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Pulmonary immune responses to respirable elongate mineral particles of asbestiform and non-asbestiform morphologies

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Pulmonary immune responses to respirable elongate mineral particles of asbestiform and non-asbestiform morphologies

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Dissertation submitted to the School of Medicine at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in
Cellular & Integrative Physiology

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Morgantown, West Virginia

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ABSTRACT

Pulmonary immune responses to respirable elongate mineral particles of asbestiform and non-asbestiform morphologies

Timur Oskarovich Khaliullin

Inhalation exposure to asbestiform elongate mineral particles (EMPs) may cause pulmonary fibrosis, lung and pleural cancer. At the same time, epidemiological evidence for non-asbestiform EMP pathogenicity is scarce, with little if any risk of cancer, but consistent findings of pneumoconiosis and increased mortality. Globally, conditions caused by asbestos exposures contribute most to the occupational cancer burden and are responsible for the increasing mortality for occupational respiratory diseases. Immunological components of the fiber-induced lesions are extremely understudied, partly due to the lack of comparable test articles. In this study I utilized respirable preparations of asbestiform and non-asbestiform riebeckite and tremolite to assess pulmonary immune responses, following exposures to studied EMPs in the alveolar macrophage (AM) cell culture model and a A/J mice. First, respirable samples of asbestiform fibers and respective non-asbestiform cleavage fragments (CF) were harvested, and detailed information on the length-width ratios, variability in metal ion concentrations and chemical composition of the studied EMPs were obtained. In the *in vitro* study alveolar macrophage-like cells were treated with mass-, surface area- (s.a.), and particle number- (p.n.) equivalent concentrations of the test articles for 24 hours. Equal mass-based dosing resulted in markedly different responses between asbestiform and non-asbestiform EMPs; at equal “critical fiber” surface area-based doses asbestos and corresponding CF had similar cytotoxic effects, but distinct transcriptional reprogramming patterns and DNA damage response, also specific for the mineral type. For the *in vivo* study I used A/J mouse strain that is prone to lung tumors, originating from atypical hyperplastic lesions in the periphery of the lung, similar to humans. Mice were exposed via repeated pharyngeal aspiration to the mass- and s.a.-based doses of the studied EMPs. Neoplastic manifestations at 1 year were much more frequent in asbestos-treated animals. Histopathology also revealed hyperplasia, mild fibrosis and immune cell infiltration, but no extensive lung fibrosis in asbestiform EMP groups. CF provoked very little histological changes. DNA damage response was evoked by asbestiform EMPs with the foci of γ H2AX staining found in the vicinity of deposited fibers, but not CF. Overall, the immune response to asbestos particles was characterized as leaning towards T_H2 - or T_H17 type. There was relative depletion of resident AMs by asbestiform EMPs and increase in the mean fluorescence intensity of inflammatory CD11b surface marker on AM surface in all treatment groups compared to controls at 12 months. Exposure to asbestiform and non-asbestiform EMPs led to discrete pulmonary immune responses at 3- and 12-months post-exposure, leading to specific long-term fibroproliferative and carcinogenic outcomes. The principal findings from this work confirm that alveolar macrophages play a major role in responding to EMPs; the preservation of resident “homeostatic” pool of AMs in the lungs might led to the lack of fibrotic and neoplastic outcomes in CF-treated animals. In conclusion, this work supplements the notion that in addition to habit and biodurability, chemical composition, unique for different minerals, plays major role in the immunological responses and respective long-term pulmonary outcomes. The work presented here calls for further investigations of the pulmonary and systemic immune cell population dynamics in the development of long-term carcinogenic and non-carcinogenic outcomes following exposures to well-characterized reference materials.

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LIST OF ABBREVIATIONS

EMP	Elongate Mineral Particles
AM	Alveolar Macrophage
BAL	Bronchoalveolar Lavage
CCL2	C-C Chemokine Ligand 2
CD	Cluster of Differentiation
CF	Cleavage Fragment
DEG	Differentially Expressed Gene
DSB	Double Strand DNA Breaks
ECM	Extracellular Matrix
EDS	Energy-Dispersive Spectroscopy
EPA	Environmental Protection Agency
FESEM	Field Emission Scanning Electron Microcopy
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
GO	Gene Ontology
H ₂ O ₂	Hydrogen Peroxide
IFN- γ	Interferon-gamma
IL-17	Interleukin-17
IL-1 α	Interleukin-1-alpha
IL-1 β	Interleukin-1-beta
IL-6	Interleukin-6
IL-8	Interleukin-8

IL-9	Interleukin-9
IPF	Idiopathic Pulmonary Fibrosis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDH	Lactate Dehydrogenase
LDH	Lactate Dehydrogenase
LMP	Lysosomal Membrane Permeabilization
MARCO	Macrophage Receptor with Collagenous Structure
MCE	Mixed Cellulose Ester
MDSC	Myeloid-Derived Suppressor Cell
MNP	Minimal Number of Particles
MPI	Max Plank Institute
MSHA	Mining Safety and Health Administration
NIEHS	National Institute of Environmental Health Sciences
NIOSH	Nation Institutes for Occupation Safety and Health
NLRP3	Nod-like Receptor Family, Pryin Domain Containing-3
OSHA	Occupations Safety and Health Administration
PBS	Phosphate Buffered Saline
PCM	Phase Contrast Microscopy
PDGF	Platelet-derived Growth Factor
PLM	Polarized Light Microscopy
PMN	Polymorphonuclear leukocytes
ROS	Reactive Oxygen Species
SR-A	Scavenger Receptor A

TEM	Transmission Electron Microscopy
TGF β	Transforming Growth Factor-beta
TNF- α	Tumor Necrosis Factor-alpha
UICC	Union Internationale Centre le Cancer
VEGFA	Vascular Endothelial Growth Factor-A
WHO	World Health Organization

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Chapter 1. Introduction

Since ancient times, people have noticed that working in a particularly dusty environment leads to morbidity and early death [1]. Throughout the history of human civilization, natural scientists and physicians registered and described the symptoms occurring in workers, proposed treatments, and prevention methods. Several thousand years ago humans discovered minerals that looked like fabric and could be woven into sheets. Since this material was essentially a rock, it was fireproof, and thus came the name – “asbestos” – from “indistinguishable” in Greek. In early XX century asbestos was proclaimed a “miracle mineral” [2], the material of the future for its properties; too late came the recognition of potential adverse health outcomes and calls for a worldwide ban [3]. In modern times nanotechnology could share that fate, but thankfully, research community recognized early the need to investigate the potential toxicity of nanomaterials [4]. Despite the findings, asbestos is still relevant in modern society: human exposures occur frequently, coupled to the long latency of the associated diseases. Issues with asbestos definitions, exposure scenarios, persisting regulatory arguments, still-understudied mechanisms of toxicity and their relation to the properties of fibers, have no end in sight as of today. Some of these issues have been at least partially addressed: we know that the health burden can be prevented [5] and there are newer substitute materials [6] even if the safety of the substitutes may be disputed [7]. This dissertation addresses the immune responses to not only asbestos, but also similar long and thin particles that are not designated as asbestiform (looking like asbestos).

1.1. Definitions and a little bit of mineralogy

“Asbestos” is the commercial term applied to the asbestiform varieties of six minerals: chrysotile, belonging to the serpentine group and five amphiboles - amosite, crocidolite, anthophyllite, tremolite, and actinolite. The term intentionally specifies asbestiform nature (or habit) of the minerals in question, meaning that they are comprised of very thin fibers with high lengths (longer than five μm) and aspect ratios (ratio of length to width); in addition, particles must have at least two of the following: parallel fibers occurring in bundles, fiber bundles displaying splayed ends, matted masses of individual fibers and/or

fibers showing curvature. To encompass the full range of fiber-like mineral particles that have an aspect ratio of more than 3:1, the term “elongate mineral particles” (EMP) is now widely used in professional literature [8].

Figure 1.1. Representation of the different mineral particles as a function of their morphology/dimensions. WHO – World Health Organization. From: *Opinion of the French Agency for Food, Environmental and Occupational Health & Safety on "Health Effects and the identification of cleavage fragments of amphiboles from quarried minerals"* (<https://www.anses.fr/en/system/files/AIR2014sa0196RaEN.pdf>).

Crystal habit is another widely used term, that helps to understand how the mineral particle assumed the elongated shape. In short, habit is how singular mineral crystal, or an aggregate of crystals, look and behave if subjected to physical stress, both micro- and macroscopically. During the crystal forming phase, asbestiform minerals naturally grow into the long and thin (meaning high aspect ratio) fibrils with parallel sides, that can stick together, forming a fibrous aggregate. In comparison, non-asbestiform minerals grow in several spatial directions, and can only produce high aspect ratio particles when broken into such, along the so-called cleavage planes; hence the term “cleavage fragments” (Figures 1.1. and 1.2.). The topic of habit is covered in much greater detail by Belluso, Cavallo and Halterman [9].

Figure 1.2. Asbestiform and non-asbestiform varieties of riebeckite and tremolite minerals.

Chemical composition of EMPs can be very diverse. For instance, natural minerals that are considered asbestos, are all silicates, built from silicate tetrahedral unit cells, but also incorporating various metals and other atoms into the lattice. Artificial fibers can be made of glass, clay, silicon carbide or just carbon, with a substantial variety of other elements. This diversity means that the particles can have unique or common chemical reactivities, with downstream biological consequences [10].

Chrysotile is a serpentine mineral that always occurs as fibers with nanometer width dimensions (henceforth nanofibers) or in bundles of larger overall size. Most asbestos products were made with this material. Fibrous silicate minerals that can be associated with chrysotile and antigorite include rare carlosturanite and balangeroite, and

the much more common amphiboles tremolite and anthophyllite. Amphibole-group minerals can crystallize as large crystals, fine prismatic or acicular crystals, or truly nanofibrous asbestos. The main amphiboles which crystallize in nanofibers are the commonly exploited crocidolite (fibrous riebeckite), amosite, anthophyllite and tremolite-actinolite. However, other amphibole minerals have been found to crystallize in the nanofibrous habit. For instance, winchite and richterite, which contaminate vermiculite from Libby, MT, and have health effects identical to asbestos, were formerly included within the definition of tremolite but are not specifically named in Occupational Health and Safety Administration (OSHA) and Mine Safety and Health Administration (MSHA) regulations [11, 12]. Other examples include sodic amphiboles in Nevada and Arizona [13, 14], and a new nanofibrillar amphibole, glaucophane, found in the Franciscan mélange of CA [15].

1.2. Exposure to asbestiform and non-asbestiform EMPs

Exposure to asbestiform and non-asbestiform mineral varieties occurs both at workplaces and in the natural environment [16]. While asbestos is no longer mined in the U.S. as a primary commodity, it can be encountered along with additional EMPs as an accessory mineral in deposits that are routinely mined [17]. EMP's are not confined to exploitable deposits and are geologically widely spread. This can lead to potentially hazardous exposures in mining almost any commodity mineral. In 2015, 1.32 billion metric tons of crushed stone valued at more than \$13.8 billion was produced by 1,430 companies operating 3,700 quarries, 82 underground mines, and 187 sales/distribution yards in 50 states [18]. The National Occupational Health Survey of Mining recorded detectable asbestos fibers in settled dust collected from mines involved in the extraction of 21 different non-asbestos commodities [19]. In samples collected during 2000-2007, the Mining Safety and Health Administration (MSHA) found that 3% of personal samples had asbestos fiber concentrations in excess of the OSHA-regulated concentration of 0.1 fibers/cc. 12% of those same samples had non-asbestos EMP concentrations above 0.1 fibers/cc. A range of 0.004–16.0 asbestos fibers/cc in the air has been reported during maintenance and replacement of vehicle brakes [20]. In 2011, NIOSH estimated that 44,000 mine workers may have been exposed to EMPs, in addition to 568,000 and

114,000 workers in the service and construction industries, respectively. Nearly 1.3 million employees in general industry could face significant asbestos exposure in occupational settings [8]. A number of studies reported that disturbance of gravel and soils may be a source of exposure [21, 22], which carry risks even at low concentrations of EMPs released by construction, road building, or dust storms. Non-asbestiform tremolite when mixed with talc enhances its usefulness for the production of ceramic appliances. Most of the naturally occurring asbestiform EMPs are amphiboles, which require lower dosages to cause asbestosis than chrysotile [23]. A recent review found that off-road vehicles can generate quantifiable airborne concentrations of mineral fibers in areas where they are naturally occurring [24]. Exposure to asbestos and asbestiform fibers is also occurring naturally, since rock types containing asbestos are common across the US, i.e. Coastal Ranges of the Rocky Mountains i.e. CA, OR, WA and AK and in the Appalachians from VT all the way down to AL, but can be found in almost every State, including, for example, TX, CO and MN, and several other countries [25, 26]. As Americans more and more prefer to build homes in forests, mountains and arid areas, an increasing part of general population is being affected. Aside from the more obvious consumer exposures through the brake pads and construction materials, the extreme cases also include asbestos fibers in cosmetic talc [27], and even toys [28]. Transplacental transfer was described for several asbestos species [29].

As of today, researchers in the field have not reached a consensus as to whether distinctions between “regulated” and “unregulated” amphibole EMPs should exist [30]. Bearing in mind the importance of EMP dimensions, most of the exposure assessment methods for regulated EMPs are based on “counts”. Thus, it becomes extremely important to know exactly what must be counted and what should be “censored” during the counting process. This was emphasized in the NIOSH Roadmap and brought up in a recent extensive review by Egilman et al. [31]. US OSHA believes that the epidemiological studies provide insufficient evidence to inform as to the carcinogenicity of non-asbestiform asbestos-group minerals and was unable to conclude that non-asbestiform minerals present a significant risk of non-malignant respiratory diseases. The American Thoracic Society (ATS) reviewing the same studies concluded that “although the role of chrysotile versus tremolite in producing disease in these patients could not be clearly sorted out,

the (...) data appear to indicate that fairly low aspect ratio fibers of tremolite are capable of causing disease, probably in fairly low concentrations in the case of pleural plaques, but certainly only in very high concentrations in regard to mesothelioma and asbestosis" (Ex. 525, p. 10). Stating that for most mineral deposit asbestos and non-asbestiform habit are distinguishable (populations of cleavage fragments and asbestos can be distinguished), OSHA treats cleavage fibers as physical irritants (occupational exposure limit of 5 mg/m³ for respirable dust). NIOSH acknowledges different risk of non-asbestiform amphiboles might compared to asbestos, but at the same time insists that recommended exposure level (REL) for airborne asbestos fibers also encompass EMPs from the non-asbestiform analogs of the asbestos minerals due to the inconclusive epidemiological evidence for cancer risk associated with exposure to cleavage fragments. NIOSH deems that as a precautionary approach. The NIOSH REL for airborne asbestos fibers and related EMPs is "0.1 countable EMP from one or more covered minerals per cubic centimeter, averaged over 100 minutes, where a countable elongate mineral particle is any fiber or fragment of a mineral longer than 5 µm with a minimum aspect ratio of 3:1 when viewed microscopically with use of NIOSH Analytical Method 7400 ('A' rules) or its equivalent." Some other agencies worldwide also adhere to this principle. French Agency for Food, Environmental and Occupational Health and Safety deemed that "...in the current state of knowledge concerning their health effects, cleavage fragments (...) meeting the WHO's dimensional criteria for fibres (L > 5 µm; D < 3 µm and L:D > 3:1) should not be distinguished from their asbestiform counterparts; there is no reason to make a distinction between the cleavage fragments meeting the "WHO" dimensional criteria for fibres (L > 5 µm; D < 3 µm and L:D > 3:1) and asbestiform fibres of calcic and sodic-calcic EMPs."

