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Ins and Outs in Environmental Safety Studies of Asthma and Engineered Nanomaterials

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Abstract

According to the Centers for Disease Control, approximately 25 million of Americans suffer from asthma. The disease total annual cost is about $ 56 billion, and includes both direct and indirect costs of medications, hospital stays, missed work and decreased productivity. Air pollution with xenobiotics, bacterial agents, and industrial nanomaterials, such as carbon nanotubes, contribute to exaggeration of this condition and are a point of particular attention in environmental toxicology as well as occupational health and safety research. Mast cell degranulation and activation of Th_2 cells triggered either by allergen-specific IgE or alternative mechanisms, such as locally produced neurotransmitters, underlie the pathophysiological process of airway constriction during an asthma attack. Other immune and non-immune cell types, including basophils, eosinophils, Th_1, Th_{17}, Th_9, macrophages, dendritic cells, and smooth muscle cells, are involved in the inflammatory and allergic responses during asthma, which under chronic conditions may progress without mast cells, the key trigger of the acute asthma attack. To decipher complex molecular, cellular and genetic mechanisms, many researchers attempted to develop in vitro and in vivo models to study asthma. Herein, we summarize advantage and disadvantages of various models and their applicability to nanoparticle evaluation in asthma research. We further suggest that a framework of both in vitro and in vivo methods should be used to study the impact of engineered nanomaterials on asthma etiology, pathophysiology, and treatment.

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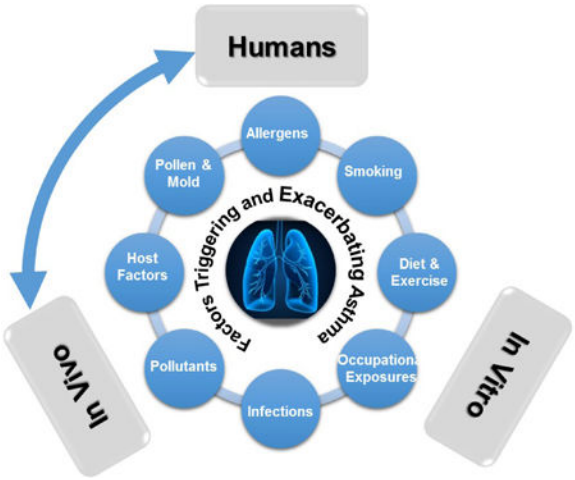
Graphical Abstract



Keywords

asthma; carbon nanotubes; air pollution; nanomaterials; nanoparticles; immune cells; inflammation

Asthma is an inflammatory disorder occurring in the lungs as a result of repeated immediate-type hypersensitivity and late-phase allergic reactions to the inhaled allergen(s). It is characterized by repeated and reversible airway obstruction, chronic bronchial inflammation, hyperreactivity to bronchoconstrictors and smooth muscle cell hypertrophy of bronchi. Asthma often co-exists with the chronic obstructive pulmonary disorder (COPD). The combination of these conditions frequently leads to irreversible airway obstruction, morbidity, and even death. According to the Centers for Disease Control (CDC), asthma affects ~8% (about 25 million) of Americans [1]. Other reports suggest that 15.7 % (or 1.9 million) of asthma cases among adults in the US are work-related [2]. Overall, about 300 million people worldwide suffer from this disorder [3], and 11% of these cases are associated with the workplace conditions, such as exposure to fumes, gases or dust [4]. The asthma prevalence rate is similar in industrialized countries but lower in other nations with the less developed industrial base. According to the Global Initiative for Asthma (GINA) the prevalence rate is increasing by 50% every decade and is accountable for 250,000 deaths annually [3]. Another organization whose research efforts focus on this disorder, the Asthma and Allergy Foundation of America conveyed that direct (hospital stays and medications) and indirect (missed work hours and decreased worker productivity) annual costs of asthma in the US total over $56 billion [5]. These data emphasize the impact of the disorder on modern society and justifies extensive research in this area.

