Advanced Drug Delivery Reviews 71 (2014) 15–33

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Challenges for inhaled drug discovery and development: Induced alveolar macrophage responses ☆

Ben Forbes [a,c], Raegan O'Lone [b], Philippa Pribul Allen [d], Anthony Cahn [d], Chris Clarke [e], Mark Collinge [f], Lea Ann Dailey [c], Louise E. Donnelly [g], Joseph Dybowski [f], David Hassall [d], Deon Hildebrand [d], Rhys Jones [f], Joanne Kilgour [h], Jan Klapwijk [d], Curtis C. Maier [i], Tim McGovern [j], Kristen Nikula [k], Joel D. Parry [d], Matthew D. Reed [l], Ian Robinson [m], Lindsay Tomlinson [f], Alison Wolfreys [n]

[a] *Academy of Pharmaceutical Sciences of Great Britain, UK*
[b] *Health and Environmental Sciences Institute, USA*
[c] *King's College London, Institute of Pharmaceutical Science, London SE1 9NH, UK*
[d] *GlaxoSmithKline R & D, Gunnels Wood Road, Stevenage SG1 2NY, UK*
[e] *Amgen, USA*
[f] *Pfizer R & D, USA*
[g] *Imperial College London, UK*
[h] *AstraZeneca R & D, Mereside, Alderley Park, Macclesfield SK10 4TG, UK*
[i] *GlaxoSmithKline R & D, USA*
[j] *SciLucent LLC, 585 Grove Street, Herndon, VA 20170, USA*
[k] *Seventh Wave Laboratories, USA*
[l] *Lovelace Respiratory Research Institute, USA*
[m] *AstraZeneca R & D, Pepparedsleden 1, SE-431 83 Mölndal, Sweden*
[n] *Novartis Horsham Research Centre, Horsham RH12 5AB, UK*

ARTICLE INFO

Article history:
Accepted 3 February 2014
Available online 13 February 2014

Keywords:
Respiratory
Inhalation
Nonclinical
Regulatory toxicology
Drug delivery
Safety

ABSTRACT

Alveolar macrophage (AM) responses are commonly induced in inhalation toxicology studies, typically being observed as an increase in number or a vacuolated 'foamy' morphology. Discriminating between adaptive AM responses and adverse events during nonclinical and clinical development is a major scientific challenge. When measuring and interpreting induced AM responses, an understanding of macrophage biology is essential; this includes 'sub-types' of AMs with different roles in health and disease and mechanisms of induction/resolution of AM responses to inhalation of pharmaceutical aerosols. In this context, emerging assay techniques, the utility of toxicokinetics and the requirement for new biomarkers are considered. Risk assessment for nonclinical toxicology findings and their translation to effects in humans is discussed from a scientific and regulatory perspective. At present, when apparently adaptive macrophage-only responses to inhaled investigational products are observed in nonclinical studies, this poses a challenge for risk assessment and an improved understanding of induced AM responses to inhaled pharmaceuticals is required.

Contents

1. Introduction . . . 16
1.1. Safety challenges in developing inhaled medicines . . . 16
1.2. Regulatory considerations. . . . 17

Abbreviations: 3D, Three dimensional; AM, Alveolar macrophage; APSGB, Academy of Pharmaceutical Science of Great Britain; BAL, Bronchoalveolar lavage; CAD, Cationic amphiphillic drugs; COPD, Chronic obstructive pulmonary disease; DLCO, Diffusing capacity of the lung for carbon monoxide; ERK, Extracellular signal-related kinase; FDA, Food and Drug Administration; FFPE, Formalin-fixed paraffin-embedded; H&E, Hematoxylin and eosin; HESI, Health and Environmental Sciences Institute; HIV, Human immunodeficiency virus; IL, Interleukin; INF, Interferon; iNOS, Inducible NO synthase; IM, Interstitial macrophages; LPS, lipopolysaccharide; MAP, p38 mitogen-activated protein; MDM, Monocyte derived macrophage; MMP, matrix metalloprotease; NHP, Non human primate; NOAEL, No observed adverse effect level; OSWG, Oligonucleotide Safety Working Group; PAP, Pulmonary alveolar proteinosis; PCR, Polymerase chain reaction; PFT, Pulmonary function test; PK/PD, Pharmacokinetics/pharmacodynamics; PM, Particulate matter; STP, Society of Toxicologic Pathologists; TGF, Transforming Growth Factor; TNF, Tumor necrosis factor.

☆ This article is based upon an international workshop held by the Academy of Pharmaceutical Sciences Great Britain and Health and Environmental Sciences Institute on 30–31 October 2012. The meeting addressed the challenge of induced alveolar macrophage responses facing those undertaking inhaled product development. Details of the workshop program, participants, presentations, discussions and the consensus achieved are freely available on the APSGB website http://www.apsgb.co.uk/FocusGroups/DrugsInTheLungs/. This article by the meeting organizers and expert speakers aims to deliver a more detailed perspective on the topics discussed and conclusions reached at the meeting.

http://dx.doi.org/10.1016/j.addr.2014.02.001
0169-409X/

1.3. Aim and scope of the article . . . 18
2. Alveolar macrophage biology and response to inhaled particulate matter . . . 18
2.1. AM biology and polarization . . . 18
2.1.1. Lung dendritic cells . . . 19
2.2. AM responses to particulate matter . . . 19
2.2.1. Acute interactions between AMs and inert, insoluble particles . . . 19
2.2.2. AM responses to chronic insoluble particle exposure . . . 20
3. AM responses to inhaled medicines . . . 20
3.1. Terminology: 'foamy' macrophages . . . 20
3.2. Adaptive responses . . . 20
3.3. Adverse pulmonary responses to inhaled medicines . . . 21
3.4. Mechanisms of AM response to inhaled medicines . . . 21
3.5. AM responses in nonclinical studies . . . 22
3.5.1. Case study 1: poorly soluble compounds . . . 22
3.5.2. Case Study 2: Inhaled oligonucleotides . . . 22
4. Advances in nonclinical safety assessment . . . 23
4.1. Harmonizing terminology in nonclinical inhalation toxicology . . . 23
4.2. Modeling accumulation of poorly soluble compounds using toxicokinetics . . . 24
4.3. Advanced analysis of formalin-fixed paraffin embedded samples . . . 25
4.4. Recognizing species differences in sensitivity and recovery . . . 25
4.4.1. Species sensitivity . . . 25
4.4.2. Species differences in reversibility of response . . . 26
4.5. *In vitro* models for nonclinical inhalation toxicology . . . 26
4.5.1. Macrophage cell lines . . . 27
4.5.2. Primary human macrophages . . . 27
4.5.3. Reconstituted tissue . . . 28
5. Progress to the clinic . . . 28
5.1. Biomarkers for safety monitoring in humans . . . 28
5.2. Case Study 3: biochemical biomarkers to support progress to the clinic . . . 28
5.3. Case Study 4: functional biomarkers to support progress to the clinic . . . 29
6. Conclusions and future perspectives . . . 29
Declaration of interests . . . 30
Acknowledgments . . . 30
References . . . 30

1. Introduction

Delivery of drugs by inhalation has a proven track record for safe and effective treatment of human respiratory diseases, principally asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis and infection [1,2]. The development of new and improved inhaled medicines, however, presents a number of challenges that have been reviewed previously [3]. This article considers induced alveolar macrophage (AM) responses, the interpretation of which is a significant challenge for safety assessment in inhaled product development. The commentary is based on a workshop held in October 2012, organized by the Academy of Pharmaceutical Science of Great Britain (APSGB) 'Drugs in the Lungs Network' in collaboration with the Health and Environmental Sciences Institute (HESI). This meeting comprised a series of structured debates which were led by the authors and informed by workshop participants [4]. In accordance with the principles of the APSGB and HESI organizations, the report reflects multisector perspectives and emphasis is given to the scientific developments and collaborative approaches required for a more efficient paradigm for developing inhaled medicines.

1.1. Safety challenges in developing inhaled medicines

Compound failures during development are costly and contribute to the industry-wide high rate of attrition during drug development [5]. Safety is an important cause of attrition during the development of inhaled medicines. For example, AstraZeneca reported at the workshop that over the last seven years safety was the second-most common reason (30% of 33 cases) for halting further development of inhaled compounds (small molecules targeted at local activity in the lung) which had reached the stage of repeat dosing with a range of doses in one or more species in nonclinical studies. Others have suggested that safety failures may be, in part, because the design considerations for improved lung-targeted medicines (*i.e.*, high molecular weight, lipophilic compounds) have resulted in poorly soluble compounds which generate lung pathology findings related to an excess of undissolved drug [6]. As new classes of molecules are developed as inhaled medicines, including biopharmaceuticals, compounds for new targets in the lung or for systemic delivery *via* inhalation, and compounds requiring novel advanced delivery systems such as nanoparticle or liposomal systems, safety assessment may provide greater challenges [7].

Regulatory guidelines dictate well-defined nonclinical (formerly referred to as preclinical) and clinical phases of inhaled medicine development [8]. At present, Good Laboratory Practice inhalation toxicology studies supporting clinical trials utilize histopathological examination of hematoxylin and eosin (H&E) stained tissue sections as the primary endpoint [9,10]. The most common responses to aerosol administration in nonclinical studies are nasal and laryngeal irritation in rats, which are generally accepted to have little relevance for human orally inhaled drug products as they result from obligate nasal breathing and species-specific airway geometry, respectively [11]. Lung irritation, observed in acute studies as changes to the epithelium at the bronchial or alveolar level (*i.e.* epithelial degeneration, ulceration, necrosis) may be seen as a high-dose effect in short-term studies. However, these effects are rarely seen with chronic dosing as doses are likely to be lower or the drug will already have been discontinued without progressing to long term toxicology studies if this occurs at lower doses.

Lung histology typical of that observed in nonclinical studies is illustrated in Fig. 1. The significance of the common histology finding of an increase in macrophage numbers in the lung and/or alterations in macrophage morphology is not clear. The challenge to toxicologists, pathologists, clinicians and regulatory scientists is to determine at what point a normal adaptive response to foreign inhaled materials becomes a pathological process in animals and at what point the response is predictive of a potentially adverse consequences for treated patients. One complication is that AM responses are often seen in control groups. For example, analysis of control animals in nonclinical studies revealed

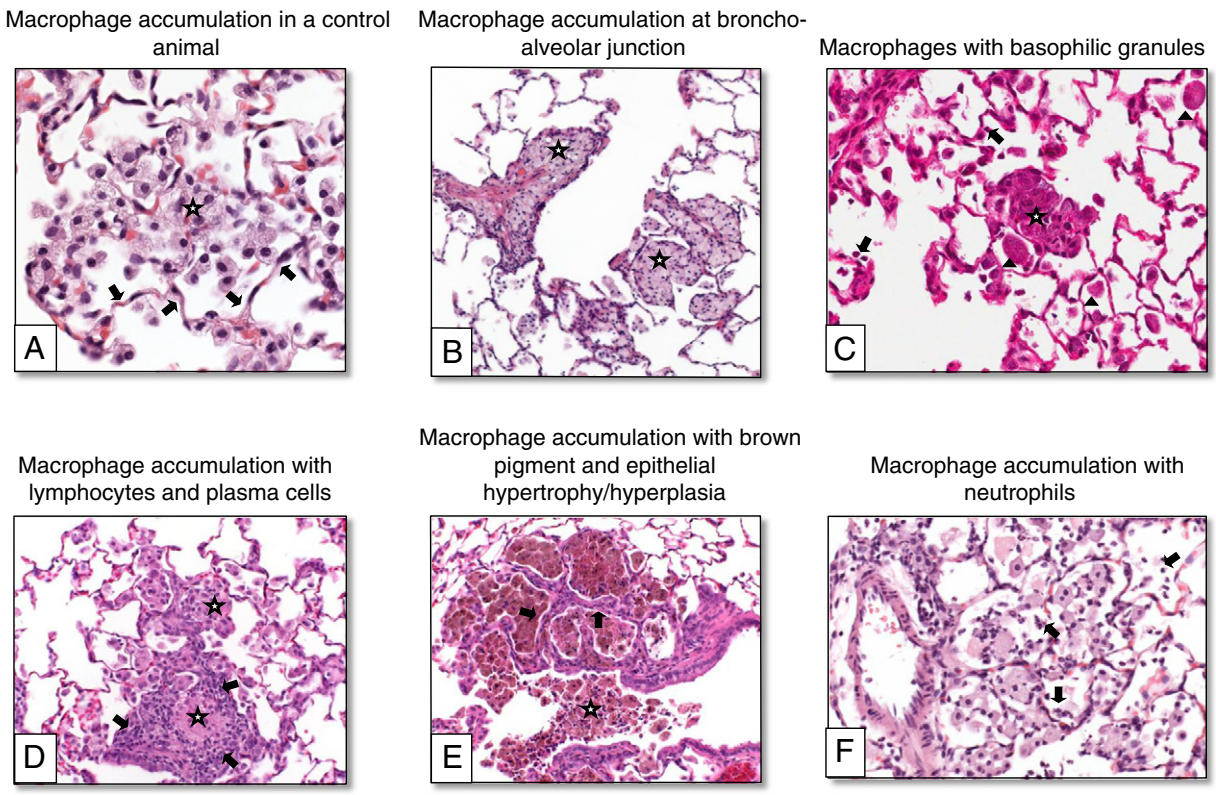

Fig. 1. Typical examples of macrophage responses to inhaled medicines observed by histopathological examination of lung sections from nonclinical studies. A: Macrophage accumulation in a control animal. The accumulation (star) is small and composed exclusively of macrophages with abundant foamy cytoplasm, neutrophils and lymphocytes are absent and local alveolar walls (arrows) are normal, histologically. H&E. ×200. B: Macrophage accumulation at bronchoalveolar junction. The accumulation (stars) is larger than those seen in control animals but still composed exclusively of large foamy macrophages without neutrophils or lymphocytes. In addition, local alveolar walls are normal, histologically. H&E. ×100. C: Macrophages with basophilic granules. Intra-alveolar macrophages are large due to abundant basophilic granular cytoplasm (arrow heads) and fill two neighbouring alveolar lumens (star). Individual neutrophils are scattered amongst the macrophages (arrows). H&E. ×200. D: Macrophage accumulation with lymphocytes and plasma cells. A disorganised admixture of macrophages (stars) and lymphocytes and plasma cells (arrows) within alveolar lumens and alveolar walls. H&E. ×200. E: Macrophage accumulation with brown pigment and epithelial hypertrophy/hyperplasia. Macrophages have abundant brown granular cytoplasmic pigment. Many are degenerated and/or necrotic (star). The local alveolar walls are thickened due to epithelial hypertrophy/hyperplasia (arrows). H&E. ×200. F: Macrophage accumulation with neutrophils. The accumulated intra-alveolar macrophages are admixed with many neutrophils (arrows). H&E. ×200.

macrophage accumulations as spontaneous findings in air-only control cynomolgus monkeys in 32 cases, 5.6% of animals; range of 0–40%, in 55 studies [12]. Another concern is that the inhaled medicine is most often for the treatment of respiratory disease, *i.e.* patients who *de facto* have underlying lung pathologies and may be more sensitive than healthy animals or human volunteers to inhaled particles.

A continuum of responses involving AMs can be recognized in association with the nature, degree and duration of inhaled stimuli. This spectrum of histological findings extends from minimal increases in AMs disseminated within the pulmonary parenchyma, through gradually escalating numbers and densities of AMs, sometimes associated with hypertrophy. Such changes are graded by pathologists (*e.g.* minimal, mild, moderate, etc.) in order to facilitate comparison between treated and control groups. It has been proposed that simple increases in qualitatively similar AMs typically constitute adaptive, physiological responses that are not adverse [6]. In contrast, some stimuli, such as drug accumulation above a certain level, may drive pathologic, adverse responses involving AMs in association with combinations of other changes including infiltrations of inflammatory cells (*e.g.* neutrophils, lymphocytes), epithelial and interstitial changes, and fibrosis [11,13].

While it is generally assumed that certain responses to inhaled particles constitute a normal physiological response that is reversible and distinct from a pathologic response, at present there is no clear agreement for determining where this threshold occurs and how it can be defined objectively using available methodologies. This uncertainty can lead to delays or non-approval for a drug to enter clinical studies and can place a limit on the doses that can be evaluated clinically. A question raised previously [3] is whether toxicological data are obtained and reported similarly between companies or is inconsistent reporting of histopathology findings creating a more complex picture than necessary? The diagnostic criteria published by the INHAND (International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice) initiative of the North American, European and Japanese toxicological pathology societies should assist in partially alleviating this concern [14]. Proposed refinement of the INHAND terminology specifically to address increases in alveolar macrophages when they are observed in nonclinical studies of inhaled pharmaceutical compounds [15] may further promote consistency in reporting results.

1.2. Regulatory considerations

Nonclinical toxicology studies required to support the development of inhaled drugs are generally the same as for other routes of administration [11]. Development plans usually follow the recommendations outlined in the relevant International Conference on Harmonization guidelines [8]. Where possible, repeat dose toxicology studies should employ the inhaled route of administration to mimic the intended clinical route of administration and to ascertain any potential for adversity.

Due to the high frequency of induced AM responses in nonclinical studies, any developer of inhaled drugs is likely to have observed test article-related increases in macrophage numbers, considered the impact of this for their clinical program and engaged in dialogue with the US FDA or other regulatory agency. Regulatory guidance specific to interpretation of alveolar macrophage responses is not currently available.

Interpretation of an inhaled drug-induced macrophage response is an important consideration with regard to authorizing progression of products from nonclinical to clinical phases, especially in terms of trial dose and duration, and for clinical indications involving lung disease. The nonclinical/clinical interface is where the interpretation of adversity is critical. If the principle that any increase in macrophage numbers should be considered a potential early indication of inflammation is applied, due to the lack of a monitoring tool in clinical studies, this impacts on the determination of the 'no observed adverse effect level' (NOAEL). A lower NOAEL value, in turn, affects safety margin calculations required for transfer to the clinic, thus limiting both the starting and maximal allowable dose in human trials, potentially restricting the ability to fully explore the relevant pharmacology in humans.