1.3. Health burden of asbestos and non-asbestiform EMPs

It is well known that inhalation exposure to respirable asbestiform EMPs leads to chronic fibrotic lung disorders, cancer, including malignant mesothelioma and other non-carcinogenic outcomes [4]. This signifies a substantial healthcare and economic burden worldwide. NIOSH, OSHA, and MSHA all agree on exposure limits of less than 0.1 countable fiber/cc. Nevertheless, substantial risk for adverse health effects remains even

at this exposure limit, which was established based on limitations of the phase contrast microscopy analytical method for counting asbestos fibers. At the current Permissible Exposure Limit (PEL) applied to airborne fibers of the asbestos minerals, OSHA predicts an excess risk of 2.3 cancers per thousand over a 20-year period, increasing to 3.4 per thousand over a 40-year working life [32]. This PEL is also implemented by MSHA. The acceptable cancer risk for workplaces is 1 per thousand, but the PEL must be based on technological feasibility factors in addition to risk. The risk at the PEL is very much higher than that tolerated in the ambient environment; for example, the Environmental Protection Agency (EPA) typically considers 1 in 10,000 to be the point at which intervention is warranted no matter the local opinion. NIOSH recommends that cleavage fragments, crystallized in massive form, be counted as asbestos fibers, if they meet dimensional criteria. However, these EMPs are not currently included as asbestos under MSHA and OSHA regulations. For instance, winchite and richterite, which contaminate vermiculite from Libby, MT, and have health effects identical to asbestos, were formerly included within the definition of tremolite but are not specifically named in OSHA and MSHA regulations [11, 12]. Other examples include sodic amphiboles in Nevada and Arizona [13, 14], non-asbestiform EMPs in taconite, and talc and gold mining [33]. NIOSH estimated that 44,000 mine workers may be exposed to EMPs, in addition to 568,000 and 114,000 workers in the service and construction industries, respectively.

Experimental and epidemiologic data indicates an increased risk for mesothelioma, lung fibrosis, interstitial and pleural abnormalities as well as lung cancer in humans exposed to asbestiform fibers. Peak of asbestos consumption in the USA was in 1951, but the long latency period means that the disease in populations persists for decades. The disease was predicted to claim the lives in excess of 29,000 individuals, between the years 2005 and 2027 [34]. There were 25,413 asbestosis deaths in the USA between 1970 and 2004, with 6.9 per million population maximum age-adjusted death rate, 35-fold greater for men than for women [35]. The areas of the country with the highest death rates were the coastal regions, with the shipbuilding industry sustaining an expected proportionate mortality ratio. Some estimates range between 55,100 [36] and 76,700 [37] EMP-caused lung cancer deaths between the years of 1980 and 2009. Another source lists a total of 13,024 deaths due to asbestosis in the US in 2005-2014,

accounting for 120,905 years of potential life lost to life expectancy [38]. Globally, conditions caused by asbestos exposures contribute most to the occupational cancer burden and are responsible for the increasing mortality for occupational respiratory diseases [39], with 243000 deaths were attributable to asbestos in 2017 worldwide [40]. A total of 13,024 deaths might be attributed to asbestosis in the US in 2005-2014, accounting for 120,905 years of potential life lost to life expectancy [38]. Recent papers showed that mortality rates of nasopharyngeal and laryngeal cancer, lung cancer, intestinal cancer, and mesothelioma can be significantly influenced by naturally occurring asbestos [22, 41]. One should also take into account the observed reappearance of progressive pneumoconiosis cases in the United States, with silicates, including silicate EMPs being implicated [42]. Productivity losses due to asbestos-related diseases in the US amount to approximately 0.36% of GDP, or 86,100 million USD [43]. It was found the total cost of asbestos damage payments in the US will eventually reach \$200 billion [44]. In Canada economic burden of asbestos-related lung cancer in 2011 was estimated to be almost 2 billion Canadian dollars (1,99 billion USD)[45]. In Australia estimated hospital and primary care costs were found to be 130,5 million USD per year, and indirect costs arising from time out of the workforce to be 321 million USD per year. Globally the diseases caused by asbestos are estimated to cost 300 billion USD in compensations over the coming years [5].

1.4. Pathological outcomes of exposures to asbestiform and non-asbestiform EMPs

Both carcinogenic and non-carcinogenic health outcomes such as pulmonary fibrosis (asbestosis), pleural fibrosis and plaques, have been extensively described in asbestos-exposed populations [16, 46]. Verified studies established a structure-activity relationship paradigm for long and thin particles with high aspect ratio and demonstrated that exposure to asbestos fibers causes many lung diseases including pleural and lung fibrosis (asbestosis), lung cancer, and pleural and peritoneal malignant mesothelioma (MM) [47-50]. Although not technically classified as asbestos, erionite [51] – a fibrous zeolite having analogous physical, chemical and morphological properties as asbestos fibers – causes a spectrum of asbestos-associated lung diseases, including pleural fibrosis, plaques and MM [52-54]. A recent study also described erionite evoking T_H17

responses and inducing autoimmune responses in mice [55, 56]. Exposure to silicon carbide (SiC) has not been indicted of causing the lung cancer [57].

Epidemiological evidence for the pathogenicity of non-asbestiform mineral varieties is scarce, but it seems that there is little if any risk of lung cancer or mesothelioma [58-60]. This is in part due to the lack of reliable exposure assessment methods for non-asbestiform EMPs, however, there is evidence for exposures during taconite, talc and gold mining associated with an increased risk for non-malignant respiratory diseases, such as as pneumoconiosis, and the excess of cardiovascular and respiratory mortality [33, 61]. There is some epidemiological evidence for non-asbestiform nephrite to be the cause of pulmonary fibrosis and lung cancer [62, 63]. A case of lung cancer was described following exposure to tremolite cleavage fragments in gravel quarry [64]. At the same time, in rat carcinogenicity study asbestiform tremolite had much higher carcinogenic potential compared to non-fibrous tremolite after intrapleural injections [65]. Practically all outcomes are tightly dependent on the immunological responses, both innate and adaptive, local and systemic [66-68].

1.5. The role of EMP dimensions and chemistry

Substantial evidence suggests that fiber dose, number, dimensions, durability, surface reactivity, surface area, and their propensity to split into thin fibers *in vivo* are important mediators of asbestos-related diseases [69, 70]. Relatively few EMPs with diameters larger than $\sim 3\ \mu\text{m}$ will penetrate into the lungs [71], but those with diameters less than $0.1\ \mu\text{m}$ are also less retained in the lungs compared to larger diameters [72]. Longer fibers deposit more readily in the airways compared to alveolar region due to the interception mechanism [73]. The World Health Organization (WHO) considers EMPs longer than $5\ \mu\text{m}$ “critical” for lung pathology development [74]; however, there are propositions to only consider fibers longer than $10\ \mu\text{m}$ [75, 76] as dangerous for human health. In a comprehensive review Lippmann (2014) states that thin fibers up to $5\ \mu\text{m}$ cause pleural fibrosis and MM, while those $20\ \mu\text{m}$ and longer – lung cancer. [77]. The disease-promoting potential of the various fiber morphologies is summarized on Figure 1.3., taken from the NIOSH Manual of Analytical Methods [78].

Figure 1.3. Comparison of proposed size ranges of asbestos fibers causing specific diseases compared with the fiber sizes detected using TEM and PCM techniques.

Bearing in mind the inferred importance of fiber dimensions, most of the exposure assessment methods for regulated EMPs are based on “counts”. Thus, it is crucial to know exactly what must be counted and what should be “censored” during the counting process. This was emphasized in the NIOSH roadmap on EMP research [8] and brought up in a recent extensive review by Egilman et al. [31]. There was a limited batch of *in vitro* studies on non-asbestiform EMP varieties [79, 80], whereas significant number of research projects focused on the shorter vs. longer asbestos fibers rather than asbestiform vs. non-asbestiform EMP comparisons. The earliest evidence led to a prevailing opinion that long asbestos fibers are the most dangerous, i.e. shown to be more cytotoxic to macrophages *in vitro* than short ones [81]. For non-fibrogenic fibers Castranova et al. found that short and thin glass fibers are more cytotoxic than thick and long [82] while Padmore et al. discovered that shorter fibers are less potent in inducing the inflammatory responses [83]. However, both short and long riebeckite asbestos fibers were able to induce H₂O₂ production by macrophages and cause cell death [81]. The

situation can be quite different *in vivo*, since EMPs longer than 20 μm rarely penetrates deep into the alveolar region. In the critical review Dodson et al. (2003) emphasizes the need to account for shorter fibers when assessing the potential hazard of asbestiform EMPs [84]. It is confirmed that short fibers still remain in significant proportions in the lungs of workers with occupational asbestos exposure [85]. And apparently, short fibers may be quite cytotoxic *in vivo* when the clearance of these fibers is prevented [81, 86].

The early evidence of surface area importance was highlighted by Lippmann (1988), who stated that "...asbestosis is most closely related to the surface area of retained fibers" [87]. In the study by Davis et al. (1991) total surface area of tremolite samples were highly correlated to the incidence of mesothelioma in rats [65]. Mossman et al. (Mossman, Lippmann et al. 2011) concluded that the surface area of retained fibers rather than the phagocytized particle numbers, mass or volume correlated best with inflammatory responses *in vivo* [88]. *In vitro* studies, such as the one done by Dunkan et al. (2014) show good association between the asbestiform fibers surface area and inflammatory responses [89]. The reason surface area must be accounted for is the presence of certain reactive sites on the exterior of individual EMPs. Those could be dangling bonds, silanol groups or transition metals, all capable of participating in redox reactions with the biological molecules. Iron is considered one of the major players in the EMP toxicity. Change from ferric iron to ferrous increases activity and vice versa. Even outside of the direct cytotoxic events, iron status influences macrophage polarization – overload leads to M2 responses, whereas deficiency – to M1 [90]. Heme Oxygenase -1 (HO-1) expression in the lungs of rats exposed to crocidolite [91]. However, redox potential of the fibers is not completely related to the iron content. For instance, in A549 lung adenocarcinoma cells both tremolite and crocidolite had comparable toxic effects despite 10-20 times difference in iron content between samples [92]. Later the same authors have evaluated the surface reactivity of the same amphibole samples and concluded that the reactivity of studied fibers was due to specific iron sites, and the radical activity persists throughout the long dissolution process [93]. The importance of fiber surface area in fiber toxicity mechanisms, i.e. active sites, surface redox-active iron, milling effect etc. is explored in detail by Aust et al. (2011) [10]. A good example of the intersection between chemical reactivity and distant pathological outcome is the

production of peroxynitrite, one of the most powerful oxidants, and a product of chemical reaction between $\bullet\text{NO}$ and superoxide anion (O_2^-), during asbestos lung injury (Figure 4.). Increased levels of peroxynitrite are associated with tumour progression in many types of cancer [94, 95], which has been linked with T-cell unresponsiveness.

Further, fibers that dissolved in lung fluid in a matter of weeks or months appear to be somewhat less toxic than more insoluble fibers. Studies performed in rats produced the evidence of high biodurability of crocidolite and tremolite fibers [96]. Conversely, low-toxicity wollastonite (a calcium inosilicate mineral CaSiO_3) fibers are much less biopersistent with very short half-time of 15 to 21 days [97]. If the fiber is durable and biopersistent, there is a chance it penetrates the epithelium translocates to the interstitium/pleura. [98]. Another evidence for the importance of mineral type rather than the EMPs size was provided by Wylie et al. (1997), who compared the toxicity of fibrous talc, chrysotile, and asbestiform riebeckite, each sample having significant proportion of long and thin durable fibers, but causing toxic responses of different magnitude [99].

The evolution of knowledge about toxic properties of various mineral fibers naturally led to an idea that dimensions alone should not be considered the primary metric critical for the hazard assessment.

1.6. Immune component of the biological response to EMPs

1.6.1. Overview of the immune system involvement

It is clear now that alterations of the immune system functions play important role in the development of asbestos pathology. For instance, lung cancer and mesothelioma following pulmonary exposure to all types of asbestos, are directly associated with immunosuppression [100]. The evidence for development of autoimmunity after asbestos exposure starts building up [14], akin to chemically similar silica, which is already recognized as a provoker of systemic autoimmune disease [101]. The impairment of phagocytic function, constant recruitment of neutrophils and cellular damage can lead to an increased secretion of host-derived antigens, that drive the autoantibodies production upon exposure to asbestos [102]. Exposure to EMP leads to changes in the population of AMs, which have a long turnover rate in mice [103], and recruitment of phagocytes that actively participate in clearance mechanisms, proinflammatory and pro/antifibrotic

signaling, contributing to the polarization of immune response. AM depletion can also enhance antigen-presenting function of resident phagocytes (parenchymal dendritic cells) and their migration, leading to re-activation of the T-cells [104]. There is also indication that asbestos-initiated chronic oxidative stress contributes to carcinogenesis and fibrosis by promoting oxidative DNA damage and regulating redox signaling pathways [105-109]. Increased biopersistence and low clearance, leading to bioaccumulation of asbestiform fibers in the lungs can not only elicit proliferative and immune response, but also directly or indirectly affect the function of the immune system. The hyperplastic outcomes thus may be anticipated based on the immune cell functionality as well as on physicochemical properties of particles. There is also a strong role of immune system in the development of pulmonary fibrosis following asbestos exposures.