Factors triggering and exacerbating asthma include both immune (i.e. allergen-specific immunoglobulins E (IgE)) and non-immune (cold, certain drugs and exercise) stimuli (Figure 1). Allergen-specific IgE binding to mast cells and basophils and activation of Th_2 cells reacting to allergens trigger the pathophysiologic chain of events culminating in an allergic asthma attack. Mast cell degranulation and release of cytokines by Th_2 cells result in

recruitment of progressively increasing numbers of T-cells, eosinophils, and basophils. While the T-cell population in asthma is dominated by Th_2 cells, other T-cell subtypes are also involved and include Th_1, $Th_{17,}$ and Th_9 cells. The infiltrating airway lymphocytes continue to stimulate the inflammatory response by secreting cytokines and chemokines, which also support the cellular infiltration into the airway. The inflammatory response finally results in hypertrophy of smooth muscles, thickening of basement membranes, hyperplasia of mucous glands, and continuing cellular infiltration. These alterations of the airway, or airway remodeling, may lead to fibrosis and irreversible airway obstruction in some patients. Collectively, cytokines and mediators secreted by lymphocytes trigger hypertrophy and hyperreactivity of bronchial smooth muscle cells, while that released by eosinophils, basophils and mast cells result in constriction of airway smooth muscles.

In contrast to the engagement of different cell populations, the molecular mechanisms underlying allergic and non-allergic asthma are insufficiently understood thus representing the major gaps towards deciphering the pathogenesis of the disease. One of such gaps is related to the role of environmental factors and conditions. Specifically, it is well established that air pollution with xenobiotics, bacterial ligands (e.g. lipopolysaccharide) and some industrial nanomaterials (carbon nanotubes (CNT), carbon black, and some diesel exhaust particles) have a significant influence on the development and progression of asthma. Moreover, the effects of these environmental factors also depend on the age of exposed individuals. So-called hygiene hypothesis proposes that more frequent bacterial infections, lower air pollution with xenobiotics and diesel exhaust, and higher exposure to commensal flora in a farm setting lead to regulated maturation of the immune system and promote less frequent occurrence of asthma and other Th_2-driven responses to non-environmental antigens in less industrialized areas. The same hypothesis states that a cleaner bacterial environment accompanied by a greater air pollution in industrialized areas are responsible for the higher prevalence of atopic diseases in these regions. However, it is also known that both bacterial and viral infections of respiratory tract predispose both to the development of asthma and exacerbation of pre-existing asthma. The hygiene hypothesis does not explain how such infections promote Th_2 and mast cell responses, neither does it clarify how non-immune mediators (cold, physical and mental stress, chemicals, medications) trigger this disease. Genetic differences may also affect susceptibility to asthma, as well as responsiveness to asthma medications. Therefore, more research is needed to understand both cellular and molecular mechanisms underlying this complex disorder.

The respiratory system represents the main route of exposure to aerosolized nanomaterials, by accident or by occupational factors. Although many studies have explored the respiratory effects of engineered nanomaterials, including CNT, in different animal models, very few publications documented the effects of respiratory nanomaterials in experimental models of pulmonary disease. Environmental exposure in sensitized individuals is a critical inducer of airway inflammatory response, which is a hallmark of the asthmatic lung. Although a variety of factors can initiate inflammation via different cellular and molecular pathways, their cumulative effects lead to increased bronchial reactivity. In the area of environmental toxicology and occupational safety, engineered nanomaterials, and particularly carbon-based particles, have recently attracted significant attention as potential triggers and contributors to asthma [6]. Due to their high aspect ratio and biopersistance, several studies emphasized the

role of CNT in asthma (e.g. [7–10]). A recent report demonstrated the presence of CNT in the bronchoalveolar fluid of children with asthma [11]. Another study, comparing gene expression profiles in the blood of individuals exposed to CNT to that of naïve workers in the same facility, revealed significant alterations in the expression of the genes implicated in diverse cellular processes such as apoptosis, cell-cycle regulation, and proliferation [12]. These findings further fueled the public alertness and triggered more thorough investigations of CNT involvement in asthma pathogenesis, including detailed and mechanistic in vivo studies [7–12]. A variety of in vitro and in vivo models have been employed to improve both understanding of the disease and contribution of environmental nanomaterials to its pathogenesis. The advantages and limitations of these models are discussed below.

