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Environmental Significance

In recent decades, particulate matter (PM) has been associated with several diseases. PM mass concentrations are typically used as the metric to estimate the health effects caused by PM. However, investigations in the past two decades have focused on the idea that mass might not be an accurate measure of PM toxicity, arising the need to find a better metric that could more accurately represent the health effects of PM. Accordingly, several assays have been developed to assess toxicity of PM which include both cell-based and acellular methods. Among the two, cellular assays allow a more comprehensive assessment of PM toxicity. However, there are numerous types of cells and cell lines differing widely in their physiology, functions, and responses to toxicants, which have been used in these studies. The choice of cell types along with other experimental factors such as exposure duration, dose, and chemical composition of PM, dictate the results of cell-based assays. These aspects of cell-based assays are often ignored in PM toxicological studies, which tend to generalize the results derived from single types of cells or cell lines. Thus, a comprehensive review focusing on the differences among the responses of various cell types (and cell lines) observed in current toxicity studies could help to highlight the pitfalls of such generalizations and provide effective guidelines for better interpretation of the results in future studies.

In this review, we discuss various aspects of the *in-vitro* studies, focusing on the morphological and immunological differences among various macrophage and epithelial cells, belonging to the respiratory system of human and murine species, used in the *in-vitro* studies evaluating PM toxicity. We also review the current state of knowledge on the role of different PM chemical components influencing PM toxicity and the relevance of atmospheric processing and aging of aerosols in PM toxicity. We anticipate that our review will guide future research towards the development of more physiologically relevant cellular models for studying PM toxicity, which will eventually lead to a better understanding of the health effects of PM exposure.

Review of *in-vitro* studies evaluating respiratory toxicity of aerosols: impact of cell types, chemical composition, and atmospheric processing

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Abstract

In recent decades, several cell-based and acellular methods have been developed to evaluate ambient particulate matter (PM) toxicity. Although cell-based methods provide a more comprehensive assessment of PM toxicity, their results are difficult to comprehend due to the diversity in cellular endpoints, cell types, assays, and the interference of PM chemical components with some of the assays' techniques. In this review, we attempt to clarify some of these issues. We first discuss the morphological and immunological differences among various macrophage and epithelial cells, belonging to the respiratory system of human and murine species, used in the *in-vitro* studies evaluating PM toxicity. Then, we review the current state of knowledge on the role of different PM chemical components and the relevance of atmospheric processing and aging of aerosols in the respiratory toxicity of PM. Our review demonstrates the need to adopt more

physiologically relevant cellular models such as epithelial (or endothelial) cells instead of macrophages for oxidative stress measurement. We suggest limiting macrophages for investigating other cellular responses (e.g., phagocytosis, inflammation, and DNA damage). Unlike monocultures (of macrophages and epithelial cells) which are generally used to study the direct effects of PM on a given cell type, the use of co-culture systems should be encouraged to investigate a more comprehensive effect of PM in the presence of other cells. Our review has identified two major groups of toxic PM chemical species from the existing literature, i.e., metals (Fe, Cu, Mn, Cr, Ni, and Zn) and organic compounds (PAHs, ketones, aliphatic and chlorinated hydrocarbons, and quinones). However, the relative toxicities of these species are still a matter of debate. Finally, results of the existing studies investigating the effect of aging on PM toxicity are ambiguous, with varying results due to different cell types, different aging conditions, and the presence/absence of specific oxidants. More systematic studies are necessary to understand the role of different SOA precursors, interactions between different PM components, and aging conditions, on the overall toxicity of PM. We anticipate that our review will guide future investigations by helping the researchers in choosing appropriate cell models resulting into a more meaningful interpretation of the cell-based assays, and thus ultimately leading to a better understanding of the health effects of PM exposure.

1 Introduction

In recent decades, ambient particulate matter (PM) has been associated with several respiratory diseases such as asthma[1], wheeze[2], chronic obstructive pulmonary disorder[3] and lung cancer[4] Globally, PM mass is used as a metric in epidemiological models to estimate the morbidity and mortality caused by PM[5–9]. However, investigations in the past two decades have focused on the idea that mass might not be an accurate measure of the respiratory toxicity of PM, raising the need

to find a better metric that could more accurately represent the health effects of PM. Oxidative stress has been speculated to be the underlying pathology for a number of diseases and it has been proposed that measuring the ability of PM to induce oxidative stress could be used as a surrogate for certain health-related damages[10–14].

Accordingly, several assays have been developed to measure the oxidative potential (OP) of PM which include both cell-based and acellular methods. Rapid progress has been made in acellular assays as they are less labor-intensive and cumbersome and provide high throughput as compared to cell-based assays. They can also be easily employed in developing online instruments for real-time measurement of OP[15–18]. In recent years, a few articles have conducted a comprehensive review of the acellular assays[19–21], which demonstrate the utility of OP in providing a preliminary assessment of the health effects of PM. These articles have also discussed the role of different components of PM in driving the response of these OP assays. However, despite all the advantages of cell-free assays, they have faced some criticism in the recent past due to several reasons. First, most acellular assays focus on a single biochemical reaction (or a set of reactions) involving only a couple of compounds of biological relevance and thus perhaps oversimplify the complex mechanisms involved in the toxicity of the aerosols. Second, the biological relevance of measuring OP of the PM in a purely chemical environment is yet to be fully established and, in many cases, poor correlation has been found between acellular OP and cellular responses[22–25]. Third, although oxidative stress is presumably the underlying pathology of many pulmonary diseases, there are several oxidative stress-independent toxicity mechanisms[26–29], and it is unknown if PM can trigger these mechanisms as well. Finally, cells have often shown the ability to protect, recover, and repair themselves in the events of an assault by a foreign agent[30,31] and the assumption that pulmonary toxicity has a linear relationship with a single triggering event is not entirely true. Since the

pathology of pulmonary diseases often involves complex mechanisms and a cascade of events which might be complementary to one another[32,33], studying the expression of all important biological markers is essential to fully understand PM toxicity mechanisms.

Cell-based assays, on the other hand, allow a more comprehensive assessment of PM toxicity. These assays help in evaluating precise biological markers and investigating the mechanisms involved in the expression of these markers. Additionally, they can also be used to assess the impact of PM (and its constituents) on the specific physiological system of interest. For example, using cell-based assays, one might be able to investigate the specific impact of PM on the cardiovascular or respiratory system by using cells or cell lines belonging to those systems. Consequently, cell-based assays have been used in several studies in the past few decades to evaluate the respiratory toxicity of PM. Such studies have revealed crucial details about the mechanisms of toxicities of various PM chemical species[34–37]. However, there are numerous types of cells and cell lines differing widely in their physiology, functions, and responses to toxicants, which have been used in these studies[38]. The choice of cell types along with other experimental factors such as exposure duration, dose, and chemical composition of PM, dictate the results of cell-based assays[39–42]. This aspect of cell-based assays is often ignored in PM toxicity studies, which tend to generalize the results derived from single types of cells or cell lines. Thus, a comprehensive review focusing on the differences among the responses of various cell types (and cell lines) observed in current toxicity studies could help highlight the pitfalls of such generalizations and provide effective guidelines for better interpretation of the results in future studies.

Several review articles have been published in the last decade on different aspects of PM toxicity[43–63]. For example, Peixoto et al.[52] reviewed the mechanisms involved in PM-induced cell death

without delving into the effect of PM chemical composition. They also discussed different cell models used in PM studies investigating cell death while focusing majorly on human cell lines. Similarly, Chen et al.[55] also focused their discussion only on the cell death mechanism induced by PM while including some discussion regarding the PM chemical species that are responsible for triggering those pathways. Several other similar articles have reviewed specific cellular responses such as oxidative stress[53], genotoxicity[62], cell signaling[59], inflammation[44,57,59,63], mitochondrial function[43], and metabolic dysregulation[49] focusing more on the mechanisms involved in these responses rather than the PM components responsible for triggering them. Nemmar et al.[50] discussed the role of PM in inducing various mechanisms responsible for diseases such as cancer and Chronic obstructive pulmonary disease (COPD) based on *in-vitro* and *in-vivo* studies, but dedicated most of their discussion to specific particles such as diesel exhaust (DEP) and engineered nanoparticles. Jia et al.[45] discussed the effect of $PM_{2.5}$ chemical composition on toxicity in different types of cell models such as macrophages, epithelial cells, endothelial cells, and co-cultures, but without discussing the role of atmospheric processing in altering chemical composition and toxicity. Kermani et al. discussed the effect of metals and PAHs on cytotoxicity while limiting their discussion only to epithelial cells[46]. Pardo et al. also discussed the role of metals and PAHs in PM-induced toxicity, but only in the context of triggering the Nrf2/antioxidant system[51]. Finally, Liu and Ng et al. provided an introductory overview of a few *in vitro* and *in vivo* techniques to assess PM toxicity and the various cell models used in PM toxicological studies, with an intention to serve as a primer of the various methodologies and assays used in the toxicity research of atmospheric aerosols[47]. There have been other articles as well which have focused on reviewing existing literature on the effect of PM in inducing certain specific diseases such as asthma and rhinitis[58], neurogenerative diseases[43,54], and cardiovascular diseases[60,61]. Although these reviews

provide valuable insights into cellular responses to PM, there are several aspects which are left unaddressed. For example, a majority of these articles provide limited discussion on the morphological and immunological differences among the most important cell types belonging to the respiratory system (i.e., macrophages and epithelial cells) used in *in-vitro* toxicological studies evaluating PM toxicity. Moreover, the role of murine cell lines (which are widely used in PM studies) and the differences in their responses as compared to human cell lines have seldom been explored. Finally, there is also a lack of reviews focusing on the effects of aerosol aging on the toxicity of secondary organic aerosol (SOA). Thus, there is a need for a review of existing literature that gives a broad perspective on the fundamental aspects influencing PM toxicity such as cell types, role of PM chemical species, and the atmospheric transformation of PM. The primary objective of this review is to discuss the cell-type-dependent responses to PM and summarize the vast differences in results one might encounter while employing various macrophage and epithelial cells of the respiratory system.

In this review, we first discuss the morphological and immunological differences among various macrophage and epithelial cells, belonging to the respiratory system of human and murine species, used in *in-vitro* studies (section 2.1) evaluating PM toxicity. Next, we briefly discuss the relevance of different co-culture systems using these cells and their advantages for a better understanding of PM toxicity mechanisms (section 2.2). We then review the current state of knowledge on the role of different PM chemical components influencing PM toxicity (section 3). Finally, we explore the relevance of atmospheric processing and aging of aerosols in PM toxicity (section 4). To maintain focus, we limit our discussion to the studies involving macrophage and epithelial cells belonging to the respiratory system of human and murine species. We anticipate that our review will guide future research towards better choice of cell models, interpretation of cell-based toxicity studies,

and the development of more physiologically relevant models for studying cellular PM toxicity, which will eventually lead to a better understanding of the respiratory effects of PM exposure.