Quantification of cellular and humoral immune responses as a function of physical/chemical/structural properties of asbestos fibers and their cleavage fragments is an essential step to determine factors and mechanisms by which these fibers exert toxicity and carcinogenesis. The early phase of lung innate immune response to foreign agents is characterized by the onset of inflammation mediated by resident phagocytic cells. The inflammatory milieu in the lung following exposure to the mineral fibers provides a range of signals required for activation and potential polarization of macrophages, which shape the subsequent T cell response and the whole local immune microenvironment. These signals are presented in the context of cytokines and chemokines, damaged cells/cell fragments, and, perhaps, specific features of the asbestos/asbestiform fibers themselves. However, little data exist on the transcriptional reprogramming that takes place upon the contact with asbestiform or non-asbestiform EMPs. Understanding of the macrophage function and lymphocytes involvement in EMP-induced lung injury will significantly contribute to the EMP immunotoxicity paradigms.

1.6.2. Alveolar macrophages responses and role in pathogenesis

Numerous cell populations are involved in asbestos-related pathology, with resident alveolar macrophages being one of the primary mediators of the host immune response. Their depletion during asbestos-induced lung disease exacerbates fibrosis and worsens overall prognosis [110]. Accordingly, macrophage responses, including patterns of cell damage, cytokines, chemokines, and changes in gene expression could be related to the

specific features of the fibers, meaning toxicological studies must go hand in hand with detailed EMP characterization. Macrophage receptor with collagenous structure (MARCO) and scavenger receptor A (SR-A) are implicated in asbestos recognition [111], through sensing polyanionic ligands. AMs mostly express SR-A, but MARCO also is heavily involved in the disease progression and immune polarization [112]. Complement activation also plays role in chemotaxis of AMs towards deposited EMPs [113]. The uptake of cylindrical microparticles particles in murine macrophages usually happens within the first 1.5-3 hours [114] and requires a proper orientation – so that the macrophage could approach from one of the ends [115]. The efficiency and speed of phagocytosis depends heavily on particle shape [116]. A paradigm is proposed that “SR-A and MARCO amplify responses of both intracellular Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), possibly increasing ligand availability in intracellular compartments.” [117]. After the recognition and (un)successful phagocytosis, asbestos fibers with different morphology and chemistry have been shown to activate NLRP-3 inflammasome through different mechanisms [118-121]. This inflammasome participates in initial inflammatory response and the chronic inflammatory conditions arising in fiber exposure settings, including driving the IL-17 responses [122], implicated in amphibole asbestos pathogenicity [56] and specifically in the associated reduction in antitumor immunity [123]. Successful internalization is actually not always necessary, since crystalline structures can activate NLRP3 through the surface contact [124]. The example of inert glass fibers is very characteristic – longer ones cannot be phagocytized, and cause inflammatory mediators production, but little toxicity *per se* [83]. In this case another mechanism may kick in – foreign body giant cells (FBGC) arise when dealing with poorly soluble large foreign materials [125]. Foreign bodies reaction may lead to the influx of monocytes from the bloodstream (mostly Ly6C⁺), more prominent in severe inflammation. At the site monocytes mature into macrophages [126].

The same mechanisms of NLRP3 activation were proposed for both asbestos and nanoscale fibers, such as carbon nanotubes (CNT) [127]. Probably one of the most important downstream consequence of EMP internalization by AMs is lysosome membrane permeabilization (LMP), leading to the leakage of lysosomal enzymes,

potassium influx, and activation of apoptotic cascades. As an alternative mechanisms, if mitochondrial damage occurred foremost, release of mitochondrial ROS into the cytosol may also act as a mediator for LMP induction [128]. Ultimately, it seems that AM death through necrosis/necrosis/apoptosis is what drives the disease further. Asbestos materials have been proven to be potent proapoptotic agents in alveolar macrophages [129].

Another important function of AMs is secretion of biologically active molecules, including cytokines, chemokines, growth factors etc. Normally the “tolerant” nature of the majority of AMs prevents overreaction and excessive activation. However, AM population is not homogenous and includes genotypically and functionally different varieties. [130-132]. Not all of them are strictly immunosuppressive and as early as in 1996 researchers hypothesized that the disbalance of suppressive vs. reactive subtypes (i.e. caused by the excessive apoptosis of the former subtype) is what constitutes the development of lung disease [133]. Polarization of Immune response heavily impacts the resulting outcomes by shaping the immune responses to EMPs. It is established that, in humans with asbestosis, alveolar macrophages secrete pro-inflammatory cytokines and are overresponsive to the other stimuli [134, 135]. Driscoll et al. reported considerable IL-1 and TNF- α secretion by alveolar macrophages obtained from exposed rats at day 3 post exposure to crocidolite, which decreased substantially on days 7, 14, and 28 [136]. The increased cytokine secretion observed in humans and animals, however, has not been reproduced *in vitro* [134]. There is huge amount of data on the role of TGF- β 1 in AM homeostasis [137, 138] and AM-related lung pathology [139], including upregulation of TGF- β 1 production in asbestosis [140]. AMs themselves may act as a source of PDGF (IFN- γ , IL-1 β and IL-13 implicated). Additionally, proinflammatory cytokines (IL-1b, TGF-b.) upregulate PDGFR-alpha [141]. PDGF along with TGF- β 1 are the cornerstones of fibrotic lung diseases.

1.6.3. Role of lymphocytes: immunosuppression and autoimmunity

Role of lymphocytes in asbestos-related pathology can vary greatly depending on whether we look at carcinogenic or non-carcinogenic outcomes. Pulmonary fibrosis in general is accompanied by the influx of T-lymphocytes, engaging in pro- or antifibrotic

activities [142]. For example, CD4+ and esp. CD8+ are capable of producing CCL3 (MIP-1a), involved in fibrocytes recruitment. Activated T-lymphocytes express CD40L that binds to CD40R on fibroblasts, stimulating their proliferation. Several important predictive/prognostic parameters have been suggested, such as CD4+/CD8+ relative proportion, and accumulation of quiescent versus activated T-cells. In idiopathic pulmonary fibrosis patients CD4/CD8 ratios in BAL are variable, and higher CD4/CD8 ratio means better response to the therapy, while elevated CD8+ is unfavorable [143]. If fibers are translocated to lymph nodes, which happens in exposed humans [144], there is a potential for CD8 differentiation into CTL suppression by asbestos (chrysotile and crocidolite), compared with glass wool fibers, leading to decreased IFN- γ and TNF- α production [145]. Disbalance of Th17 and T_{reg} lymphocytes can also be a major event in the development of asbestos-driven lung pathology [123].

Autoimmunity caused by EMP exposure is a pressing issue. A comprehensive set of studies have been performed by Dr. Pfau and colleagues, following the news of Libby amphibole (6-mix) exposure-related surge in autoimmune conditions in workers and general population: rheumatoid arthritis, systemic lupus erythematoses, systemic scleroderma, and lamellar pleural thickening were diagnosed at higher prevalence in LA-exposed populations [146-148]. At the same time, negative correlation of asbestos exposure and autoantibody titers have also been described [149].

1.6.4. Role of other immune cell types

Polymorphonuclear leukocytes (PMN), including neutrophils, myeloid-derived suppressor cells (MDSCs), mast cells and dendritic cells Our previous *in vivo* study comparing pulmonary effects of tremolite, crocidolite, and wollastonite EMPs revealed differential innate inflammatory responses. In particular, it was found that tremolite elicited inflammatory responses with polymorphonuclear leukocytes (PMN) influx peaking at day 1 and eosinophil response at day 7 post-exposure. In contrast, exposure to crocidolite fibers induced a "delayed" inflammatory response with maximal influx of PMNs and eosinophils occurring on day 7 post exposure [150]. MDSCs are emerging as potent immunosuppressive agents, participating in the pathogenesis of many lung diseases [151], including mesothelioma induced by asbestos exposures [152]. Higher eosinophils

count was previously observed in animal models and humans exposed to asbestos [153, 154], and eosinophils are known to facilitate lymphocytes-driven T_H2 -type response. Steady increase in mast cells (including submesothelial) was observed in rats, inhaling both chrysotile and amphibole fibers [155, 156], which can facilitate ECM deposition, T_H2 and autoimmune responses. Suppression of the dendritic and other cells contributes to asbestos-induced tumorigenesis [157].

1.7. Knowledge gaps

Although it is not easy to recapitulate human asbestos-induced lung disease in rodents, first murine asbestosis models were proposed in 1980s [158, 159]. They utilized BALB/C mice and chrysotile asbestos administered via inhalation or instillation. However, despite the knowledge of major outcomes, it is still unclear from existing literature what characteristics of fibers are the major determinants of toxicity and how does it contribute to the (non-) carcinogenic outcomes associated with their exposure. The evidence of knowledge gap lies in the fact that Immunological components of the fiber-induced lesions are extremely understudied; especially important is determining whether similar long-term health outcomes are reached through disparate immunological mechanisms in the case of different types of EMP. If the lung immune responses play decisive role in pulmonary and extrapulmonary long-term outcomes following various EMP exposures; what is the role of antigen-presenting cells and T_H polarization in asbestos exposures; how significant is the immune suppression from EMP of different chemical composition and growth habit; what is the significance of alterations in the alveolar macrophage populations following exposures to various EMPs? Recent discoveries of new occupational and incidental exposures to EMPs, emergence of new technologies and new engineered materials, continuous use of old-fashioned methods for EMP quantification and characterization makes studies aimed at elucidating precise immune mechanisms of EMP-related pathology extremely timely.

1.8. Concluding remarks:

There is a strong need for research on fundamental mineralogical properties of EMPs in their relevance to inhalation toxicity, which can be used as a basis for exposure assessment and workers' protection. Ultimately, the knowledge of what EMP

characteristics are major determinants of health outcomes will improve our understanding of dose-response relationships and help us to better determine how much of a reduction in exposure is needed to prevent adverse health effects. Data on immunotoxicity of various EMP is critical for the occupational health specialists, including clinical doctors, who deal with the adverse health effects in overexposed workers. There are still many questions remaining on exposure assessment, measurements and health effects for asbestos fibers and other EMPs, and these questions are a large source of confusion for many stakeholders in the mining, construction (building and road), and manufacturing industries. Unambiguous identification and characterization of accurate and reliable biomarkers of lung diseases associated with EMP exposures in animals or humans has not been achieved. This is due, at least in part, to the lack of comprehensive comparative studies that evaluate the adverse effects of asbestiform and non-asbestiform fibers in relation to their physical, chemical, morphological and surface characteristics, as well as their bioavailability and biopersistence. It is currently impossible to present comprehensive model of the immune responses to elongate mineral fibers due to the sheer diversity of the latter. In his letter to editor Dr. Gualtieri, a renowned asbestos researcher, states (talking about carcinogenesis, as an example outcome) that creating such models requires "...the intersection of different perspectives, ..., comparison of experimental evidence from other research fields (e.g. biochemistry, mineralogy, physics, and toxicology) to take into account the complex synergy of all the factors at play in defining the toxicity and pathogenicity of mineral fibers (e.g. fiber morphometry, frustrated phagocytosis, production of reactive oxygen species/nitric oxide synthase, inflammasome models, genetic factors, and many more)" [160]. Occasionally, complex schemas are provided, such as the one by Otsuki et al. [161], but they usually include limited test articles (i.e. only "asbestos"), as well as specific humoral (certain cytokines/chemokines) and cellular components (for instance, lymphocytic origin cells only). Studying the dosimetry and mode of action of multiple types of asbestiform minerals and respective cleavage fragments is essential to understanding the specific exposure responses. Consequently, quantification of cellular and humoral immune responses as a function of physical/chemical/structural properties of asbestos fibers and their cleavage fragments is an essential step to determine factors and mechanisms by which these fibers exert

toxicity and carcinogenesis. The early phase of lung innate immune response to foreign agents is characterized by the onset of inflammation mediated by resident phagocytic cells. The inflammatory milieu in the lung following exposure to the mineral fibers provides a range of signals required for activation and potential polarization of macrophages, which shape the subsequent T cell response and the whole local immune microenvironment. These signals are presented in the context of cytokines and chemokines, damaged cells/cell fragments, and, perhaps, specific features of the asbestos/asbestiform fibers themselves. However, little data exist on the transcriptional reprogramming that takes place upon the contact with asbestiform or non-asbestiform EMPs. Understanding of the function of antigen-presenting cells and lymphocytes activation patterns will significantly contribute to the EMP immunotoxicity paradigms.

1.9. Objective and Aims of this dissertation study

The broad, long-term objective of this project focuses on the assessment of local and systemic immune responses to two distinct types of elongate mineral fibers (EMPs): asbestiform fibers and their non-asbestiform mineral analogue cleavage fragments in order to identify the critical cellular and humoral mediators. The central research question of the dissertation was: how do the asbestiform and non-asbestiform respirable EMP preparations influence the immune landscape in the mouse lungs, in regard to pulmonary fibrotic/neoplastic outcomes after repeated pharyngeal aspiration exposure?

To answer the central research question, three specific aims were proposed:

Aim 1: To prepare respirable test samples of two amphiboles – riebeckite and tremolite, and their respective cleavage fragments, with comparable dimensions, and assess their size distribution, morphology, composition and structural make-up, using polarized light microscopy, transmission electron microscopy, and X-ray spectroscopy.

Aim 2: To determine the contribution of innate tissue resident macrophages to the development of specific responses to asbestiform and non-asbestiform EMPs, alveolar macrophage-like cells will be incubated with studied EMPs to determine cytotoxic responses, and transcriptional reprogramming.

Aim 3: To evaluate the myeloid lineage cellular composition and humoral component of the immune response in the mouse lungs upon exposure to the tested

EMPs, in relation to the histopathological changes, mouse animal model of repeated pharyngeal aspiration will be utilized, where animals are sacrificed at 3 and 12 months post final exposure day, and cytokine production/release will be assessed in the lungs. Lungs will be also enzymatically digested to produce a single cell suspension that will be stained for respective immune cells surface markers and analyzed on Flow Cytometer. Histopathology and immunohistochemistry analysis to be performed by board-certified pathologist.

1.10. References

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Chapter 2. Characterization and harvesting of the Riebeckite and Tremolite Elongate Mineral Particles for Toxicological Studies

2.1. Introduction

Currently it is not exactly clear from existing literature what characteristics of fibers are the major determinants of toxicity and how do they contribute to pathological outcomes associated with EMP exposures. Accumulated data suggests that fiber dose, number, dimensions, durability, surface reactivity, surface area, and their propensity to split into thin fibers *in vivo* are important mediators of asbestos-related diseases [1, 2]. The World Health Organization (WHO) considers EMPs longer than 5 μm “critical” for lung pathology development [3]; however, there are propositions to only consider fibers longer than 10 μm [4, 5] as dangerous for human health. Bearing in mind the inferred importance of fiber dimensions, most of the exposure assessment methods for regulated EMPs are based on “counts”. Thus, it is crucial to know exactly what must be counted and what should be “censored” during the counting process. This was brought up in a recent extensive review by Egilman et al. [6]. In 2011 the National Institute for Occupational Safety and Health (NIOSH) published its Current Intelligence Bulletin 62 - Asbestos Fibers and Other Elongate Mineral Particles: State of the Science and Roadmap for Research. In the concluding part, this document has indicated two major research needs that have to be addressed: “...(1) for the asbestos minerals, development of a clearer understanding of the important dimensional and physicochemical determinants of pathogenicity; (2) for other EMPs, such as those from non-asbestiform habits of the asbestos minerals and erionite, development of a deeper understanding of the determinants of toxicity...”[7].