The benefits of in vivo studies include the ability to account for particle biodistribution, metabolism, repeated exposure and more closely mimic chronic inhalation conditions existing in environment contributing to lung inflammation and asthma (Table 1). For instance, an in vivo study in a model of asthma demonstrated that pulmonary exposure to carbon nanoparticles (CNP) induces a significant release of pro-inflammatory cytokines, modulates proliferation of splenic T cells, and alters the pattern of inflammatory cells in the lung [13]. Growing evidence demonstrates that the adverse effects of inhaled CNT are increased under conditions of pre-existing inflammation such as in allergic asthma. Recent data revealed that exposure to CNT exacerbates ovalbumin-induced allergic airway inflammation in mice by causing airway fibrosis, increasing ovalbumin-induced T-cell proliferation, amplifying lung Th2 cytokines and chemokines. Interestingly, induction of allergic responses exaggerated by CNT involved activation of B cells and was accompanied by the production of IgE in the absence of pre-exposure to allergen [14–16]. This effect is not unique to CNT as the exposure to another carbon-based nanomaterial, graphene oxide, resulted in potentiation of ovalbumin-triggered asthma through the mechanism involving an increase in chitinase production by macrophages [13]. Collectively, these and other in vivo studies in animals models suggest that similar to the findings in animals, humans with allergic asthma may be more susceptible to the immune responses and airway remodeling induced by carbon-based nanomaterials and that CNT alone may serve as allergens. However, due to genetic diversity between different species and even different strains of animals of the same species as well differences in the immune system between mouse and human, extrapolation of in vivo data to the human population is often limited, and it is still unknown whether carbon nanotubes can cause or exacerbate asthma in humans.

Additional technical nuances have to be considered when analyzing and interpreting complex in vivo results. Specifically, re-distribution of airborne particles from the lungs to other organs and tissues depends on the physicochemical properties of nanomaterials, including composition, size, shape, surface properties, and aspect ratio [17–19]. Routes of nanoparticle entry into the body, their biodistribution, and metabolism may also contribute significantly to the test results. Administration routs - inhalation vs intratracheal instillation - may affect the outcomes even in the same animal strain and for the same nanomaterial. For example, inhaled iridium nanoparticles were found in the lung and other organs (liver, spleen, brain, and heart) of experimental rats [20, 21]. In contrast, these nanomaterials administered via intratracheal instillation were not found in secondary organs [22]. The potential explanation for the difference was associated with their partial ingestion after the

inhalation; as such some of the inhaled particles may be ingested and distributed to the liver and spleen via gastrointestinal absorption [21]. This emphasizes that a study design is critical for data interpretation [21]. Intratracheal instillation, on another hand, may lead to the lung overload, thus causing the exaggerated picture of pulmonary toxicity of nanomaterials administered via this route. The importance of the administration route and its relevance to the effects of nanoparticles on humans has been recently emphasized [23].

Several other factors of special relevance to the nanoparticle exposure are the change in nanoparticle composition and physicochemical properties following the particle interaction with proteins and other biomolecules and the dose. The changes in nanoparticle composition and physicochemical properties are triggered by adsorption of different biomolecules on the nanoparticle surface, biodegradation of nanomaterials via enzymatic machinery of inflammatory cells, and by direct pro-oxidant effects of nanoparticles. Absorption of biomolecules on nanoparticle surface leads to the formation of so-called protein and lipid “coronas” whose composition is dynamic and changes during the redistribution of nanoparticles between different compartments in the body [24–25]. For example, it has been demonstrated that different protein and lipid components of the lung surfactant, initially integrated in the nanoparticles surface after inhalation, were replaced with plasma proteins and lipids after the nanoparticles transfer to systemic circulation [26–28]. Such “coronas” of nanoparticles may contribute to the particle recognition as pro-asthmatic signals by different types of the immune cells [29–31]. The biodegradation of nanoparticles is also essential for their role in the immune responses and pathogenesis of asthma. The major oxidative enzymatic pathways of inflammatory cells, including myeloperoxidase/NADPH oxidase of neutrophils and iNOS/NADPH oxidase of macrophages, were reported to catalutically degrade carbon-based nanomaterials [32]. In addition to destroying nanoparticles and lowering their concentrations in tissues, these oxidative effects may also lead to the appearance of oxidized proteins, lipids and carbohydrates thus affecting their immunogenic potential [33]. Although the exact role and significance of these metabolic transformations of nanoparticles in the pathogenesis of asthma remain to be elucidated, the ability of nanomaterials to affect other enzymatic pathways in an experimental asthma model in mice has been already reported. For example, studies of the mechanisms of up-regulated airway responsiveness following pulmonary graphene oxide (GO) exposure in the animal model of OVA sensitization uncovered an intriguing role of pulmonary macrophage-derived chitinase and chitinase-like molecules [13]. The chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin. These enzymes were found to improve allergic responses in the murine models of allergy via a mechanism involving the increase in pulmonary macrophages, reduction in alveolar eosinophils and decrease in the serum IgE levels. Interestingly, although the GO-induced up-regulation of chitinase expression in the lung, the enzymatic activity of this enzyme was decreased [13]. Based on the molecular modeling studies, the authors suggested that GO binds to the entrance of the catalytic site of chitinase, mimicking interactions with chitinase inhibitors. Thus, by interfering with chitinase, nanomaterials may restrict its role in allergic inflammation and asthma [13].