2 Cell models used to evaluate respiratory toxicity of PM

Although it is widely established that PM is associated with several diseases, the specific mechanisms through which PM causes these diseases are not yet well-established. Moreover, it is also not yet clear which of the PM chemical species are most hazardous to human health. Investigating these aspects of PM has been a topic of research for several decades. Consequently, several experimental techniques have been established using animals such as rodents and monkeys to study the effects of PM exposure. However, these experiments are rather complex, expensive, and often complicated by ethical issues. Therefore, *in-vitro* studies could play an important role. One of the major advantages of *in-vitro* studies is their reductionist approach, which allows for a detailed investigation of toxicity mechanisms using a variety of doses, experimental conditions, chemical species and cell models. They are also cheaper, quicker, and easier to handle compared to *in-vivo* and clinical trials. Most *in-vitro* studies involve measuring 4 broad groups of fundamental cellular responses to PM exposure. These are oxidative stress, inflammatory or immune response, cell death, and mutagenicity or genotoxicity. The various assays and methods to measure these responses are given in Table 1. As is evident from Table 1, there are several assays and techniques to measure a variety of cellular responses. Several publications have already reviewed these responses extensively in the past[64–78]. However, very few reviews have discussed the various cell models used in PM *in-vitro* studies investigating these cellular responses.

There are several cell models derived from different organisms which could be used in PM toxicity studies and given the diversity in the origins of these cell models, it is reasonable to expect a

diverse range of cellular responses leading to varied interpretations about PM toxicity. In this section, we will discuss the current state of knowledge on the various cell models being used in PM studies. Specifically, we will first discuss the differences between macrophages and epithelial cells belonging to the respiratory system, mostly focusing on the fundamental differences between these two cell types observed in monocultures (Section 2.1). We will limit our discussion to macrophages and epithelial cells of human and murine origin only. Next, in Section 2.2, we will discuss various co-culture systems used in PM studies. Here also, we will focus our discussion on only those systems which use macrophages and/or epithelial cells, while briefly touching on more complex and advanced co-culture systems containing other cell types.

2.1 Respiratory macrophages and epithelial cells used in PM toxicity studies

Given the differences in the uptake and metabolism of the PM chemical components by different cell lines, the results of PM toxicity analysis are prone to depend on the type of cell line chosen (discussed further in section 3 and 4 of the paper). Two species − human and murine − have been most widely used in PM studies. Within these species, a number of cell lines have been established and used by researchers based on the ease of availability and reliability of the results. Nearly 25 different human and murine cell lines of respiratory origin, as listed in Table 2, have been used in PM studies so far. Note, unlike murine cell lines, there are currently no human macrophage cell lines available for the *in-vitro* studies. Hence, a lot of *in-vitro* studies evaluating the effect of PM on human pulmonary macrophages use either primary macrophages (i.e., macrophage cells derived freshly from patients) or two non-pulmonary macrophage cell lines: THP-1 (derived from blood) and U937 (derived from pleural fluid), to study respiratory toxicity caused by PM. We have included both of these cell lines in this review. On the other hand, there exist only two murine macrophage cell lines of respiratory origin − NR8383 and MH-S, which have been used in PM

studies. In addition to these, we have also included two other murine macrophage cell lines of non-respiratory origin, RAW264.7 and J774 (established from tumors in mice), in our review as they have also been widely used as a proxy for macrophages to study respiratory toxicity. Note, a majority of cell lines shown in Table 2 are derived from cancerous tissues. Therefore, although they do exhibit the properties of real macrophages and epithelial cells, there are slight physiological differences, which could make them behave differently from the "true" macrophages/epithelial cells. Nevertheless, they are crucial in investigating the toxicity mechanisms of PM because they are easy to handle and are immortalized (i.e. they can be cultured "theoretically" forever in laboratories).

We will first briefly discuss the differences between various respiratory macrophage cells, followed by the difference between different epithelial cells and finally compare macrophages with epithelial cells.

2.1.1 Differences among different macrophage cell models

Both human and murine macrophages perform a similar set of functions, such as neutralizing infectious agents (e.g., microbes), clearing foreign particles, dead cells, and cell debris, and assisting in wound healing[79]. Indeed human and murine pulmonary macrophages show great similarities[80] and murine macrophages are considered good predictive models for estimating macrophage responses in humans to different toxicants[81–83]. However, macrophages of the two species also have some distinct characteristics that could influence their *in-vitro* responses to PM exposure. For example, primary murine alveolar macrophages are 4 times smaller in size than primary human alveolar macrophages, which could result in different phagocytic abilities of inhaled particles[84]. Similarly, there is a marked difference between the pulmonary injury responses (expression of RNS and related enzymes) in murine vs. human macrophages. For example,

inducible nitric oxide synthase and nitric oxide, which are essential participants in pulmonary injuries, were found to be expressed more explicitly in primary rat alveolar macrophages than in primary human alveolar macrophages[85]. Moreover, the phagocytic ability of the primary murine macrophages is more resistant to acidic environment as compared to the phagocytic ability of primary human macrophages[86]. Lastly, there also exist differences between the phenotypic and genome markers of murine and human macrophages. For example, murine and human macrophages show different gene expression levels for chemokines. The expression of mannose receptor (a macrophage membrane protein) is seen only in M2 macrophages [macrophages that have been modified to perform specific functions (a process also called polarization) such as promoting cell proliferation and tissue repair] of mice, unlike human macrophages, where both polarized and unpolarized versions express this receptor[87]. These differences between the cells of the two species are perhaps responsible for the distinct responses observed when both are exposed to the same chemical species. For example, primary human alveolar macrophages have shown greater ROS activity as compared to primary murine alveolar macrophages when both were exposed to TiO_2 and silica particles of similar size and concentrations[88]. Similarly, primary human alveolar macrophages showed a greater inflammatory response [expression of a protein called tumor necrosis factor-α (TNF-α)] and particle uptake as compared to primary murine alveolar macrophages when exposed to TiO_2 particles[89]. Thus, a comparison of the responses between murine and human macrophage responses requires a consideration of all these differences in their phagocytic abilities, secretion of specific enzymes, gene expressions, and their individual sensitivity to different chemical components of PM.

Note, even among macrophages of the same species, the organ from which they are derived could substantially influence their responses to a toxicant. For example, in comparison to primary

interstitial macrophages (macrophages residing in the spaces between alveoli and blood vessels surrounding them) derived from BALB/c mice, primary alveolar macrophages from the same animal have been shown to secrete higher amounts of inflammatory cytokines and greater ROS (reactive oxygen species) and RNS (reactive nitrogen species) production[9091]. Similarly, there is also a marked difference in some physiological and immunological properties of human macrophages derived from different pulmonary regions. For example, alveolar macrophages in humans are typically larger (2 times) and show higher phagocytic activity as compared to interstitial macrophages[92]. Moreover, among U937 and THP-1 (both cell lines of human origin), U937 shows greater similarity to primary cells with respect to the nature of their interaction with particles[93].

Lastly, while comparing the responses of different macrophages, it may also be beneficial to consider their polarization to pro-inflammatory (M1) and anti-inflammatory (M2) forms induced by the toxicant. For example, certain toxicants (e.g., cypermethrin, an insecticide found in ambient PM[94]) can stimulate M2 polarization resulting in the growth and progression of cancer in the human body[95]. On the other hand, cigarette smoke can stimulate M1 polarization leading to the development of chronic obstructive pulmonary disease (COPD)[96]. However, currently, the role of ambient PM in inducing macrophages' polarization is not yet clear. Limited studies conducted on primary cells have demonstrated that ambient PM could induce both M1 and M2 polarizations[97,98]. However, the ability of PM to cause such a change in behavior needs to be explored for other commonly used immortalized macrophage cell lines. Overall, all of these aspects indicate that one has to be careful in generalizing the results obtained from these studies employing a single macrophage cell line (or a single type of primary cells).

2.1.2 Differences among different epithelial cell models

Similar to macrophages, human and murine epithelial cells too have several morphological and biochemical similarities[99,100], as well as some differences[101–105]. For example, murine airway epithelial cells show a more enhanced expression of Ca^{2+} activated chloride channels (a group of proteins present in cell membranes responsible for transporting ions into and out of the cell) as compared to human epithelial cells[104]. Moreover, although both A549 (human) and MLE-15 (murine) cell lines represent the alveolar TYPE-II epithelial cells, MLE-15 cells are more efficient in forming selectively permeable monolayers that are more representative of physiological conditions[105], whereas A549 cells are comparatively more resistant to hyperoxia[103]. Similarly, although both A549 and RLE-6TN (murine) also represent alveolar TYPE-II epithelial cells, they show different phenotypes such as differential secretion levels of certain important proteins [e.g., actins (a group of proteins responsible for providing structural support to the cell)][102] which affects how they respond to PM exposure. Human epithelial cell lines (A549 and BEAS-2B) also have shown lower sensitivity to the toxicity of dioxin-like compounds as compared to murine epithelial cell lines (MLE-12 and RLE-6TN) and this could be attributed to the distinct binding affinities of murine and human aryl hydrocarbon receptor (AhR; an important protein that regulates the enzymes which metabolize xenobiotic chemicals)[101]. Similarly, primary human bronchial epithelial cells (PHBE) have also shown lower sensitivity to Ag particle-toxicity as compared to murine epithelial cells (RLE-6TN) and this was attributed to the differential expression of the metallothionein gene (a protein which plays a substantial role in metal detoxification within cells) between the two species[106]. Thus, these differences between human and murine cells must be considered while interpreting the results from the studies employing murine epithelial cell lines using PM with high concentrations of metals and organic species such as PAHs.

However, we must also be cautious even when employing different human epithelial cell lines as different cell lines display different characteristics due to the differences in their origin (e.g., whether the cell line was derived from cancerous tissue or it was turned into cancerous cells by using a virus to inactivate the tumor suppression genes in healthy cells). For example, although Calu-3, 16HBE14o$^-$ (16HBE), H292, and BEAS-2B cell lines are all representative of human epithelial cells, it has been observed that only Calu-3 cells have the ability to retain the monolayer structure and maintain a strong tight junction in long-term air-liquid interface (ALI) cultures to study the toxicity of inhaled aerosols[107]. In another study comparing PHBEs with BEAS-2B, BEAS-2B was observed to be functionally very different from primary cells[108]. For example, compared to primary cells, BEAS-2B was significantly inefficient in forming tight junctions [the adhesion between epithelial cells playing a crucial role in regulating the selective movement of molecules (e.g., water, ions, soluble components of PM) across the epithelial barrier]. Moreover, although 16HBE14o$^-$ and NuLi-1 are both considered representative of bronchial epithelial cells, they have been demonstrated to differ widely in their sensitivity to oxidative stress induction by dust particles[109]. Comparisons between A549 and BEAS-2B cell lines' response to $PM_{2.5}$ demonstrated clear chemical composition-dependent variations in the response of A549 cells, but not so much in BEAS-2B cells[110]. Similarly, comparisons between A549 and BEAS-2B show that the former is more resistant to cytotoxicity and cell-cycle arrest (stopping of cell-cycle and ceasing the ability to duplicate and divide), while BEAS-2B shows more pronounced activation of PAHs[111]. Similarly, A549 cells have shown more resistance to Palladium(Pd)-toxicity as compared to PHBE and this was due to the susceptibility of PHBE to caspase-dependent apoptosis triggered by Pd[112], which is not exhibited by A549 cells. All these differences between different cells show that although they are all representative of epithelial cells, their applicability in PM studies may

largely depend on the chemical composition of PM. More studies are needed to establish appropriate chemical-composition-based cell models for PM studies.