Considerations for interpretation of alveolar macrophage findings in a regulatory context include the nature and extent of the potentially inflammatory response and the clinical population (*e.g.* whether the respiratory system is compromised by disease). Macrophage responses not evolving into more involved inflammatory responses with increasing duration could potentially be considered non-adverse, while responses that become more involved over time, or in combination with chronic active inflammation, lymphoproliferative change, and/or fibrosis, could impact the NOAEL identification.

Interpretation and understanding of macrophage responses may be aided by improving consistency in terminology used. Terminology can differ across labs, drug sponsors, peer reviewers and Pathology Working Groups, and inconsistency can contribute to uncertainty in evaluating alveolar macrophage findings. Other considerations in interpretation of macrophage responses include evaluation of responses across doses and duration of exposure, reversibility of findings, and evaluation of historical control data from the laboratory conducting a given toxicity study.

1.3. Aim and scope of the article

The interpretation of AM responses arising during inhaled drug toxicology studies is a major scientific challenge in the development and registration of new inhaled therapies. Issues include how to identify, report and interpret findings related to induction of AMs during nonclinical and clinical development, and how to discriminate adaptive lung responses from adverse events. This article elaborates discussion at the recent APSGB-HESI workshop [4] covering risk assessment and risk management with regard to observed AM responses in nonclinical toxicology studies and suggests initiatives to facilitate the future development of safe inhaled medicines.

Emerging scientific understanding of AM biology and macrophage responses to inhaled pharmaceuticals is reviewed. The need to define and improve current best practices for measurement and interpretation is addressed by considering currently available assays and their utility in distinguishing adverse from adaptive AM responses. Potential improvements to existing assays and emerging new methodologies are matched to gaps in knowledge and lack of tools (*e.g.* biomarkers, lung function tests for safety monitoring) that currently hinder progress of projects into the clinic. Recommendations are made for improvements in measuring and reporting induced AM changes, research priorities for basic macrophage bioscience research and the development of discriminatory measurement techniques. Finally, the advantages of collaborative approaches towards data sharing, harmonization of methods and reporting and the development of novel biomarkers are identified.

2. Alveolar macrophage biology and response to inhaled particulate matter

2.1. AM biology and polarization

Two distinct populations of tissue resident macrophages are present in the lungs: interstitial macrophages (IM) and AM. Both are generally considered to originate from bone marrow-derived precursor cells which develop into circulating blood monocytes prior to recruitment into the lungs and maturation into macrophages [16–20]. However, a recent study by Hashimoto et al. [21] reports evidence from murine parabiosis and genetic fate-mapping studies to support the hypothesis that the baseline population of tissue-resident macrophages, including AM, proliferate locally throughout adult life with minimal replenishment from circulating monocytes [21], although there is no evidence to support this hypothesis in human studies. They suggest that tissue macrophages are recruited to the lung from the circulating monocyte population only in response to a pathology or stimulus. In contrast to AMs, IM are half the diameter of AMs (~8 *versus* 16 μm), are more heterogeneous in morphology, and exhibit a lower phagocytic activity than AMs, although in contrast they express higher levels of complement C3, intercellular adhesion molecule-1 (ICAM-1), and are better at antigen presentation than AMs [18,22]. However, since changes in IM are not as commonly observed in nonclinical inhalation toxicology studies during the development of inhaled pharmaceuticals (especially in the absence of concurrent AM effects) this article will concentrate on AMs.

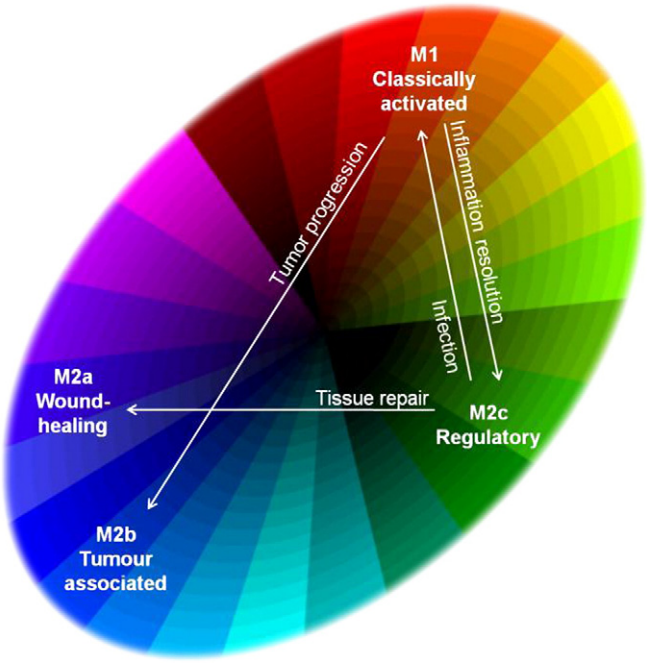

Fig. 2. Illustration of macrophage polarization states according to the current classification system. (The figure has been adapted from [24]).

AMs have two important functions in the peripheral lung: modulation of immunological homeostasis and host defense [23]. In their 'quiescent' state, AMs play an important immunosuppressive role in the lungs. Without immunosuppressive regulation, mediated through secretion of anti-inflammatory agents such as IL-10, the adaptive immune system would be activated continually by the wide range of harmless airborne antigens causing substantial tissue damage to the lungs over time [23]. Due to their immunosuppressive role in maintaining immunological homeostasis, 'quiescent' AMs are considered to be in an alternatively activated (M2) polarization state and, more specifically, display characteristics of regulatory (M2c) macrophages (Fig. 2, Table 1). AM polarization towards the regulatory M2c phenotype is thought to be maintained by epithelial secretion of TGF-β, which promotes $\alpha_v\beta_6$ integrin expression on the macrophage surface and results in adhesion to the epithelial cell layer and secretion of anti-inflammatory mediators. Regulatory macrophages are heterogeneous, but a defining feature is the concurrent upregulation of IL-10 and downregulation of IL-12. Phagocytic activity and antigen presenting capability is not necessarily impaired in this polarization state [24].

Polarization away from the regulatory phenotype is stimulated by changes to the microenvironment which occur during pathogen

Table 1
A summary of the characteristics of macrophage polarization [20,23,24,27].

	M1	M2a	M2c	M2b
	Classically activated	Wound-healing	Regulatory	Tumor-associated
Drivers of phenotype	INF-γ + LPS/TNF-α	IL-4 and IL-13	TGF-β and $\alpha_v\beta_6$ integrin	Dependent on tumor microenvironment
Cytokine secretion	Pro-inflammatory (IL-12, IL-1β, IL-6, IL-23)	Reduced cytokine secretion	Anti-inflammatory (IL-10)	Pro-angiogenic (TNF-α, VEGF, IL-1), IL-10
Enzyme activity	iNOS, MMP9	Arginase, chitinases	–	MMP9
Phagocytosis	Enhanced	Reduced	Normal	Dependent on tumor microenvironment
Physiological role	Host defense	Tissue repair	Immunological homeostasis	–
Associated pathologies	Chronic inflammation; tissue damage promoting neoplasia	Fibrosis; asthma; susceptibility to infection	Susceptibility to infection	Neoplasia

invasion, which induces the classically activated phenotype M1, or events that trigger other M2 polarization states (Fig. 2, Table 1). AMs typically express high levels of pattern recognition receptors (*e.g.* mannose or toll-like receptors for recognition of various microbial components) and scavenger receptors (*e.g.* cell surface receptors that selectively recognize polyanionic surfaces, such as those found on low density lipoproteins), which enable them to recognize and phagocytize a wide range of inhaled foreign matter, including bacteria, viruses and environmental particles [20]. During infection, elevated levels of interferon gamma (IFN-γ) combined with the triggering of pattern recognition receptors activates AMs according to the M1-like classically activated pathway. This macrophage polarization state is characterized in the AM population by a detachment from the alveolar epithelial surface, an enhanced phagocytic capability, the production of reactive oxygen and nitrogen radicals, as well as secretion of pro-inflammatory cytokines (*e.g.* IL-12, IL-1, IL-6, IL-23) and chemotactic agents [23,24]. M1 activation is associated with substantial changes to gene transcription, expression and epigenetic regulation [25], which direct the cells to kill invading pathogens and enhance their antigen-presenting capabilities.

Tissue damage or pathophysiological conditions resulting in elevated levels of IL-4 and IL-13 will, in contrast, polarize AMs towards a wound-healing (M2a) phenotype, which is characterized by increased arginase activity (an enzyme that promotes collagen formation through conversion of arginine to ornithine) and elevated expression of chitinase-like molecules, which play a role in cell matrix reorganization. M2a macrophages have been shown to produce minimal amounts of pro-inflammatory cytokines, reactive oxygen and nitrogen species, and have low phagocytic capability. For this reason they are less effective at clearing or killing infective agents compared to other macrophage phenotypes [24,26]. Furthermore, prolonged or uncontrolled stimulation of macrophages towards the M2a phenotype can lead to tissue fibrosis and is thought to play an important role in the progression of fibrotic pulmonary diseases, including airway remodeling in asthma [26].

The M2b phenotype is a heterogeneous population of tumor-associated macrophages which promote tumor initiation, growth and development. Tumor-associated macrophages are currently thought to play a role in creating and maintaining an anti-inflammatory environment within the tumor and promoting angiogenesis. However, the variety of M2b sub-populations, with both distinct and overlapping characteristics, does not lend itself to simple biological categorization and the clinical relevance of different M2b polarization states are not clearly defined [27].

Current understanding of the role of AM polarization in respiratory health and disease is basic, although reviews summarizing the current understanding of AM involvement in asthma, COPD, pulmonary fibrosis, cystic fibrosis and lung cancer have been published [22,27–31]. Ongoing research in this area has the potential to identify new therapeutic targets or biomarkers for the assessment of therapeutic intervention. At the same time it is widely acknowledged that studying AM biology is complicated by the plasticity of these cells, which means that functional activity, protein and gene expression patterns, as well as epigenetic regulation can change very quickly in response to sometimes very subtle changes in the microenvironment [25]. It is clear that a deeper understanding of AM biology and a wider range of validated assays to determine AM polarization state would be useful to guide interpretation when macrophage responses arise during inhaled drug toxicology studies.

2.1.1. Lung dendritic cells

In addition to lung alveolar macrophages, a population of dendritic cells is also present, first identified by Sertl and co-workers [32]. Similar to any organ facing the outside milieu, the lung's network of dendritic cells aids in the recognition of exogenous threats. There are at least three resident populations of dendritic cells in humans, based upon phenotypic markers and up to four in mice [33]. They form a well-developed network in the epithelial layers of conducting airways with long cellular extensions between the mucosal surface and the basal layer. They are also located in the alveolar septa and patrol the walls of the pulmonary arterial vasculature [34]. Whilst they play a crucial role in sensing pathogens and potential hazards, their role, if any, in the development of macrophage responses is uncertain so the following discussion will focus on the macrophage alone.

2.2. AM responses to particulate matter

The lungs have evolved to protect themselves against a wide range of inhaled environmental (including man-made) particulate matter (PM). There is compelling epidemiological evidence that inhaled PM exacerbates respiratory diseases such as COPD and asthma [35,36]. Much research has been driven by concerns over the presence of air pollutants, especially cigarette smoke, engine exhaust fumes and fossil fuel smoke. These PM in complex mixtures are generally classified as insoluble, although it is recognized that they may have soluble components. Studies of the deposition of PM in the respiratory tract demonstrate that inhaled particles with sizes of less than 100 μm deposit in the upper respiratory tract and larger airways by impaction and gravitational sedimentation [37,38]. Inhaled particles of 6 μm diameter or less tend to deposit in the mucus lining the airways where they are cleared by the mucociliary escalator. Smaller particles deposit *via* diffusion in the smaller airways and the alveolar ducts and sacs. PM between 1 and 3 μm in aerodynamic diameter are the optimal particle size range for deposition in the peripheral lung, representing an important particle population which will come into contact with AMs. Submicron-sized particles have also been shown to deposit throughout the entire respiratory tract, despite a significant portion being exhaled, and therefore may also interact with AMs [39–41].

2.2.1. Acute interactions between AMs and inert, insoluble particles

AMs have a density of 12–13 per alveolus in a healthy person and play a key role in removing PM [39]. It is well known that the physicochemical properties of PM are closely linked with macrophage responses, yet the exact mechanisms by which macrophages can recognize different PM and tailor their response (*i.e.* instructive phagocytosis) is still poorly understood. Underhill and Goodridge [42] provide an evocative description of instructive phagocytosis as occurring in four phases: 'tasting', 'feeling', 'swallowing', and 'digestion'. Tasting usually involves interactions between the particle and cell surface receptors. For example, AMs possess a broad family of scavenger receptors that will bind to polyanionic motifs and will be triggered by a wide variety of particles possessing this chemistry. In particular, macrophage receptor with collagenous structure

(MARCO), a Class A scavenger receptor, has been shown to be directly involved in the uptake of titanium dioxide (TiO_2), silica, diesel particles, and latex beads by AMs [43,44]. Specific receptor involvement for binding to highly cationic surfaces has not been reported; however, non-specific electrostatic interactions are often cited as a mechanism for the high binding and cytotoxic effects observed for cationic particles [45]. Finally, inhaled particles depositing in the alveolar lining fluid are likely to be opsonized by lung lining fluid components (*i.e.* albumin, surfactant proteins, glycoproteins and phospholipids), which can also bind to a range of macrophage surface receptors [46].

'Feeling' or 'mechanosensing' involves coordination of a number of triggered surface receptors which instigate membrane re-arrangement around the particle, with the response depending on properties such as size, rigidity and shape of the particle [42,47,48]. The number and type of receptors triggered by this process dictate the response, which may explain why inert PM with a size and shape similar to common micro-organisms can be highly effective at activating macrophages, while other inert PM is not. Interestingly, in some instances 'feeling' can occur independently of receptor binding through direct interaction between crystalline materials and cholesterol-rich regions of the cell membrane. Uric acid crystals (a well-known immunostimulatory adjuvant) has been reported to interact strongly with cholesterol-rich membrane regions of dendritic cells, inducing lipid sorting, phagocytosis and activation *via* a Syk kinase-dependent signaling pathway [49].

The subsequent phases of 'swallowing' and 'digestion' (*i.e.* phagosomal processing and signaling) are not well described for inert particles; this is in contrast to a sizeable literature on phagosomal processing of microorganisms and dead cells. It is known that AMs respond to certain inhaled PM in a pro-inflammatory manner (similar to M1 activation) *via* generation of oxygen radicals, increased protease activity and release of pro-inflammatory mediators (TNFα, IL-1β, IL-6, IL-8, MIP-1, MMP, GM-CSF) [50]. These mediators can recruit neutrophils into the alveolar spaces and tissues and stimulate other immune responses, thereby establishing an inflammatory response and potentially causing tissue damage. For certain types of PMs this activation may result from phagosomal processing (*i.e.* acidification and enhanced enzymatic activity in the lysosome) which can alter the physical or chemical properties of the ingested material, resulting in release of soluble chemical species, disruption of normal phagosomal functions or even phagosomal rupture. An example of this phenomenon is Nlrp3 inflammasome activation following macrophage exposure to inert particles [51]. Even for inert, insoluble, PM that does not induce a pro-inflammatory response, there is concern that they may impair normal AM functions, including the ability to recognize and phagocytose pathogens [39], especially in compromised populations with existing airway diseases [52]. In addition, PM composed of certain materials, such as silica, can be directly cytotoxic to AMs [53,54].

2.2.2. AM responses to chronic insoluble particle exposure

A large body of literature has accumulated reporting the outcomes of inhalation toxicology studies investigating the effects of chronic exposure to inert, insoluble PM on respiratory health. The outcomes of inhalation studies using insoluble particles can be used as guidance in interpreting non-specific responses to inhaled particulate pharmaceuticals. From a historical perspective, the materials investigated in many of these studies (*e.g.* titanium dioxide, carbonyl iron, and carbon black) were chosen to investigate the phenomenon of lung overload and overload-related carcinogenesis in rats. However, in studies where lower dose groups were included, increases in AMs were observed without pulmonary inflammation and were considered adaptive and non-adverse.

For example, an inhalation study using rats exposed to titanium dioxide or carbonyl iron for four weeks [55] demonstrated that low dose exposure (5 mg/m^3) resulted in an increase in alveolar macrophages without inflammatory or other lung changes and the increases in macrophages resolved during the recovery period. Similar findings have been reported for exposure of rats to carbon black at 1 mg/m^3 for 13 weeks [56,57]. At higher concentrations, exposures to these insoluble particles induced accumulations of particle-laden macrophages, hypertrophy and hyperplasia of alveolar epithelium, neutrophilic inflammation and, in some carbon black exposure groups, additional changes such as degeneration and necrosis, alveolar proteinosis, and fibrosis. At the higher concentrations these changes either partially resolved, showed no resolution, or, in the case of fibrosis, progressed. The degree of resolution or progression depended on the amount of exposure, resulting particle lung burden and the severity of the response.

In a longer term (2 year) study, inhalation of titanium dioxide at 10 mg/m^3 [58] resulted in a small increase in alveolar macrophages (observed in clusters), slight local hyperplasia of type II epithelial cells, and an increase in macrophages in local lymphoid tissue. There was no change in body weight, clinical signs, morbidity, or mortality compared with untreated animals. This study demonstrated that a particle exposure which induced slight type II cell hyperplasia in association with accumulation of alveolar macrophages over the lifetime of the rats did not have an identifiable adverse effect on the lungs or the health of the animals.

3. AM responses to inhaled medicines

AM responses to inhaled medicines are generally observed in nonclinical studies as a focal or multifocal increase in numbers in the lungs and/or a change in the appearance. Changes in AM morphology are sometimes referenced confusingly in the literature and a note on terminology is included below.

3.1. Terminology: 'foamy' macrophages

'Foamy macrophage' is a term used by pathologists to describe lung macrophages that have taken on a granular or vacuolated cytoplasmic appearance when viewed by light microscopy (Fig. 3). These AMs are also typically enlarged compared to non-vacuolated AMs. As it is used currently, the foamy macrophage descriptor is applied to a number of different macrophage phenotypes, including AMs that respond adaptively, but non-adversely to inhaled medicines (Section 3.2) and AM responses that are associated with a variety of different etiologies and pathophysiologic processes (Section 3.3).