Accordingly, particle preparation is a crucial part of any studies aimed at investigating the toxicological properties, be it cell culture or animal models. In this chapter I would like to address the preparation and characterization of the asbestiform and non-asbestiform varieties of riebeckite and tremolite minerals, with an ultimate goal of obtaining respirable fiber size ranges similar to those encountered in workplace air and potentially respirable by the exposed human populations. Two amphiboles have been chosen for the study – riebeckite and tremolite. Both exist in asbestiform and non-

asbestiform varieties, both have been implicated in the most severe health outcomes in humans, such as asbestosis, lung cancer, and mesothelioma. However, while riebeckite asbestos (also known as crocidolite) mostly causes lung cancer, tremolite is a much more potent inducer of mesothelioma. The two minerals are dissimilar in their chemical structure, specifically, iron content. Utilizing test articles of distinct crystal growth habits allowed us to investigate another important issue: only a handful of studies previously addressed the biological effects of EMPs with similar chemistry, but different geometry.

Since “counted” fibers are by protocol supposed to be greater than 5 μm in length and have an aspect ratio (length to width) of 3:1 or greater. We have deemed necessary to achieve less than 3.0 μm in diameter for all fibers, since this is a cutoff for reliable deep lung penetration. The procedure involved crushing massive mineral samples using a hydraulic press, sieving crushed minerals to harvest fine fractions, and hand-grinding using a mortar and pestle to obtain/produce fibers similar to those found in the workplace. Following the generation of fibers, parameters concerning fiber length, width, and aspect ratio (length:width) was determined by Phase Contrast Microscopy (PCM) and Transmission Electron Microscopy (TEM). Altogether, a combination of light microscopy, electron microscopy, and X-ray methods was used to characterize physical parameters of particles and chemical composition.

2.2. Materials and Methods

2.2.1. Raw materials description and sources

Obtaining relevant test articles relies heavily on the relevant initial bulk samples source. For this study we have chosen four reliable sources of representative asbestiform and non-asbestiform EMPs. In 1990 U.S. Bureau of Mines prepared non-asbestiform tremolite samples for the National Institute of Environmental Health Sciences (NIEHS). they were used in rat oral ingestion studies to investigate the oral toxicity [8]. Union Internationale Centre le Cancer (UICC) riebeckite asbestos (crocidolite) was supplied by Research Triangle Institute (RTI International). Non-asbestiform riebeckite was originally obtained by NIOSH from Pikes Peak in Colorado Springs, Colorado. Tremolite asbestos was sourced from Lone Pine, California, also by NIOSH.

2.2.2. Preparation of the test articles

Cleavage fragments were prepared from the bulk material as follows: each bulk specimen was crushed to centimeter-sized fragments using a hydraulic press. The material was sieved progressively through 1 mm (No. 18), 364 μm (No. 45), and 250 μm (No. 60) sieve pans. The material passing through the finest sieve was then weighed and divided into aliquots approximately 2 grams each. These were then ground by mortar and pestle with minimal hand pressure. Periodically mineral grains were mounted on a glass slide in a refractive index oil and examined by polarized light microscopy (PLM) to observe the range of particle sizes present. When a minimum of 5 μm long and a maximum of 3 μm wide particles were observed, the grinding was stopped. The resultant material was weighed and reserved for suspension and filtration. Suspensions were poured into a 2 L graduated cylinder with a magnetic stir plate, made to a volume of 2 L of deionized water (with two drops of Liquinox detergent), and stirred for 5 minutes. A 10 mL aliquot of the suspension was drawn from the 1200 mL level of the graduated cylinder at increments of 5, 7, 10, 20, and 30 minutes, and 1, 2, 5, and 21 hours. Each aliquot was placed in a clean 50 mL beaker, and then 1 mL aliquots of each drawn suspension were immediately pipetted on to the filtration system. Aliquots were deposited on 25-mm diameter 0.45 μm mixed cellulose ester (MCE) filters using a 6-station filtration manifold. Filters were allowed to dry on a slide warmer. A quarter of each filter was placed on a labeled slide and collapsed using an acetone vaporizer. Slides were examined to note the characteristics of the particle population, the presence of elongate particles and cleavage fragments, and the density of the particle population. Following the exploratory phase of the work, the sample was re-stirred for 5 minutes, and the clock was restarted. The sample was allowed to settle for 30 minutes, at which time ten 20-mL aliquots were placed in the vacuum filtration funnel and filtered for harvest and for analysis by TEM. This process was repeated five times to harvest at least 100 milligrams. Schematic representation of the process is presented on Figure 2.1.

Riebeckite asbestos particles were already supplied in respirable range and thus did not require mechanical preparation, but only characterization. Tremolite asbestos bulk material was crushed by a hydraulic press, sieved, and ground using a ball mill until sufficient amount of EMPs with the desired dimensions were observed under polarized

light microscopy. Samples were then suspended in a mixture of deionized water and isopropanol and filtered through 0.45 μm mixed cellulose ester (MCE) filters.

Figure 2.1. Schema for preparation and harvesting of the cleavage fragments.

2.2.4. Polarized Light Microscope Alignment

PCM analysis (400x) - to estimate the length and diameter during the harvesting step - was performed using a standard Walton-Beckett graticule (as described in NIOSH 740024). The Olympus BX50 polarized light microscope used for the analysis of the mineral samples for the NIOSH project was aligned prior to use per Analytical Sciences SOP No. 1609-001, "Aligning the Polarized Light Microscope," to obtain the most accurate measurements possible. The alignment included centering the objectives, aligning the substage condenser, aligning the polarizer and analyzer, and aligning the ocular crosshairs. Photomicrographs acquired using an Olympus BX50 polarized light microscopes (Olympus America, Center Valley, PA), were used to estimate optical properties of the fibers such as extinction angle and refractive indices of several fibers ($n \sim 10$ -20). Further, spindle stage analysis under PLM was used to determine and

confirm whether individual fibers from the samples contain polycrystalline bundles or single fibrils, or cleavage fragments from larger crystals.

2.2.5. TEM measurements:

TEM particle characterization was performed using a Hitachi 7000 analytical electron microscope with AMT XR280 digital camera. Particles were measured at an on-screen magnification ranging from 4570X to 21,900X. Three TEM grids were prepared from each of the mineral samples. Each grid square analyzed was traversed from left to right. At least 700 particles were measured in each sample. Length and width measurements in microns and aspect ratio for each particle were registered. From that data approximate surface areas of individual particles were calculated using the formula $4 * (length * width) + 2 * (width * width)$. The length and width of each fiber were measured using a calibrated image from the AMT XR280 digital camera. We were basing our calculations on the established numbers. 550 would be the Minimal number of particles (MNP) necessary for determination of the percentage distributions. 100 would be the MNP required for determination of the shape descriptors [9]. Figure 2.2. contains the example of microphotograph used for measurements. Elemental makeup of the studied EMPs was determined by energy-dispersive spectroscopy (EDS) detector. Such analysis allowed determining the similarities and differences in the cleavage planes of these fibers, which are critical for obtaining parameters concerning their cleavage fragments.

Figure 2.2. Measuring the lengths and widths of tremolite EMPs harvested during the study using TEM microphotographs.

2.2.6. Gravimetric Analysis

The Mettler PG503 balance was used to weigh the subsamples before and after each step of sample reduction. It was calibrated before each use as prescribed by SOP No. 0100-001 "Calibration and Operation of Mettler PG503 Balance." The balance is calibrated using a pair of NIST-traceable 0.020-gram and 200-gram reference weights that are recertified annually by Troemner.

2.3. Results and discussion

2.3.1. Morphology of the acquired samples

TEM analysis offers the highest resolution and the most accurate identification capabilities in the case of EMPs. It allows detection of particle shape and structure down to the individual fibrils. Hence, we used it to calculate the approximate surface areas and masses of individual particles, based on known density values. Representative microphotographs of the harvested EMPs can be seen on Figure 2.3.

Figure 2.3. A – Riebeckite asbestos. B - Riebeckite CF. C – Tremolite asbestos, 4,000x Magnification. D – Tremolite CF, 10,000x Magnification.

Detailed particle characteristics summaries and size distribution charts were prepared after measuring at least 700 individual particles on TEM photographs. The summaries are listed in Tables 2.1-2.4.

**Table 2.1. Summary Statistics for Riebeckite Asbestos Particle Size Data
TEM (2,000X to 10,000X Magnification)**

<i>Length (microns)</i>		<i>Width (microns)</i>		<i>Aspect Ratio</i>	
Mean	6.36	Mean	0.34	Mean	23.72
Standard Error	0.24	Standard Error	0.01	Standard Error	1.18
Median	4.43	Median	0.26	Median	15.68
Mode	15.60	Mode	0.15	Mode	14.14
Standard Deviation	6.61	Standard Deviation	0.29	Standard Deviation	32.71
Sample Variance	43.74	Sample Variance	0.08	Sample Variance	1070.01
Kurtosis	19.66	Kurtosis	27.56	Kurtosis	166.18
Skewness	3.57	Skewness	4.06	Skewness	10.12
Range	62.53	Range	3.23	Range	633.66
Minimum	0.67	Minimum	0.03	Minimum	2.70
Maximum	63.20	Maximum	3.26	Maximum	636.36
Sum	4885.58	Sum	258.77	Sum	18215.96
Count	768	Count	768	Count	768

**Table 2.2. Summary Statistics for Riebeckite CF Particle Size Data
TEM (2,000X to 10,000X Magnification)**

<i>Length (microns)</i>		<i>Width (microns)</i>		<i>Aspect Ratio</i>	
Mean	6.09	Mean	1.88	Mean	3.69
Standard Error	0.11	Standard Error	0.04	Standard Error	0.07
Median	5.45	Median	1.74	Median	3.20
Mode	10.20	Mode	1.18	Mode	3.07
Standard Deviation	3.28	Standard Deviation	1.02	Standard Deviation	1.89
Sample Variance	10.75	Sample Variance	1.03	Sample Variance	3.58
Kurtosis	2.88	Kurtosis	0.55	Kurtosis	7.84
Skewness	1.32	Skewness	0.78	Skewness	2.13
Range	22.74	Range	6.44	Range	17.38
Minimum	0.96	Minimum	0.14	Minimum	1.12
Maximum	23.70	Maximum	6.58	Maximum	18.50
Sum	5052.01	Sum	1556.76	Sum	3063.01
Count	829	Count	829	Count	829

**Table 2.3. Summary Statistics for Tremolite Asbestos Particle Size Data
TEM (2,000X to 10,000X Magnification)**

<i>Length (microns)</i>		<i>Width (microns)</i>		<i>Aspect Ratio</i>	
Mean	6.56	Mean	0.31	Mean	25.73
Standard Error	0.21	Standard Error	0.01	Standard Error	0.92
Median	4.65	Median	0.25	Median	17.20
Mode	1.75	Mode	0.17	Mode	10.30
Standard Deviation	6.13	Standard Deviation	0.22	Standard Deviation	26.29
Sample Variance	37.53	Sample Variance	0.05	Sample Variance	691.38
Kurtosis	11.67	Kurtosis	12.11	Kurtosis	40.12
Skewness	2.69	Skewness	2.63	Skewness	4.47
Range	54.40	Range	2.12	Range	360.00
Minimum	0.61	Minimum	0.02	Minimum	2.00
Maximum	55.00	Maximum	2.14	Maximum	362.00
Sum	5362.24	Sum	253.29	Sum	21024.96
Count	817	Count	817	Count	817

**Table 2.4. Summary Statistics for Tremolite CF Particle Size Data
TEM (2,000X to 10,000X Magnification)**

<i>Length (microns)</i>		<i>Width (microns)</i>		<i>Aspect Ratio</i>	
Mean	7.71	Mean	1.83	Mean	6.51
Standard Error	0.24	Standard Error	0.07	Standard Error	0.29
Median	5.59	Median	1.11	Median	4.93
Mode	10.50	Mode	1.19	Mode	3.33
Standard Deviation	6.38	Standard Deviation	1.95	Standard Deviation	7.57
Sample Variance	40.71	Sample Variance	3.81	Sample Variance	57.35
Kurtosis	2.99	Kurtosis	4.73	Kurtosis	116.48
Skewness	1.71	Skewness	2.04	Skewness	8.99
Range	33.00	Range	13.39	Range	125.07
Minimum	0.70	Minimum	0.01	Minimum	1.00
Maximum	33.70	Maximum	13.40	Maximum	126.07
Sum	5429.00	Sum	1289.09	Sum	4580.69
Count	704	Count	704	Count	704

Size distribution charts for all 4 tested materials are available in the Appendix A. The output data included frequency distribution of lengths, widths, and aspect ratios of the studied test articles.

Relatively few EMPs with diameters larger than $\sim 3 \mu\text{m}$ will penetrate into the lungs [10], but those with diameters less than $0.1 \mu\text{m}$ are also less retained in the lungs compared to larger diameters [11]. All EMPs had widths less than $3 \mu\text{m}$, with much smaller median numbers for asbestiform varieties, which was unsurprising. However, if applied to the macrophage functions, diameter of the fiber does not affect phagocytosis or internalization [12]. It was obvious that the surface areas of individual EMPs will be quite dissimilar between respective habits. TEM measurements data allowed us to find approximate volumes, masses and surface areas of individual EMPs, using the respective formulas:

$$\text{Volume} = \text{length} * \text{width} * \text{width}$$

$$\text{Mass} = \text{Volume} * \text{density}$$

$$\text{Surface Area} = 4 * (\text{length} * \text{width}) + 2 * (\text{width} * \text{width}).$$

The comparative surface areas for “critical” fibers and non-fibers are listed in Table 2.5. Again, unsurprisingly, asbestiform varieties had much higher surface areas per unit of weight.

Table 2.5. The total number and surface areas of particles in the test articles, including “critical” fibers, having length $> 5 \mu\text{m}$ and aspect ratio of $\geq 3:1$, per $120 \mu\text{g}$ of dry bulk preparations. CF – Cleavage Fragments.