2.1.3 Comparing the responses of macrophage and epithelial cells

As discussed earlier, both macrophages and epithelial cells perform different functions in the respiratory system. Accordingly, there are differences in the way these cells interact with inhaled PM. However, interpreting the results of existing PM toxicity studies employing these cells gets complicated because these studies vary widely in the techniques they adopt for exposing PM to the cells. For example, the cells could either be directly exposed to the particles as happens in the lungs, or the cells could be exposed to water, or organic extracts of PM collected over a filter. The responses of the two cell types in both cases could be vastly different given differences in the ability of cell lines to interact with particles. Thus, it is possible for these two cell types to show contrasting results when exposed directly to the particles, but similar results when exposed to PM extraction and ALI.

Comparisons among different cell lines of macrophage vs. epithelial origin raise the question of which of these would be a better model for the *in-vitro* studies. The answer is complicated given a lot of studies have used a combination of murine and human cell lines. Some of these studies show a similarity in the response between human epithelial and murine macrophage cell lines to ultrafine particles[113,114]. However, there are studies showing murine macrophages to be more sensitive to particle-induced effects than human epithelial cells when the cells were directly exposed to particle suspensions[115]. Other studies in which the cells were exposed to PM extracts have shown that human epithelial cells had a higher ROS activity[116,117]. One of the reasons for these differences could be that macrophages and epithelial cell lines respond differently to soluble vs. non-soluble components owing to the different PM collection methods. For example, BEAS-

2B cells showed higher secretion of interleukins and TNF-α when exposed to particle suspensions (containing both soluble and non-soluble components) versus PM filter extracts (containing only soluble components)[118]. It is hypothesized that macrophages are better at internalizing the particles than epithelial cells[115,119] and hence they might respond better when exposed to particle suspensions, whereas the inability of epithelial cells to convert thiol agents to glutathione leads them to be more vulnerable to cytotoxicity and producing greater inflammatory cytokines when exposed to PM extracts[116]. However, there are a few studies which also show that human macrophages are more sensitive than human epithelial cells when exposed to both particle suspensions[40] and PM extracts[40,120]. Therefore, it is not entirely clear what exact mechanisms drive the varied responses of macrophages vs. epithelial cells to PM exposure.

It appears that certain types of cells are more sensitive to specific groups of PM constituents. For example, early evidence regarding the susceptibility of different types of lung cells to metal ions indicated alveolar macrophages to be more affected than alveolar type II cells[121]. Also, studies comparing A549 cells (human epithelial cell line) and RAW264.7 (murine macrophage-like cell line) showed that human epithelial cells respond better to endotoxins and hydrocarbon components, whereas the murine macrophages are more responsive to metals[122,123]. However, such detailed comparison between macrophages and epithelial cells is currently lacking in PM studies. Therefore, more studies are required to understand the endpoint-specific responses of similar cell types obtained from different species when exposed to different PM components. It must also be recognized that most of the studies so far have used cell cultures with only one type of cells (monocultures). Although monocultures provide excellent insights into cell-specific characteristics and behavior, they do not represent physiological conditions where several types of cells are in contact with each other. Therefore, it is necessary to investigate PM toxicity using co-

culture techniques to get a more realistic picture of PM toxicity. A few cell models which are generally considered physiologically more relevant, are discussed in the next section.

2.2 Co-culture models of macrophages and epithelial cells used in PM toxicity evaluation

It is well known that different cells of the body act in tandem with each other and their responses are often interconnected. Moreover, not all types of cell lines may be directly exposed to particles *in-vivo* and their responses could be due to cellular communication alone. Therefore, co-culturing different cells could provide a physiologically more relevant cell model to assess the biological effects of PM exposure. Three major types of *in-vitro* co-culture models have been used in PM studies: 2D co-culture models, air-liquid interface (ALI), and lung organoids (both ALI and lung organoids are 3D co-culture models). Although there are other *in-vitro* cell models such as lung-on-a-chip (a microfluidic device mimicking complex mechanical and biochemical processes of our respiratory system)[124,125] and precision-cut-lung slices (thin 3D slices cut from real lungs which maintain a majority of tissue structure and functions for *in-vitro* studies)[126], these are not very popular and are rather in their infancy stage. Therefore, in this section, we will provide an overview of only those three models by first discussing the 2D co-culture models followed by 3D co-culture models.

2D co-culture systems are well-established and have been reviewed extensively by several publications in the recent past[127–133]. Here, we will focus on the differences observed in these systems as compared to monocultures of macrophages and epithelial cells. 2D co-cultures involve two (and sometimes three) different types of cells cultured together in the same petri dish or well of a multi-well plate[127]. Essentially, in these models, the cells are all grown in a single layer as a flat sheet, mostly submerged in a cell culture medium. The different types of cells could either be layered on one another (e.g., macrophage layer over a layer of epithelial cells) or be cultured as a

mixed population (mixing two different cell types and culturing them as a monolayer of cells) or be separated using a membrane while being submerged in the same culture medium (indirect co-culture method). These arrangements of the cells could depend on the type of interactions between cell types that are being investigated. For example, if cell-to-cell interactions are being studied, then the layered method and mixed population method would be preferable. However, if the effect of cellular communication which affects only nearby cells is to be studied then the indirect co-culture method is preferable.

2D co-cultures have provided interesting insights into the probable behavior of different cell types in a real physiological system and how different they could be as compared to *in-vitro* monoculture systems most commonly used in PM studies. For example, different types of cells could have a synergistic relationship with each other in the expression of some biomarkers (such as chemokines) when exposed to a toxicant. Such interactions between macrophages and epithelial cells have been seen in both murine[134,135] and human cell lines[136,137], and lead to an amplification of inflammatory responses. Co-cultures could also reveal interesting aspects of the induction of bystander effect. Bystander effect refers to the response observed due to the transmission of the stimuli from one cell type to the adjacent cell types which are not directly interacting with the stimulation. An example of this is a reduction in adhesivity and cell motility in the unexposed neighboring cells, which is induced by the cells exposed to PM[138]. Certain cell lines can even protect others from toxicants and reduce the damage. For example, co-cultures of macrophages with lung epithelial cells show that macrophages have a protective effect on the epithelial cells against ROS-induced DNA damage and this could be due to the higher ability of macrophages to resist oxidative damage and/or due to their non-proliferative nature as compared to the epithelial cells[136,139]. The protective effect induced by co-culture models of macrophages and epithelial cells is also perhaps due to the

changes in the expression of cytokines such as LIF and the activation of related pathways such as the TNF-signaling pathway[140]. However, some co-culture models have also shown that the cytotoxicity observed in a monoculture of A549 cells was much lower than in a co-culture of A549 and MRC-5 cells (which are fibroblasts) when both cultures were exposed to similar concentrations of $PM_{2.5}$[141]. Thus, monocultures could also sometimes underestimate PM toxicity. Overall, a 2D co-culture provides a more physiologically relevant system to evaluate PM toxicity. However, similar to monocultures, cells in the 2D co-culture are submerged in the culture medium and are often grown in monolayered flat structures. Moreover, these culture techniques are not airway-specific, meaning, the same culture technique is applied to all types of cells, irrespective of the organ to which they belong. For example, both liver cells and lung cells are grown using similar *in-vitro* techniques, although they are physiologically exposed to the external environment very differently. Moreover, in the lungs, the cells are multilayered in 3-D structures with a portion of the cells in contact with the inhaled air. Therefore, to be more physiologically relevant, more complex systems are needed which can capture these characteristics of lung physiology. ALI and lung organoids generally serve as two of such complex 3D co-culture systems. Both of these systems, i.e. ALI[142–146] and lung organoid systems[147–152] have been also discussed extensively in recent publications. Here, we will only briefly discuss some important features of these systems relevant to PM toxicity evaluation.

ALI is a special type of cell culture in which the base of the cells is in contact with the culture medium and the top of the cells is exposed to air. Most importantly, ALI systems try to mimic stratification in the epithelium (i.e., the multilayer nature of epithelial tissue)[153] which is absent in normal *in-vitro* cell cultures. ALI systems facilitate the investigation of the phenomena caused by PM that would be impossible to evaluate using monoculture or 2D co-culture models. For example,

ALI makes it possible to investigate the effect of varying chemical composition of PM on the permeability of the epithelial barrier and the locations of epithelial damage (whether basal or apical)[154] which would not be possible with a 2D co-culture model. Similarly, integrating pulmonary epithelial cell lines (such as A549) with cardiovascular cell lines (such as EA.hy926) in an ALI system could help us investigate the proportion of fine and ultrafine PM penetrating the epithelial barrier and causing direct damage to cardiovascular cells[155]. These investigations are possible because ALI systems promote the differentiation of cells (to a more specialized cell type)[143], given the differences in cell culture technique and culture medium composition. For example, epithelial cells in the bronchial region display a number of characteristics such as mucus production, display of cilia, formation of tight junctions [structural formations between epithelial cells that permit (and prevent) the transport of ions, particles, and water], and polarization[146]. Although these characteristics are not displayed by most epithelial cell lines in commonly used monocultures[146], they are encouraged in ALI cultures as the cells are exposed to greater concentrations of oxygen which is crucial for these processes[156].

Among the ALI systems used to evaluate the toxicity of PM so far in the literature, the most common choices for epithelial cell lines are A549 and BEAS-2B[142,157,158]. Moreover, among the various epithelial-macrophage combinations used in ALI cultures, the combination of A549 and THP-1 is one of the most frequently used combination[157]. These ALI studies have revealed some distinctive aspects regarding the cellular toxicity of PM. For example, there were varied results when A549 and THP-1 cells were co-cultured in submerged cultures (PM suspensions) vs. when they were cultured in ALI[159]. The ROS response and release of inflammatory markers were much higher in submerged cultures vs. ALI. In another study investigating the toxicity of Saharan dust and crystalline quartz on A549 and THP-1 ALI cultures, it was found that ALI cultures could lead

to the production of pulmonary surfactants that could influence the solubilization of certain compounds, thus affecting the overall particle toxicity[160]. Some studies have also used ALI cultures featuring a combination of epithelial cells (A549 or BEAS-2B) and endothelial cells (e.g., EA.hy926). These studies have also revealed important differences in the results obtained from mono-cultures vs. ALI co-cultures. For example, when BEAS-2B cells were co-cultured with EA.hy926, BEAS-2B cells showed both higher cell proliferation and higher permeability as compared to monocultures[161]. However, an opposite trend was observed for A549 co-cultured with EA.hy926, indicating the importance of considering cell-line-dependent differences in ALI studies. Overall, it can be concluded that ALI systems are crucial in capturing several important cellular responses, although the choice of cell lines might also play a major role in the expression of those responses. Moreover, ALI protocols are still quite complex compared to monocultures and 2D co-cultures and this is one of the reasons for a majority of studies showing lesser preference for ALI. Thus, there is a need to investigate ways to simplify the ALI culture protocols to make them more accessible.