The ability of nanoparticles to alter the activity of different enzymatic systems is not limited to chitinase and has also been reported for other enzymes, for example, alpha-chemotrypsin [34]. Additionally, the dose of the tested nanomaterials should also be considered. It has

been found that DCs exposure to a low and medium dose of fullerenes preceding antigen (OVA) challenge, promoted the ability of DCs to stimulate OVA-specific T cells. In contrast, the higher dose of fullerenes down-regulated antigen presentation by DCs [35], whereas carbon nanoparticles did not affect the ability of DC to stimulate antigen-specific T cells [35]. These differential effects of carbonaceous nanoparticles on DC may be associated with their capacity to alter expression of the main proteins involved in antigen processing machinery, such as immunoproteasomes [35]. These results support the notion that nanoparticles may modify the ability of antigen-presenting cells to process and present antigens (allergens) and thus control Th_1/Th_2 balance in exposed individuals.

Finally, nanoparticles containing transition metals such as biologically common Fe, Cu, Mn, and Co as well as normally not present in the body Cr, Ni, Ce can all affect the redox environment and cause oxidative stress and injury. In the context of asthma and Th_2 inflammation, pro-oxidant conditions can be particularly essential factors that influence exacerbations of asthma episodes and worsen its general course and outcomes [36]. These effects may be related to the lowered effectiveness and levels of reduced glutathione (GSH) and GSH-driven enzymes such GSH-Peroxidases [37]. It is clear that all of the conditions discussed above emphasize the complexity of biological responses to nanoparticles and nanoparticle interaction with various cells, proteins, and other macromolecules. The inherent complexity of in vivo responses to nanopartciels exposures limits the value and the accuracy of the in vitro studies.

One of common criticisms of the animal studies, however, is their relevance to human population [23]. Among the other limitations of in vivo models are their high cost and relatively low throughput. In vitro models help to overcome some of these constraints. They offer higher throughput, are less cost-intensive, allow better insights into the contribution of an individual cell type and isolation of specific molecular pathways. Their major drawbacks include the inability to account for particle distribution and metabolism, accurately mimic repeated exposure to multiple, often fluctuating, environmental factors and represent relevant regulatory interactions between various cells, organs, and tissues. Altogether, these limitations lower the predictive value of in vitro models. To overcome the limitations of in vitro models pertinent to their inability of mimicking chronic inflammation, a recent study by Chortarea S. et al. published in ACS NANO, suggested to use an air-liquid interface cell exposure (ALICE) system [38]. This system is efficient in aerosolization of both spherical and fibrous nanomaterials and permits repeated aerosol exposures of cultured bronchial epithelial cells to CNT. The cells harvested from healthy individuals and asthmatic patients were cultured at the air-liquid interface and treated in vitro with an aerosolized form of CNT at concentrations relevant to those coresponding to human occupational lifetime exposures [38]. The results showed that chronic CNT exposure elicited a duration-dependent pro−inflammatory and oxidative stress response as well as a significant alteration of the mucociliary clearance mechanism in both healthy and asthmatic cultures. The latter displayed stronger and more durable long-term effects compared to healthy cells, indicating that individuals with asthma may be more prone to adverse effects from CNT exposure compared to non-asthmatic populations [17]. The results also highlight the importance of occupationally relevant sub-chronic exposures when in vitro models are used in the nanotoxicity hazard assessments. Another recently proposed in vitro systems reconstitute

organ-level lung functions on a chip [39]. This is a biomimetic microsystem that retains the critical functional alveolar-capillary interface of the human lung. The micro-device reproduces complex integrated organ-level responses to bacteria and inflammatory cytokines introduced into the alveolar space. The lung-mimicking model has been applied to nano-toxicology studies and demonstrated adequate responses to silica nanoparticles regarding toxic and inflammatory reactions as well as enhanced epithelial and endothelial uptake of nanoparticles and their transport into the underlying microvascular channel.