3.2. Adaptive responses

Nonclinical inhalation toxicity studies, particularly those conducted with drugs designed to maximize effects in the lung through low solubility, often report an increase in AMs as the primary histopathology observation. In the absence of any other indication of inflammation, an increase in AMs is consistent with a non-specific physiological response to the delivery of particles to the alveolus and may be considered non-adverse (Nikula et al. [15]). In such circumstances, development may progess into the clinic even though it is currently not possible to monitor and characterize potential macrophage accumulation in man due to the absence of clinical measurement techniques and discriminatory biomarkers (Section 5). If the particulate burden exceeds the clearance mechanism of the alveolus this may result in additional findings in nonclinical studies indicative of inflammation. In this case the clinical relevance of the response to excessive doses utilized in nonclinical studies may be queried, but if additional findings are observed at the lowest dose investigated a NOAEL to support progress to the clinic has not been established.

A further complication is that the pharmacological properties of the drug being tested may induce changes in the alveolus [59]. It is often difficult to distinguish primary and secondary pharmacology responses from non-specific effects associated with the particle burden of poorly soluble drugs. Direct pharmacological effects upon pneumocytes (surfactant production, modulation of cytokine release) or macrophages (inhibition of phagocytosis, cell migration or apoptosis) and any relevant secondary pharmacology may induce a macrophage response or modify the ability of alveolar macrophages to clear particles.

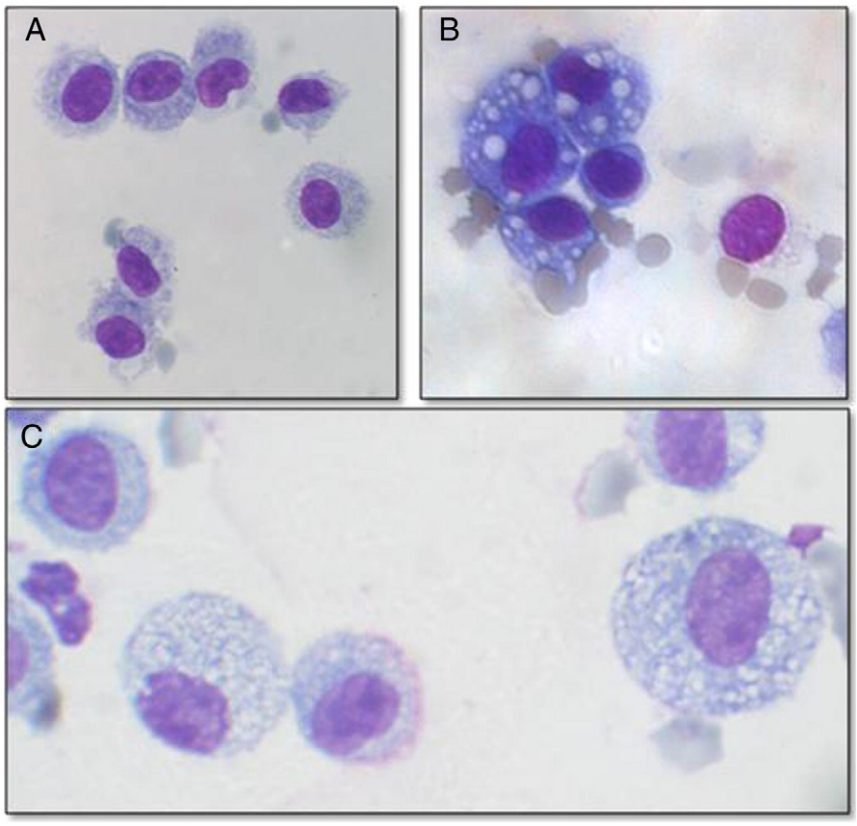

Fig. 3. Light microscopy images (×40) of murine alveolar macrophages (AM) recovered from bronchoalveolar lavage illustrating (a) AM from an untreated animal, (b) AM with coarsely vacuolated cytoplasm after exposure to particulate matter, (c) AM with finely vacuolated cytoplasm after exposure to particulate matter. The particulate matter was 150 nm nanoparticles formed of polyvinyl acetate with different degrees of hydroxylation: 40% (image b) and 20% (image c).

3.3. Adverse pulmonary responses to inhaled medicines

Although macrophage accumulation and/or the presence of foamy macrophages in the absence of other findings are not necessarily indicative of toxicity, when these responses are seen alongside additional markers of inflammation or distinct histopathological changes, the overall response is likely to be interpreted as adverse. The determination and reporting of pathology findings are critical to this categorization and recommendations to assist in distinguishing and reporting adaptive *versus* adverse findings have recently been published (Section 4.1). Typical pathology findings indicative of inflammation are the presence of inflammatory cells with epithelial and interstitial changes, including those characteristic of chronic inflammation and fibrosis.

Aside from inflammation, a number of distinct pathologies associated with foamy AMs have a clear clinical relevance. These include pulmonary alveolar proteinosis (PAP) and phosholipidosis. PAP is described particularly in humans and features AMs that have a foamy appearance, with the additional conspicuous presence of intra-alveolar eosinophilic material that can be demonstrated to be lipid-rich *via* Oil Red O or adipophilin staining (sometimes associated with tubular myelin). Evidence suggests that impairment of the ATP-binding cassette ABCG1 is implicated as its loss is associated with an inability to control cellular sterol levels, especially in pulmonary macrophages [60,61]. PAP appears to be related to certain exposures, such as the inhalation of silica dust [13].

AM phospholipidosis is caused by an excessive accumulation of phospholipids in lysosomal lamellar bodies resulting in a foamy phenotype [62]. The change is not restricted to the lungs and can also be seen in other cells across multiple tissues, although often the AM is the most sensitive cell type affected and most readily demonstrates the change on light microscopic examination. Phospholipidosis is a common feature in several lung diseases, and can be seen in surfactant protein D (SP-D)-deficient mice and in response to infections such as tuberculosis [63,64] and human immunodeficiency virus [65]. AM phospholipidosis also develops in the lungs in an emphysema model induced by chronic cigarette smoke exposure [66] in which the foamy macrophages are thought to play a key role in the development of the emphysema and are associated with increased production of matrix metalloproteases (MMP), TNF and GM-CSF and reduced presence of SP-D. AM phospholipidosis can be induced directly by drugs of certain chemical classes, *e.g.* cationic amphiphillic drugs (CADs) such as chloroquine and amiodarone [62,67,68]. It is thought that CADs form intracellular complexes with phospholipids, which become resistant to intracellular digestion and accumulate in the cytoplasm of macrophages. Typically the exposure to CAD is systemic or oral, rather than by inhalation, and the response is not restricted to alveolar macrophages.

The functional consequences of AM phospholipidosis include slow clearance of the engulfed phospholipidosis-inducing material which results in AM recruitment and increases in macrophage numbers [62,69]. The cellular changes can progress to lysosomal fragility and proteolytic enzyme leakage, causing cell damage, death and associated inflammation. Drug-induced phospholipidosis which does not advance to the severity described above may be reversible, although this reversal can be a lengthy process. The observation of AMs with a 'foamy' appearance, but without lysosomal lamellar bodies should not be confused with phospholipidosis.

3.4. Mechanisms of AM response to inhaled medicines

The principal responses of AMs to inhaled aerosol medicines are phagocytosis and biochemical activation as discussed in Section 2. AMs act rapidly to phagocytose/neutralize and remove unusual material from the alveolar space [13]. These mechanisms underpin the typically observed AM-related findings in nonclinical toxicology studies; accumulation through recruitment of additional macrophages and/or changes in AM appearance. Accumulations of macrophages are

sometimes observed as aggregates although the reason for this is unclear. When the rate of inhaled particle deposition exceeds the rate of clearance, material accumulates potentially leading to the overloading of macrophages with adverse consequences [11].

The foamy AM morphology may arise though a number of different mechanisms and therefore herald different consequences. For example, the macrophages may present neutral lipid in droplets or lipid vacuoles or exhibit lysosomal lamellar bodies indicative of phospholipidosis [70]. Surfactant, which is rich in phospholipids, is constitutively produced and cleared by alveolar epithelial cells, but in situations when an excess is produced (as may arise after corticosteroid inhalation) surplus surfactant is phagocytosed by AMs which develop a foamy appearance. Inhalation of saliva can produce a similar change. Exogenous materials associated with the development of foamy AMs include high-fat diets, liposomal delivery vehicles, misdosed oral drug delivery vehicles such as methylcellulose, and drug particulate matter in the form of therapeutic aerosols. The vacuolated cytoplasm in AMs exposed to inhaled oligonucleotides contains basophilic granules thought to be oligonucleotide-related material. Foamy macrophages are also symptomatic of some disease states including Niemann–Pick disease and some infections, including tuberculosis and human immunodeficiency virus (HIV).

When foamy cytoplasm is observed by light microscopy, transmission electron-microscopic examination of macrophage contents may assist in discriminating distinctive features such as insoluble PM, crystalline formations (related to drug crystals) or multilamellar bodies (suggestive of phospholipidosis). As the development and functional consequence of a foamy morphology is poorly understood, it is difficult to interpret. For example, the observation of foamy phenotypes following ingestion of PM composed of poorly soluble drug material by AMs may simply be an adaptive response to an increased particle burden. However, continued exposure could lead to a situation where the adaptive response is overcome as deposition exceeds clearance and an adverse response may ensue. Differentiating between an adaptive response and a response that is indicative of toxicity is critical to the safety assessment of a material [71], and the use of definitive terms such as phospholipidosis should be used selectively to describe clear cases where an effect associated with lipid lamellar inclusions is confirmed.

If AM accumulation and/or foamy appearance in response to acute inhalation are reversible, this may be indicative of an adaptive rather than an adverse response. However, under the condition of chronic dosing, even in the absence of accumulation of PM, the AM response may lead to other non-resolvable changes in the lungs over time.

3.5. AM responses in nonclinical studies

The frequency of AM findings in nonclinical inhalation toxicology studies and uncertainty in the interpretation of these makes the design of inhaled product development programs complex. Two case studies are presented to illustrate study design, findings related to AMs and their interpretation.

3.5.1. Case study 1: poorly soluble compounds

Prototypical 'inhalation by design' drugs have high molecular weight and are highly lipophilic with limited solubility in aqueous solutions. These properties result in long residence times that maximize activity in the lungs and minimize systemic toxicity potential through low systemic bioavailability and high unbound clearance. This case study describes the pulmonary findings observed in repeat dose inhalation toxicology studies of an adenosine A_{2a} receptor agonist.

Adenosine A_{2a} receptor agonists can inhibit neutrophil activation degranulation [72] which provides an attractive therapeutic strategy for COPD. To avoid cardiovascular effects associated with activation of adenosine A_{2a} receptors [73] an A_{2a} agonist, Compound X, was developed for inhaled delivery with properties designed to maximize anti-inflammatory activity in the lungs, while minimizing systemic exposure. Pivotal, repeat-dose inhalation toxicology studies of 2 weeks duration were conducted in Sprague–Dawley rats and Beagle dogs with a dry powder formulation of 10% Compound X in lactose monohydrate. Due to poor tolerance observed in dogs at high dose, the NOAEL was considered to be 138 μg/kg and doses were reduced for a 3-month study (Fig. 4).

In subsequent 3-month inhalation toxicity studies, the highest daily dose in beagles of 931 μg/kg was well tolerated with no evidence of local or systemic toxicity making this the NOAEL. In rats, the highest daily dose of 4754 μg/kg was associated with minimal to slight accumulations of alveolar macrophages in the lungs at the broncholaveolar junction, indicative of incomplete clearance of inhaled particles. These macrophage accumulations were sometimes accompanied by neutrophils/cell debris and/or secondary epithelial hyperplasia of alveolar ducts, and in some animals, epithelial hyperplasia of terminal bronchioles. The inflammatory response in the lungs was associated with minimal to slight hypercellularity and foci of macrophages in the tracheobronchial lymph node. The presence of an inflammatory response and epithelial hyperplasia in animals exposed to 4754 μg/kg was considered adverse and the NOAEL was concluded to be 539 μg/kg.

The pulmonary changes observed in the rat were consistent with "lung overload", *i.e.* expected changes when the amount of particle deposited exceeds pulmonary clearance mechanisms [40,74]. At the bronchoalveolar interface, where few cilia are present, the primary route of clearance for poorly soluble particles is *via* macrophages. An overload of macrophage clearance capacity can lead to activation, with inflammatory consequences. The lung deposition of Compound X at 4754 μg/kg/day was calculated to be approximately 0.1 mg/g lung weight, which was at the lower end of the estimated lung burden threshold of 0.1–1 mg/g reported to be the point of transition between adaptive and adverse changes in the lung [6].

An additional 6-week study in rats was necessary to characterize better the NOAEL using intermediate doses and establish a NOAEL of 2910 μg/kg which provided adequate safety margins needed to support the proposed maximum clinical dose. The pulmonary findings observed in the rats exposed to Compound X could not have been predicted prior to initiation of the 3-month inhalation studies as the deposition levels were below the threshold at which an adverse change would be predicted and the known primary and secondary pharmacology of Compound X did not provide an obvious explanation for this apparent increase in sensitivity of the rat to particulate burden. However, further development of Compound X was halted for reasons unrelated to the rat pulmonary findings, limiting the ability to further investigate the mechanism of toxicity.

3.5.2. Case Study 2: Inhaled oligonucleotides[1]

Inhaled oligonucleotide-based drugs for respiratory diseases are being developed by several companies [75]. For example, Pharmaxis with combination RNAse H-mediated antisense for the treatment of asthma [76] and Alnylam with small interfering RNA for the treatment of respiratory syncytial virus infection [77] have already completed Phase II clinical trials for inhaled oligonucleotide formulated in simple physiological buffers. A number of oligonucleotide class-related toxicities (*i.e.* actions independent of pharmacology) have been identified in nonclinical studies [78–84], a number of which are relevant for the inhaled route [85]. These include increased presence of basophilic granules assumed to be oligonucleotide-related material in tissue macrophages (*e.g.* Kupffer cells in the liver).

Lung responses to oligonucleotides in nonclinical toxicology studies, and potential markers that can be assessed in the clinic to monitor changes in the lungs have been identified for inhaled oligonucleotide treatment [85]. Lung findings are generally reminiscent of the effects reported with systemic oligonucleotide administration [86,87]. Key findings in short-term inhalation toxicology studies include AMs with vacuolated cytoplasm containing basophilic granules and mononuclear cell infiltration, mainly in the interstitium but also in the local lymphoid

[1] On behalf of Inhalation Subcommittee of the OSWG.

		Sprague Dawley Rats	Beagle Dogs
Pivotal repeat dose study 2 weeks	Daily doses (μg/Kg)	45.2, 433, 3716	57.5, 138, 2432
	Observations	Well tolerated – no sign of local or systemic toxicity	Highest dose reduced to 2238 from day 5 and terminated at day 10 due to severe clinical signs (e.g. decreased food intake, weight loss)
	NOAEL (μg/Kg)	3716	138.1

⇩ ⇩

		Sprague Dawley Rats	Beagle Dogs
Inhalation toxicology study 3 months	Daily doses (μg/Kg)	58.9, 539,4754 μg/Kg	40.4, 204, 931 μg/Kg
	Observations	Aggregates of macrophages at bronchoalveolar junctions, accompanied by neutrophils and some epithelial hyperplasia at the highest dose	Well tolerated – no sign of local or systemic toxicity
	NOAEL (μg/Kg)	539 Insufficient to support the clinical dose	931 Adequate safety margin to support maximum clinical dose

⇩

		Sprague Dawley Rats
Additional study (intermediate doses) 6 weeks	Daily doses (μg/Kg)	742, 1490, 2910
	Observations	Well tolerated – no sign of local or systemic toxicity
	NOAEL (μg/Kg)	2910 Adequate safety margin to support maximum clinical dose

Fig. 4. Schematic illustrating the challenge posed by the nonclinical requirement to establish NOAEL in two species with sufficient safety margin to support the maximum dose in humans (safety margins are 10× in rats, 6× in dogs). This case study is based on a poorly soluble A2 receptor agonist developed by Pfizer (Section 3.5.1).

tissues and upper airways. The 'foamy' appearance of the vacuolated cytoplasm should not be confused with phospholipidosis. *In vitro* data in the murine monocyte RAW264.7 cell line suggest that single stranded phosphorothioate (PS) oligonucleotide exposure does not result in obvious activation, cytotoxicity or impaired phagocytosis [88].

It is possible that PS-backbone-mediated chemotaxis partly underlies the macrophage accumulation observed in many tissues following oligonucleotide administration, including AMs in the lungs. Baek et al. [89] reported that single stranded immunostimulatory CpG oligonucleotides caused concentration-dependent activation of murine peritoneal macrophages *in vitro* independent of backbone chemistry (PS or PO). However, in a chemotaxis assay, only CpG oligonucleotides with a PS backbone increased migration of peritoneal macrophages across an 8 μm membrane in a chemotaxis chamber [89]. Overall, inhaled oligonucleotides for the treatment of respiratory diseases are well tolerated clinically [85], although the nonclinical safety knowledge base is limited. At present, it is unclear whether the AM response is part of an inhaled oligonucleotide-related toxicity and the activation status of AMs after acute exposure and the chronic effects of oligonucleotides are unknown. However, it is encouraging that with systemic oligonucleotide administration, similar morphological changes in tissue macrophages appear to be benign.

4. Advances in nonclinical safety assessment

Nonclinical inhalation toxicology is a challenge, especially for new chemical entities or novel compound classes. A number of recent or on-going initiatives include efforts to harmonize methods and reporting and to develop new techniques to inform our understanding of macrophage-related responses to inhaled medicines.