Similar to ALI, lung organoids are other major co-culture systems which could provide crucial insights into physiologically relevant toxicity mechanisms of PM. Lung organoids are miniature 3D models of lungs and are among the most accurate *in-vitro* cellular models possible so far[151]. Lung organoids are more complex systems compared to ALI and can be generated through several routes, most commonly involving different forms of stem cells[151]. Limited studies have evaluated the toxicity of PM using lung organoids. A few of these studies have focused on the role of PM exposure on viral infections and demonstrated that high PM exposure could possibly exacerbate the infectivity of these viruses[162,163]. Other studies have focused on specific health effects of PM. For example, a study investigating the effect of $PM_{2.5}$ exposure on fetal lung development used

lung organoids to mimic the early stages of fetal lung development and found that $PM_{2.5}$ exposure severely hampered the expression of several important transcriptional factors that are crucial for lung development[164]. A few more studies have used lung organoids for investigating the toxicity mechanisms of specific types of particles such as diesel exhaust[165] and tire wear[166] particles. Both of these studies revealed some important aspects related to the effect of PM on the expression of certain genes that are critical in the detoxification and elimination of ROS. However, further research is required to fully understand how PM exposure affects the interactions among different cell types in lung organoids. To summarize, lung organoids are still in their early stages of development and their complex culturing protocol has led to their slow adoption in the *in vitro* studies.

Overall, it can be concluded from the discussion in this section that although there are several cell lines representing macrophages and epithelial cells, they widely differ from one another and display different responses to the same chemical components. Moreover, despite immunological and biochemical similarities between murine and human cell models, there are important differences which might yield misleading interpretations about PM toxicity when relying only on murine cell lines. Finally, the use of co-culture studies could provide significant advantages in understanding the toxicity of PM. However, interpretations of such models should be made with caution considering the influence of the specific cell line used in the model.

It must also be noted that besides factors such as cell types, species and organs of origin, and culturing techniques, cellular responses observed in PM studies are also highly influenced by particle properties such as size, shape, and chemical composition. In the next section, we will discuss how different cell lines differ in their responses to differences in the physicochemical properties of PM.

3. Physicochemical properties of PM

The physical properties of particles such as size and shape affect their transport and fate (i.e. site of action) in the human respiratory tract. Along with physical characteristics, the chemical composition of the particles is also important in determining the biochemical reactions and subsequent toxicity of the particles. In this section, the role of physicochemical properties of PM in inducing various cellular responses in macrophage and epithelial cells of human and murine origin is discussed.

3.1 Effects of physical properties

The most important physical property of particles (with respect to pulmonary diseases) is size, which primarily influences their site of deposition and clearance mechanisms and is thus responsible for their different pathophysiological pathways[167–170]. Size also determines their ability to cross the lung epithelium barrier of the respiratory tract and their transportation to various organs of the body via blood. Interestingly, size has also been found to influence the interaction of non-soluble particles with phagocytes. Renwick et al.[171] found in their study on the J774 cell line that the phagocytotic activity of the cells was hindered to a greater extent when they were exposed to ultrafine TiO_2 and Carbon Black particles (mean size = 29 nm) in comparison to the exposure to fine particles with same chemical composition (mean size = 250 nm). Particles which are smaller than the typical size of phagocytes such as macrophages and neutrophils (5-10 μm) have been shown to induce greater toxicity than bigger particles[172]. Generally, the clearance efficiency of pulmonary macrophages for various particles has also been shown to depend on their size, with the efficiency being much lower for ultrafine particles as compared to fine particles[173,174]. Experiments on rat macrophages (J774 and RAW264.7) have revealed that macrophages could show a preferred recognition for a particular size range (0.5-3 μm)[175–177]. Such bias in particle

attachment and phagocytosis is also perhaps responsible for the differences in the inflammatory responses (e.g., cytokine (TNF-α) and mRNA expression) in murine macrophages (J774 and NR8383) when exposed to different size fractions of particles having similar chemical composition[173,178]. Similarly, exposing NR8383 cells to ultrafine TiO_2 particles (mean size = 25 nm) induced extracellular ROS, heme-oxygenase mRNA, and TNF-α expression but a similar response was not obtained when the cells were exposed to fine TiO_2 particles (mean size = 250 nm)[178]. These studies indicate a clear relationship between particle size and inflammatory responses, with fine and ultrafine particles inducing different cytokine and gene expression behaviors in the macrophages.

Along with particle size, particle shape could also play an important role in phagocytosis. It has been shown that murine macrophages (J774 and RAW264.7) show a preferential recognition of rod or oblate ellipsoid-shaped particles[176,179]. Some studies even indicate that a particle's shape could be more important than its size in determining the response of macrophages[180,181].

It is important to note here that the studies cited above involved engineered/artificial particles of a single chemical composition whereas ambient PM are often mixtures of different chemical species with varying solubilities in water. Major chemical species in ambient PM such as transition metals, organic carbon, and inorganic salts (e.g., ammonium, sulfate, nitrate, chloride, etc.) have been shown to be highly soluble in water[182–185]. Therefore, once PM is inhaled, the particle morphology (shape, size, and surface area) may no longer be relevant due to the dissolution of a major portion of the PM in the respiratory tract lining fluid. Moreover, it has been shown that the chemical composition of ambient particles varies with size (different sources emit different-sized particles)[186–189]. Therefore, in case of ambient PM, it is complicated to attribute the differences in cellular responses to merely particle size. This is further evident from the studies conducted on

ambient PM for assessing the role of different-sized particles on cytotoxicity, inflammatory responses, and oxidative stress. Many of these studies have found that the relationship between inflammatory response and cytotoxicity with particle size depends on season, location, and time of the day[190–193], with generally both coarse and fine particles dominating cellular responses during different seasons. This spatiotemporal effect could be attributed to the presence of specific chemical species that drive a particular cellular response and therefore the physiological relevance of these results should probably be interpreted in the context of chemical composition rather than size alone. It implies that the overall toxicity of the ambient PM is influenced more by chemical composition, which could partly be determined by the size but is not an explicit function of it.

3.2 Effects of chemical composition

PM consists of a broad range of chemical components including metals, organic, and inorganic species. Usually, coarse particles are dominated by crustal elements compared to fine particles, which consist of a greater fraction of combustion-derived organic and inorganic species. But the chemical composition even within a single size fraction of PM could show substantial spatiotemporal variations. The constituents of PM could also be divided based on their water-solubilities, based on which they could induce very different biological responses in the cells. In this section, we will first briefly discuss the *in-vitro* studies involving macrophages and epithelial cells of human and murine origin which show differential toxicities of water-soluble vs. non-soluble PM chemical species followed by the studies that discuss the toxicities of metals and organic species.

3.2.1 Water-soluble versus insoluble chemical species

Both water-soluble and insoluble fractions of PM have been observed to be capable of inducing genotoxicity[194]. Some studies have found the water-soluble fraction of PM to be largely associated

with oxidative stress[195,196] and inflammation[197] in both epithelial and macrophage cells, whereas the insoluble fraction has been found to induce cell membrane damage[195], cell cycle dysregulation[197] and in some cases oxidative DNA damage and apoptosis[198]. Certain studies have shown that aqueous extracts of $PM_{2.5}$ (containing metals and inorganic ions) were found to be more cytotoxic to epithelial cells (A549) as compared to the dicholoromethane (DCM) extracts (organic extracts) which had the majority of PAHs[110,199]. Moreover, these aqueous extracts also induced more pro-inflammatory cytokines as compared to DCM extracts[110]. Note, the water-soluble and DCM-soluble extracts used in these studies were extracted separately, thus the DCM extracts may also contain an appreciable portion of water-soluble compounds, making it difficult to compute the actual contribution of water-insoluble compounds to PM toxicity. However, a study by Ma et al.[200] compared the toxicities of water-soluble and insoluble fractions separately by sequentially extracting the PM, i.e. first extracting the water-soluble fraction in water, followed by DCM to extract the remaining water-insoluble fraction. Interestingly, the water-soluble fraction turned out to be more efficient in inducing apoptosis in BEAS-2B cells than the DCM extracts, thus indicating that water-soluble components were more toxic to epithelial cells.

However, we must be careful in making such generalizations because several other studies have suggested that water-insoluble fractions may sometimes contribute to PM toxicity more than water-soluble fractions. For example, it was found in a study comparing different urban, rural, and industrial sites in France that the DCM extracts were more closely associated with the cell membrane and DNA damage of BEAS-2B cells than water-soluble extracts[201]. In another study, the water-insoluble fraction of urban dust aerosols was observed to contribute to most of the LDH release in A549 cells[202]. Mohseni Bandpi et al.[203] had also reported higher DNA damage by organic [DCM/methanol (v/v) = 3] extracts of $PM_{2.5}$ as compared to water-soluble components in A549

cells[203]. Similarly, in a study conducted in rural China, it was found that the organic [DCM/acetone (v/v) = 1] fraction induced significantly higher cell death and cellular ROS response as compared to water-soluble fraction in A549 cells[204]. Finally, the organic (heptane) fraction of diesel emission particles (DEP) has also been found to induce a significantly higher release of pro-inflammatory cytokines in BEAS-2B cells[205]. The assessment of contributions of water-soluble and insoluble species can be indirectly used to yield some insights into the relative roles of metals and organic species, respectively. This is primarily based on a rough assumption that organic compounds are concentrated in the organic extracts (e.g., DCM which is water-insoluble), while aqueous extracts largely contain metals and other inorganic ions, although chemical composition analysis of organic and aqueous extracts was not conducted in those studies. However, this assumption may not always be true because, as indicated earlier, the organic extracts may also contain significant amounts of water-soluble compounds[206]. Therefore, to gain better insights into the relative contributions of water-soluble and insoluble components, it is better to adopt sequential extraction (i.e., water extraction followed by extraction of the same PM filter in an organic solvent) of PM instead of extracting the two fractions separately. However, currently, there is a lack of PM toxicity studies adopting such procedures. It is perhaps also beneficial to chemically analyze individual species (such as metals, water-soluble organic carbon, PAHs, etc.) in these two fractions to confirm their contribution to the overall PM toxicity. Therefore, in the next sub-section, we will discuss the *in-vitro* studies involving macrophages and epithelial cells comparing specific roles of metals and organic species.