To get better mechanistic insights into pathophysiological events during asthma development, analysis of in vitro-in vivo correlation can be performed (Figure 1). The dominant clearance mechanism of inhaled nanoparticles is particle uptake by the lung resident macrophages. As such, oxidative stress, cytokine secretion, and consequent inflammation were commonly reported in studies with inhaled nanomaterials [40–42]. Particle aggregation to a larger micron sized materials and presence of other environmental factors, for example, endotoxin, were shown to contribute to inflammatory reactions to inhaled nanomaterials. For example, certain airborne particulates are not inflammatory alone but enhance the inflammation caused by endotoxin [43–46]. Therefore, mitigation of the inhalation toxicity of environmental nanoparticles should include control over contributing contaminants such as endotoxin. Oberdorster et al. suggested to include several end-points (reactive oxygen species, lactate dehydrogenase leakage, protein oxidation induction, the number of polymorphonuclear neutrophils in bronchoalveolar fluid) in the study design to correlate in vitro and vivo toxicology data [47]. Another group suggested that hemolysis correlates with inflammation in vivo [48]. In vitro-in vivo correlation of the hemolysis tests have been demonstrated by several studies (reviewed by Brown at al,[49]). From a mechanistic standpoint, stroma-free hemoglobin can act as a peroxidase, provided a source of oxidizing equivalents is available [50, 51]. Under conditions of enhanced inflammation, superoxide radicals generated by macrophages and to a lesser extent neutrophils [52], can dismutate to yield H_2O_2 that will feed the peroxidase activity of free hemoglobin. Once formed, this poorly controlled pro-oxidant catalytic center can cause severe oxidative stress and oxidative damage to many cells, including red blood cells thus perpetuating release of hemoglobin and creating a vicious cycle of pro-oxidant reactions [53].

Collectively, available data indicate that current model do not accurately mimic the complex network of cellular, biochemical and molecular pathways involved in the development of asthma, especially in different environmental conditions. The advantages and disadvantages of each of the mentioned approaches are summarized in Table 1. Recognizing the limitations and non-ideal nature of each of the approaches is important to arrive at accurate conclusions. Future studies would benefit from better understanding of the nanomaterial effects on various types of immune cells beyond alveolar macrophages. More profound insights into nanoparticle effects on mast cells, neutrophils, eosinophils, basophils, and multiple subsets of the T- cells (Th_1, Th_2, Th_{17}, Th_9), as well as their communications with and bi-directorial regulation by macrophages and other antigen-presenting cells, is needed. Systematic approaches based on both in vitro and in vivo studies would fulfill this need (Figure 1).