4.1. Harmonizing terminology in nonclinical inhalation toxicology

Unless care is taken when selecting terminologies, the manner in which incidental and potentially drug-related lung changes are recorded in inhalation studies may make toxicology data tables difficult to interpret. Scientific evaluation relies on consistent and objective recording of data, and this is particularly important in regulatory science. Inconsistency in terminology (between different pathologists describing the same lesion or the same pathologist looking at the same lesion at different

times) leads to unnecessary confusion. It is also important to appreciate that generic terms, *e.g.* 'foamy macrophages', may be used for findings which, although morphologically similar by light microscopy, nevertheless are quite different from a biochemical/pathogenic point of view. In nonclinical safety assessment, it is important to identify adaptive responses and to distinguish them from those likely to be adverse. Terminology used by pathologists in summary data tables reporting inhalation studies should distinguish non-adverse, adaptive increases in numbers of AMs from potentially adverse changes complicated by inflammation, cytotoxicity, or other changes in the pulmonary parenchyma [15].

To this end, a position paper [15] endorsed by the Society of Toxicologic Pathologists (STP) has recommended use of the term "increased alveolar macrophages" to describe an uncomplicated increase in the number or number and size of AMs, regardless of whether the increase occurs in naïve or vehicle control animals or those that inhale test article. This "no threshold" approach to recording all instances of increased macrophages above the expected number of randomly scattered, individual AMs has been proposed for several reasons that include facilitating the use of historical control data and the fact that it shows that an increase in AMs is part of a normal adaptive response and, in the absence of secondary changes, should not be considered adverse. The STP group recommended use of "increased alveolar macrophages" as a stand-alone diagnosis not meant to include other findings such as inflammation, degeneration, hyperplasia, or fibrosis. Additional findings, when they occurred, would be entered separately. The STP group acknowledged that in some situations where additional findings, such as inflammation and Type II pneuomocyte hyperplasia, are consistently colocalized with accumulations of macrophages, it may be more appropriate to use a single term, such as chronic inflammation, but the group recommended against using increased alveolar macrophages as an umbrella term to include additional findings. The pathologist must also be alert to non-test article-related findings and use alternative terminology to distinguish these, *e.g.* findings which may present when food or bedding material is inhaled.

4.2. Modeling accumulation of poorly soluble compounds using toxicokinetics

There is a significant body of evidence within the literature linking inhaled particulate burden to adaptive changes and adverse pathology in the lungs with particular reference to increases in AMs. This includes guidance on estimated thresholds of inhaled PM that will result in adaptive or adverse changes within the lungs [55,58].

Deposition, dissolution, absorption and distribution within the lungs following inhaled delivery is complex (Fig. 5). Furthermore, absorption from the lungs may involve transport *via* and localization in the pulmonary lymphatics, especially for particles and macromolecules [90–92]. Although direct quantitative measurement of non-dissolved particles in the alveolar compartments is unfeasible, an estimate of lung particulate burdens can be made by evaluating plasma toxicokinetic data from nonclinical studies. When the intrinsic pharmacokinetics of a compound in a nonclinical species of interest are understood (*i.e.* intravenous clearance, volume of distribution, systemic elimination half-life, oral absorption and lung absorption rate following solution administration to the lungs) the toxicokinetic data can provide an insight into the likely events occurring within the lungs and enable an estimate of lung particulate burden over time.

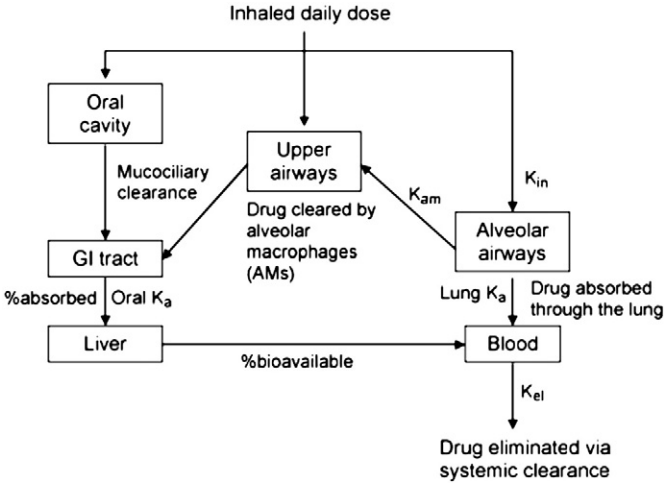

Fig. 5. Schematic of inhaled absorption from the lungs [figure reprinted with permission from [6]].

Typically in rats and dogs the intravenous systemic half-life of an inhaled molecule is less than the inhaled dosing interval (typically once daily) and therefore enhanced systemic drug concentration in the absence of lung accumulation is unlikely to occur. It is therefore likely that any systemic accumulation (*i.e.* increase in systemic drug concentration) observed during an inhaled toxicology study will be driven by slow absorption from the lungs into the systemic compartment of drug from an accumulation of slowly dissolving particles. This rationale is supported by the general accord that adverse lung findings are strongly linked with molecules of lower solubility and slower absorption rates throughout the lungs.

Using basic pharmacokinetic principles, an estimate of the dissolution half-life in the lungs can be made by taking into account the dosing frequency and observed systemic accumulation during the dosing period. Using an estimated lung dissolution rate, daily dose administered and estimated deposition fraction a lung particulate burden can be estimated over the time course of the inhalation study (Fig. 6) using Eq. (1) below.

Eq. (1): Estimating alveolar particulate burden

$$LB_n = \left(D * 1 - \exp^{-(Ka+Kam).t.(n+1)} / 1 - \exp^{-(Ka+Kam).t}\right) - D$$

Adapted from [93]

$\mathbf{LB_n}$	lung particulate burden on the `nth' day (μg/g lung tissue)
D	lung dose (μg/g lung tissue) (assuming FPD of 10% in rat)
$\mathbf{K_a}$	lung absorption rate constant (day^{-1})
$\mathbf{K_{am}}$	AM lung particulate clearance rate constant (day^{-1}). Value of 0.007 day^{-1} based on rat clearance half life of 100 days in rat [94]
t	dosing interval (days)
n	number of doses given

Although this remains a simplistic approach and incorporates a number of assumptions, the modeling can aid interpretation of lung findings where it is unclear whether they are adaptive *versus* adverse and can guide the selection of doses for an inhaled toxicology study. Proposed thresholds above which responses would be expected to be observed are: (i) >0.1 mg/g lung to induce an adaptive response, and (ii) >1 mg/g lung to induce adverse changes. The ability to estimate lung particulate burdens can provide data to help deconvolute whether an observed pathology is more likely to be driven by particulate burden or the physicochemistry/pharmacology of the molecule.

Based on discussion at the APSGB-HESI workshop, the majority of the pharmaceutical industry evaluates total lung concentrations over time routinely. Generally, this is not captured as part of inhalation toxicology studies but primarily as a separate pharmacokinetic evaluation to aid the understanding of pharmacology studies. However, the information provided by measuring drug concentration in homogenized lungs is limited by the inability to discriminate between non-dissolved and dissolved drug. Comparison of total lung concentrations between compounds will also be influenced by the specific molecular properties and lung tissue affinity/distribution coefficient. Although toxicokinetic data may not provide precise concentrations in different lung compartments, it does

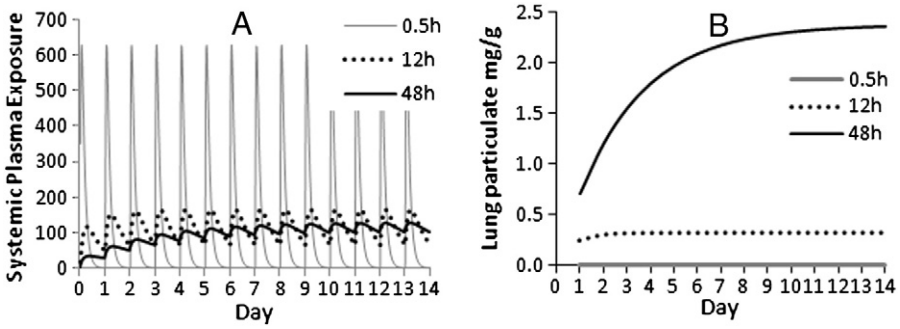

Fig. 6. Simulation of systemic plasma exposure (A) and lung particulate burdens (B) for a molecule with differing dissolution rates in the lung following a 1 mg/g deposited lung dose administered daily for 14 days. Grey line—0.5 h dissolution half-life. Black dotted line—12 h dissolution half-life. Solid black line—48 h dissolution half-life.

provide a means of evaluating an overall elimination half-life of drug from the lungs. Examples of plasma and lung exposure data have been published recently demonstrating the ability to compare plasma and lung half-lives to provide further information on the molecule of interest [95].

In summary, plasma toxicokinetics are routinely measured in nonclinical studies following inhaled administration to provide a measure of systemic exposure during the dosing period to enable systemic exposure margins to be estimated for inhalation delivery to humans. Plasma Cmax and AUC measurements are made, and the difference in exposure from the first to the last dose can be evaluated to provide information on accumulation. However, although these measurements are useful from a systemic perspective, they do not provide a direct link to the concentrations within the lungs. Generally, lung concentration data is not routinely part of an inhalation toxicology study due to the limited interpretation that can be made and the additional cost and complexity in study design. In this absence, the plasma toxicokinetic data can provide additional utility for molecules that are of low solubility and exhibit a plasma AUC that indicates accumulation from the first to the last dose. Under these criteria, pharmacokinetic principles can be applied to estimate lung particulate burden providing the overall disposition of the molecule after inhaled delivery is well understood.

4.3. Advanced analysis of formalin-fixed paraffin embedded samples

Formalin-fixed paraffin embedded (FFPE) samples of lungs in nonclinical inhalation toxicology studies enable macrophage accumulations to be studied in detail using multiple analytical techniques. Longitudinal analysis of samples collected using this technique allows the onset of responses and effects after cessation of dosing to be evaluated. The morphological features that can be discerned in FFPE include the number and size of AM accumulations in a histological section, the presence or absence of any other changes, such as inflammation, necrosis and epithelial hypertrophy/hyperplasia. The evaluation of the number and average size of macrophage profiles in histological sections can be semi-automated by immunohistochemical staining with a macrophage marker such as ED1 [96] and analysing the stained sections with an image analysis platform.

A major advantage of FFPE samples is the additional insights into AM accumulations that may be elicited. The expression levels of genes in samples may be analysed by polymerase chain reaction (PCR) [97] and, if required, specific cell types can even be sampled from the lung sections using laser capture microdissection [98]. Several commercial kits allow extraction of total RNA from FFPE samples and convert it to cDNA for the measurement of biologically relevant gene expression data [99]. Gene arrays are available that can identify changes in aspects of lung biology, *e.g.* immune balance [100] or the activation status of macrophages [24]. *In situ* hybridization is a useful adjunct to PCR analysis because it localizes significant changes in gene expression *in situ* [101]. FFPE samples of lung are also amenable to quantification of protein levels by mass spectrometry [102], which can be combined with immunohistochemistry to localize the measurements. The additional information about macrophage accumulations available through such analyses has the potential to provide powerful evidence to discern whether an induced AM response to inhaled material is adaptive or adverse.

Advantages of the FFPE technique include the ability to generate several different data sets from the same FFPE sample. This reduces animal usage as additional analyses can be performed on the FFPE samples of lung that are processed routinely for analysis by light microscopy in toxicology studies. Further, the novel data from additional analyses can be compared directly to the morphology of the lung obtained by routine microscopy. Other advantages are that the residual wax blocks can be stored and repeatedly sampled and analysed as studies evolve, allowing samples from control, non-adverse and adversely affected lungs to be revisited. Archiving of FFPE samples will enable retrospective analyses to be carried out on large sample sets from previous toxicity studies, with the potential to accelerate the acquisition of data and knowledge [103]. The noteworthy disadvantage of the FFPE approach is that all data are generated from thin histological sections of lung, raising concerns regarding how representative the data are of the lungs as a whole. Analytical considerations include the limitations of 2 dimensional compared to 3 dimensional counting methods [104] and the impact of degradation of RNA during storage on the quality of gene expression data [105].

4.4. Recognizing species differences in sensitivity and recovery

Risk assessment during the development of inhaled medicines requires an understanding of the sensitivity of the species used in nonclinical toxicology studies to inhaled particles. This enables the relevance of findings in different species to be evaluated and compared. The species for which most data is available are rodents and nonhuman primates.

4.4.1. Species sensitivity

Rats are more sensitive than mice and hamsters to pulmonary overload with poorly soluble particles, such as pigmentary titanium dioxide (TiO_2) [106] and other inhaled toxic substances like diesel exhaust particles [107]. This sensitivity is associated with differences in physiologic responses. After exposure to TiO_2 for 13 weeks, cell turnover at alveolar sites was consistently high in hamsters, consistently low in mice, and there was an increase in turnover at the mid and high doses at one or more time points in the rat [106]. There was a similar clearance of TiO_2 in mouse and rat lung, compared to the more substantial clearance seen over 52 weeks of recovery in hamster. However, rats had substantially higher amounts of TiO_2 accumulated in lung-associated lymph nodes than mice or hamsters. Rats also had the highest percentage of neutrophils in the lung, an endpoint that did not recover over the 52 week recovery period. The percentage of neutrophils in hamsters was high (~55% of total cellularity) at the beginning of the recovery period but it dropped to less than 10% after 52 weeks of recovery. These findings suggest that there are differences in the ability of various rodent species to clear insoluble particles, and the authors concluded that these differences predispose rats to chronic inflammation and an increased susceptibility to tumor formation.

Species differences have also been identified between rats, Syrian hamsters, and mice after chronic exposure to diesel exhaust [106,107] and silica [108]. Rats develop more prominent alveolar epithelial hyperplasia, chronic-active inflammation, focal areas of fibrosis, and epithelial metaplasia and are more prone to develop lung tumors [109]. Hamsters developed mild bronchiolar–alveolar hyperplasia but no lung tumors. While some strains of mice may be susceptible to tumor formation after exposure to diesel exhaust, CD-1 mice exposed to conditions that were carcinogenic to F344 rats did not develop lung tumors [110].

Non-human primates have more physiological similarities to humans than to rats and other rodents. Despite having a higher fractional deposition and slower rate of clearance of particles than rodents [41], pulmonary overload is not common in humans. Mice and rats have simple acini (alveoli without respiratory bronchioles) while monkeys and humans have larger more complex acini (similar respiratory bronchiole anatomy and alveolar ducts) [111,112]. Humans have thicker pleura, more abundant pleural lymphatics and more interlobular connective tissue than rats. Non-human primates have little interlobular connective tissue but tend to accumulate macrophages in the interstitium in response to inhaled coal dust and diesel exhaust, similar to humans [109,113,114]. In the study of Nikula and colleagues, AMs were less prominent in monkeys than in rats with less prominent epithelial hyperplasia, fibrosis, and alveolar proteinosis [109]. This can be explained in part by the presence of lymphatics adjacent to the respiratory bronchioles and alveoli in primates, decreasing the amount of luminal particles which initiate the inflammatory response [115].

Sensitivity to a single output, such as epithelial hyperplasia in response to inhaled silica in rats, cannot be extrapolated to other lesions. In humans, silica results in high grade fibrosis, but that is not the case in rats [116]. Thus, rats are more sensitive than humans to developing epithelial hyperplasia and tumor formation in response to inhaled silica, whereas humans are more sensitive than rats to developing fibrosis. This example illustrates that a finding in one species cannot be assumed to be predictive of another finding in another species as the underlying physiological responses may be different. Greater understanding of species-specific macrophage responses (*e.g.* polarization) could help explain differences in responses to inhaled pharmaceuticals.

4.4.2. Species differences in reversibility of response

Species differences in physiology and response will also impact the recovery of responses to inhaled particles. Normally, PM is removed from the lung slowly, with the main route of removal being mucociliary clearance [39]. The recovery from adverse responses to inhaled PM in the lung will depend on the resolution of inflammation as well as removal of the PM.

In species that respond to PM with a low to modest influx of neutrophils into the lungs, recovery occurs over a long period of time. Since rats respond to inhaled toxicants by influx of high numbers of neutrophils and accumulate the majority of particles within AMs rather than in the interstitium, they may incur significant tissue disruption and develop chronic-active inflammation more readily than other species. Under these conditions, irreversible lesions such as ongoing inflammation and fibrosis develop. When the rat is subject to particulate overload, *e.g.* with carbon black dust, the chronic-active inflammatory process has been followed for up to 15 months without evidence of recovery [117]. The hamster develops the same character of lesions but clears the inflammation over time, with fewer ongoing and irreversible changes [106]. Thus, the same response to PM in different species can have dramatic differences in reversibility. This is probably because alveolar macrophages in the hamster are more efficient at phagocytosis of particles and produce less reactive oxygen species compared to rat [118,119], which leads to less ongoing damage and inflammation.

Species differences in other physiological aspects may influence recovery from lung injury associated with inhaled PM. In iNOS −/− mice, resolution of LPS-induced acute lung injury was delayed. This effect could be alleviated by introducing wildtype (iNOS +/+) bone marrow-derived monocytes, indicating the importance of iNOS in the resolution of lung injury [120]. In contrast, hamsters fail to produce iNOS protein or form nitric oxide in response to *in vitro* exposure to LPS, IFN-gamma or TNF-alpha [121], indicating that the mechanisms governing recovery are multifactorial or different in different species.

There is a paucity of information on recovery from inhaled particles in monkeys. Interstitial accumulations of macrophages in monkeys would be expected to recover very slowly based upon the long half-life of retention of insoluble particles (~700 days for dogs, and presumably similar for monkeys and humans) during the slow phase of clearance [41]. A percentage of these macrophages will be transported to local lymph nodes [41]. However, the lack of inflammation associated with the accumulations of interstitial macrophages makes the recovery time less of a concern.

In summary, the differences between rodent and human lung anatomy and physiology are significant. The changes in lungs associated with inhaled PM and toxicants are not consistent across different species, even among rodent species, and are not necessarily directly predictive of human response to the same particles or toxicants. Models, including *in vitro* assays, that are more predictive of human responses are desirable for improved risk assessment.

4.5. In vitro models for nonclinical inhalation toxicology

In vitro AM models which are predictive of the human response to inhaled medicines would be a potentially valuable tool to provide supplementary data to inform the design or interpretation of nonclinical inhalation toxicology studies and support progress to the clinic. Several cell culture models of AMs are currently in use or are being developed to investigate the biology and role of macrophages in human health and disease. *In vitro* models are also used extensively in inhaled medicine discovery research, although their role in product development programs is currently limited. A number of different *in vitro* models are available and their selection depends on the suitability of the system for the application.