Per 120 μg	Total number		Total surface area of all particles, cm^2/g	Total surface area of critical fibers, cm^2/g
	Fibers	Non-fibers		
Tremolite	1.48E+07	4.68E+08	2.43E+04	2.02E+04
Riebeckite	4.88E+06	4.21E+08	1.15E+04	9.91E+03
Tremolite CF	2.83E+05	1.10E+06	2.94E+03	1.68E+03
Riebeckite CF	7.77E+05	3.88E+06	5.11E+03	2.47E+03

The importance of surface area calculation stems from the nature of mineral-cell interactions, that depend on active sites present on that surface, microscopic topography

and surface charge of the mineral particles [13]. Dangling bonds, silanol groups, and unoccupied cation coordination sites can all lead to drastic changes in redox environment around EMPs. Previously it was found that both riebeckite and tremolite fibers exhibit sustained surface reactivity, and in both studied minerals free radical production does not depend on total iron content, but rather on specific Fe^{2+} and Fe^{3+} sites on the surface [14]. Upon incubation with H_2O_2 – simulating the lysosomal conditions – hydroxyl radical yield from tremolite significantly dropped by 24 hours, as opposed to significant increase in riebeckite fibers, but sustained radical reactivity of tremolite continued for quite a long time (especially the $\text{COO}^{\bullet}$ production) [14].

2.3.2. Chemical composition.

The EDS analysis showed elemental signatures to be predominantly Si, Fe, Mg, Na, and O for riebeckite EMPs, and Si, Ca, Mg, and O with only trace Fe for tremolite particles (Figure 2.4.). Cu signal comes from the copper grids utilized in the process. Previous studies on crocidolite toxicity suggested that the chemistry, presumably the high iron content, is important not only for cytotoxicity *per se*, but also for a set of biological effects, such as transcriptional activation of iNOS.

Figure 2.4. Representative EDS spectra of the studied EMPs. The highest peaks have the associated element identifications. RF – riebeckite asbestos, RC – riebeckite cleavage fragments, TF – tremolite asbestos, TC – tremolite cleavage fragments

2.3.3. Dose calculations for toxicological studies

For the *in vitro* experiments with murine alveolar macrophages (MPI cells): Based on the TEM calculations, we recalculated the amount of “critical” fibers (>5 μm in length, aspect ratio of >3:1) in the used EMP preparations per macrophage in the plates. 1 μg of riebeckite asbestos contained 4.07×10^4 fibers, 1 μg of tremolite asbestos contains 1.23×10^5 . For the surface areas of individual wells of 9.6 cm² and 0.32 cm² and seeding densities of 1×10^6 and 3×10^4 per well for 6-well plate and 96-well plate respectively, riebeckite asbestos dose 9 μg/cm² had 3.5 fibers per cell. 6 μg/cm² – 2.5 fibers per cell; tremolite asbestos dose 5 μg/cm² had 6 fibers per cell. 1.2 μg/cm² – 1.5 fibers per cell. From Stone et al. [15] – 6×10^9 AMs per lungs in humans. $9\text{--}36 \times 10^9$ total fibers – within the lifetime of significant population of workers as listed in Chapter 6.2 of the WHO

guidelines on air quality [3]. For *in vitro* studies usually higher fiber doses are needed to observe the cytotoxic effects, but for mechanistic studies and gene expression profiling lower doses are more suitable [16].

For *in vivo* study we based off our dosing calculations on the suggested cancer risks at specific numbers of critical fibers per gram dry weight of the lung tissue – a threshold for cancer causation in humans. While there are no established limits in the US, Japan uses the following number of fibers for establishing the asbestos exposure and cancer risks: more than 2×10^6 per gram of dry weight for fibers longer than $5 \mu\text{m}$ [17]. In the Finnish study researchers deemed that the highest odds ratio (9) for lung cancer in asbestos-exposed workers was achieved at fiber concentrations of more than 3×10^6 per gram of dry weight [18]. Assuming the human lungs weight of $\sim 840 \text{ g}$ [19] and dry weight of 26% $\sim 218.4 \text{ g}$ [20], it is more than 4.37×10^8 fibers $> 5 \mu\text{m}$ in length per pair of lungs if using Japanese threshold value and 6.55×10^8 fibers $> 5 \mu\text{m}$ if using the Finnish study value. Again, it fits the workers' accumulated lifetime concentration. We used mouse wet lung weight of 0.13 g and dry weight of 0.0325 g [21]. Assuming the 20% loss of the microparticles administered via pharyngeal aspiration [22], and the riebeckite asbestos retention data [23] as 35% for fibers longer than $5 \mu\text{m}$, at 1 year post-exposure, the mice receiving $120 \mu\text{g}$ of riebeckite asbestos test article will have 4.2×10^7 critical fibers per gram of dry lung weight left. There was no data on what cleavage fragments retention would be, but if using the same approach as used for riebeckite fibers, tremolite cleavage fragment (having the least number of critical fibers per unit of mass) will have $8.72 \times 10^6 \times 0.8 \times 0.35 = 2.44 \times 10^6$ critical fibers per gram of dry lung weight, meaning that we can use $120 \mu\text{g}$ as an equal mass-based dose. Considering the importance of surface area parameter in fiber toxicity paradigm, we also added to critical fiber surface-area equivalent doses for riebeckite and tremolite asbestos and respective cleavage fragments ($10 \mu\text{g}/\text{mouse}$ and 120 for tremolite asbestos and CF, $30 \mu\text{g}/\text{mouse}$ and 120 for riebeckite asbestos and CF).

2.4. Conclusions

A detailed characterization of physical, chemical and structural characteristics of asbestiform fibers and corresponding mineral analog cleavage fragments is an essential

step in determining the factors and mechanisms by which these fibers exert toxicity. Our approach allowed to: 1) harvest the required amount of the material for the testing, 2) obtain detailed information on the length-width ratios, variability in metal ion concentrations and chemical composition of the studied riebeckite and tremolite EMPs. Prepared EMP samples were characterized by the uniform median lengths and the sufficient amount of EMPs longer than 5 μm , which is a requirement for the “critical” fibers. Using the TEM measurements of particles, surface areas of the critical fibers were calculated to provide additional reference doses for the toxicological studies. Test articles were deemed appropriate for use in toxicological experiments in cell culture models and *in vivo* repeated pharyngeal aspiration exposure in mice.

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Supplementary Materials

Size distribution charts for the studied EMP preparations

Frequency distribution of aspect ratios, lengths and widths for riebeckite asbestos particles. Measured by TEM (2,000 to 10,000X Magnification), n =768

Frequency distribution of aspect ratios, lengths and widths for riebeckite cleavage fragments. Measured by TEM (2,000 to 10,000X Magnification), n = 829

Frequency distribution of aspect ratios, lengths and widths for tremolite asbestos particles. Measured by TEM (2,000 to 10,000X Magnification), n = 817

Frequency distribution of aspect ratios, lengths and widths for tremolite cleavage fragments. Measured by TEM (2,000 to 10,000X Magnification), n = 704

Chapter 3. Differential responses of murine alveolar macrophages to elongate mineral particles of asbestiform and non-asbestiform varieties: cytotoxicity, cytokine secretion and transcriptional changes

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3.1. Abstract

Human exposures to asbestiform elongate mineral particles (EMP) may lead to diffuse fibrosis, lung cancer, malignant mesothelioma and autoimmune diseases. Cleavage fragments (CF) are chemically identical to asbestiform varieties (or habits) of the parent mineral, but no consensus exists on whether to treat them as asbestos from toxicological and regulatory standpoints. Alveolar macrophages (AM) are the first responders to inhaled particulates, participating in clearance and activating other resident and recruited immunocompetent cells, impacting the long-term outcomes. In this study we address how EMP of asbestiform versus non-asbestiform habit affect AM responses. Max Planck Institute (MPI) cells, a non-transformed mouse line that has an AM phenotype and genotype, were treated with mass-, surface area- (s.a.), and particle number- (p.n.) equivalent concentrations of respirable asbestiform and non-asbestiform riebeckite/tremolite EMP for 24h. Cytotoxicity, cytokines secretion and transcriptional changes were evaluated. At the equal mass, asbestiform EMP were more cytotoxic, however EMP of both habits induced similar LDH leakage and decrease in viability at s.a. and p.n. equivalent doses. DNA damage assessment and cell cycle analysis revealed differences in the modes of cell death between asbestos and respective CF. There was an increase in chemokines, but not pro-inflammatory cytokines after all EMP treatments. Principal component analysis of the cytokine secretion showed close clustering for the s.a. and p.n. equivalent treatments. There were mineral- and habit-specific patterns of gene expression dysregulation at s.a. equivalent doses. Our study reveals the critical nature of EMP morphometric parameters for exposure assessment and dosing approaches used in toxicity studies.

3.2. Introduction

The history of prolonged and sometimes uncontrolled exposures in occupational and natural settings has proved that, upon inhalation, airborne elongate mineral particles (EMPs) can cause pulmonary inflammation, fibrosis and cancer [1-4], signifying the healthcare and economic burden worldwide. Adverse health outcomes following exposures to asbestos – a commercial term encompassing asbestiform varieties of six different minerals – were studied the most. However, EMP itself is a much broader term, defined as “any mineral particle with a minimum aspect ratio of 3:1” [5]. That includes particles with varied crystal growth habits, such as asbestiform (long, thin, splayed ends) and non-asbestiform (usually short, thick, brittle) [6]. During mining, milling, and processing, non-asbestiform mineral varieties can break down into elongate particles, also known as cleavage fragments (CF). Sometimes that patterned breakage results in dimensions akin to asbestiform fibers. CF might be chemically identical to asbestiform variety of the same mineral, but there is no consensus between different groups (regulators and scientific communities) on whether to treat them exactly as asbestos in terms of their potential health effects. Inability to reliably predict the occurrence and development of health outcomes after exposure to inhaled EMPs poses a significant challenge to health professionals. The latest professional conference on EMPs has shown that there is still uncertainty, whether some EMPs should be “regulated” (treated similar to asbestos by regulatory agencies) or not [7]. Of particular concern are the emerging cases of workplace and incidental exposures to EMPs such as those present in vermiculite in Libby, Montana, USA [8, 9], erionite in Oregon, USA and Turkey [10], silicon carbide fibers in Norway [11], sodic amphiboles in Nevada and Arizona, USA [12, 13], and non-asbestiform EMPs in taconite, talc and gold mining [14].

Epidemiological studies in talc, taconite, and gold mine workers exposed to non-asbestiform EMPs revealed little if any risk of lung cancer or mesothelioma [15-17], but consistent findings of pneumoconiosis and cardiovascular mortality excess occur in each of these mining types [14, 17]. Limited data exist on the health effects of tremolite and riebeckite CF exposure. There is some epidemiological evidence for tremolite CFs and short (shorter than 10 μm) tremolite asbestos fibers to be the cause of pulmonary fibrosis and lung cancer in nephrite carvers and quarry workers [18-20].

A number of studies have investigated the role of dimensions and physicochemical properties of asbestos particles [21-24]. There were, however, very limited efforts in elucidating the potential determinants of toxicity of non-asbestiform EMPs, mostly dating back to 1970-90s and the major biological effects found in the studies related not to the number of particles or mass, but to the surface area of the samples [25, 26]. Studies comparing short and long asbestos fibers were much more prevalent. For instance, short riebeckite asbestos fibers, fully phagocytosed by macrophages caused comparable cytotoxicity to long fibers at 24 hours when compared at similar number-based dose [27]. There is also an indication that asbestos particles shorter than 5 microns may be critical for health outcomes from the *in vivo* retention and translocation standpoints [28-30]. No study, however, has utilized well-characterized respirable EMP preparations.

Numerous cell populations are involved in asbestos-related pathology, with resident alveolar macrophages being one of the primary mediators of the host immune response. Their depletion during asbestos-induced lung disease exacerbates fibrosis and worsens overall prognosis [31]. Accordingly, macrophage responses, including patterns of cell damage, cytokines, chemokines, and changes in gene expression could be related to the specific features of the fibers, meaning toxicological studies must go hand in hand with detailed EMP characterization. Macrophage-based *in vitro* systems are uniquely important for the EMP hazard prediction [32]. They can also help minimize the comprehensive toxicity testing based on long-term animal studies and epidemiological evaluations of each type of EMP.

We hypothesized that alveolar macrophage responses (response defined as a change from the normal state) to respirable non-asbestiform EMPs that fall in the WHO “critical” fiber range are similar to asbestiform variety of the same minerals, especially when compared at equal surface area or number, rather than mass. To test this hypothesis, we exposed primary mouse alveolar macrophage-like cells (MPI cells) for 24 h to preparations of asbestiform (asbestos) and non-asbestiform (cleavage fragments) respirable EMPs, at doses equivalent for total mass, surface area, or number of the particles with length > 5 μm and aspect ratio > 3:1, and evaluated the cytotoxicity, DNA damage, cytokine secretion and differential gene expression.

3.3. Methods

3.3.1. Particle preparation and characterization

Specific particles that were investigated as part of this study included non-asbestiform tremolite, prepared by the U.S. Bureau of Mines for the National Institute of Environmental Health Sciences (NIEHS) for use in oral ingestion studies [33], Union Internationale Centre le Cancer (UICC) riebeckite asbestos (crocidolite), supplied by Research Triangle Institute (RTI International), non-asbestiform riebeckite obtained by NIOSH from Pikes Peak in Colorado Springs, Colorado, and tremolite asbestos from Lone Pine, California, also provided by NIOSH. Sample preparation is described in detail in the Chapter 2. Briefly, the bulk material was crushed by a hydraulic press, sieved, and ground by hand until sufficient amount of EMPs with the desired dimensions were observed under polarized light microscopy (PLM). For PLM counting purposes small aliquots were suspended in a mixture of deionized water and isopropanol and filtered through 0.45 µm mixed cellulose ester (MCE) filters. Particle characterization was performed using a Hitachi 7000 analytical electron microscope with AMT XR280 digital camera. Particles were measured at an on-screen magnification ranging from 4570X to 21,900X. Three TEM grids were prepared from each of the mineral samples. Each grid square analyzed was traversed from left to right. At least 700 particles were measured in each sample. Length and width measurements in microns and aspect ratio for each particle were registered. From that data approximate surface areas of individual particles were calculated using the formula $4 * (length * width) + 2 * (width * width)$. EMP elemental composition was assessed using SEM coupled with the energy-dispersive X-ray spectroscopy (EDS).

