stress. However, there is no clarity as to when does oxidative stress becomes detrimental. Although oxidative stress is assessed for nanomaterials, a clear link between nanomaterial induced oxidative stress and a pathological outcome is yet to be demonstrated.

Response: The impact of oxidative stress on AOP 173 has been modified, and it has been described as an associative event potentiating a number of KEs within the AOP. Please see Comment # 4; 1.0 Overall Comments and Revisions.

11. The description of the KEs and the graphical illustration imply that MIE and then the cell and tissue effects occur sequential. However, as I understand it - and what is partially described in the text - inflammation is chronic and other KEs may occur simultaneously. This aspect should be better discussed to also guide researchers for future investigations.

Initial Response: In our opinion, nothing in biology is sequential. Everything (positive and negative trajectories) is initiated in parallel. It is the exposure, material property and changing microenvironment consequential to signalling is what determines the fate. This is very important to take into consideration for AOP development. The AOP framework recognises that there are parallel processes and feedback loops. But the AOP will depict the most important set of events required for the AO to occur. If we add all those parallel processes and feedback loops, it will be a MOA and not an AOP.

12. For in vivo studies, little information is given about the time-line of the occurrence of different KEs. Could this be expanded to include recommendation on the duration of an experiment? It is obvious that in humans or animals the final KE, which

proliferation / activation of fibroblasts and deposition of ECM, only occurs after months / years.

Initial Response: None given.

Response: The quantitative understanding behind two of the KERs was expanded, and time-scale data was provided indicating the timespan in which the two KEs are induced. Furthermore, weight of evidence tables created for each KER list the timepoint in which key observations were made, providing a measure of KE occurrence.

Revisions: Text in blue indicates clickable hyperlinks which lead to the weight of evidence tables for each KER. The two other links lead to the Time-scale sections of two KERs containing relevant quantitative information.

MIE → KE1 WoE Table

KE1 → KE2 WoE Table

KE2 → KE3 WoE Table

KE3 → KE4 WoE Table

KE4 → KE5 WoE Table

KE5 → KE6 WoE Table

MIE → KE1 (Time-Scale)

KE2→KE3 (Time-Scale)

13. An overview about the challenges and limitations of animal vs cell models to study this AOP could be given, this would also help to identify optimal experimental approaches for additional relevant data to fill some of the gaps. For instance, KE3 is more difficult to study in vivo, while the influx of immune cells (KE2) is difficult to mimic in vitro.

Response: Unfortunately, AOPs are not the place for discussing the limitations. We have however, stated where necessary, some of the challenges.