The cell type should be relevant to those exposed to inhaled PM and many cell culture models based on lung cells are available [122,123], including cell lines and primary cells, co-culture and 3D culture systems [124]. It is important that such cell systems respond in a similar manner to the cells *in situ*. In the case of AMs, primary cells from the lungs and cell lines are available [125,126]. However, the less ready availability and efforts to decrease animal usage makes investigation using primary cells and tissues unrealistic for routine high throughput screening, although with sufficient cell yield and suitable assay end-points, primary cell systems may still represent an animal-sparing approach. It must be remembered that cultured AMs, like their *in vivo* counterparts, show considerable phenotypic plasticity [18,20,127,128]. This means that culture systems are dependent on how the macrophage responds in the *in vitro* microenviroment and may not accurately replicate responses to drug inhalation in a complex lung environment.

A number of assays are available to investigate responses to drugs or PM *in vitro* (Table 2). Viability assays can determine the potential of the drug in its intended inhaled form to cause cellular damage/death. AM activation may be evaluated by the production of inflammatory markers, but these are dependent on the selection of analytes and the interpretation of responses is complicated by questions regarding how responses seen *in vitro* may relate *in vivo,* and whether changes seen *in vitro* can be predicted to drive pathology *in vivo*. The function of AMs can be used as a marker for the potential of a drug to disrupt homeostasis in the lungs. AMs serve as important sentinels, phagocytosing particles and clearing pathogens, but if their functional capacity is compromised, there is the potential for reduced clearance capacity and inability to respond appropriately to pathogen challenges. Changes in AM morphology *in vitro* in response to drugs or PM may reflect responses *in vivo*. Consolidating these readouts can reveal the potential for drugs or PM to cause detrimental changes to the cells of the lungs

Table 2
Assays commonly used with *in vitro* models to investigate macrophage responses to drugs or particulate matter.

Cell response	Assay	Limitations
Viability	AM viability can be assessed easily [179,182] at low to high throughput by a variety of readily available cost effective kits [183]. Drugs can be compared and ranked relative to their potential to cause cytotoxicity. This is used to screen out compounds that have clear unequivocal toxicity issues.	Viability endpoints do not provide any mechanistic understanding of cellular response. In addition, cells in culture do not include the dynamics of lung residency, so whilst compounds may be identified with potential risk, cellular responses may differ when other lung physiology is included.
Activation	AM activation state may complement *in vitro* cytotoxicity readouts and forewarn of the potential for any marked *in vivo* inflammatory response triggered by drug stimulation of AMs. Activation can be measured by cytokine/chemokine induction and immuno-phenotypic changes [71].	The choice of which cytokines/chemokines to measure remains speculative. Responses may vary between different chemical classes or be modulated by the pharmacological activity of the drug, making direct interpretation to lung responses challenging.
Function	Phagocytic function can be quantified using flow cytometric assays [184] and immune functions such as oxidative stress can be assessed by measuring reactive oxygen species (ROS) production, glutathione (GSH:GSSG), lipid peroxidation and heme oxygenase-1 (HO-1) expression. Early identification of responses to drugs or PM *in vitro* may help to guide the appropriate studies to undertake *in vivo*.	Early functional measures in response to drug/particle challenge may be influenced directly by pharmacological effects. Often culture systems do not accommodate the chronic dosing aspects of *in vivo* toxicology studies.
Morphology	Low resolution and confocal microscopy can reveal detailed changes, particle accumulation and foamy phenotypes. Flow cytometry can quantify changes in cellular granularity associated with drug accumulation.	Changes in surface or internal characteristics only describe the phenotype crudely with little detailed understanding of the intracellular changes taking place.

at reduced cost, and can help inform discussions on candidate selection, reducing the risk of taking forward a potentially toxic or unintentionally immune disrupting compound to further development.

4.5.1. Macrophage cell lines

Macrophage cell lines are used widely as models for AMs to study mechanisms of macrophage responses to a wide variety of stimuli, including PM [129–131], inhaled pathogens [132], and to a lesser extent pharmaceuticals [133]. Certain cell lines appear more popular than others. RAW 264.7 (mouse leukemic monocyte-derived continuous macrophage cell line; [134]), J774.A1 (mouse reticulum cell sarcoma-derived continuous macrophage cell line; [135]), and differentiated THP-1 (human leukemic monocyte cell line differentiated to macrophage-like cells through phorbol ester treatment; [136]) are used extensively.

The preferential use of these particular cell lines may be explained by such diverse reasons as ease of handling, to facilitate comparison to *in vivo* models by using the same species [132] or simply precedence and the amount of benchmarking data already available for these cell lines. There is currently a paucity of literature which critically reviews the characteristics, advantages and disadvantages of macrophage cell lines as a tool to understand macrophage responses to inhaled pharmaceuticals. Difficulties are often encountered in using *in vitro* assessment to predict *in vivo* response to particles. The extent to which particular cell lines are able to exhibit responses that are predictive of *in vivo* toxicity is an aspect that requires clarification if *in vitro* studies are to produce valuable data for use in human safety assessment.

4.5.2. Primary human macrophages

Despite the challenge in gaining access to sufficient numbers of primary human macrophages from broncho-alveolar lavage (BAL) or tissue samples, the use of primary human AM culture systems to investigate the role of AMs in health and disease is becoming more prevalent. The increased use of primary cells isolated from human donors is driven by concerns related to the species differences described above when investigating mechanisms of health, disease and therapeutic interventions.

Most investigations using primary human AMs are studies into disease mechanisms and biomarker identification. For example, it has been shown that AMs isolated from COPD patients have an impaired ability to phagocytose both microorganisms and apoptotic cells, and release higher levels of cytokines, chemokines and matrix metalloproteinase-9 (MM9) compared to AMs isolated from non-COPD smokers and healthy volunteers [22,137]). Similar studies have been conducted for other major respiratory disease areas including asthma, respiratory infection, cystic fibrosis, and pulmonary fibrosis [22, 28–31,138].

An alternative human model of AMs uses circulating monocyte-derived macrophages (MDMs). Monocytes are isolated from the blood of human donors and differentiated in suspension with granulocyte–macrophage colony-stimulating factor (GM-CSF) to produce a cell population with a consistent and stable phenotype which exhibits lineage markers, Fc gamma receptors, adhesion molecules, antigen presentation co-receptors, and scavenger receptors that are claimed to be representative of AMs [139,140]. Studies using MDMs are advantageous in that they provide a plentiful and consistent supply of cells for experimentation. MDMs from healthy donors have been demonstrated to respond to stimuli such as cigarette smoke in ways that recapitulate responses described for AMs *in vivo* or *ex vivo*. These include activation of extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinase, production of interleukin 8 (IL-8, CXCL8) and inhibition of phagocytosis. Similar transcriptional profiles of regulated genes between derived cultures and *ex vivo* cells have also been noted [141,142]. MDMs derived from different patient groups have also been used to examine macrophage pathophysiology in diseases, such as asthma and COPD.

The extent to which MDMs truly represent AM responses has been investigated recently. With regard to phagocytic behavior, Taylor et al. [137] demonstrated that AMs and MDMs derived from the same donor and cultured under similar conditions were equally efficient at uptake of different particles (polystyrene beads, bacteria and dead cells). Notably, AMs and MDMs from COPD patients exhibited significantly impaired phagocytosis compared to healthy controls and non-COPD smokers [137].

The comparison of cell surface marker expression between AMs and MDMs in response to disease state or therapeutic intervention has been widely studied with mixed outcomes. For example, expression patterns of markers such as toll-like receptors in healthy donors [143] or human leukocyte antigen (HLA-DR) and CD80 in COPD patients [144] show distinct differences between AM and MDM populations. In contrast, Poliska et al. [145] identified overlapping, COPD-specific gene expression signatures (including surface markers) in AM and MDM populations, which correlated with lung function [145]. These studies show that careful selection and validation of biomarkers is required in studies where MDMs are used as surrogates for AMs. Although the use of MDM from patients with respiratory disease is an attractive option, the processes used to induce differentiation *in vitro* into AM-like cells may alter the characteristics of these cells compared to AMs of patients.

Notably, Tomlinson et al. [140] reported that human AMs collected from BAL and subsequently cultured demonstrated a transient pro-inflammatory transcriptional signature with elevated secretion of the pro-inflammatory mediators, IL-6 and TNF-α, which peaked at 4 h post collection and diminished over 24 h before returning to a non-inflammatory 'resting' state (both in terms of transcriptional regulation and cytokine secretion). MDMs from healthy volunteers did not show a pro-inflammatory phenotype over 48 h in culture [140]. This study highlights an important limitation associated with most *in vitro* AM

models, in that the isolation procedures associated with BAL collection and subsequent culture conditions influence the macrophage activation state, thus making it difficult to determine how well the *in vitro* study results correlate with *in vivo* responses [140,146].

4.5.3. Reconstituted tissue

Several advances in *in vitro* models using cultured cells have recently been reported, which aim to improve *in vitro* and *in vivo* correlations. Notably, triple [147,148] and tetra [149] co-cultures mimicking the alveolar region and containing lung epithelial, endothelial and macrophage cells have been reported. Three dimensional (3D) cell culture models provide realistic growth conditions for a variety of tissues including the lung, thus directing gene expression patterns and cell function towards a more physiological state [150]. To date there are few examples of 3D cultures for the lung, although preliminary studies show evidence of providing a more physiological cell phenotype compared to monolayer cultures. For example, Carterson et al. [151] report that a 3D culture of the lung alveolar epithelial-like A549 (adenocarcinoma-derived) cells increased expression of epithelial cell-specific markers, whilst decreasing expression of cancer-specific markers compared to monolayer grown cells. Further, 3D culture also promoted tight junction formation and cell polarity in comparison to cell monolayers [151]. However, most of the models reported to date do not incorporate macrophages [123,152], which renders them unsuitable for investigating AM-mediated adverse responses. Recently, the Vitrocell™ system, a tetraculture composed of A549 cells, differentiated macrophage-like cells (THP-1), mast cells (HMC-1) and endothelial cells (EA.hy 926), seeded in a 3D-orientation on a microporous membrane and grown under air-interface conditions, was reported as an improved testing system for *in vitro* screening of PM. The developers of the Vitrocell™ system claim that the model provides a much more physiologically relevant system in which to test inhaled PM, due to observations such as the accumulation of PM preferentially in the CD14+ THP-1 macrophage cells and more moderate inflammatory response to low toxicity, insoluble PM compared to monolayer systems.

Another notable development in *in vitro* systems is the so-called 'lung on a chip', which mimics the alveolar-capillary barrier through co-culture of lung epithelial and endothelial cell monolayers grown on a semi-permeable, flexible membrane. The serosal chamber is permeated with a flow of cell culture medium, whilst the mucosal side is grown under air interface conditions. The innovation of this system is the ability to apply a vacuum-driven fluctuation in air pressure to the mucosal chamber, which stretches the cells grown on the flexible membrane and mimics physiological breathing. The authors have shown that the mechanical deformation applied to the cell layers represents a more physiologically relevant system with regard to barrier permeability and response to exogenous stimuli [124,153–155]. Further, the model has recently been shown to mimic pulmonary edema, through perfusion of IL-2 *via* the serosal chamber, resulting in liquid leakage into the alveolar (air-filled) microchamber and a loss of barrier integrity [156]. Macrophages have yet to be incorporated into the 'lung on a chip' system, but this may be possible to provide a potentially useful inhalation toxicology screening technique.

5. Progress to the clinic

The outcomes of nonclinical toxicology investigations, including induced AM effects, are critical in determining whether to progress a drug candidate into human clinical studies and for the design of those studies, especially dose selection. Although a consensus is emerging about the interpretation of different AM responses in nonclinical studies using healthy animals [15], Regulatory Authorities and clinicians conducting clinical trials must satisfy themselves regarding the likelihood of adverse effects in humans where an increase in AMs has been observed nonclinically, *i.e.* are changes involving AMs likely to occur in human trial subjects (healthy volunteers or patients)? In patients with respiratory disease, an important question is how the disease background may reduce or enhance the ability of AMs to respond to inhaled drug in the way that was observed in nonclinical species.

If nonclinical observations do translate into human findings, the concern is whether AM induction will impact respiratory disease/pathology, *i.e.* the lung disease for which the inhaled medicine is being delivered. Although further increases in cellularity in an already inflamed airway such as asthma may be undesirable, it is not clear whether an adaptive or physiological macrophage response to PM would be of clinical significance compared to the inflammatory background. There are also potential effects of altered AM function as a result of inhaling PM such as compromised immune surveillance, impaired bacterial clearance and tissue remodeling and repair. Another concern is the possibility that non-adverse effects at doses used in nonclinical or human/patient studies may become adverse over a life-time of treatment, as in COPD.

Where uncomplicated nonclinical AM changes were reversible, most of these questions would not apply to healthy human volunteer studies and the level of concern by clinicians responsible for these studies would be minimal. The uncertainties regarding studies in patients with respiratory disease, are mitigated by the pragmatic approach of using large safety margins in dose setting and dose escalation in the clinic. Issues around the safety margins used in inhaled product development have been discussed previously [3]. A more scientific approach to dose setting and enabling progression from nonclinical testing to human testing would be the development and use of meaningful safety biomarkers; *i.e.* biomarkers that predict drug-induced changes before they became adverse/irreversible.

5.1. Biomarkers for safety monitoring in humans

A number of biomarkers of lung damage or function are currently used in clinical studies, but they are non-validated and limited in specificity, sensitivity and relevance to alveolar macrophage effects. The advantages and limitations of the biomarkers currently used or in development are described in Table 3.

In addition to the limitations noted in Table 3, current biomarkers do not provide the resolution provided by histopathological evaluation in animal studies. In addition, equivalent endpoints have not generally been evaluated in nonclinical studies; hence, there is currently no direct comparison between clinical and nonclinical data. Combining BAL biomarkers with some of the novel endpoints advocated elsewhere in this paper (*i.e.* genomic/proteomic markers of macrophage activation) may provide a translatable endpoint in the future. Another prospect is to label blood monocytes and use imaging to determine whether and how they are recruited into the lung. These approaches have the potential to provide step changes in the ability to translate nonclinical studies into humans, but will require extensive validation in nonclinical and clinical settings to realize their potential.

5.2. Case Study 3: biochemical biomarkers to support progress to the clinic

BAL has been used for several decades to identify the hazard of inhaled materials in nonclinical studies and to investigate clinical response and diagnoses in patients [157–159]. As analytes have expanded from simple toxicity assays (*e.g.*, total protein, and lactate dehydrogenase) to include cytokine arrays and other biochemical markers, a wider range of more sensitive information is being obtained. This information may be used in concert with other biomarkers to define early signs of adversity in nonclinical toxicology studies.

In the following case study, several dextran polymers of differing molecular size, dextran succinate 5 KDa (D5), 10 KDa (D10)and 20 KDa (D20), and side chain substitution (dextran 5 propanyl-succinate; D5PS) were compared in 7-day repeat dose inhalation rat studies (6–10 animals per group) at matched doses, aerosol concentrations, exposure times, and particle sizes (1.1 mg/kg/day; 0.3 mg/L; 60 min; ~2.4 μm MMAD) [160,161]. Further, D10 doses were expanded to include lower (0.1 mg/kg/day) and higher (8.7 and 34.4 mg/kg/day) levels. Subsequent to

Table 3
Biomarkers and functional markers for safety monitoring in humans.

	Biomarkers and functional markers	Advantages	Limitations
Cell-based	• Differential counts of inflammatory cells, macrophages and other cells in induced sputum	• Sputum samples are easy to obtain	• Sputum samples do not contain representative numbers of AMs • Routine evaluations of cytokines or activation states is not performed due to expense
	• Bronchoscopy and BAL	• Improved sampling of AMs compared to sputum • Cell counts plus cytokine assays/activation assays are routinely performed	Invasive sampling procedure Numbers of cells recovered is often variable and operator-dependent More representative of the airways rather than the alveolar region of the lungs
	• Lung tissue biopsy	• High cell numbers • Cell counts plus cytokine assays/activation assays are routinely performed, plus evaluation of other biomarkers, *e.g.* SP-D and SP-A, KL-6, HGF	Invasive sampling procedure Tissue from the bronchial wall; alveolar tissue sampling is rare Cells isolated from tissue biopsies may be more representative of IMs rather than AMs
Functional	Spirometry Forced vital capacity (FVC) and forced expiratory volume (FEV)	Well established method	Primarily reflects airway rather than alveolar function Not pathognomonic for specific pathologies Unlikely to be sensitive enough to detect modest effects, especially in animal models
	• Gas diffusion tests Diffusing capacity of the lung for carbon monoxide (DLCO)	• Well established method	• Not pathognomonic for specific pathologies • Unlikely to be sensitive enough to detect modest macrophage effects, especially in animal models • High variability endpoints

exposure, BAL was performed on the left lung while standard H&E slides were prepared from the right lung for histopathology. BAL analyses included cell differentials, LDH, total protein and a cytokine array (IL-2, 4, 6; TNF-α, IL-18, GRO-KC and RANTES).

AM responses were similar to control groups at all levels and physical parameters were unaffected in all groups. However, relative increases in total protein, LDH, total cells, PMNs, and cytokines (primarily IL-4, 6 and TNFα) in BAL reflected those groups that tended to have mixed inflammatory incidence that were above background air controls. The materials inducing these responses were D5, D5PS and D20. D10 produced responses at or above 8.7 mg/kg/day, but at 1.1 mg/kg day findings reflected control values. These data suggest that BAL parameters can be used to screen candidates for inflammatory potential, with these signals above the background variation in uncomplicated macrophage response across control and test groups providing an index of safety/toxicity in a nonclinical setting. Specifically this study suggests that selection and 'tuning' of compounds using BAL analysis in concert with histopathology as an index of safety may by possible.