3.2.2 Role of metals

Table 3 summarizes the most important elemental species including metals showing strong associations with cellular responses in various ambient PM studies. From this table, Fe, Cu, Mn,

Cr, Ni, and Zn seem to be the most important metallic species which play a major role in PM toxicity as consistently suggested by the current literature. A conventional method for assessing the role of various chemical components in biological responses is to correlate the spatiotemporal variation in cellular responses with that in PM chemical composition. Studies investigating these correlations have revealed metals to be largely associated with cell membrane damage, DNA damage, lower cell viability, and oxidative stress. For example, in some studies, the high potency of coarse particles to induce cellular responses in human epithelial cells (BEAS-2B and A549) and murine macrophages (RAW264.7), such as the release of specific pro-inflammatory cytokines[111,207,208] was primarily associated with the presence of crustal metals such Al and Fe, which have been shown to induce pulmonary inflammations. Similarly, coarse particles from industrial and traffic sources were also found to contain large fractions of Fe and Al as compared to the finer-sized (< 2.5) particles and exhibited higher LDH release and reduction in ATP in both murine J774 cells and human A549 cells[209]. Lag et al.[210] found As, Zn, and Cd to be associated with more inflammatory response and oxidative stress whereas Fe, Mn, and Al have been positively correlated with pro-inflammatory cytokines such as IL-6 and IL-8 in BEAS-2B cells[211]. Similarly, more adverse biological responses such as lower cell viability, higher LDH release, and higher IL-6 induced in A549 cells exposed to particle suspensions in winter vs. summer samples, were attributed to certain specific metals such as Cu, Mn, As, Zn, and Al, which were at least two times higher in the winter samples[212,213]. Species such as As and Ni have been found to be associated with altering the cell cycle in human bronchial epithelial (HBE) cells[214]. Such significant correlations between metals and various cellular responses have also been found in RAW264.7 cells and A549 cells, with one group of metals (Al, Fe, Mg, Co, V, Mn, and Ca) more closely associated with inflammatory and cytotoxic responses, while another group (Zn, Cd, Cu,

and Pb) associated with the induction of oxidative stress[122,215]. Among the metals, Fe and Cr seem to have the most consistent association with PM toxicity. Fe has been associated with both ROS response and DNA damage in BEAS-2B cells[216] and its water-soluble form is believed to play an important role in PM-mediated hydroxylation of DNA through Fenton reaction in A549 cells[194]. Moreover, Fe has been associated with the induction of higher cytotoxicity and inflammatory response when A549 cells were exposed to $PM_{2.5}$ collected during dust storms[217], $PM_{2.5}$ emitted from coal combustion[218], and $PM_{2.5}$ from a photochemical smog event[219]. Similarly, Cr is also shown to be toxic to a variety of cell lines such as A549, BEAS-2B, and NR8383 (see Table 3). Other than Fe and Cr, Mn, Cu, and Zn are the metals most commonly associated with cellular toxicity in PM studies.

There are few studies showing the correlation of some other metals as well with toxicity. For example, in a study on the impact of snowfall events on the toxicity of aerosols, metals, and semi-metals like Sr, Ni, V, and As were found to be strongly associated with inflammatory markers such as TNF-α and IL-6[220]. Se was found to be more strongly correlated with oxidative stress than Fe and Cu when A549 cells were exposed to solid fuel combustion-derived $PM_{2.5}$[221]. Ni and Pb were more significantly associated with inflammatory response than Fe, Mn, and Cu in A549 cells[217]. However, more studies are needed to investigate the specific toxicity mechanism of different metals to establish their relative importance. Moreover, a lack of correlation between an element and a cellular response may not always mean that it is non-toxic as these studies have not mechanistically investigated the reasons behind correlations (or lack of correlations). Note, oxidation state is a major parameter influencing the reactivity of metals and it has been shown in some studies that it could impact the overall toxicity of PM. For example, Fe (II) has been shown to induce greater ROS response in NR8383 cells as compared to Fe (III)[222]. Similarly, in a study

using 16HBE cells, it was shown that Cr (VI) induced a larger reduction in cell viability as compared to Cr (III)[223]. Further investigations into the significance of the oxidation state of metals might better clarify their toxicity mechanisms.

3.2.3 Role of organic compounds

Several studies have also shown the importance of organic compounds in inducing cellular ROS and cytotoxicity in both macrophages and epithelial cells[207,224–226]. Organic compounds supposedly play an important role in γH2AX generation (a phosphorylated protein indicating DNA damage), LDH release, and ROS generation in human macrophages and epithelial cells[224,227]. In a study comparing the toxicity of metals and organic compounds in human small airway epithelial cells (SAEC), organic compounds induced greater expression of genes related to certain antioxidants (SOD-1 and -2, catalase, HO-1, NQO-1)[228] as compared to metals. Water Soluble Organic Carbon (WSOC) has been widely studied for its role in inducing oxidative stress as its distribution across the size-spectrum of PM seems to drive the size-segregated biological response of the particles. For example, in the study conducted by Wang et al.[229], a two-times higher concentration of WSOC in the finest fraction of PM (<0.4 μm) than the coarse fraction was speculated to be the chief driver of the highest inflammatory effects in NR8383 cells of that size fraction. Besides being directly responsible for the induction of oxidative stress in NR8383 cells, WSOC has also been demonstrated to play an indirect role through the complexation of Fe[230]. WSOC has also been associated with other cellular responses such as apoptosis in BEAS-2B cells[200] and DNA methylation in A549 cells[110]. However, it is not yet clear how toxic WSOC is relative to the metals. WSOC has been found to be more strongly associated with the induction of ROS in NR8383 cells[231] and NF-κB activation in THP-1 cells[232] than metals such as Cu, Mn, and

Ni, although the association of inflammation in A549 cells with WSOC was weaker in comparison to most metals[220].

Among the organic compounds, polycyclic aromatic hydrocarbons (PAHs) have received great attention and have often been cited as the primary driver of oxidative stress and cytotoxicity[224,233–235] in macrophages and epithelial cells of both human and murine origin. In fact, PAHs have been shown to be more strongly associated with cytotoxicity than metals in A549 cells[236]. Higher organic carbon and PAH content have been speculated to be responsible for depleted glutathione in cells as well as the expression of heme oxygenase-1[237] in RAW264.7 and BEAS-2B cells. PAHs have also been shown to be highly associated with altering cell cycles in HBE cells[214] and THP-1 cells[197]. Experiments on human lung epithelial cells exposed to PM have revealed that PAHs could elicit the expression of mRNA genes such as cytochrome P450 (cyp) 1a1, cyp2e1, and cyp2f1, which are necessary for the metabolic transformation of these organic compounds to induce cytotoxicity[238,239]. Studies comparing emissions from traditional fossil fuels and biodiesel blends have found that the reduction in cytotoxicity and genotoxicity in U937 and A549 cells by PM emitted by vehicles using biodiesel could primarily be due to a lower PAH content[233,240]. However, the exact role of PAHs in inducing inflammatory responses is not entirely clear. In a study conducted on the $PM_{2.5}$ collected in both industrial and urban areas, Chen et al.[241] found a largely negative correlation for the expression of IL-6 and TNF-α in A549 cells with the majority of PAHs in the industrial area. However, in urban areas, these biological responses were positively correlated with several PAHs, despite a negative correlation between PAHs and LDH release. Another study investigating the correlation between PAHs and inflammatory cytokines such as IL-6, IL-8, and IL-1β in a co-culture of A549 and THP-1 cells found no significant relationship between PAHs and cytokine release[242]. In fact, PAHs have also been found to be negatively

correlated with inflammatory responses in BEAS-2B cells[211] in one study. However, at least two different studies using the same cell line have found that PAHs were positively correlated with the release of inflammatory cytokines such as IL-6[243] and the expression of genes related to inflammation and myocardial fibrosis[244].

There are other organic compounds that probably play a more significant role than PAHs in inducing biological responses. In a study investigating the genotoxicity of organic extracts of $PM_{2.5}$, Oh et al.[245] showed that compared to PAHs and their alkyl derivatives, aliphatic and chlorinated hydrocarbon fractions caused partially greater DNA breakage in BEAS-2B cells. Moreover, compounds such as nitro-PAHs, ketones, and quinones were almost similar to PAHs in genotoxicity[245]. Oxygenated PAHs and nitro-PAHs have been found to be more toxic than PAHs in a few studies[246–248] and this toxicity seems to have been linked to their ability to exert direct toxic effects, in contrast to parent PAHs, which first require bio-activation by certain enzymes[249]. Between nitro-PAHs and oxygenated PAHs, it is possible that nitro-PAHs are perhaps more toxic[221], although more studies systematically comparing the toxicity of the two groups of compounds in lung cell lines are needed. It has been previously shown that quinones could be more efficient than PAHs in $O_2^{-\bullet}$ generation[250]. This redox cycling induced by quinones could ultimately play an important role in the cytotoxicity of PM. Few studies have suggested that the transformation of PAHs into quinones through atmospheric processing often induces more cytotoxicity and oxidative potential. Further discussion in this regard is included in section 4. Other compounds such as n-alkanes, hopanes, and steranes have also been shown to have a good correlation with several biological responses such as oxidative stress and inflammatory damage in A549 cells[241,251]. N-alkanes have also displayed a strong correlation with neutrophilic inflammation in BEAS-2B cells[252].

Note, none of the studies cited above have considered the interactions between various chemical components of PM in cytotoxicity. There is very limited work conducted in this area. Studies exploring the relationship between the chemical composition of aerosols and cellular responses have found that the response of the total PM is not equal to the sum of responses of the individual fractions[224,253]. Moreover, interactions between different metals may also influence PM toxicity. For example, Al^{3+} has been shown to have an antagonistic effect on Cr^{-}, Pb^{-}, and Zn^{-} induced oxidative stress in 16HBE cells[254]. Therefore, further research into these interactions is needed to advance our understanding of the net effect of PM chemical composition on oxidative stress, toxicity, and health effects.

Apart from the interaction among different chemical species, varying exposure doses and durations used in different studies could also contribute to the disparities in the observed toxicities of different chemical species. Table 4 summarizes the different exposure concentrations and durations used by different studies evaluating the toxicity of ambient PM. The extract concentrations used in these studies ranged from 1 to 1100 μg/mL, while the exposure duration ranged from 2.5 to 72 h for oxidative stress evaluation and 6 to 72 h for cytotoxicity and other cellular responses. This could also influence the interpretation of the observations made in those studies in two ways. First, the toxicity thresholds for various chemical species vary among different cell lines[255,256], so it is possible that the lack of toxicity observed for a specific chemical species at a particular exposure concentration could be simply due to a higher toxicity threshold for that chemical to that cell line. Second, the toxicity kinetics for different chemical species also vary for different cell lines[257,258]. Moreover, different cell lines may also differ widely in their growth or proliferation rate profiles[259]. Thus it is difficult to compare the inhibitory effects of PM on different cell lines if their exposure durations are different. In conclusion, we should be cautious

in comparing the results of the studies using varying exposure durations and doses. Future studies might consider testing the toxicity of PM using a range of concentrations (e.g., 1-1000 μg/mL) and exposure durations (e.g., 6-72 h) rather than a single concentration and exposure duration to remove such biases.

Finally, it is also necessary to remember that the chemical composition of the aerosols itself is highly dynamic and evolves with atmospheric processing, which eventually affects its toxicological properties. Therefore, to properly assess the health impacts of aerosols, it is necessary to also understand the effects of atmospheric processing on aerosol toxicity. In the next section, we discuss the current state of knowledge on how the toxicity of aerosols could vary due to various environmental factors.