3.3.2. Cell culture

The experiments were conducted *in vitro* and utilized alveolar macrophage-like cells, obtained and differentiated from the murine fetal livers (Max Planck Institute a.k.a. MPI cells) [34]. MPI cells are phenotypically close to alveolar macrophages, can self-renew in culture, and respond to respirable particulates [35]. MPI cell culture was a generous gift by Dr. J. Pestka from the University of Michigan, originally obtained from the fetal livers of C57BL/6J mice following the standard protocol. Cells were cultured in Roswell Park

Memorial Institute (RPMI) medium with 10% fetal Bovine Serum (FBS), 1% L-glutamine (HyClone), 1% penicillin-streptomycin mixture (HyClone), and 30 ng/ml of mouse recombinant GM-CSF (R&D Systems). Humidified incubator (Fisher Scientific) at 37 °C and an atmosphere of 5% v/v CO₂ was used. Cells were routinely passaged when reaching 75% confluence, using Accutase (Innovative Cell Technologies, San Diego, CA) detachment solution. Passages between 4 and 8 were used for the studies. Phenotypic profile of MPI cells was confirmed using flow cytometry as follows: cells at passage 5 were detached using Accutase, suspended in staining buffer and stained with fluorochrome-conjugated antibodies versus CD45 (Clone: 30-F11, BD), Siglec F (Clone: E50-2440, BD Horizon™), CD11c (Clone: N418, eBioscience™), CD64 (Clone: X54-5/7.1, Biolegend) and CD24 (Clone: M1/69, BD) (all obtained from Fischer Scientific, Pittsburgh, PA). Samples were run on Amnis FlowSight imaging flow cytometer (Luminex Corp., Austin, TX) and analyzed using IDEAS 6.2 software. Alveolar macrophage genotype was confirmed using the Mouse Cell Atlas [36] by uploading the raw counts from bulk Next Gen RNA Sequencing.

3.3.3. *Treatment details*

Dry EMP preparations were heated to 220°C for 2 h to inactivate endotoxin. Endotoxin presence was further investigated in the stock suspensions using Limulus amoebocyte lysate (LAL) chromogenic endpoint assay kit (Hycult biotech, Inc., Plymouth Meeting, PA). Stock particle suspensions were prepared in Ca²⁺, Mg²⁺-free phosphate-buffered saline (PBS, pH 7.4) at the concentrations of 1.0 mg/ml. The EMPs were serially diluted in phenol-free RPMI medium with 1% FBS, 1% penicillin/streptomycin and 30 ng/ml of GM-CSF (treatment medium) at appropriate concentrations. Immediately before dilution, stock suspensions were sonicated in a 20kHz Q500 sonicator with 5.5" cup horn (QSonica LLC, Newtown, CT). The settings were set to six pulses, ten seconds each, with five seconds intervals between, at 30% amplitude, for a total delivered energy of ~625 Joules/ml per sample.

The current consensus is that EMPs with greater than 5 µm lengths should be prioritized in terms of the possible health effects, thus for dosing we accounted for the number and total surface area of the fibers of sufficient length and greater than 3:1 aspect

ratio, using the approach described in the Chapter 2. The importance of the surface area of the critical fibers parameter have been established by a number of robust and highly cited studies [23, 37-39]. The example of critical fiber number and surface area values per unit of mass (120 μg) is shown in Table 3.1. MPI cells, grown in 6-well and 96-well plates, were washed with PBS and treated with the mass-equivalent (9 $\mu\text{g}/\text{cm}^2$ for riebeckite asbestos and CF and 5 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF), fibers surface area-equivalent (9 and 40 $\mu\text{g}/\text{cm}^2$ for riebeckite asbestos and CF respectively, 5 and 60 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF respectively), and fibers number-equivalent (6 and 40 $\mu\text{g}/\text{cm}^2$ for riebeckite asbestos and CF respectively, 1.2 and 60 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF respectively) doses of asbestiform/non-asbestiform riebeckite or tremolite EMPs for 24 hours. There were no equal mass comparisons between riebeckite and tremolite minerals, but 5 $\mu\text{g}/\text{cm}^2$ tremolite asbestos and 9 $\mu\text{g}/\text{cm}^2$ riebeckite asbestos doses had similar total surface areas of critical fibers between each other. The fiber number for all equivalent doses utilized in our study were within the number concentrations used in other studies and relevant to exposed workers' lifetime-accumulated number of fibers (See Chapter 2: Dose calculations).

3.3.4. Cytotoxicity assessment

For cytotoxicity assessment MPI cells were plated at a density of 3×10^4 cells per well in 96-well microplates one day prior to EMP treatments. 24 hours after the start of treatment, cell membrane damage was assessed using commercially available lactate dehydrogenase (LDH) assay (Pointe Scientific, Lincoln Park, MI) by monitoring the reduction of NAD⁺ in the presence of lactate in the supernatants at 340 nm in a Synergy H1 plate reader (Biotek, Winooski, VT). Multi-Tox Fluor kit (Promega, Madison, WI) was used to evaluate the viability following EMP treatment per the assay instructions. 1 μM staurosporine (Sigma-Aldrich, St. Louis, MO) was chosen as positive control, since apoptosis was suspected to be the major cell death mechanism following exposure to the studied EMPs. Staurosporine was also previously used in murine macrophage *in vitro* LDH and viability assays [40]. Cytotoxicity experiments were performed three times, with at least eight technical replicates (wells) per treatment per experiment.

3.3.5. Assessment of DNA double-strand breaks via 2-color flow cytometric analysis

Formation of double strand DNA breaks (DSB) triggers phosphorylation of the histone variant H2AX at Ser-139, producing γ H2AX. By measuring the amount of phosphorylation, we can estimate the extent of DNA damage. MPI cells at passage six were seeded in 6-well plates at the density of 1×10^6 cells per well one day prior to EMP treatments. 24 hours after the start of treatment, supernatants from each well were taken for the cytokine measurements, while cells were washed with PBS and detached using the Accutase detachment solution. After collection, 20 μ l of cell suspension was taken for counting using Trypan Blue exclusion method on a Countess FL II automated cell counter, and the rest were centrifuged at 300 x g, and washed once with PBS. Cells were then fixed and permeabilized using FXP3 Fix/Perm solution (Invitrogen, Carlsbad, CA), and stained with anti-Phospho-Histone H2A.X(Ser139) antibody conjugated with eFluor® 660 fluorophore (Invitrogen, Carlsbad, CA) together with Hoechst 33342 nuclear dye (Invitrogen, Carlsbad, CA) per supplied protocols. Samples were processed on an Amnis FlowSight imaging flow cytometer (Luminex Corp., Austin, TX) and analyzed using IDEAS 6.2 software. 50 000 cells per sample were collected for analysis. The median Max Pixel intensity parameter in the cells exhibiting higher than threshold amount of γ H2AX (greater than 98.5% of control cells) was recorded. 500 μ M H₂O₂ treatment was used as a positive control. Sub-G1 cell populations were additionally evaluated as a marker of apoptosis/secondary necrosis. Gating strategy is visualized in Figure 3.1. Three technical replicates (three wells across three 6-well plates) per treatment were used.

Figure 3.1. Gating strategy for the flow cytometric analysis. Individual cells were gated out from debris using Area vs. Aspect ratio chart. Signal Gate was based on the cell population having pH2AX signal intensity >98.5% of control cells. Y axis – γ H2AX fluorescence signal intensity, X axis – nuclear dye (Hoechst 33342) signal intensity. CF – cleavage fragments

3.3.6. Cytokine secretion in the supernatants

Cell supernatants from the 6-well plates used for γ H2AX staining (see above) were collected and snap-frozen until the further analysis. Secreted cytokine profiles were determined in the supernatants using Bio-Plex Pro Mouse Cytokine 23-plex Assay on a Bio-Plex 200 Reader (Bio-Rad Laboratories, Hercules, CA) following manufacturer's directions, with three technical replicates allocated per each of three wells per sample. Prior to measurements supernatant samples were centrifuged at 2500 g. Cytokine quantities was normalized per total number of live cells in each well at the collection time. For each cytokine, out of range (OOR)-low results were set equal to 80% of the minimum within-range value, while OOR-high results were set equal to 125% of the maximum within-range value, if applicable. Hierarchical clustering analysis of the secreted cytokines was performed using Clustvis [41], with Euclidean distance similarity and Ward's clustering method.

3.3.7. Next generation RNA sequencing

A different set of cells was plated in 6-well plates and treated with an equivalent surface area of the critical fibers-based doses, with three replicates (wells) per each treatment group. 24 hours post-exposure cells were washed with PBS, lysed with the TRIzol™ reagent (Invitrogen, Carlsbad, CA) and stored at -80° C in RNase/DNase-free tubes for further analysis. RNA was purified using RNeasy MiniKits (Qiagen, Mississauga, ON, Canada) as described by the manufacturer, quantified using Thermo Scientific™ Nanodrop™ ND-1000 and qualified by agarose gel electrophoresis. The mRNA was enriched by oligo(dT) magnetic beads. Illumina kits were used for the RNA-seq library preparation, which included procedures of RNA fragmentation, random hexamer primed first strand cDNA synthesis, dUTP based second strand cDNA synthesis, end-repairing, A-tailing, adaptor ligation and library PCR amplification. Finally, the prepared RNA-seq libraries were qualified using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and quantified by qPCR absolute quantification method. The sequencing was performed on Illumina NovaSeq 6000 (Illumina, San Diego, CA). The expression level (FPKM value) of known genes and transcripts were calculated using ballgown through the transcript abundances estimated with StringTie. Principal Component Analysis was

performed with genes that have the ANOVA p value ≤ 0.05 on FPKM abundance estimations. The number of identified genes and transcripts per group was calculated based on the mean of FPKM in group ≥ 0.5 . Genes showing expression changes of at least 1.5-fold in either direction compared to their controls, having $p < 0.05$ and q (false discovery rate adjusted p -value) ≤ 0.1 were considered significantly differentially expressed and were considered for further analysis. All gene expression data were uploaded to NCBI's Gene Expression Omnibus and are accessible via accession number GSE157922.

3.3.8 Enrichment of KEGG pathways and GO enrichment analysis

Pathway analysis for the differentially expressed genes was carried out based on the latest KEGG (Kyoto Encyclopedia of Genes and Genomes) database. The analysis allows users to determine whether the differentially expressed mRNAs are enriched in certain biological pathways. The p -values calculated by Fisher's exact test were used to estimate the statistical significance of the enrichment of the pathways between the two groups. A p value cutoff of <0.05 was considered significant. We also investigated whether specific Gene Ontology (GO) terms were more likely to be associated with the DEGs. A Fisher's exact test is used to estimate the statistical significance of such enrichment of terms between the two groups (EMP and control). The p -value denotes the significance of GO Term enrichment in the differentially expressed gene list. All analyses with the differentially expressed genes were performed in R, Python or shell environment for statistical computing and graphics.

3.3.9. Statistical analysis

Statistical analysis was performed using SigmaPlot 14.0 (San Jose, CA). Treatment related differences were evaluated using Student's t -test or one-way ANOVA, as appropriate. A $p < 0.05$ was considered to be statistically significant. Data are presented as Mean $\pm$ SE. Principal component analysis and hierarchical clustering analysis were performed using online tool (www.clustvis.org).

3.4. Results

3.4.1. Particle characteristics

Summary of the EMP size and dimensions is listed in Table 3.1. Detailed tables for every sample can be found in Chapter 2. Asbestos samples had smaller median widths and higher aspect ratios, compared to cleavage fragments, but median lengths of all samples were around 5 μm as seen on the size distribution charts (See Appendix A.). The EDS analysis showed elemental signatures to be predominantly Si, Fe, Mg, Na, and O for riebeckite EMPs, and Si, Ca, Mg, and O with only trace Fe for tremolite particles. Cu signal comes from the copper grids utilized in the process.

Table 3.1. Morphological characteristics of the studied EMPs, calculated from the detailed TEM analysis of at least 700 particles per sample. A.R. – aspect ratio

	Riebeckite	Tremolite	Riebeckite CF	Tremolite CF
Median length, μm	4.43	4.65	5.45	5.59
Length Range (Min-Max), μm	0.67-63.2	0.61-55	0.96-23.7	0.7-33.7
Median width, μm	0.264	0.254	1.74	1.11
Width Range (Min-Max), μm	0.03-3.26	0.02-2.14	0.14-6.58	0.01-13.4
Median A.R.	15.7	17.2	3.2	4.93
A.R. Range (Min-Max)	2.7-636.4	2-362	1.12-18.5	1-126.07

3.4.2. EMP cytotoxicity

Cytotoxicity was observed for all treatments at 24h with the normalized ratio of viable-to-dead cells significantly decreasing to 90-70% of untreated controls (Figure 3.2).

Figure 3.2. Normalized viable/dead cells ratios, 24h exposure to riebeckite (A) or tremolite (B) EMPs. Values are expressed as mean $\pm$ SEM from 3 independent experiments. Stauro – 1 μ M of staurosporine. Equal mass: 9 μ g/cm² for both riebeckite asbestos and cleavage fragments (CF); 5 μ g/cm² for both tremolite asbestos and CF; Equal surface area of critical fibers: 9 and 40 μ g/cm² for riebeckite asbestos and CF, 5 and 60 μ g/cm² for tremolite asbestos and CF; Equal number: 6 and 40 μ g/cm² for riebeckite asbestos and CF, 1.2 and 60 μ g/cm² for tremolite asbestos and CF. α p <0.05 vs. control; * p <0.05 vs. cleavage fragments equivalent.

Riebeckite cleavage fragments were less cytotoxic than asbestos at the equivalent mass doses, however significant difference between the growth habits was observed only for the equal mass doses of riebeckite (Figure 3.2.A.). At the equal mass, asbestiform EMPs clearly caused significantly greater LDH leakage (Figure 3.3.).

Figure 3.3. LDH activity in the supernatant, as percentage of untreated cell values. 24h exposure to riebeckite (A) or tremolite (B) EMPs. Values are expressed as mean $\pm$ SEM from 3 independent experiments. Stauro – 1 μ M of staurosporine. Equal mass: 9 μ g/cm² for both riebeckite asbestos and cleavage fragments (CF); 5 μ g/cm² for both tremolite asbestos and CF; Equal surface area of critical fibers: 9 and 40 μ g/cm² for riebeckite

asbestos and CF, 5 and 60 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF; Equal number: 6 and 40 $\mu\text{g}/\text{cm}^2$ for riebeckite asbestos and CF, 1.2 and 60 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF. $^{\alpha}$ $p < 0.05$ vs. control; * $p < 0.05$ vs. cleavage fragments equivalent.

For riebeckite EMPs, LDH leakage from the cells was similar at the calculated surface area equivalent doses. When treated with equal particle numbers, CF trended to have more pronounced cytotoxic effects, compared to respective asbestiform analogues, however never reaching statistical significance.

3.4.3. Relative amount of the γH2AX staining and nuclear staining analysis

Isolated particles were excluded from the analysis through initial size/aspect ratio scatterplot. Further, the imaging flow cytometry allows checking the visuals of every registered event. We spotted only several large enough isolated particles, all cleavage fragments, in the data collection gates amongst thousands of cells across all samples. Hoechst 33342 binding to the particles was negligent. Individual cell γH2AX signal was highest in asbestos-treated cells, specifically riebeckite asbestos. There was no difference between tremolite asbestos and CF treatments at number-equivalent dose. Cleavage fragments were not as efficient in inducing the H2AX phosphorylation in general (Figure 3.4.).