5.3. Case Study 4: functional biomarkers to support progress to the clinic

The use of pulmonary function tests (PFT) has long been a standard of care in patients with COPD, asthma, and fibrosis [162–165]. PFT are used routinely to assess the efficacy of new formulations of 'old drugs' (*e.g.*, beta agonists, muscarinic antagonists) and novel treatments in clinical studies. In a nonclinical setting, it is generally expected that these assays will be adversely affected during toxicology studies only at extreme burdens of inert particles where substantial histopathology is observed. However, it remains to be demonstrated whether or not more sensitive PFT assays (*e.g.*, DLCO and vital capacity) could be used as a translational marker in nonclinical and clinical development of drugs in which a macrophage response is observed.

The following example illustrates a study design in which the most sensitive PFT available, DLCO and vital capacity, were used to provide enhanced reassurance for progression of drug into clinical testing. A novel antibody targeting αvβ6 integrin has previously been administered subcutaneously to mice (murine form) and cynomologus macaques to provide dose ranging information for Good Laboratory Practice toxicology studies and clinical dosing [166–168]. Anti-αvβ6 is currently in clinical development for the treatment of pulmonary fibrosis based on its potential inhibition of cleavage of latent TGF-β by αvβ6. The latter is essential in the deposition of collagen matrix in the fibrotic process.

Doses of 0.3, 0.6, 1.0 and 20 mg/kg/day and 0.1 and 10.0 mg/kg/day were administered by inhalation for 28 consecutive days in the mouse and NHP studies, respectively. Consistent with earlier murine studies, a response was induced that was indistinguishable by microscopic examination from the minimal to mild uncomplicated AM response seen upon inhalation of inhaled pharmaceuticals or inert PM [169,170]. A threshold was observed at levels at or above 10 mg/kg. At these doses, a minimal to mild mixed cell inflammatory response was induced. When DLCO and vital capacity were assessed after treatment and a 4 week recovery period, no adverse effect of treatment was observed in either species. These data were used to support further development and eventual clinical administration of anti-αvβ6. This study illustrates how sensitive PFT measures can be used to support progress to the clinic.

6. Conclusions and future perspectives

The APSGB-HESI workshop highlighted many areas in which progress has been made in our understanding of AM biology. This included a number of assays that are currently being used or are being developed in an attempt to predict much earlier those AM responses which are likely to affect the development pathway for inhaled pharmaceuticals. However, there remains a long way to go to optimize the models and batteries of tests currently being used for this purpose, and to utilize fully the scientific and technical knowledge available to our best advantage in this endeavor. A summary of the current unknowns and the research approaches that might help address these is provided in Table 4.

A greater role for *in vitro* testing is currently limited by the absence of reliable or validated assays for AM responses to inhaled medicines. There is a lack of multicellular/organotypic cellular models which include an AM component. Since it is clear that the response of AMs to inhaled particles depends not only on the nature of the material itself, but also on the cross-talk with other cells of the immune system and the respiratory tract, further advances in this area are needed to provide models which more closely resemble the *in vivo* situation. There are examples of single cell [53,171], and multicellular systems in use [172–176], but none can be considered optimized for studying and predicting the response of AMs to inhaled materials *in vivo* and information on their human relevance is uncertain. In addition, it is clear that cell type (cell line *versus* normal or patient-derived primary cells) and culture conditions within these systems can influence the phenotype exhibited by cultured cells. For example, air–liquid interface *versus* submerged cultures [175], substrate type and stiffness [177], and medium components influence macrophage

Table 4
A summary of the research approaches that may help address the challenge of measuring and interpreting macrophage responses to inhaled medicines.

Challenge	Unknown/unmet need	Research approach
Scientific understanding	Macrophage phenotyping	Develop methods to define macrophage responses as adaptive or adverse, *e.g.* based on biochemical, morphometric or functional indicators.
	Longitudinal studies	Utilize study designs that address the question of whether macrophage responses resolve, stabilize or progress over time, *e.g.* long term studies, use of imaging to track responses.
	Mechanisms of response	Understand the biological mechanisms that lead to different types of macrophage responses and the consequences of this. Define the underpinning cellular pathways that indicate an adverse macrophage response through the use of appropriate investigative tools
Candidate selection and lead optimization for industry	Predictive science	Develop algorithms for predicting adversity/safety based on dose, material, and macrophage responses observed *in vitro* and *in vivo* during non-clinical development.
	Discriminatory assays	Define discriminatory screens that provide early assessment of whether compounds will induce macrophage responses during non-clinical and clinical development, *e.g.* improved *in vitro* assays (cell systems, biomarkers, toxicological or functional end-points).
Clinical relevance and monitoring	Translation	Establish how responses to different inhaled materials translate between species and between healthy lungs and those with respiratory disease.
	Clinical monitoring	Develop validated non-invasive monitoring techniques and biomarkers to enable clinical development of projects where induced macrophage responses have been observed.

polarization and our understanding of how best to control this needs to be improved. Likewise, the differences between MDMs and resident AMs need to be better understood in the context of developing optimal models for studying AM responses—are MDMs a good surrogate? There is also a need for nisms of, and sequelae to, the cellular response that leads to the 'foamy' appearance. There is also a lack of *in vitro* models which support the translation of responses in nonclinical species to humans. Most recent advances in cell and tissue culture have focused on the use of human cells/tissues [176], but systems are also required to understand how nonclinical species will react to inhaled materials so that this can be related to responses observed in human volunteers and patients. In addition to more complex models, a truly high throughput method is needed for rapid screening during drug discovery.

One of the most critical issues with respect to AM findings during inhaled drug development is to establish and define clearly what is considered to be an adverse AM response as differentiated from an adaptive response. Some progress has been made here in rationalizing the adaptive nature of an uncomplicated macrophage-only response [15] and making recommendations for reporting pathology results consistently and appropriately. However, methods are unavailable to monitor induction of macrophages in the clinic and provide confirmatory safety reassurance. Existing pulmonary function tests are not sufficiently sensitive to detect early signs of an adverse response, thus selective and sensitive cell or molecular biomarkers are still required. Ideally, such assays would be minimally-invasive, *e.g.* based on blood/plasma biomarkers or imaging techniques. Building on recent advances in our understanding of the various markers of AM phenotype (*e.g.* cell surface markers, cytokine release profiles) may go some way towards achieving this goal. The use of disease models for toxicology studies is a complex and under-developed field and has the potential to investigate questions regarding the impact of respiratory disease on AM responses to inhaled drugs.

In the field of particle toxicology much work has been done to understand how the physical and chemical characteristics of particles can influence toxicity, and to identify some of the pitfalls when studying these effects experimentally [178,179]. Coupling these new approaches with a better understanding of PK/PD in the lung (*e.g.* dissolution rates, transfer rates across respiratory epithelium—see Section 4.2) could lead to an improved ability to predict the behavior of inhaled drugs and specifically their likely effects on AMs in animals, healthy human volunteers and patients.

A number of initiatives designed to reduce attrition due to AM-related safety concerns are in various stages of advancement and have been highlighted in this review. These efforts begin with harmonization of terminology in pathology reporting across the pharmaceutical industry, an important step which will enhance both internal development and regulatory processes. A bolder step in this direction would be the development of a cross-industry consensus on nonclinical study designs to evaluate responses and agree upon cut-offs/dosing limits for studies to aid regulatory comparisons and allow a unified critical regulatory review process.

There are currently a plethora of *in vitro* methods and culture systems available which are, or could be, used for increasing our fundamental understanding of AM biology. However, none are currently qualified to predict likely adverse effects which may occur later in development, and so enable better candidate selection decisions early in development. It is clear that many pharmaceutical companies have invested varying degrees of effort in evaluating some of these systems, but that no clear definition of an optimum system or battery of systems currently exists which addresses this. Recent examples of pre-competitive data sharing initiatives [153,180] have shown the value of sharing experiences, methodologies and data, and of working together on the scientific issues that surround such problems. We suggest that this approach would be invaluable here, allowing bigger data sets to be analysed, experiences of what works and what does not to be shared, and ultimately to achieve the advancement of predictive science in inhaled drug development with the goal of reducing safety related attrition. To this end, organizations are in the early stages of initiating a collaboration to enable longitudinal and non-invasive assessment of inflammation and foamy macrophages in the same animal through a series of dose escalation stages [181].

In summary, an improved essential understanding of AM responses in the context of inhaled drug development is fundamental to reducing safety-related attrition during inhaled drug development. Advances in macrophage bioscience would underpin the development of better methods for early identification of compounds with adverse safety profiles and, by enabling evidence-based decision making, ease the development pathway for drug candidates or formulations that induce non-adverse AM responses.

Declaration of interests

This publication stems from a subgroup of the HESI Immunotoxicology Technical Committee, whose work is funded through ILSI HESI, in collaboration with the 'Drugs in the Lungs' Network of the Inhalation Focus Group of the Academy of Pharmaceutical Sciences of Great Britain.

Acknowledgments

Thank you to Megan Harries (HESI) for support with the referencing for this manuscript.

References

[1] A.J. Hickey, Back to the future: inhaled drug products, J. Pharm. Sci. 102 (2013) 1165–1172.
[2] C.A. Ruge, J. Kirch, C.-M. Lehr, Pulmonary drug delivery: from generating aerosols to overcoming biological barriers? Therapeutic possibilities and technological challenges, Lancet Respir. Med. 1 (2013) 402–413.

[3] B. Forbes, B. Asgharian, L.A. Dailey, D. Ferguson, P. Gerde, M. Gumbleton, L. Gustavsson, C. Hardy, D. Hassall, R. Jones, R. Lock, J. Maas, T. McGovern, G.R. Pitcairn, G. Somers, R.K. Wolff, Challenges in inhaled product development and opportunities for open innovation, Adv. Drug Deliv. Rev. 63 (2011) 69–87.
[4] Academy of Pharmaceutical Sciences, APS Inhaled Drug Development, accessed September 1, 2013, available at http://www.apsgb.org/drugsinthelungs2012/.
[5] J. Arrowsmith, A decade of change, Nat. Rev. Drug Discov. 11 (2012) 17–18.
[6] R.M. Jones, N. Neef, Interpretation and prediction of inhaled drug particle accumulation in the lung and its associated toxicity, Xenobiotica 42 (2012) 86–93.
[7] M.C. McElroy, C. Kirton, D. Gliddon, R.K. Wolff, Inhaled biopharmaceutical drug development: nonclinical considerations and case studies, Inhal. Toxicol. 25 (2013) 219–232.
[8] International Conference on Harmonization, Topic M3(R2): non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals, report CPMP/ICH/286/95, ICH, Geneva, Switzerland, 2009.
[9] R.K. Wolff, M.A. Dorato, Inhalation toxicity studies, in: J. Lamb (Ed.), Comprehensive Toxicology, Elsevier, Oxford, 2010, pp. 226–245.
[10] K.L. Hentz, Safety assessment of pharmaceuticals, in: J. Lamb (Ed.), Comprehensive Toxicology, Elsevier, Oxford, 2010, pp. 18–28.
[11] K. Owen, Regulatory toxicology considerations for the development of inhaled pharmaceuticals, Drug Chem. Toxicol. (2012).
[12] R. Chamanza, H.A. Marxfeld, A.I. Blanco, S.W. Naylor, A.E. Bradley, Incidences and range of spontaneous findings in control cynomolgus monkeys (*Macaca fascicularis*) used in toxicity studies, Toxicol. Pathol. 38 (2010) 642–657.
[13] D.J. Lewis, T.P. McKevitt, Respiratory system, in: P.J.A. Sahota P., J.F. Hardisty, C. Gopinath (Eds.), Toxicological Pathology: Non-Clinical Safety Assessment, CRC Press, 2013, pp. 367–420.
[14] R. Renne, A. Brix, J. Harkema, R. Herbert, B. Kittel, D. Lewis, T. March, K. Nagano, M. Pino, S. Rittinghausen, M. Rosenbruch, P. Tellier, T. Wohrmann, Proliferative and nonproliferative lesions of the rat and mouse respiratory tract, Toxicol. Pathol. 37 (2009) 5S–73S.
[15] K.J. Nikula, J.E. McCartney, T. McGovern, G.K. Miller, M. Odin, M.V. Pino, M.D. Reed, STP position paper: interpreting the significance of increased alveolar macrophages in rodents following inhalation of pharmaceutical materials, Toxicol. Pathol. (October 31 2013) (Available online).
[16] D.H. Bowden, I.Y.R. Adamson, Pulmonary interstitial cell as immediate precursor of alveolar macrophage, Am. J. Pathol. 66 (1972) A56.
[17] D.H. Bowden, The alveolar macrophage, Environ. Health Perspect. 55 (1984) 327–341.
[18] M. Fathi, A. Johansson, M. Lundborg, L. Orre, C.M. Skold, P. Camner, Functional and morphological differences between human alveolar and interstitial macrophages, Exp. Mol. Pathol. 70 (2001) 77–82.
[19] D.L. Laskin, B. Weinberger, J.D. Laskin, Functional heterogeneity in liver and lung macrophages, J. Leukoc. Biol. 70 (2001) 163–170.
[20] S. Gordon, P.R. Taylor, Monocyte and macrophage heterogeneity, Nat. Rev. Immunol. 5 (2005) 953–964.
[21] D. Hashimoto, A. Chow, C. Noizat, P. Teo, M.B. Beasley, M. Leboeuf, C.D. Becker, P. See, J. Price, D. Lucas, M. Greter, A. Mortha, S.W. Boyer, E.C. Forsberg, M. Tanaka, N. van Rooijen, A. Garcia-Sastre, E.R. Stanley, F. Ginhoux, P.S. Frenette, M. Merad, Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes, Immunity 38 (2013) 792–804.
[22] P.J. Barnes, Alveolar macrophages in chronic obstructive pulmonary disease (COPD), Cell. Mol. Biol. 50 (2004) 627–637.
[23] P.G. Holt, D.H. Strickland, M.E. Wikstrom, F.L. Jahnsen, Regulation of immunological homeostasis in the respiratory tract, Nat. Rev. Immunol. 8 (2008) 142–152.
[24] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol. 8 (2008) 958–969.
[25] L. Cassetta, E. Cassol, G. Poli, Macrophage polarization in health and disease, Sci. World J. 11 (2011) 2391–2402.
[26] A. Munitz, E.B. Brandt, M. Mingler, F.D. Finkelmant, M.E. Rothenberg, Distinct roles for IL-13 and IL-4 via IL-13 receptor alpha 1 and the type II IL-4 receptor in asthma pathogenesis, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 7240–7245.
[27] J.G. Quatromoni, E. Eruslanov, Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer, Am. J. Transl. Res. 4 (2012) 376–389.
[28] C. Sorio, P. Melotti, Editorial: the role of macrophages and their scavenger receptors in cystic fibrosis, J. Leukoc. Biol. 86 (2009) 465–468.
[29] R.J. Homer, J.A. Elias, C.G. Lee, E. Herzog, Modern concepts on the role of inflammation in pulmonary fibrosis, Arch. Pathol. Lab. Med. 135 (2011) 780–788.
[30] J. Balhara, A.S. Gounni, The alveolar macrophages in asthma: a double-edged sword, Mucosal Immunol. 5 (2012) 605–609.
[31] K.C. Patterson, K. Hogarth, A.N. Husain, A.I. Sperling, T.B. Niewold, The clinical and immunologic features of pulmonary fibrosis in sarcoidosis, Transl. Res. 160 (2012) 321–331.
[32] K. Sertl, T. Takemura, E. Tschachler, V.J. Ferrans, M.A. Kaliner, E.M. Shevach, Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura, J. Exp. Med. 163 (1986) 436–451.
[33] B.N. Lambrecht, H. Hammad, Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology, Annu. Rev. Immunol. 30 (2012) 243–270.
[34] M.A. Willart, H. Jan de Heer, H. Hammad, T. Soullie, K. Deswarte, B.E. Clausen, L. Boon, H.C. Hoogsteden, B.N. Lambrecht, The lung vascular filter as a site of immune induction for T cell responses to large embolic antigen, J. Exp. Med. 206 (2009) 2823–2835.
[35] C.A. Pope, D.W. Dockery, J. Schwartz, Review of epidemiological evidence of health-effects of particulate air-pollution, Inhal. Toxicol. 7 (1995) 1–18.
[36] F.J. Kelly, J.C. Fussell, Air pollution and airway disease, Clin. Exp. Allergy 41 (2011) 1059–1071.
[37] J. Heyder, Deposition of inhaled particles in the human respiratory tract and consequences for regional targeting in respiratory drug delivery, Proc. Am. Thorac. Soc. 1 (2004) 315–320.
[38] M.B. Snipes, R.O. Mcclellan, J.L. Mauderly, R.K. Wolff, Retention patterns for inhaled particles in the lung—comparisons between laboratory-animals and humans for chronic exposures, Health Phys. 57 (1989) 69–78.
[39] M. Geiser, Update on macrophage clearance of inhaled micro- and nanoparticles, J. Aerosol. Med. Pulm. Drug Deliv. 23 (2010) 207–217.
[40] M.B. Snipes, Species comparison for pulmonary retention of inhaled particles, in: R.O. McClellan, R.F. Henderson (Eds.), Concepts in Inhalation Toxicology, Hemisphere Publishing Corporation, New York, 1989, pp. 193–227.
[41] M.B. Snipes, Long-term retention and clearance of particles inhaled by mammalian species, Crit. Rev. Toxicol. 20 (1989) 175–211.
[42] D.M. Underhill, H.S. Goodridge, Information processing during phagocytosis, Nat. Rev. Immunol. 12 (2012) 492–502.
[43] L. Kobzik, Lung macrophage uptake of unopsonized environmental particulates—role of scavenger-type receptors, J. Immunol. 155 (1995) 367–376.
[44] R.F. Hamilton, S.A. Thakur, J.K. Mayfair, A. Holian, MARCO mediates silica uptake and toxicity in alveolar macrophages from C57BL/6 mice, J. Biol. Chem. 281 (2006) 34218–34226.
[45] S. Bhattacharjee, L.H. de Haan, N.M. Evers, X. Jiang, A.T. Marcelis, H. Zuilhof, I.M. Rietjens, G.M. Alink, Role of surface charge and oxidative stress in cytotoxicity of organic monolayer-coated silicon nanoparticles towards macrophage NR8383 cells, Part. Fibre Toxicol. 7 (2010) 25.
[46] C. Muhlfeld, B. Rothen-Rutishauser, F. Blank, D. Vanhecke, M. Ochs, P. Gehr, Interactions of nanoparticles with pulmonary structures and cellular responses, Am. J. Physiol. Cell Physiol. 294 (2008) L817–L829.
[47] J.A. Champion, S. Mitragotri, Role of target geometry in phagocytosis, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 4930–4934.
[48] X. Banquy, F. Suarez, A. Argaw, J.M. Rabanel, P. Grutter, J.F. Bouchard, P. Hildgen, S. Giasson, Effect of mechanical properties of hydrogel nanoparticles on macrophage cell uptake, Soft Matter 5 (2009) 3984–3991.
[49] G. Ng, K. Sharma, S.M. Ward, M.D. Desrosiers, L.A. Stephens, W.M. Schoel, T.L. Li, C.A. Lowell, C.C. Ling, M.W. Amrein, Y. Shi, Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells, Immunity 29 (2008) 807–818.
[50] R. Miyata, S.F. van Eeden, The innate and adaptive immune response induced by alveolar macrophages exposed to ambient particulate matter, Toxicol. Appl. Pharmacol. 257 (2011) 209–226.
[51] N. Riteau, L. Baron, B. Villeret, N. Guillou, F. Savigny, B. Ryffel, F. Rassendren, M. Le Bert, A. Gombault, I. Couillin, ATP release and purinergic signaling: a common pathway for particle-mediated inflammasome activation, Cell Death Dis. 3 (2012).
[52] J. Karavitis, E.J. Kovacs, Macrophage phagocytosis: effects of environmental pollutants, alcohol, cigarette smoke, and other external factors, J. Leukoc. Biol. 90 (2011) 1065–1078.
[53] S. Lanone, F. Rogerieux, J. Geys, A. Dupont, E. Maillot-Marechal, J. Boczkowski, G. Lacroix, P. Hoet, Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines, Part. Fibre Toxicol. 6 (2009) 14.
[54] K.M. Waters, L.M. Masiello, R.C. Zangar, B.J. Tarasevich, N.J. Karin, R.D. Quesenberry, S. Bandyopadhyay, J.G. Teeguarden, J.G. Pounds, B.D. Thrall, Macrophage responses to silica nanoparticles are highly conserved across particle sizes, Toxicol. Sci. 107 (2009) 553–569.
[55] D.B. Warheit, J.F. Hansen, I.S. Yuen, D.P. Kelly, S.I. Snajdr, M.A. Hartsky, Inhalation of high concentrations of low toxicity dusts in rats results in impaired pulmonary clearance mechanisms and persistent inflammation, Toxicol. Appl. Pharmacol. 145 (1997) 10–22.
[56] A. Elder, R. Gelein, J.N. Finkelstein, K.E. Driscoll, J. Harkema, G. Oberdorster, Effects of subchronically inhaled carbon black in three species. I. Retention kinetics, lung inflammation, and histopathology, Toxicol. Sci. 88 (2005) 614–629.
[57] K.E. Driscoll, J.M. Carter, B.W. Howard, D.G. Hassenbein, W. Pepelko, R.B. Baggs, G. Oberdorster, Pulmonary inflammatory, chemokine, and mutagenic responses in rats after subchronic inhalation of carbon black, Toxicol. Appl. Pharmacol. 136 (1996) 372–380.
[58] K.P. Lee, N.W. Henry III, H.J. Trochimowicz, C.F. Reinhardt, Pulmonary response to impaired lung clearance in rats following excessive TiO2 dust deposition, Environ. Res. 41 (1986) 144–167.
[59] K.M. Giles, K. Ross, A.G. Rossi, N.A. Hotchin, C. Haslett, I. Dransfield, Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac, J. Immunol. 167 (2001) 976–986.
[60] A. Baldan, P. Tarr, C.S. Vales, J. Frank, T.K. Shimotake, S. Hawgood, P.A. Edwards, Deletion of the transmembrane transporter ABCG1 results in progressive pulmonary lipidosis, J. Biol. Chem. 281 (2006) 29401–29410.
[61] A.J. Wojcik, M.D. Skaflen, S. Srinivasan, C.C. Hedrick, A critical role for ABCG1 in macrophage inflammation and lung homeostasis, J. Immunol. 180 (2008) 4273–4282.
[62] M.J. Reasor, S. Kacew, Drug-induced phospholipidosis: are there functional consequences? Exp. Biol. Med. 226 (2001) 825–830.
[63] D. Ordway, M. Henao-Tamayo, I.M. Orme, M. Gonzalez-Juarrero, Foamy macrophages within lung granulomas of mice infected with *Mycobacterium tuberculosis* express molecules characteristic of dendritic cells and antiapoptotic markers of the TNF receptor-associated factor family, J. Immunol. 175 (2005) 3873–3881.
[64] P.J. Cardona, R. Llatjos, S. Gordillo, J. Diaz, I. Ojanguren, A. Ariza, V. Ausina, Evolution of granulomas in lungs of mice infected aerogenically with *Mycobacterium tuberculosis*, Scand. J. Immunol. 52 (2000) 156–163.
[65] H. Muller, S. Kruger, Immunohistochemical analysis of cell composition and in situ cytokine expression in HIV- and non-HIV-associated tuberculous lymphadenitis, Immunobiology 191 (1994) 354–368.
[66] N. Hirama, Y. Shibata, K. Otake, J. Machiya, T. Wada, S. Inoue, S. Abe, N. Takabatake, M. Sata, I. Kubota, Increased surfactant protein-D and foamy macrophages in smoking-induced mouse emphysema, Respirology 12 (2007) 191–201.
[67] Z. Hruban, Pulmonary and generalized lysosomal storage induced by amphiphilic drugs, Environ. Health Perspect. 55 (1984) 53–76.