4 Role of atmospheric processes in altering the cellular activity of PM

Physicochemical properties of PM not only depend on emission sources but also on environmental conditions such as relative humidity, temperature, and mixing height, along with prevalent aerosol and gaseous mixtures, which result in the physicochemical transformation of aerosols. Processes like gas-particle partitioning towards the particle phase (which is often favored at lower temperatures) and the presence of background organic aerosols [which could provide the surfaces for condensational uptake of the volatile organic compounds (VOCs)], also play a crucial role in atmospheric processing of aerosols. Higher background concentrations of organic aerosol often lead to greater partitioning towards the particle phase[260]. Recent studies have also shown a significant influence of aerosol liquid water (ALW) content on the chemical composition of the aerosols, particularly SOA[261]. ALW could also cause a change in aerosol acidity, enhancing the dissolution of certain metals and thus affecting their oxidative potential[262]. Note, ALW is

dependent on both the relative humidity (RH) and hygroscopicity of the aerosol which in turn is influenced by the presence of other species such as isoprene and sulphate in urban environments[261]. Similarly, the adsorption and condensation of VOCs could further alter the chemical composition of the aerosols[263,264]. Such modifications in the chemical composition due to atmospheric processing and aging could significantly alter the toxicity of aerosols. To the best of our knowledge, only one article (Weitekamp et al.[265]) has reviewed the existing studies investigating the effects of aging and atmospheric processing on the toxicity of aerosols. However, Weitekamp et al.[265] has majorly focused on *in-vivo* studies while briefly discussing the effect of aging only on human lung cells (such as A549), and with a very limited discussion on the toxicity of secondary organic aerosols (SOA) on human and murine lung macrophages and epithelial cells. Therefore, in this section, we will discuss the results of *in-vitro* studies using human and murine cells of respiratory origin, that have assessed the impact of atmospheric processing on the toxicity of ambient aerosols. We will also discuss existing literature on the toxicity of SOA.

4.1 Aging of carbonaceous aerosols

The chemical composition of carbon-containing aerosols dramatically changes with aging. There is an increase in the molecular weight of organic compounds during the initial stages with a corresponding decrease in the volatility[266]. Moreover, organic compounds such as catechol, maleic acid, and oxalic acid could also react with transition metals such as iron to form metal-organic complexes[267]. Such a change in chemical composition could further lead to alteration in toxicity and oxidative potential of aerosols. For example, an increased cytotoxicity and inflammatory response in RAW 264.7 cells when they were exposed to aged biomass burning aerosols was attributed to the change in PAH composition[268] which were most likely oxidized to oxy or nitro-PAHs when the aerosols were aged in the presence of ozone.

The influence of oxidation of carbonaceous particles on cellular responses has been a subject of great interest in recent studies. An appreciable increase in ketonic, carboxylic, and quinone-like functional groups has been reported during the oxidation of carbonaceous particles[269]. The presence of such oxygenated functional groups in aged aerosols as compared to fresh particles has been found to increase their oxidative potential and lower cell viability[270]. Investigations on aged gasoline exhaust PM at atmospherically relevant inhaled concentrations showed that there were significant consequences such as increased cell death and impaired pro-inflammatory cytokine release (such as IL-6, IL-8, and monocyte chemotactic protein (MCP)-1 which are essential in immunological responses) in BEAS cells and Human Bronchial epithelium[271]. A similar increase in cytotoxicity was also observed when human epithelial A549 cells were exposed to photochemically oxidized products of 1,3-butadiene (BD)[272,273] and isoprene[273] compared to the cases when the cells were exposed to BD and isoprene. Such an increase in toxicity due to aging was also found for other carbonaceous aerosols such as black carbon. For example, experiments conducted on A549 cells using oxidized black carbon showed a significant dose-dependent increase in the expression of the heme oxygenase-1 (HO-1) protein, mitochondrial damage, activation of apoptosis, and accumulation of autophagy[274]. Similarly, when oxidized black carbon was exposed to A549 cells co-cultured with THP-1 cells, there was a significant increase in genotoxicity and immunosuppression[275].

However, the elevated cellular responses to oxidized particles have not been ubiquitous for all endpoints. For example, oxidation of soot has been reported to cause at least a 37% decrease in cell viability as compared to fresh soot, but only a negligible difference in IL-8 response in 16HBE cells[276]. However, another study using the same cell line found that oxidation of black carbon in the presence of ozone did not cause any significant change in cell viability[277]. Oxidized carbon

particles have also been shown to induce greater LDH activity and lower cell proliferation in J774 cells, but they did not induce a significant change in their metabolic activity[278]. In A549 cells, fresh and oxidized black carbon particles did not differ much in their cytotoxicity, although DNA damage was higher in the case of fresh particles as compared to oxidized particles[274] and the genes related to oxidative stress, inflammation, and autophagy were largely different in both cases[279,280]. Another study conducted using 16HBE cells showed that although cell viability and COMET length were significantly higher in the case of ozone-oxidized carbon black particles, the ROS response did not vary significantly as compared to the case of fresh carbon black particles[281]. Such distinct results could probably be explained by different experimental conditions used in these studies. For example, the concentrations of particles used in these studies ranged from as low as 10 μg/mL to as high as 2 mg/mL. Moreover, the exposure duration of the cells to particles varied from 2-48 hours, although most studies maintained an exposure time of 24 hours. Most importantly, the studies have employed a variety of cell lines which include macrophages and epithelial cells of both human and murine origin, which further complicates the interpretation of responses due to the reasons discussed earlier in section 2.1.

4.2 Secondary Organic Aerosols (SOA)

SOA are important contributors to ambient $PM_{2.5}$ mass (7-60%)[282–284] and elevated SOA concentrations have been linked to increased aerosol toxicity and premature mortality[285,286]. For example, an increase in the oxidative potential of the ambient PM during the afternoon hours and in foggy conditions has been attributed to the formation of SOA[287,288]. SOA could have both biogenic and anthropogenic origin and the presence of specific anthropogenic gaseous pollutants such as SO_2, NO_x, and O_3 could further alter their formation pathways[289–291]. Besides, SOA consists of a variety of chemical compounds such as alcohols, ketones, aldehydes, organic peroxides, and

esters, and the chemical composition is dictated by an abundance of precursors and particular environmental conditions[292,293]. Thus, the complex chemistry of its formation pathways and higher prevalence in ambient PM emphasizes the importance of evaluating the toxicity of SOA. However, the toxicity studies related to SOA are scarce and often limited to the most abundantly found precursors of SOA in ambient environments such as α-pinene, naphthalene, m-xylene, and isoprene. Some of these studies include comparisons between anthropogenic and biogenic SOA, all of which show that anthropogenic SOA causes more oxidative stress in A549, NR8383, and MH-S cells[294–296] and greater expression of inflammatory cytokines such as IL-6, IL-8, and TNF-α in MH-S, A549, and BEAS-2B cells[120,294,297] compared to the biogenic SOA. This difference in the toxicity of the two types of SOA is perhaps due to the presence of more oxidized and aromatic compounds in SOA of anthropogenic origin as indicated by Offer et al.[298] although more studies are needed to confirm this hypothesis.

Other studies focusing on specific precursors and their products reveal that a number of factors, such as RH, precursors of hydroxyl radicals, the presence of oxidative gases such as ozone, and aging could influence the toxicity of SOA. The effect of various factors on the toxicity of SOA observed in the studies so far is summarized in Figure 3. Note, one has to be cautious in generalizing these results because some of these factors have been investigated in a limited number of studies. For example, only one study has explicitly investigated the effect of RH and it demonstrated that the production of inflammatory cytokines such as TNF-α and IL-6 in MH-S cells when exposed to naphthalene and pentadecane SOA was directly proportional to the RH at which the SOA was formed[294]. Similarly, studies exploring the influence of aging on SOA are also limited and currently, they provide conflicting results. For example, it has been demonstrated in some studies that toxicity and cellular ROS production in A549 cells from SOA from

naphthalene[296,299], α-pinene[296,299], and anisole[300] precursors increased with aging in NOx-rich environments. On the other hand, in a separate study, naphthalene SOA displayed a reduction in cytotoxicity in BEAS-2B cells, when it was aged in an ozone-rich environment[301]. Similarly, the cytotoxicity and oxidative stress induced in A549 cells by phenol and guaiacol SOA have also been shown to decrease with aging (in NO_X-rich environments) and this reduction was attributed to the formation of ring-opening products which are relatively less toxic compared to their parent compounds[302]. Overall, these results show that the toxicity of SOA is dependent on the chemistry of the reaction products (which are in turn highly influenced by environmental factors) and perhaps, also on the cell type being used to evaluate the toxicity.

Since in most of the studies cited above oxidative stress has been speculated as the major pathway for the toxicity of SOA, detailed information regarding the specific ROS formed during the interaction of SOA with cells, and the correlation of these ROS with cytotoxicity and inflammatory responses could help us understand which organic compounds drive SOA toxicity. For example, Liu et al.[303] found in their investigations on naphthalene SOA that H_2O_2 was the main ROS driving oxidative stress in MH-S cells. This information could then be combined with the ability of different organic compounds to generate H_2O_2 measured either through cellular or acellular assays to understand their toxicity. There could also be other factors influencing the toxicity of SOA such as the oxidation state of carbon which has been shown to be directly proportional to the ROS response and caspase 3/7 activity of the murine macrophage (MH-S) cells[304]. There is also a need for more studies including other cell types such as murine epithelial (e.g., MLE-12 and RLE-6TN) and human macrophage (such as THP-1, U937) cells to confirm whether the observations made about SOA are not biased by species-specific cellular responses. For assessing the macrophage's response to SOA exposure, more emphasis should be placed on the induction of oxidative stress

and phagocytosis as these are crucial characteristics of macrophages. On the other hand, for epithelial cells, inflammatory response measurements combined with oxidative stress and different modes of cell death (apoptosis and necrosis) could provide a more thorough analysis of SOA toxicity. In addition to these, investigating the effect of SOA on tight junctions of epithelial cells using ALI cultures could provide an even more physiologically relevant evaluation of SOA toxicity as these properties of epithelial cells are crucial in the transport of PM components to other parts of the body[305]. In summary, a more comprehensive and systematic evaluation of particle toxicity using a variety of cell lines with different endpoints is required to properly assess the cellular responses of SOA formed from different precursors under varied atmospheric conditions.

5. Conclusions

In this review, we have discussed various macrophage and epithelial cell models used in PM studies and the current state of knowledge on several experimental and environmental factors, such as PM extraction procedure, exposure duration and concentration, and aerosol aging that affect PM toxicity. We first discussed the differences among various macrophages and epithelial cells of human and murine origin belonging to the respiratory system. There are marked genotypic and phenotypic differences between different cell lines and conclusions of toxicity should not be based on a single cell line, but rather verified with other relevant cell lines. While comparing responses of commonly used macrophage cell lines, the effect of polarization induced by the PM needs to be considered. Moreover, future studies should also investigate the association between the chemical composition of PM and the polarization state induced in different macrophage cell lines.

Comparison of different cell lines raises two important questions – 1. Do murine macrophages provide a suitable model to understand PM effects on human pulmonary system? 2. Within the

same species, are macrophages more sensitive to PM-induced effects than epithelial cell lines, and if such sensitivity is dependent on specific PM components? Since these questions are yet to be answered, it would be wise to design PM toxicity experiments based on specific role of the cells in the pulmonary system and the relevance of their biological response to the actual function of the cell in the human body. For example, the primary function of macrophages is phagocytosis and therefore, how efficiently macrophages respond to particle intrusion into the human pulmonary system depends on how efficiently they can phagocytose the particles as well as express related inflammatory cytokines. Thus, combining oxidative stress measurements in macrophages with other cellular responses such as phagocytosis and change in inflammatory responses could help us understand the consequences of PM-induced oxidative stress. We recommend the use of epithelial cells instead of macrophages for oxidative stress measurement and limit macrophages for investigating phagocytosis, inflammatory responses, and other cellular responses such as DNA damage and cell death when using monocultures.