Figure 3.4. Median Max Pixel intensity (arbitrary units) of the γH2AX fluorescence staining in the signal gate, 24h exposure to riebeckite (A) or tremolite (B). Values are expressed as mean of three biological replicates $\pm$ SEM. Equal mass: 9 $\mu\text{g}/\text{cm}^2$ for both riebeckite asbestos and cleavage fragments (CF); 5 $\mu\text{g}/\text{cm}^2$ for both tremolite asbestos and CF; Equal surface area of critical fibers: 9 and 40 $\mu\text{g}/\text{cm}^2$ for riebeckite asbestos and CF, 5 and 60 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF; Equal number: 6 and 40

$\mu\text{g}/\text{cm}^2$ for riebeckite asbestos and CF, 1.2 and 60 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF. $^{\alpha}$ $p < 0.05$ vs. control; * $p < 0.05$ vs. cleavage fragments equivalent.

Nuclear staining revealed increased number of cells found in the sub G-1 region of the dot plots (Figure 3.5.) for riebeckite CF, tremolite CF, and riebeckite asbestos.

Figure 3.5. % of cells in the sub-G1 gate, relative to total cells, 24h exposure to riebeckite (A) or tremolite (B). Values are expressed as mean of three biological replicates $\pm$ SEM. Equal mass: 9 $\mu\text{g}/\text{cm}^2$ for both riebeckite asbestos and cleavage fragments (CF); 5 $\mu\text{g}/\text{cm}^2$ for both tremolite asbestos and CF; Equal surface area of critical fibers: 9 and 40 $\mu\text{g}/\text{cm}^2$ for riebeckite asbestos and CF, 5 and 60 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF; Equal number: 6 and 40 $\mu\text{g}/\text{cm}^2$ for riebeckite asbestos and CF, 1.2 and 60 $\mu\text{g}/\text{cm}^2$ for tremolite asbestos and CF. $^{\alpha}$ $p < 0.05$ vs. control; * $p < 0.05$ vs. cleavage fragments equivalent.

Riebeckite CF had lower sub-G1 cells than riebeckite asbestos at mass-equivalent doses, and higher at surface area-equivalents. Tremolite asbestos treatment did not significantly increased the Sub-G1 population of cells compared to controls.

3.4.4. Cytokine secretion

All EMP treatments provoked significant secretion of IL-1 α , MCP-1, MIP1 α and MIP1 β and KC (mouse analog of IL-8), but not other cytokines, such as IL-6 or TNF- α (Table 3.2.). Hierarchical cluster analysis of the cytokine/chemokine secretion was performed for the cytokines/chemokines, showing significant difference ($p < 0.05$) in at least 3 different treatments, compared to respective controls: IL-1 α , MCP-1, MIP1 α , MIP1 β and KC cytokines, and showed close clustering for the surface area and fiber number equivalent treatments (Figure 3.6.).

Table 3.2. Pooled cytokine values for MPI cells, following treatment with various elongated mineral particles (EMPs) in pg/10⁶ viable cells. RCF - Riebeckite Cleavage Fragments, RAsb - Riebeckite Asbestos, TCF - Tremolite Cleavage Fragments, TAsb - Tremolite Asbestos. OOR = Out of Range; OOR> = Out of Range Above; OOR< = Out of Range Below. The cytokines/chemokines values, showing significant difference (p<0.05) compared to respective controls have a red font color. Cytokines with at least 3 different EMP samples, significantly different, compared to respective controls, are highlighted in yellow.

Value type	Treatment	IL-1a	IL-1b	IL-2	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12(p40)	IL-12(p70)	IL-13	IL-17	Eotaxin	G-CSF	IFN-g	KC	MCP-1	MIP-1a	MIP-1b	RANTES	TNF-a
Mean	Control	8.56	10.56	16.51	6.33	11.27	8.98	20.38	21.98	23.90	43.40	138.85	86.26	13.49	16.93	29.37	18.46	45.11	268.57	408.09	259.39	7.36	45.72
	RCF 40 µg/cm ²	15.01	10.92	15.80	6.61	12.51	8.47	12.74	20.21	23.98	36.14	148.06	62.95	12.59	15.57	28.72	15.35	83.98	614.23	751.20	464.64	6.13	43.51
	RCF 9 µg/cm ²	8.68	9.44	14.06	5.90	11.02	7.23	11.09	18.46	20.23	24.26	115.38	64.62	11.27	14.26	26.78	15.48	30.83	261.72	348.10	259.55	6.29	38.72
	RAsb 9 µg/cm ²	18.49	11.31	14.72	6.20	13.07	6.76	11.34	19.70	22.38	29.51	142.47	62.75	11.48	15.03	25.46	12.60	76.46	728.08	1233.12	1115.46	6.12	37.41
Standard Error of Mean (SEM)	RAsb 6 µg/cm ²	13.78	9.01	12.97	5.45	11.06	6.39	9.25	18.10	19.60	21.57	123.79	57.78	10.40	12.84	23.05	12.42	56.05	500.06	785.75	761.88	5.28	35.11
	Control	0.40	0.44	0.70	0.24	0.57	0.47	2.79	1.92	1.24	1.53	9.25	6.22	0.62	1.02	2.16	1.04	5.36	13.40	14.67	22.98	0.80	3.24
	RCF 40 µg/cm ²	0.48	0.43	1.07	0.21	0.37	0.85	0.98	1.18	1.80	1.19	7.85	4.99	0.80	1.39	1.25	1.57	4.99	16.13	42.75	11.44	0.19	2.98
	RCF 9 µg/cm ²	0.19	0.37	0.71	0.17	0.21	0.71	0.58	0.89	1.27	2.23	6.25	3.71	0.33	0.73	0.47	1.04	1.57	12.21	11.41	12.28	0.12	1.41
Value type	RAsb 9 µg/cm ²	0.55	0.29	0.88	0.25	0.37	0.51	0.28	1.01	1.32	0.29	7.65	5.02	0.73	1.14	1.04	1.39	1.26	12.04	19.37	11.81	0.18	2.32
	RAsb 6 µg/cm ²	0.23	0.37	0.49	0.16	0.40	0.25	0.59	0.93	0.59	1.04	2.41	2.67	0.36	0.85	0.85	0.67	1.81	3.59	35.04	17.61	0.09	1.58
	Control	9.80	7.14	8.60	2.99	2.31	10.53	19.75	24.93	23.30	20.42	96.11	112.59	2.27	10.91	25.54	18.71	29.41	161.76	639.15	92.07	1.97	32.33
	TCF 60 µg/cm ²	29.22	12.98	11.72	5.93	9.18	12.17	22.63	26.63	25.81	24.72	119.16	98.93	3.07	11.32	14.81	15.69	117.05	873.09	2042.70	473.89	2.89	40.10
Mean	TCF 5 µg/cm ²	15.37	10.84	12.55	4.11	3.55	15.52	22.11	29.87	31.48	23.11	134.19	168.62	3.55	16.82	32.91	25.02	45.02	272.87	839.78	147.86	2.59	40.86
	TAsb 5 µg/cm ²	37.11	14.51	7.14	2.15	3.10	2.95	21.17	21.88	24.85	26.58	115.13	76.41	3.18	10.47	15.87	11.76	130.75	1323.66	3160.84	1177.37	2.82	28.32
	TAsb 1.2 µg/cm ²	20.39	10.10	8.91	2.78	2.71	8.87	18.62	24.35	23.62	20.38	113.45	101.30	3.09	10.10	19.92	16.44	50.56	478.11	1005.84	402.49	2.58	32.70
	Control	0.57	0.54	0.86	0.52	0.12	1.52	1.89	3.31	1.55	1.29	5.98	12.64	2.27	1.16	2.25	2.06	2.53	11.48	22.40	2.60	0.22	3.57
Standard Error of Mean (SEM)	TCF 60 µg/cm ²	2.16	0.69	0.44	0.60	0.54	1.49	2.04	2.22	1.53	1.10	6.29	8.66	3.07	0.30	3.10	2.22	0.62	38.92	190.69	11.59	0.15	1.84
	TCF 5 µg/cm ²	1.35	1.48	1.97	0.79	0.61	4.23	3.62	5.71	3.27	3.10	11.89	27.92	2.90	2.56	8.36	4.08	4.77	12.21	17.17	2.55	0.25	5.85
	TAsb 5 µg/cm ²	0.60	0.43	0.14	0.09	0.23	0.73	0.97	2.01	0.61	1.07	3.85	1.76	3.18	0.23	2.40	1.36	4.35	10.53	84.50	8.60	0.02	2.96
	TAsb 1.2 µg/cm ²	0.52	0.63	0.60	0.21	0.20	0.42	0.51	1.40	1.04	1.78	3.20	4.46	3.09	0.58	1.03	0.31	1.38	13.33	17.31	4.32	0.16	1.15

Figure 3.6. Hierarchical clustering of the secreted cytokine profiles for the MPI cells exposed to riebeckite (A) or tremolite (B) EMPs. RF – riebeckite asbestos, RC – riebeckite cleavage fragments, TF – tremolite asbestos, TC – tremolite cleavage fragments. Rows are centered; unit variance scaling is applied to rows. Both rows and columns are clustered using Euclidean distance and Ward linkage. Heatmap reflects unit variance.

3.4.5. Next generation RNA sequencing

Principal component analysis (PCA) showed a distinguishable gene expression profiling among the samples (Figure 3.7.).

Figure 3.7. Principal component analysis of the gene expression profiles in MPI cells, treated with critical fibers' surface area-equivalent doses of riebeckite and tremolite asbestiform and non-asbestiform EMPs. CF – cleavage fragments.

PCA also demonstrated expected grouping among replicates within samples and also sample groups with different treatment spread across the first two PC dimensions. The Pearson R² correlation of gene expression between replicates was well over 0.92 (Figure 3.8.) suggesting an inter- and intragroup variability, consistent with each group belonging to the same cell and exposure type.

Figure 3.8. Heatmap of Pearson R2 correlation of gene expression between biological replicates. Blue colors indicate high Pearson correlation, and white colors indicate low correlation. Per ENCODE Consortium guidelines RNA sequencing experiments should be performed with two or more biological replicates, unless there is a compelling reason why this is impractical or wasteful. Typically, the Pearson R2 correlation of gene expression between two biological replicates should be over 0.92. CF – Cleavage Fragments

Detailed comparative analysis of all differentially expressed genes (DEG) revealed 492 $\uparrow$ /302 $\downarrow$, 374 $\uparrow$ /220 $\downarrow$, 224 $\uparrow$ /70 $\downarrow$ or 258 $\uparrow$ /118 $\downarrow$ up ($\uparrow$)/down ($\downarrow$) regulated genes with a

significant fold change $\geq \pm 1.5$ in response to riebeckite asbestos, riebeckite CF, tremolite asbestos and tremolite CF, respectively (Figure 3.9).

Figure 3.9. Venn Diagrams for upregulated (A) and downregulated genes (B) of MIP cells, treated with critical fibers' surface area-equivalent doses of riebeckite and tremolite asbestiform and non-asbestiform EMPs, compared to untreated cells.

There were more uniquely dysregulated genes common for the riebeckite EMP samples (198), compared to tremolite exposures (10). Among the top 10 DEGs, all 4 materials up-regulated 5 (*H2-M2*, *Inhba*, *Cfb*, *Acp5*, *Ear2*) and down-regulated 5 (*Cfh*, *Clec4a*, *Ifitm6*, *Sell*, *Clec12a*) common genes, with several others common for 2 or 3 of the EMPs (Table 3.3). *H2-M2* (MHC-II) was the most upregulated gene. *Acod1* was highly upregulated in asbestos and less so in cleavage fragments. Complement factors B (*Cfb*) and H (*Cfh*) had opposite regulation status.

Table 3.3 The top 10 most significantly up- and down-regulated genes. Bold font indicates dysregulated genes common for all treatments.

Gene Symbol			
Riebeckite	Riebeckite CF	Tremolite	Tremolite CF
Top 10 UP Regulated Genes (Log ₂ Fold Change)			
<i>H2-m2</i> (4.783)	<i>H2-m2</i> (3.406)	<i>H2-m2</i> (3.969)	<i>H2-m2</i> (2.837)

<i>Acod1</i> (3.622)	<i>Ear2</i> (3.157)	<i>Cfb</i> (2.575)	<i>Inhba</i> (2.723)
<i>Inhba</i> (3.475)	<i>Acp5</i> (2.908)	<i>Acod1</i> (2.503)	<i>Ear2</i> (2.488)
<i>Cfb</i> (3.396)	<i>Cfb</i> (2.688)	<i>Inhba</i> (2.415)	<i>Krt79</i> (2.428)
<i>Acp5</i> (3.186)	<i>Inhba</i> (2.614)	<i>Ear2</i> (2.321)	<i>Cfb</i> (2.298)
<i>Hp</i> (2.846)	<i>Krt79</i> (2.588)	<i>Tmsb15b2</i> (1.948)	<i>Tgfb1</i> (2.009)
<i>Slc6a12</i> (2.776)	<i>Cxcr2</i> (2.506)	<i>Tnfrsf9</i> (1.913)	<i>Serpine1</i> (1.974)
<i>Ear2</i> (2.766)	<i>Siglec8</i> (2.274)	<i>Acp5</i> (1.839)	<i>Mmp12</i> (1.816)
<i>Krt79</i> (2.641)	<i>Serpine1</i> (2.206)	<i>Il7r</i> (1.826)	<i>Acp5</i> (1.792)
<i>Pdpr</i> (2.544)	<i>Hp</i> (2.196)	<i>Emp1</i> (1.626)	<i>Pdpr</i> (1.791)
Top 10 DOWN Regulated Genes (Log₂ Fold Change)			
<i>Cfh</i> (-2.616)	<i>Cfh</i> (-2.423)	<i>Ifitm6</i> (-1.979)	<i>Cfh</i> (-2.132)
<i>Adgre4</i> (-2.330)	<i>Adgre4</i> (-2.253)	<i>Cfh</i> (-1.898)	<i>Ifitm6</i> (-2.085)
<i>Clec4a3</i> (-2.254)	<i>Ifitm6</i> (-2.077)	<i>Sell</i> (-1.784)	<i>Sell</i> (-1.860)
<i>Ifitm6</i> (-2.184)	<i>Sell</i> (-1.926)	<i>Pram1</i> (-1.431)	<i>Pram1</i> (-1.600)
<i>Ms4a8</i> (-2.128)	<i>Ch25h</i> (-1.911)	<i>F5</i> (-1.109)	<i>Adgre4</i> (-1.536)
<i>Pram1</i> (-2.123)	<i>Emilin2</i> (-1.870)	<i>Tarm1</i> (-1.105)	<i>Clec12a</i> (-1.373)
<i>Sell</i> (-1.949)	<i>Olfm1</i> (-1.835)	<i>Clec4a3</i> (-1.102)	<i>Ccn3</i> (-1.367)
<i>Elovl6</i> (-1.904)	<i>Clec12a</i> (-1.794)	<i>Gda</i> (-1.078)	<i>Clec4a3</i> (-1.345)
<i>Olfm1</i> (-1.770)	<i>Clec4a3</i> (-1.785)	<i>Clec12a</i> (-1.064)	<i>Ccnd1</i> (-1.271)
<i>Clec12a</i> (-1.767)	<i>Klf2</i> (-1.759)	<i>Cd300lg</i> (-1.000)	<i>Klf3</i> (-1.254)