[68] T. Nonoyama, R. Fukuda, Drug-induced phospholipidosis—pathological aspects and its prediction, J. Toxicol. Pathol. 21 (2008) 9–24.
[69] J. Pauluhn, Inhaled cationic amphiphilic drug-induced pulmonary phospholipidosis in rats and dogs: time-course and dose–response of biomarkers of exposure and effect, Toxicology 207 (2005) 59–72.
[70] S. Rajagopalan, X.P. Meng, S. Ramasamy, D.G. Harrison, Z.S. Galis, Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability, J. Clin. Invest. 98 (1996) 2572–2579.
[71] K.E. Driscoll, J.K. Maurer, Cytokine and growth factor release by alveolar macrophages: potential biomarkers of pulmonary toxicity, Toxicol. Pathol. 19 (1991) 398–405.
[72] S. Gessi, K. Varani, S. Merighi, E. Ongini, P.A. Borea, A(2A) adenosine receptors in human peripheral blood cells, Br. J. Pharmacol. 129 (2000) 2–11.
[73] E. Ongini, B.B. Fredholm, Pharmacology of adenosine A2A receptors, Trends Pharmacol. Sci. 17 (1996) 364–372.
[74] R.B. Schlesinger, Deposition and clearance of inhaled particles, in: R.O. McClellan, R.F. Henderson (Eds.), Concepts in Inhalation Toxicology, Hemisphere Publishing Corporation, New York, 1986, pp. 163–192.
[75] R.M. Seguin, N. Ferrari, Emerging oligonucleotide therapies for asthma and chronic obstructive pulmonary disease, Expert Opin. Investig. Drugs 18 (2009) 1505–1517.
[76] H. Imaoka, H. Campbell, I. Babirad, R.M. Watson, M. Mistry, R. Sehmi, G.M. Gauvreau, TPI ASM8 reduces eosinophil progenitors in sputum after allergen challenge, Clin. Exp. Allergy 41 (2011) 1740–1746.
[77] D. Devuni, G.Y. Wu, ALN-RSV01: siRNA targeting RSV nucleocapsid N gene treatment of RSV infection, Drugs Future 34 (2009) 781–783.
[78] A.A. Levin, A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides, Biochim. Biophys. Acta 1489 (1999) 69–84.
[79] S.P. Henry, R.S. Geary, R. Yu, A.A. Levin, Drug properties of second-generation antisense oligonucleotides: how do they measure up to their predecessors? Curr. Opin. Investig. Drugs 2 (2001) 1444–1449.
[80] S.K. Srinivasan, P. Iversen, Review of in vivo pharmacokinetics and toxicology of phosphorothioate oligonucleotides, J. Clin. Lab. Anal. 9 (1995) 129–137.
[81] P.L. Iversen, B.L. Copple, H.K. Tewary, Pharmacology and toxicology of phosphorothioate oligonucleotides in the mouse, rat, monkey and man, Toxicol. Lett. 82–83 (1995) 425–430.
[82] S.A. Burel, S.R. Han, H.S. Lee, D.A. Norris, B.S. Lee, T. Machemer, S.Y. Park, T. Zhou, G. He, Y. Kim, A.R. Macleod, B.P. Monia, S. Lio, T.W. Kim, S.P. Henry, Preclinical evaluation of the toxicological effects of a novel constrained ethyl modified antisense compound targeting signal transducer and activator of transcription 3 in mice and cynomolgus monkeys, Nucleic Acid Ther. 23 (2013) 213–227.
[83] S.P. Henry, T.-W. Kim, K. Kramer-Strickland, T.A. Zanardi, R.A. Fey, A.A. Levin, Toxicological properties of 2′O-methoxyethyl chimeric antisense inhibitors in animals and man, in: S.T. Crooke (Ed.), Antisense Drug Technology, CRC Press, 2008, pp. 327–363.
[84] T. Koch, H. Orum, Locked nucleic acid, in: S.T. Crooke (Ed.), Antisense Drug Technology, CRC Press, 2008, pp. 519–564.
[85] E.W. Alton, H.A. Boushey, H. Garn, F.H. Green, M. Hodges, R.J. Martin, R.D. Murdoch, H. Renz, S.B. Shrewsbury, R. Seguin, G. Johnson, J.D. Parry, J. Tepper, P. Renzi, J. Cavagnaro, N. Ferrari, Clinical expert panel on monitoring potential lung toxicity of inhaled oligonucleotides: consensus points and recommendations, Nucleic Acid Ther. 22 (2012) 246–254.
[86] M.V. Templin, A.A. Levin, M.J. Graham, P.M. Aberg, B.I. Axelsson, M. Butler, R.S. Geary, C.F. Bennett, Pharmacokinetic and toxicity profile of a phosphorothioate oligonucleotide following inhalation delivery to lung in mice, Antisense Nucleic Acid Drug Dev. 10 (2000) 359–368.
[87] A. Guimond, E. Viau, P. Aube, P.M. Renzi, L. Paquet, N. Ferrari, Advantageous toxicity profile of inhaled antisense oligonucleotides following chronic dosing in non-human primates, Pulm. Pharmacol. Ther. 21 (2008) 845–854.
[88] A. Brasey, R. Igue, L. Djemame, S. Séguin, P. Renzi, N. Ferrari, R. Seguin, The effect of in vitro exposure to antisense oligonucleotides on macrophage morphology and function, J.Nucleic Acids Investig. 2 (2011) 77–84.
[89] K.H. Baek, S.J. Ha, Y.C. Sung, A novel function of phosphorothioate oligodeoxynucleotides as chemoattractants for primary macrophages, J. Immunol. 167 (2001) 2847–2854.
[90] M.A. Videira, M.F. Botelho, A.C. Santos, L.F. Gouveia, J.J. de Lima, A.J. Almeida, Lymphatic uptake of pulmonary delivered radiolabelled solid lipid nanoparticles, J. Drug Target. 10 (2002) 607–613.
[91] F. Blank, P.A. Stumbles, E. Seydoux, P.G. Holt, A. Fink, B. Rothen-Rutishauser, D.H. Strickland, C. von Garnier, Size-dependent uptake of particles by pulmonary antigen-presenting cell populations and trafficking to regional lymph nodes, Am. J. Respir. Cell Mol. Biol. 49 (2013) 67–77.
[92] G.L. McIntire, E.R. Bacon, J.L. Toner, J.B. Cornacoff, P.E. Losco, K.J. Illig, K.J. Nikula, B.A. Muggenburg, L. Ketai, Pulmonary delivery of nanoparticles of insoluble, iodinated CT X-ray contrast agents to lung draining lymph nodes in dogs, J. Pharm. Sci. 87 (1998) 1466–1470.
[93] M. Roland, T.N. Tozer (Eds.), Clinical Pharmacokinetics and Pharmacodynamics: concepts and applications, 4th ed., Lippincott, Wiliams and Wilkins, USA, 2011.
[94] G. Oberdorster, J. Ferin, P.E. Morrow, Volumetric loading of alveolar macrophages (AM): a possible basis for diminished AM-mediated particle clearance, Exp. Lung Res. 18 (1992) 87–104.
[95] A.E. Cooper, D. Ferguson, K. Grime, Optimisation of DMPK by the inhaled route: challenges and approaches, Curr. Drug Metab. 13 (2012) 457–473.
[96] G.J. van der Brugge-Gamelkoorn, C.D. Dijkstra, T. Sminia, Characterization of pulmonary macrophages and bronchus-associated lymphoid tissue (BALT) macrophages in the rat. An enzyme-cytochemical and immunocytochemical study, Immunobiology 169 (1985) 553–562.
[97] S.M. Farragher, A. Tanney, R.D. Kennedy, D. Paul Harkin, RNA expression analysis from formalin fixed paraffin embedded tissues, Histochem. Cell Biol. 130 (2008) 435–445.
[98] S. Curran, G.I. Murray, An introduction to laser-based tissue microdissection techniques, Methods Mol. Biol. 293 (2005) 3–8.
[99] L. Roberts, J. Bowers, K. Sensinger, A. Lisowski, R. Getts, M.G. Anderson, Identification of methods for use of formalin-fixed, paraffin-embedded tissue samples in RNA expression profiling, Genomics 94 (2009) 341–348.
[100] E. Wissinger, J. Goulding, T. Hussell, Immune homeostasis in the respiratory tract and its impact on heterologous infection, Semin. Immunol. 21 (2009) 147–155.
[101] C.S. Herrington, Demystified—in situ hybridisation, Mol. Pathol. 51 (1998) 8–13.
[102] M.S. Sicchitano, D.A. Dalmas, R.W. Boyce, H.C. Thomas, K.S. Frazier, Protein extraction of formalin-fixed, paraffin-embedded tissue enables robust proteomic profiles by mass spectrometry, J. Histochem. Cytochem. 57 (2009) 849–860.
[103] K.M. Linton, Y. Hey, E. Saunders, M. Jeziorska, J. Denton, C.L. Wilson, R. Swindell, S. Dibben, C.J. Miller, S.D. Pepper, J.A. Radford, A.J. Freemont, Acquisition of biologically relevant gene expression data by Affymetrix microarray analysis of archival formalin-fixed paraffin-embedded tumours, Br. J. Cancer 98 (2008) 1403–1414.
[104] F.M. Benes, N. Lange, Two-dimensional versus three-dimensional cell counting: a practical perspective, Trends Neurosci. 24 (2001) 11–17.
[105] J.-Y. Chung, T. Braunschweig, R. Williams, N. Guerrero, K.M. Hoffmann, M. Kwon, Y.K. Song, S.K. Libutti, S.M. Hewitt, Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue, J. Histochem. Cytochem. 56 (2008) 1033–1042.
[106] E. Bermudez, J.B. Mangum, B. Asgharian, B.A. Wong, E.E. Reverdy, D.B. Janszen, P.M. Hext, D.B. Warheit, J.I. Everitt, Long-term pulmonary responses of three laboratory rodent species to subchronic inhalation of pigmentary titanium dioxide particles, Toxicol. Sci. 70 (2002) 86–97.
[107] J.L. Mauderly, Relevance of particle-induced rat lung tumors for assessing lung carcinogenic hazard and human lung cancer risk, Environ. Health Perspect. 105 (Suppl. 5) (1997) 1337–1346.
[108] U. Saffiotti, L.N. Daniel, Y. Mao, A.O. Williams, M.E. Kaighn, N. Ahmed, A.D. Knapton, Biological studies on the carcinogenic mechanisms of quartz, Rev. Mineral. Geochem. 28 (1993) 523–544.
[109] K.J. Nikula, K.J. Avila, W.C. Griffith, J.L. Mauderly, Lung tissue responses and sites of particle retention differ between rats and cynomolgus monkeys exposed chronically to diesel exhaust and coal dust, Fundam. Appl. Toxicol. 37 (1997) 37–53.
[110] J.L. Mauderly, D.A. Banas, W.C. Griffith, F.F. Hahn, R.F. Henderson, R.O. McClellan, Diesel exhaust is not a pulmonary carcinogen in CD-1 mice exposed under conditions carcinogenic to F344 rats, Fundam. Appl. Toxicol. 30 (1996) 233–242.
[111] R.F. McLaughlin, W.S. Tyler, R.O. Canada, A study of the subgross pulmonary anatomy in various mammals, Am. J. Anat. 108 (1961) 149–165.
[112] R.F. McLaughlin Jr., W.S. Tyler, R.O. Canada, Subgross pulmonary anatomy of the rabbit, rat, and guinea pig, with additional notes on the human lung, Am. Rev. Respir. Dis. 94 (1966) 380–387.
[113] J.L. Mauderly, Usefulness of animal models for predicting human responses to long-term inhalation of particles, Chest 109 (1996) 65S–68S.
[114] K.J. Nikula, V. Vallyathan, F.H. Green, F.F. Hahn, Influence of exposure concentration or dose on the distribution of particulate material in rat and human lungs, Environ. Health Perspect. 109 (2001) 311–318.
[115] J.M. Lauweryns, J.H. Baert, The role of the pulmonary lymphatics in the defenses of the distal lung: morphological and experimental studies of the transport mechanisms of intratracheally instillated particles, Ann. N. Y. Acad. Sci. 221 (1974) 244–275.
[116] F.H. Green, V. Vallyathan, F.F. Hahn, Comparative pathology of environmental lung disease: an overview, Toxicol. Pathol. 35 (2007) 136–147.
[117] B. Bellmann, H. Muhle, O. Creutzenberg, R. Mermelstein, Irreversible pulmonary changes induced in rat lung by dust overload, Environ. Health Perspect. 97 (1992) 189–191.
[118] M. Dorger, A.M. Allmeling, A. Neuber, J. Behr, W. Rambeck, F. Krombach, Interspecies comparison of rat and hamster alveolar macrophage antioxidative and oxidative capacity, Environ. Health Perspect. 105 (Suppl. 5) (1997) 1309–1312.
[119] M. Dorger, S. Munzing, A.M. Allmeling, F. Krombach, Comparison of the phagocytic response of rat and hamster alveolar macrophages to man-made vitreous fibers in vitro, Hum. Exp. Toxicol. 19 (2000) 635–640.
[120] F.R. D'Alessio, K. Tsushima, N.R. Aggarwal, J.R. Mock, Y. Eto, B.T. Garibaldi, D.C. Files, C.R. Avalos, J.V. Rodriguez, A.T. Waickman, S.P. Reddy, D.B. Pearse, V.K. Sidhaye, P.M. Hassoun, M.T. Crow, L.S. King, Resolution of experimental lung injury by monocyte-derived inducible nitric oxide synthase, J. Immunol. 189 (2012) 2234–2245.
[121] M. Dorger, N.K. Jesch, G. Rieder, M.R. Hirvonen, K. Savolainen, F. Krombach, K. Messmer, Species differences in NO formation by rat and hamster alveolar macrophages in vitro, Am. J. Respir. Cell Mol. Biol. 16 (1997) 413–420.
[122] A. Steimer, E. Haltner, C.M. Lehr, Cell culture models of the respiratory tract relevant to pulmonary drug delivery, J. Aerosol Med. 18 (2005) 137–182.
[123] K. BeruBe, M. Aufderheide, D. Breheny, R. Clothier, R. Combes, R. Duffin, B. Forbes, M. Gaca, A. Gray, I. Hall, M. Kelly, M. Lethem, M. Liebsch, L. Merolla, J.P. Morin, J. Seagrave, M.A. Swartz, T.D. Tetley, M. Umachandran, In vitro models of inhalation toxicity and disease. The report of a FRAME workshop, Altern. Lab. Anim. 37 (2009) 89–141.
[124] D. Huh, G.A. Hamilton, D.E. Ingber, From 3D cell culture to organs-on-chips, Trends Cell Biol. 21 (2011) 745–754.
[125] D. Van Soolingen, C. Moolenbeek, H.V. Loveren, An improved method of bronchoalveolar lavage of lungs of small laboratory animals: short report, Lab. Anim. 24 (1990) 197–199.
[126] H.Y. Reynolds, Bronchoalveolar lavage-obtaining biologic specimens from the respiratory tract surface, Sarcoidosis Vasc. Diffuse Lung Dis. 25 (2008) 5–9.
[127] M.A. Vega, A.L. Corbi, Human macrophage activation: too many functions and phenotypes for a single cell type, Immunologia 25 (2006) 248–272.
[128] F. Geissmann, M.G. Manz, S. Jung, M.H. Sieweke, M. Merad, K. Ley, Development of monocytes, macrophages, and dendritic cells, Science 327 (2010) 656–661.