We must also consider the use of co-cultures to study PM toxicity, as co-culture studies could help us understand the kinetics of toxicity and progression of cellular responses while considering the influence of neighboring cells in inhibiting or enhancing such responses. Thus, unlike monocultures (of macrophages and epithelial cells) which could be used to study the direct effects of PM, co-culture systems could be used to investigate the indirect effect of PM on one cell type (e.g., macrophages) in the presence of some other cell types (e.g., epithelial cells). For example, the interactions between macrophages and epithelial cells when exposed to PM suspensions have shown that macrophages inhibit the impact of PM exposure on epithelial cells. Thus, it is possible to overestimate PM toxicity when relying solely on monocultures. We recommend co-culture (e.g., macrophages + epithelial cells) studies especially when using PM suspensions to obtain a more

physiologically relevant understanding of PM toxicity. Similarly, 3D co-cultures such as ALI cultures could provide a comparatively better evaluation of PM toxicity as they allow epithelial cells to develop certain features, such as cilia and tight junctions and an ability to secrete mucus, which are absent in monocultures and 2D co-cultures.

PM exposure techniques also influence the results and robust conclusions cannot be drawn based on a single exposure technique. Comparison between different methods and a combination of different methods are required to further understand different modes (e.g., particulate vs. soluble) of PM toxicity. Currently, in most PM studies, cells are exposed to water or an organic solvent extract obtained from filters using sonication or other extraction (e.g., shaking) methodologies. Such extraction procedures do not necessarily capture the bioavailability of the PM chemical species and thus could produce a distorted picture of PM toxicity. It is also important to consider that the solubility of various PM components in pulmonary fluids could differ from their solubility in water. Moreover, although organic solvents are typically used to capture water-insoluble compounds to measure their OP, one could question the physiological relevance of using such solvents. If the solubility of PM compounds in organic solvent differs significantly than in the respiratory fluids, it will also cause an overestimation of the toxicity stemming from the compounds, which otherwise would never leach from PM into the respiratory fluid. Thus, there is a clear need for better extraction and cellular exposure procedures that can closely replicate the fate of PM in our respiratory system. Perhaps the use of a simulated lung fluid, which is not only a mixture of antioxidants present in the respiratory tract lining fluid (RTLF) but is also calibrated for parameters such as viscosity, conductivity, surface tension, and density to better mimic respiratory exposure, should be explored. Some recent studies have already been using these

simulated lung fluids to assess the solubility and ultimate fate of common anti-inflammatory drugs (such as corticosteroids) in the lungs[306–308].

Next, we discussed various chemical species responsible for PM toxicity. Studies investigating the role of chemical composition in toxicity have identified two major groups of toxic species, i.e., metals such as Fe, Cu, Mn, Cr, Ni, and Zn, and organic compounds such as PAHs, ketones, aliphatic and chlorinated hydrocarbons, and quinones. However, the relative toxicity of these species is still a matter of debate. This is partially because most studies so far have relied on a simple correlation between concentrations of PM components and cellular responses, which gives varied results due to several reasons. First, differential solubilities of PM components in the exposure medium could lead to varied cellular responses. Second, certain cells may lack the ability to activate receptors such as AhR which are essential for biotransformation of compounds like PAHs. In such cases, the lack of a cellular response does not mean PM is less toxic because it can exert equivalent toxicity in the presence of the relevant cells carrying AhR, rather indicates the need for further evaluation. Third, the toxicity of a given species could be enhanced (synergistic interaction) or inhibited (antagonistic interaction) by the presence of other species. Few studies have shown the strong interactions among species such as metals (Fe, Al, Cr, Pb, and Zn) and organic compounds. There could be more interactions, however, currently, there is a lack of studies which have explored this in detail. Therefore, various PM chemical species should not only be evaluated individually for toxicity but also in mixtures. Synthetic mixtures may be prepared with pure solutions of chemical species at ambient concentrations and analyzed for toxicity to parametrize the intrinsic toxicities of various PM components and their interactions to yield net toxicity. Moreover, it is also important that the exposure duration and PM concentrations used in

cellular toxicity studies be standardized as per the reaction kinetics and toxicity thresholds of different chemical species for various cell lines.

Finally, we discussed the role of atmospheric processing, primarily aging in modifying the toxicity of aerosols. The effect of aging on PM toxicity is unclear as the *in-vitro* studies using macrophages and epithelial cells show contradictory results, which could also be due to different SOA products formed as a result of relative humidity and the presence of oxidants (O_3, NOx, and NH_3) in the SOA chamber in these studies. Moreover, SOA studies so far have focused on limited types of macrophages (only murine cell lines: MH-S and NR8383) and epithelial (only human cell lines: A549 and BEAS-2B) cell lines, thus making it difficult to ascertain if the diversity in results is due to cell-specific responses or aging-induced changes in the PM chemical composition. More systematic studies need to be performed using a variety of precursors while applying different aging techniques and using the same cell lines to investigate the effects of aging on the resultant toxicity of PM.

In conclusion, a more nuanced approach that adopts physiologically relevant cell models and PM exposure techniques, and interactive effects of different chemical components as PM ages, is necessary for a more advanced understanding of the cellular toxicological effects of PM.

Conflicts of interest

There are no conflicts to declare.

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Table 1: Various cellular responses and techniques to measure them

Cellular response	Endpoint	Technique	Assay or Method
Oxidative stress	ROS measurements	Fluorescence-based	DCFH-DA and its many forms, Dihydroethidium (DHE), Amplex Red,
		Electron paramagnetic resonance/Electron paramagnetic spin resonance	Cyclic hydroxylamine spin probes, pyrroline-based cyclic nitrones
		Chemiluminescence-based	Luminol and lucigenin assays
	Antioxidant measurements	GSH depletion measurement	Monochlorobimane assay, o pthaldialdehyde assay, High performance chromatography (HPLC) assay
		Antioxidant enzyme activity	Superoxide dismutase (SOD) assay, catalase activity assays, peroxidase activity assays, glutathione reductase activity assays
Inflammation	Chemokine and cytokine production	Antibody specificity measurement	Enzyme-linked immunosorbent assay (ELISA)
		mRNA transcript expression measurement	Quantitative polymerase chain reaction (Q-PCR)
		Flow cytometry	Cytometric beads
	Nitric Oxide (NO) production	Colorimetric-based	Griess assay
		Chemiluminescence based	Ozone assay, luminol assay, luciferin-luciferase assay,
		Fluorescence-based	2,3-diaminonaphthalene (DAN) assay, diaminofluoresceins (DAF) assay, DAF-2-DA assay, Copper-based probes
		Other techniques	Electrochemical method, Gas chromatography, Electron paramagnetic resonance/Electron paramagnetic spin resonance
Cell death	Cell Viability	Metabolic activity	MTT, XTT, MTS, WST,

			ATP, resazurin reduction assays
		Membrane integrity	LDH assay
		Dye uptake and pH gradient maintenance	NRU assay
		Cell adherence	Crystal violet assay
		Dye exclusion	Trypan blue assay
		Protease activity	Glycylphenylalanyl aminofluorocoumarin; GF AFC assay, Western blot
	Apoptosis	Flow cytometry	Fluorescein Isothiocyanate (FITC) Annexin V, Hoechst dye, monitoring the cell size,
		Caspase detection	Caspase 3/8 Assay, caspase activity assay,
		Mitochondrial detection	Cytochrome C Assay, Mitochondrial Membrane Potential assay, JC-1 dye based assay
	Necrosis	Membrane integrity	LDH assay, propidium iodide assay
Mutagenicity and genotoxicity	DNA damage	Microgel electrophoresis	COMET assay
		DNA double-stranded break detection	γH2AX assay
		Micronucleus formation	Micronucleus assay
		structural chromosomal abnormalities	Chromosomal aberration tests

Table 2: Different macrophage and epithelial cell lines of both human and murine origin

Cell line	Species	Cell type	Cancerous origin	Representative of
16HBE14o−	Human	Epithelial	No	Bronchial epithelial cells
A-427	Human	Epithelial	Yes	General lung epithelial cells
A549	Human	Epithelial	Yes	Alveolar Type-II epithelial cells
BEAS-2B	Human	Epithelial	No	Bronchial epithelial cells
Calu-1	Human	Epithelial	Yes	Bronchial epithelial cells
Calu-3	Human	Epithelial	Yes	Bronchial epithelial cells
Calu-6	Human	Epithelial	Yes	Bronchial epithelial cells
hAELVi	Human	Epithelial	No	Alveolar epithelial cells
HCC-827	Human	Epithelial	Yes	General lung epithelial cells
HLC-1	Human	Epithelial	Yes	General lung epithelial cells
LC-2/ad	Human	Epithelial	Yes	General lung epithelial cells
NCI-H1299	Human	Epithelial	Yes	General lung epithelial cells
NCI-H1975	Human	Epithelial	Yes	General lung epithelial cells
NCI-H292	Human	Epithelial	Yes	General lung epithelial cells
NCI-H358	Human	Epithelial	Yes	Epithelial cells of the bronchioles
NuLi-1	Human	Epithelial	No	Bronchial epithelial cells
PC-14	Human	Epithelial	Yes	General lung epithelial cells
RPMI 2650	Human	Epithelial	Yes	Nasal epithelial cells
THP-1	Human	Macrophage	Yes	Blood monocyte-derived macrophages
U937	Human	Macrophage	Yes	Blood monocyte-derived macrophages
J774	Murine	Macrophage	Yes	Tissue-dwelling macrophages
MH-S	Murine	Macrophage	No	Alveolar macrophages
MLE-12	Murine	Epithelial	No	Both bronchial and alveolar epithelial cells
NR8383	Murine	Macrophage	No	Alveolar macrophages
RAW 264.7	Murine	Macrophage	Yes	Bone-marrow derived macrophages
RLE-6TN	Murine	Epithelial	No	Alveolar epithelial cells

Table 3: Elemental species showing strong associations with various cellular responses in ambient PM studies. The shaded cells here represent the metals which have shown a strong association with a particular cellular response in a particular *in-vitro* study. Unshaded cells indicate that no strong correlation was found between a particular elemental species and the corresponding cellular response. The information about the cell line used in the study has also been included. Here, only those studies are included, which have explicitly determined the correlation of cellular responses with the concentration of metals in ambient PM. Criteria chosen for strong association was $r > 0.5$ and $p < 0.05$.