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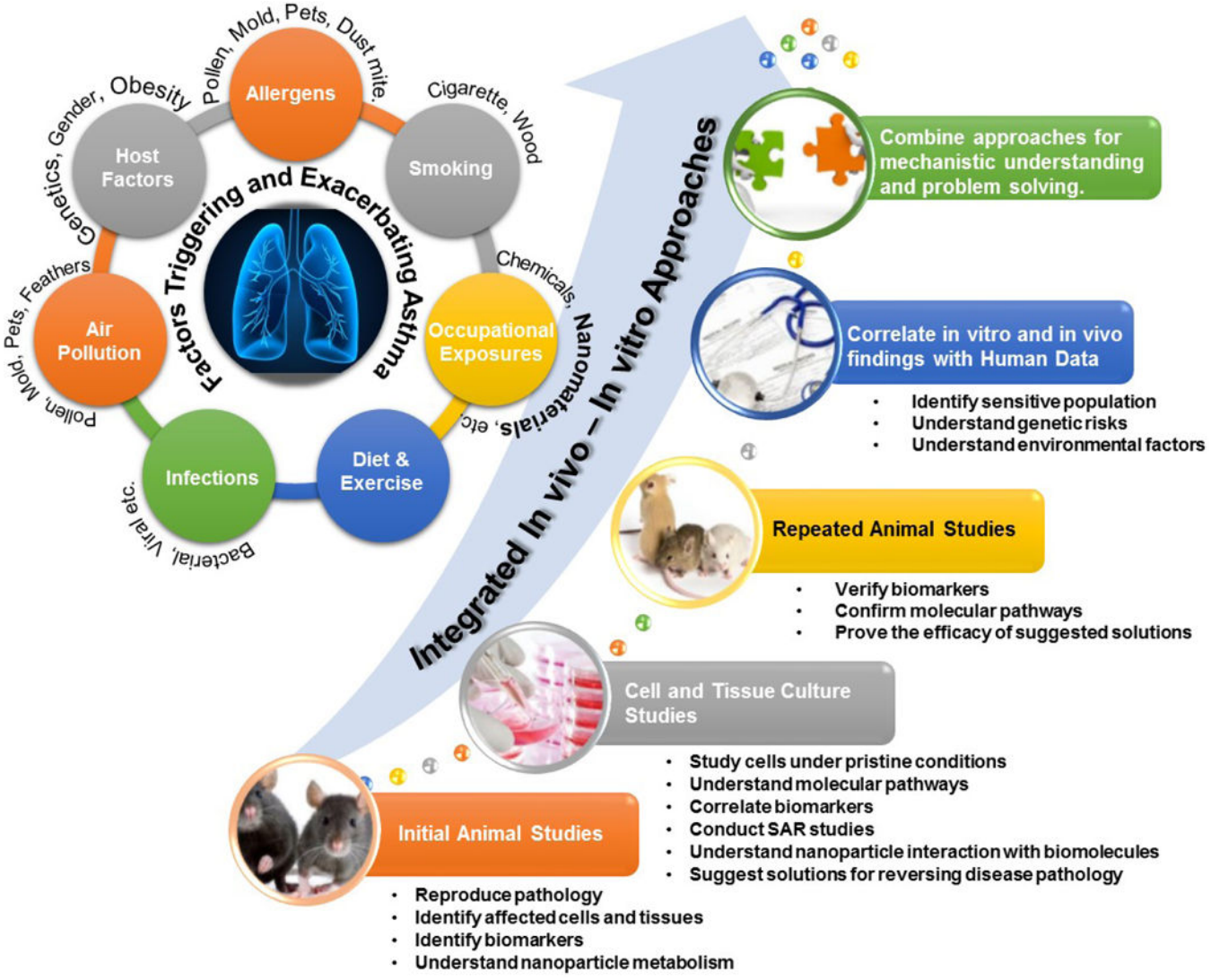

Figure 1.

Systemic approaches potentially required for evaluating the asthmatic effects of nanomaterials exposure.

Table 1.

Advantages and limitations of in vitro and in vivo methods used to study the role of engineered nanomaterials in the development of asthma.

In vitro		In vivo	
Advantages	**Limitations**	**Advantages**	**Limitations**
Allows to single out particular cell type Allows deciphering specific molecular pathway(s) Less time-consuming Less cost-intensive High throughput Ability to test human cells and biological fluids relevant to disease Possibility of testing exact doses and concentrations Opportunity of long-term cultures and cell lines Cell-specific testing of specific inhibitors, antagonists and antibodies	Does not account for particle biodistribution and homing Do not account for particle metabolism and degradation Hard to account for mechanisms involving multiple cellular players Cannot account for relevant repeated exposure Low predictive value	Allows to account for particle biodistribution Allows to account for metabolism and degradation Allows for repeated exposure Allows more close mimicking chronic inhalation Allows complex interactions between various cell types Allows modeling of different pulmonary diseases Allows working with transgenic and knockout animals to study specific cell types or molecular pathways Allows evaluation of human cells in humanized mouse models Allows testing different routes of administration	Often does not consider the real NP concentrations in occupational setting Inter-species difference in the immune system between rodents and humans makes extrapolation of data difficult Difficult to model irregular nature of repeated exposure especially in the context of other environmental factors Does not account for genetic polymorphisms More time-consuming More cost-intensive Low throughput High possibility of lung overload during intratracheal instillation Difference in biodistribution to secondary organs between inhalation and intratracheal instillation