[129] W.J. Sandberg, M. Lag, J.A. Holme, B. Friede, M. Gualtieri, M. Kruszewski, P.E. Schwarze, T. Skuland, M. Refsnes, Comparison of non-crystalline silica nanoparticles in IL-1beta release from macrophages, Part. Fibre Toxicol. 9 (2012) 32.
[130] S. Laing, G. Wang, T. Briazova, C. Zhang, A. Wang, Z. Zheng, A. Gow, A.F. Chen, S. Rajagopalan, L.C. Chen, Q. Sun, K. Zhang, Airborne particulate matter selectively activates endoplasmic reticulum stress response in the lung and liver tissues, Am. J. Physiol. Cell Physiol. 299 (2010) C736–C749.
[131] D.M. Brown, K. Donaldson, V. Stone, Effects of PM10 in human peripheral blood monocytes and J774 macrophages, Respir. Res. 5 (2004) 29.
[132] H. Zhou, L. Kobzik, Effect of concentrated ambient particles on macrophage phagocytosis and killing of *Streptococcus pneumoniae*, Am. J. Respir. Cell Mol. Biol. 36 (2007) 460–465.
[133] A. Gebre-Hiwot, G. Tadesse, S.L. Croft, D. Frommel, An in vitro model for screening antileishmanial drugs: the human leukaemia monocyte cell line, THP-1, Acta Trop. 51 (1992) 237–245.
[134] W.C. Raschke, S. Baird, P. Ralph, I. Nakoinz, Functional macrophage cell lines transformed by Abelson leukemia virus, Cell 15 (1978) 261–267.
[135] P. Ralph, I. Nakoinz, Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line, Nature 257 (1975) 393–394.
[136] S. Tsuchiya, Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, K. Tada, Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester, Cancer Res. 42 (1982) 1530–1536.
[137] A.E. Taylor, T.K. Finney-Hayward, J.K. Quint, C.M.R. Thomas, S.J. Tudhope, J.A. Wedzicha, P.J. Barnes, L.E. Donnelly, Defective macrophage phagocytosis of bacteria in COPD, Eur. Respir. J. 35 (2010) 1039–1047.
[138] D.G. Russell, P.J. Cardona, M.J. Kim, S. Allain, F. Altare, Foamy macrophages and the progression of the human tuberculosis granuloma, Nat. Immunol. 10 (2009) 943–948.
[139] E.F. Redente, D.M. Higgins, L.D. Dwyer-Nield, I.M. Orme, M. Gonzalez-Juarrero, A.M. Malkinson, Differential polarization of alveolar macrophages and bone marrow-derived monocytes following chemically and pathogen-induced chronic lung inflammation, J. Leukoc. Biol. 88 (2010) 159–168.
[140] G.S. Tomlinson, H. Booth, S.J. Petit, E. Potton, G.J. Towers, R.F. Miller, B.M. Chain, M. Noursadeghi, Adherent human alveolar macrophages exhibit a transient pro-inflammatory profile that confounds responses to innate immune stimulation, PLoS One 7 (2012).
[141] K. Karimi, H. Sarir, E. Mortaz, J.J. Smit, H. Hosseini, S.J. De Kimpe, F.P. Nijkamp, G. Folkerts, Toll-like receptor-4 mediates cigarette smoke-induced cytokine production by human macrophages, Respir. Res. 7 (2006) 66.
[142] I. Doyle, M. Ratcliffe, A. Walding, E. Vanden Bon, M. Dymond, W. Tomlinson, D. Tilley, P. Shelton, I. Dougall, Differential gene expression analysis in human monocyte-derived macrophages: impact of cigarette smoke on host defence, Mol. Immunol. 47 (2010) 1058–1065.
[143] E. Juarez, C. Nunez, E. Sada, J.J. Ellner, S.K. Schwander, M. Torres, Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes, Respir. Res. 11 (2010).
[144] A.R. Pons, A. Noguera, D. Blanquer, J. Sauleda, J. Pons, A.G.N. Agusti, Phenotypic characterisation of alveolar macrophages and peripheral blood monocytes in COPD, Eur. Respir. J. 25 (2005) 647–652.
[145] S. Poliska, E. Csanky, A. Szanto, I. Szatmari, B. Mesko, L. Szeles, B. Dezso, B. Scholtz, J. Podani, I. Kilty, L. Takacs, L. Nagy, Chronic obstructive pulmonary disease-specific gene expression signatures of alveolar macrophages as well as peripheral blood monocytes overlap and correlate with lung function, Respiration 81 (2011) 499–510.
[146] M. Bur, C.M. Lehr, Pulmonary cell culture models to study the safety and efficacy of innovative aerosol medicines, Expert Opin. Drug Deliv. 5 (2008) 641–652.
[147] F. Blank, B. Rothen-Rutishauser, P. Gehr, Dendritic cells and macrophages form a transepithelial network against foreign particulate antigens, Am. J. Respir. Cell Mol. Biol. 36 (2007) 669–677.
[148] A.D. Lehmann, N. Daum, M. Bur, C.M. Lehr, P. Gehr, B.M. Rothen-Rutishauser, An in vitro triple cell co-culture model with primary cells mimicking the human alveolar epithelial barrier, Eur. J. Pharm. Biopharm. 77 (2011) 398–406.
[149] S.G. Klein, T. Serchi, L. Hoffmann, B. Blomeke, A.C. Gutleb, An improved 3D tetraculture system mimicking the cellular organisation at the alveolar barrier to study the potential toxic effects of particles on the lung, Part. Fibre Toxicol. 10 (2013) 31.
[150] A. Birgersdotter, R. Sandberg, I. Ernberg, Gene expression perturbation in vitro—a growing case for three-dimensional (3D) culture systems, Semin. Cancer Biol. 15 (2005) 405–412.
[151] A.J. Carterson, K.H.Z. Bentrup, C.M. Ott, M.S. Clarke, D.L. Pierson, C.R. Vanderburg, K.L. Buchanan, C.A. Nickerson, M.J. Schurr, A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for *Pseudomonas aeruginosa* pathogenesis, Infect. Immun. 73 (2005) 1129–1140.
[152] L.G. Griffith, M.A. Swartz, Capturing complex 3D tissue physiology in vitro, Nat. Rev. Mol. Cell Biol. 7 (2006) 211–224.
[153] D. Huh, A. Mammoto, M. Montoya-Zavala, D.E. Ingber, A human breathing lung-on-a-chip, FASEB J. 24 (2010).
[154] D. Huh, B.D. Matthews, A. Mammoto, M. Montoya-Zavala, H.Y. Hsin, D.E. Ingber, Reconstituting organ-level lung functions on a chip, Science 328 (2010) 1662–1668.
[155] D. Huh, B.D. Matthews, A. Mammoto, M. Montoya-Zavala, D.E. Ingber, A human breathing lung-on-a-chip for drug screening and nanotoxicology applications, Drug Metab. Rev. 43 (2011) (6-6).
[156] D. Huh, D.C. Leslie, B.D. Matthews, J.P. Fraser, S. Jurek, G.A. Hamilton, K.S. Thorneloe, M.A. McAlexander, D.E. Ingber, A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice, Sci. Transl. Med. 4 (2012).
[157] B.C. Noell, K.L. Dawson, H. Seethamraju, Effect of hypogammaglobulinemia on the incidence of community-acquired respiratory viral infections after lung transplantation, Transplant Proc, 2013.
[158] A.U. Wells, The revised ATS/ERS/JRS/ALAT diagnostic criteria for idiopathic pulmonary fibrosis (IPF)—practical implications, Respir. Res. 14 (Suppl. 1) (2013) S2.
[159] S.C. Heng, O. Morrissey, S.C. Chen, K. Thursky, R.L. Manser, R.L. Nation, D.C. Kong, M. Slavin, Utility of bronchoalveolar lavage fluid galactomannan alone or in combination with PCR for the diagnosis of invasive aspergillosis in adult hematology patients: a systematic review and meta-analysis, Crit. Rev. Microbiol. (2013).
[160] D. Vodak, M. Channel, D. McClintock, M.D. Reed, Comparative repeat-exposure inhalation toxicity of dextran polymers, The Toxicologist 114 (2010).
[161] D.T. Vodak, D.E. Dobry, D. Friesen, M. Reed, A.P. Gigliotti, P.J. Kuehl, D.K. Lyon, Dextran-based materials as excipients in engineered particle formulations: tailoring physical properties to optimize performance, manufacturability, and safety, in: R.N. Dalby, P.R. Byron, J. Peart, J.D. Suman, S.J. Farr, P.M. Young (Eds.), Respiratory Drug Delivery, Virginia Commonwealth University, 2011, pp. 1–9.
[162] J.D. Leuppi, P. Schuetz, R. Bingisser, M. Bodmer, M. Briel, T. Drescher, U. Duerring, C. Henzen, Y. Leibbrandt, S. Maier, D. Miedinger, B. Muller, A. Scherr, C. Schindler, R. Stoeckli, S. Viatte, C. von Garnier, M. Tamm, J. Rutishauser, Short-term vs conventional glucocorticoid therapy in acute exacerbations of chronic obstructive pulmonary disease: the REDUCE randomized clinical trial, JAMA 309 (2013) 2223–2231.
[163] J. Corren, L.E. Mansfield, T. Pertseva, V. Blahzko, K. Kaiser, Efficacy and safety of fluticasone/formoterol combination therapy in patients with moderate-to-severe asthma, Respir. Med. 107 (2013) 180–195.
[164] J. Altenburg, C.S. de Graaff, Y. Stienstra, J.H. Sloos, E.H. van Haren, R.J. Koppers, T.S. van der Werf, W.G. Boersma, Effect of azithromycin maintenance treatment on infectious exacerbations among patients with non-cystic fibrosis bronchiectasis: the BAT randomized controlled trial, JAMA 309 (2013) 1251–1259.
[165] L. Shulgina, A.P. Cahn, E.R. Chilvers, H. Parfrey, A.B. Clark, E.C. Wilson, O.P. Twentyman, A.G. Davison, J.J. Curtin, M.B. Crawford, A.M. Wilson, Treating idiopathic pulmonary fibrosis with the addition of co-trimoxazole: a randomised controlled trial, Thorax 68 (2013) 155–162.
[166] K. Olivier, M. Reed, V. Palmer, A. Gigliotti, D. Hutto, J. Clarke, Preclinical development of a monoclonal antibody to the avb6 integrin, Toxicologist 90 (2006).
[167] STX-100 in Patients with Idiopathic Pulmonary Fibrosis, ClinicalTrials.gov, accessed 27 June 2012, available at http://clinicaltrials.gov/ct2/show/NCT01371305.
[168] M. Allison, Stromedix acquisition signals growing interest in fibrosis, Nat. Biotechnol. 30 (2012) 375–376.
[169] K. Puthawala, N. Hadjiangelis, S.C. Jacoby, E. Bayongan, Z. Zhao, Z. Yang, M.L. Devitt, G.S. Horan, P.H. Weinreb, M.E. Lukashev, S.M. Violette, K.S. Grant, C. Colarossi, S.C. Formenti, J.S. Munger, Inhibition of integrin alpha(v)beta6, an activator of latent transforming growth factor-beta, prevents radiation-induced lung fibrosis, Am. J. Respir. Crit. Care Med. 177 (2008) 82–90.
[170] J.S. Munger, X. Huang, H. Kawakatsu, M.J. Griffiths, S.L. Dalton, J. Wu, J.F. Pittet, N. Kaminski, C. Garat, M.A. Matthay, D.B. Rifkin, D. Sheppard, The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis, Cell 96 (1999) 319–328.
[171] A. Dickie, C. Roper, S. Constant, S. Huang, J. Welch, Mucilair™, an in vitro model for assessment of human airway toxicity, evaluated using model irritant sodium dodecyl sulphate (SDS), Abstract presented at the SULSA symposium, 2012.
[172] A.T. Nguyen Hoang, P. Chen, J. Juarez, P. Sachamitr, B. Billing, L. Bosnjak, B. Dahlen, M. Coles, M. Svensson, Dendritic cell functional properties in a three-dimensional tissue model of human lung mucosa, Am. J. Physiol. Lung Cell. Mol. Physiol. 302 (2012) L226–L237.
[173] S. Huang, L. Wiszniewski, S. Constant, The use of in vitro 3D cell models in drug development for respiratory diseases, in: I. Kapetanović (Ed.), Drug Discovery and Development—Present and Future, 2011.
[174] E. Alfaro-Moreno, T.S. Nawrot, B.M. Vanaudenaerde, M.F. Hoylaerts, J.A. Vanoirbeek, B. Nemery, P.H. Hoet, Co-cultures of multiple cell types mimic pulmonary cell communication in response to urban PM10, Eur. Respir. J. 32 (2008) 1184–1194.
[175] S.G. Klein, J. Hennen, T. Serchi, B. Blomeke, A.C. Gutleb, Potential of coculture in vitro models to study inflammatory and sensitizing effects of particles on the lung, Toxicol. In Vitro 25 (2011) 1516–1534.
[176] B. Rothen-Rutishauser, F. Blank, C. Muhlfeld, P. Gehr, In vitro models of the human epithelial airway barrier to study the toxic potential of particulate matter, Expert Opin. Drug Metab. Toxicol. 4 (2008) 1075–1089.
[177] A.K. Blakney, M.D. Swartzlander, S.J. Bryant, The effects of substrate stiffness on the in vitro activation of macrophages and in vivo host response to poly(ethylene glycol)-based hydrogels, J. Biomed. Mater. Res. A 100 (2012) 1375–1386.
[178] H. Johnston, G. Pojana, S. Zuin, N.R. Jacobsen, P. Moller, S. Loft, M. Semmler-Behnke, C. McGuiness, D. Balharry, A. Marcomini, H. Wallin, W. Kreyling, K. Donaldson, L. Tran, V. Stone, Engineered nanomaterial risk. Lessons learnt from completed nanotoxicology studies: potential solutions to current and future challenges, Crit. Rev. Toxicol. 43 (2013) 1–20.
[179] V. Stone, H. Johnston, R.P. Schins, Development of in vitro systems for nanotoxicology: methodological considerations, Crit. Rev. Toxicol. 39 (2009) 613–626.
[180] J. Bowes, A.J. Brown, J. Hamon, W. Jarolimek, A. Sridhar, G. Waldron, S. Whitebread, Reducing safety-related drug attrition: the use of in vitro pharmacological profiling, Nat. Rev. Drug Discov. 11 (2012) 909–922.
[181] CRACK-IT, Challenge 14: Inhalation Translation, available at http://www.crackit.org.uk/crack/2013/2013_challenges/Challenge14-inhalationtranslation.
[182] V. Stone, P.G. Barlow, G.R. Hutchison, D.M. Brown, Proinflammatory effects of particles on macrophages and epithelial cells, in: Donaldson, Borm (Eds.), Particle Toxicology, CRC Press, 2007, pp. 183–196, (Ch 9).
[183] T. Riss, R. Oravec, A. Niles, Selecting cell-based assays for drug discovery screening, Cell Notes 13 (2005) 16–21.
[184] A.K. Lehmann, S. Sornes, A. Halstensen, Phagocytosis: measurement by flow cytometry, J. Immunol. Methods 243 (2000) 229–242.