Cellular Response	Fe	Cu	Mn	Zn	Al	Pb	Cr	Ni	As	Sr	Ba	V	Cd	Co	Se	Br	Ca	Mg	Cell line	Reference
Cell death	■		■		■							■		■			■	■	RAW264.7	Lyu et al.[122]
								■											BEAS-2B	Yang etal.[216]
						■		■											A549	Zhang et al.[217]
				■			■	■					■						A549	Das et al.[219]
Inflammation	■		■		■							■		■			■	■	RAW264.7	Lyu et al.[122]
			■	■				■	■				■						BEAS-2B	Låg et al.[309]
	■		■		■														BEAS-2B	Shao et al.[211]
								■											BEAS-2B	Yang et al.[216]
								■											A549	Zhang et al. [217]
							■		■	■		■		■					A549	Huang et al.[220]
	■						■	■				■							NR8383	Hamad et al.[310]
	■						■												BEAS-2B and THP-1	Cao et al.[311]
Oxidative Stress		■		■		■							■						RAW264.7	Lyu et al.[122]
				■					■				■						BEAS-2B	Låg et al.[210]
		■		■		■	■												A549	Sun et al.[215]
	■																		BEAS-2B	Yang et al.[216]
							■												A549	Zhang et al.[217]
	■	■		■		■	■												A549	Niu et al.[218]
				■			■	■					■						A549	Das et al.[219]
										■									A549	Huang et al.[220]
			■						■						■	■			A549	Sun et al.[221]
		■		■															NR8383	Hamad et al.[310]
		■	■	■								■							NR8383	Wang et al.[229]
	■				■														NR8383	Wang et al.[312]
Genotoxicity	■						■												BEAS-2B	Yang et al.[216]
							■	■					■	■					A549	Barzgar et al.[313]

Table 4: PM extract Dose and exposure duration used in various studies evaluating cellular responses to ambient PM. Only those studies which have explicitly mentioned mass concentrations of PM extracts are included here. WS = Water soluble, WIS = water insoluble, DCM = dichloromethane, Hex = Hexane and Meth = Methanol.

Study	Cellular Response	Cell Type	Dose	Exposure Duration	Exposure Condition
Knaapen et al.[194]	DNA damage	A549	5 and 20 μg/cm^2	3 h	WS extracts
Zou et al.[195]	cell viability cellular ROS	A549	50-400 μg/mL	2-48 h	WS and WIS extracts
An et al.[197]	inflammation	THP-1	50 μg/mL	12 h	WS and DCM extracts
Yi et al.[198]	DNA damage and apoptosis	A549	10 μg/mL	24 h	WS and WIS extracts
Ma et al.[110]	inflammation	A549, BEAS-2B	16.8-90.9 μg/cm^2	72 h	WS and DCM extracts
Ma et al.[200]	Apoptosis	A549, BEAS-2B	16.8-90.9 μg/cm^2	72 h	WS and DCM extracts
Mouffareet al.[201]	Oxidative stress, inflammation	BEAS-2B	24 μg/cm^2	48 h	WS and DCM extracts
Mohseni Bandpi et al.[203]	DNA damage	A549	25-100 μg/mL	24 h	WS and DCM extracts
Lai et al.[204]	Oxidative stress, cytotoxicity	A549	50 and 240 μg/mL	5 and 24 h	WS and DCM extracts
Franzi et al.[207]	inflammation	RAW264.7	1 mg/mL	0.5-24 h	WS extracts
Gualtieri et al.[111]	inflammation	A549, BEAS-2B	25 μg/cm^2	24 h	WS extracts
Schwarze et al.[208]	inflammation	A549	0-120 μg/cm^2	24 h	PM suspension
Thomson et al.[209]	inflammation, cytotoxicity	A549, J774	0-300 μg/cm^2	4 and 24 h	Meth extracts
Chen et al.[212]	Oxidative stress, cytotoxicity, inflammation	A549	0-400 μg/mL	48 h	WS extracts

Table 4 (Contd.):

Study	Cellular Response	Cell Type	Dose	Exposure Duration	Exposure Condition
Perrone et al.[213]	cytotoxicity, DNA damage, inflammation	A549	12 μg/cm^2	24 h	WS extracts
Yang et al.[214]	cell cycle arrest	HBE	1 μg/mL	24 h	WS extracts
Lyu et al.[122]	oxidative stress, cytotoxicity, inflammation	A549, RAW264.7	50-400 μg/mL	24 and 45 h	WS extracts
Yang et al.[216]	oxidative stress, inflammation	BEAS-2B	0-300 μg/mL	12-72 h	WS extracts
Zhang et al.[217]	cytotoxicity, inflammation	A549	80 μg/mL	24 h	WS extracts
Das et al.[219]	Inflammation, oxidative stress	A549	100-1100 μg/mL	24 h	WS extracts
Huang et al.[220]	cytotoxicity, DNA damage	A549	80 μg/mL	24 h	WS extracts
Liu et al.[223]	cytotoxicity	16HBE	10-800 μg/mL	48 h	WS extracts
Landkocz et al.[224]	cytotoxicity	BEAS-2B	1.25-80 μg/cm^2	24-72 h	WS and DCM extracts
Palleschi et al.[225]	cytotoxicity	A549	500 μg/mL	24 h	WS extract
Saint-Georges et al.[226]	cytotoxicity, oxidative stress	Human alveolar macrophages	18.84-150.72 μg/mL	24-72 h	WS extract
Longhin et al.[227]	cytotoxicity, oxidative stress, DNA damage	A549, THP-1	10 μg/cm^2	24 h	WS extract
Wang et al.[312]	oxidative stress	NR8383	30 μg/mL	2.5 h	WS extracts
Wang et al.[230]	oxidative stress	NR8383	30 μg/mL	2.5 h	WS extracts
Zhang et al.[232]	inflammation	THP-1	5 μg/mL	24 h	WS extracts

Table 4 (Contd.):

Study	Cellular Response	Cell Type	Dose	Exposure Duration	Exposure Condition
Hetland et al.[234]	inflammation	primary rat alveolar macrophages	2 mg/mL	20 h	Meth extracts
den Hartigh et al.[235]	inflammation, oxidative stress	THP-1	50 μg/mL	3 h	WS extracts
Li et al.[237]	oxidative stress	RAW264.7, BEAS-2B	50 μg/mL	16 h	Water suspension
Billet et al.[238]	cytotoxicity	A549	18.84-150.72 μg/mL	24-72 h	WS extracts
Abbas et al.[239]	cytotoxicity, inflammation, genotoxicity, cell cycle regulation	BEAS-2B	1-30 μg/cm^2	6-72 h	DCM extracts
Chen et al.[241]	cytotoxicity, oxidative stress, inflammation	A549	80 μg/mL	24,48 h	DCM-Hex extracts
Li et al.[243]	oxidative stress, inflammation	BEAS-2B	50-200 μg/mL	24 h	Meth extracts
Xing et al.[244]	oxidative stress	BEAS-2B	200 μg/mL	24 h	WS extracts
Oh et al.[245]	oxidative stress, DNA damage	BEAS-2B	1-50 μg/mL	24 h	DCM-Hex, DCM-Meth, DCM, Meth extracts
Niu et al. [251]	cytotoxicity	A549	50 μg/mL	24 h	WS extracts

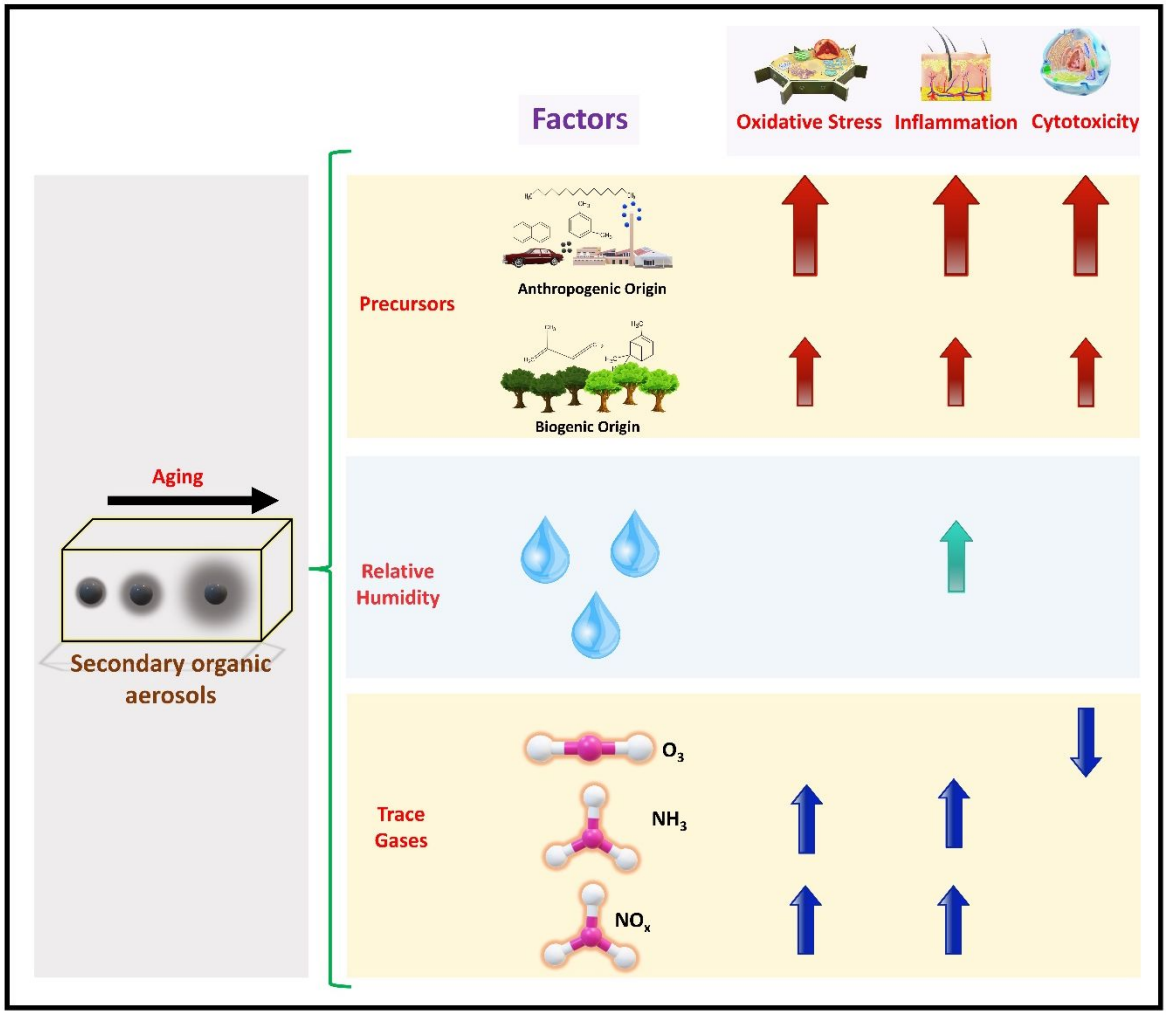

Figure 1: Effects of some important factors influencing the toxicity of SOA generated in laboratory environmental chambers and flow reactors observed in the studies so far. The up-arrow shows that the particular effect increases with increasing level of that factor, while down-arrow shows that the effect decreases with increasing level. The difference in relative size of the arrows for the effects of biogenic vs. anthropogenic precursors indicates a higher degree of the effect from anthropogenic SOA than biogenic. The effect of relative humidity shown in this figure is based on a single study.

- No primary research results, software or code have been included and no new data were generated or analyzed as part of this review.