3.4.6. KEGG pathway enrichment analysis

Pathways analysis of DEGs following 24 hours of exposure to riebeckite asbestos, riebeckite CF, tremolite asbestos and tremolite CF predicted the involvement of a total of 61, 21, 44 and 25 pathways ($p < 0.05$), respectively. 11 of them were common to all exposure groups (Supplementary Table 1). Those were associated with the immune cell differentiation, inflammation, complement activation, cancer, and granulomatous lesions. *Cytokine-cytokine receptor interaction* was one of the most uniformly enriched pathways across all groups. *NF- κ B signaling*, and *Phagosome* were enriched for all groups except riebeckite CF. *Th1 and Th2 cell differentiation* was exclusive for asbestiform EMPs. Riebeckite asbestos had the most number of uniquely enriched pathways, including

Regulation of actin cytoskeleton, Fc gamma R-mediated phagocytosis, Galactose metabolism, Natural killer cell mediated cytotoxicity, Lysosome, B cell receptor signaling pathway, MAPK signaling pathway, HIF-1 signaling pathway, and Ferroptosis. Pathway associated with *NOD-like receptor signaling* was uniquely represented only upon tremolite asbestos exposure. *JAK-STAT* and *PPAR* signaling pathways were unique for riebeckite EMPs and tremolite CF, while *PI3K-Akt signaling pathways* was shared only by riebeckite samples. There was also a marked difference between asbestiform and non-asbestiform riebeckite, the former having 41 enriched pathways, not registered in CF. There were also notable differences between tremolite asbestos and CF, and the only common term unique for tremolite mineral EMPs was *Transcriptional misregulation in cancer*.

3.4.7. Gene Ontology term enrichment analysis

GO analysis revealed significant enrichment of the upregulated DEGs in a lot of specific biological processes (BP), most of them tied with the immune responses (Supplementary Table 2). Asbestiform EMPs had overall higher enrichment scores compared to their non-asbestiform counterparts. Tremolite asbestos and cleavage fragments had more distinctions between each other than corresponding riebeckite EMPs. Specific GO biological processes, having much larger scores for the riebeckite samples were those related to apoptosis (GO:0010942, GO:0043065, GO:0097190), *intracellular transport* (GO:0071702), *response to toxic substance* (GO:0009636) and others. Tremolite had distinctively enriched BPs related to innate immunity (GO:0002252, GO:0045087) and viral defense (GO:0009615, GO:0051607, GO:0045071 etc.).

3.5. Discussion

Epidemiology of asbestos-related diseases and biological effects of asbestos fibers have been extensively studied since mid-twentieth century, resulting in a firm belief, that “a worldwide ban of asbestos would eventually virtually eliminate its associated diseases” [42, 43]. Still, decades after becoming known to general public, conditions caused by asbestos exposures contribute most to the global occupational cancer burden and are responsible for the increasing mortality for occupational respiratory diseases [44], with 243,000 deaths attributable to asbestos in 2017 worldwide [45]. In addition, while *in vitro* studies provided important information on asbestos-induced cyto- and genotoxicity, the essential knowledge of the significance and role of different features and characteristics of fibers, that define responses, is still missing.

There currently is a consensus that among critical determinants of the fibers' pathogenicity are their respirability (ability to deposit into the deep lung), geometric dimensions [46], durability and surface reactivity [1]. The latter is important for toxic effects due to the presence of reactive sites on EMP surface, which was reviewed extensively elsewhere [38]. There is evidence that surface properties of asbestiform and non-asbestiform habits are quite similar for respective riebeckite and tremolite mineral [47, 48]. Whether growth habit itself is a determinant of EMP toxicity is the critical question that exists in the field of fiber toxicology. There were very limited efforts in elucidating the potential determinants of toxicity of non-asbestiform EMPs, with the general opinion leaning towards their non-toxic nature [49]. One of the major issues with the studies so far was inadequate preparation and characterization of EMPs, which lead to comparisons between vastly different asbestiform and non-asbestiform populations. In our study we: 1) attempted to refine the treatment approach by utilizing pre-made respirable asbestiform and non-asbestiform EMP formulations with the specific dimensional parameters, 2) included three dosing approaches: equivalent mass, surface area and particle number of the critical fibers, and 3) used relevant cell model by employing MPI cells, that are phenotypically close to alveolar macrophages (allowing us to forego using multiple animals for isolating alveolar macrophages from bronchoalveolar lavage). Fiber numbers for all equivalent doses utilized in our study were well within the number concentrations

used in other studies and are within the exposed workers' lifetime-accumulated number of fibers (Chapter 2). We were not aiming at elucidating the precise mechanisms of asbestos-induced lung disease, due to its complexity and model limitations [50].

Particle dimensions are important due to their established role in fiber toxicity paradigm. In case of human asbestos exposures, thinner fibers (< 0.1 μm thick) are associated with pleural plaques and mesothelioma, whilst those having width 0.1 – 3 μm – with fibrosis and lung cancer [51]. Lengthwise, 5 μm is considered a “critical” fiber length by the health agencies [52, 53]. Our sample preparations included EMPs within respirable range, with median lengths close to 5 μm (see Appendix A for detailed distribution charts), and cumulative frequency of EMPs with diameter < 3 μm at ~90%. Although not part of this study aims, it is important to make parallels with the short vs. long asbestos fibers toxicity paradigm, especially since significantly more studies focused on the shorter vs. longer asbestos rather than asbestiform vs. non-asbestiform EMP comparisons. The earliest evidence led to a prevailing opinion that long asbestos fibers *in vitro* are more cytotoxic to macrophages than short ones [27]. The situation can be quite different *in vivo*, however, since EMPs longer than 20 μm rarely penetrates deep into the alveolar region. Thus, *in vitro* studies utilizing non-respirable EMPs should be evaluated having in mind this serious limitation. For non-fibrogenic fibers Castranova et al. found that short and thin glass fibers are more cytotoxic than thick and long [46] while Padmore et al. discovered that shorter fibers are less potent in inducing the inflammatory responses [21]. However, both short and long riebeckite asbestos fibers were able to induce H_2O_2 production by macrophages and cause cell death [27].

Airborne particles deposited in the airways and respiratory compartments can be recognized and cleared by macrophages – innate immune cells with a range of molecular tools to deal with newcomers, be it microorganisms, inorganic dust or EMPs [54]. MPI cells have been utilized before to assess the alveolar macrophage response to infectious agents, such as *P. Aeruginosa* and *S. pneumoniae* [55], *M. tuberculosis* [56], *B. thailandensis* [57], and adenoviruses [58] as well as nanoparticles [35]. Previous functional study revealed that phagocytosis of fibrous particles, even longer than the cell itself, does not lead to the immediate loss of membrane integrity and death in macrophages, but pulling the fiber out (by other macrophage, for instance) does [24]. In

our case we noticed a uniform decrease in the ratio of live/dead cells across the treatment groups, except the lower dose of riebeckite CF (mass-equivalent to riebeckite asbestos). At the same time, there was a significant increase in the LDH leaked from the cells, which was similar for riebeckite CF and asbestos if compared at the calculated surface area- and particle number-equivalent doses. Despite statistically significant decrease in the ratio of viable to dead cells for both higher and lower doses, the lower tremolite CF dose (5 $\mu\text{g}/\text{cm}^2$) did not increase the LDH activity in supernatants. The difference may have to do with the excessive LDH leakage from the cells that were not yet dead in the other treatment groups. The cytotoxicity methods utilized, does not discriminate between necrosis and the consequences of a programmed cell death – apoptosis [59].

Differences between AM responses to riebeckite and tremolite can be approached by first looking at the redox behavior of respective minerals. EDS analysis confirmed the abundance of iron in riebeckite samples, but only a very small amount in tremolite asbestos and no detectable iron in tremolite CF. However, previously it was found that both riebeckite and tremolite fibers exhibit sustained surface reactivity, and in both studied minerals free radical production does not depend on total iron content, but rather on specific Fe^{2+} and Fe^{3+} sites on the surface [47]. Upon incubation with H_2O_2 – simulating the lysosomal conditions – hydroxyl radical yield from tremolite significantly drops by 24 hours, as opposed to significant increase in riebeckite fibers, but sustained radical reactivity of tremolite continues (especially the $\text{COO}^{\bullet}$ production) for quite a long time [47]. Increased genotoxicity of riebeckite over tremolite was previously observed in A549 cells [22], but not in macrophages, which are capable of more prominent “oxidative bursts”. To look deeper into the particle-specific cellular responses, we decided to measure the extent of DNA damage. Asbestos exposures had long been found causing double-strand DNA breaks (DSBs), mostly through mechanisms involving redox stress [60-63]. DSBs are the most deleterious DNA lesions, which, if left unrepaired, may have severe consequences for cell survival, as they lead to chromosome aberrations, genomic instability, or cell death. Cells respond to DNA damage by activating the so-called DNA damage response (DDR), a complex molecular mechanism developed to detect and repair DNA damage. The formation of DSBs triggers activation of many factors, including phosphorylation of the histone variant H2AX, producing γH2AX . By measuring the

amount of phosphorylation, we can estimate the extent of DDR. It should be noted, however, that H2AX phosphorylation also occurs under basal conditions, and can increase, for example during the cell division. There was a clear surge in median γ H2AX signal in the asbestos-treated cells, compared to control and cleavage fragments, except for the lower dose of tremolite (equivalent for particle number to respective cleavage fragments). Equal surface area did not play role in this case. The plausible explanation being that relatively small but potent population of asbestiform EMPs, longer than 20 μ m (about 5% in riebeckite and 4% in tremolite asbestos samples), can cause the most deleterious lesions or affect the DNA repair processes more severely [64]. Cell apoptosis also leads to the formation of DSBs during the DNA fragmentation. In permeabilized cells, the fragmented 182bp DNA multimers may leak outside, resulting in a reduced DNA population of cells (sub-G1 peak on a cell-cycle histogram). Analysis of the nuclear staining signal revealed increased sub-G1 populations for most of the treatments, more so in riebeckite EMPs, with cleavage fragments having the highest values. However, upon closer inspection (Figure 3.1.), it can be seen that sub-G1 populations in CF samples are confined closer to G1 itself, which is characteristic of necrotic cells, and do not exhibit significant γ H2AX signal, while in riebeckite asbestos the sub-G1 cells localize away from the G1, many of them having high γ H2AX, which is characteristic of cells at different stages of programmed cell death [65, 66]. Substantial presence of apoptotic cells is further supplemented by the specific enrichment of GO BP terms related to apoptosis in riebeckite-treated cells. The combined results still do not allow us to make confident assumptions regarding the prevalent mode of death in each treatment case. More extensive testing, focusing specifically on the hallmarks of cell deaths is required, since alveolar macrophage apoptosis has been implicated in the development of pneumoconiosis [67] and asbestosis [68].

The “tolerant” nature of the AM majority prevents overreaction and excessive activation. That being said, AM population is not homogenous and includes genotypically and functionally different varieties. [69-71]. Not all of them are strictly immunosuppressive and as early as in 1996 researchers hypothesized that the disbalance of suppressive vs. reactive subtypes (i.e. caused by the excessive apoptosis of the former subtype) is what constitutes the development of lung disease [68]. MPI cells themselves are more than

capable of acute pro-inflammatory reaction to pathogens, such as live *M. tuberculosis* [56]. In our study, compared to basal conditions, EMP-treatments only slightly upregulated secretion of alarmins, such as IL-1 α , and IL-1 β , but not TNF- α or IL-6, while at the same time caused significant increase in chemokines (MIP-1a, MIP-1b, MCP-1 and KC). This was previously observed *ex vivo* as well where alveolar macrophage obtained from the healthy human volunteers secreted minuscule amount of cytokines compared to the blood monocytes from the same subjects, after stimulation with 4 different types of asbestos, which was attributed to the translational block [72]. Hierarchical cluster analysis of the cytokine/chemokine secretion pattern showed close clustering for the surface area and particle number equivalent treatments, with significantly more chemokines secreted by asbestos-treated cells compared to cleavage fragments (Table B1, Appendix B). Secretion of chemokines/chemoattractant proteins is a major event happening during cell-fiber interaction. KC in particular is responsible for neutrophils chemotaxis, which plays a major role in the asbestosis pathogenesis [73]. Although important from the macrophage response standpoint, these results cannot be reliably translated into the *in vivo* settings, as shown by multiple studies that assessed the cytokine secretion profiles of the AMs. It is established that, in humans with asbestosis, alveolar macrophages secrete pro-inflammatory cytokines and are overresponsive to the other stimuli [74, 75]. Driscoll et al. reported considerable IL-1 and TNF- α secretion by rat alveolar macrophages at day 3 post exposure to crocidolite, which decreased substantially on days 7, 14, and 28 [76]. The increased cytokine secretion observed in humans and animals, however, has not been reproduced *in vitro* [74], just like in our study.

Detectable changes in macrophage gene expression happen within 6-24 hours post-stimuli [77], thus we performed the next generation RNA sequencing of the MPI cells 24 hours post exposure to surface area-equivalent doses of riebeckite and tremolite EMPs to see if AM immediate transcriptional reprogramming patterns are intrinsic for different minerals and/or crystallization habit. We decided to go with the surface area-equivalency, since it was more uniformly predictive of cytotoxicity for both riebeckite and tremolite, than fiber number equivalent doses. RNAseq revealed that MPI cells responded to EMP treatment by upregulating a significant number of genes responsible for the acute phase response, inflammation, antigen processing etc. Riebeckite asbestos

had the most significantly dysregulated genes compared to control, followed by riebeckite CF, tremolite asbestos and tremolite CF. Genes involved in iron handling, such as haptoglobin (*Hp*), ferritin light chain (*Ftl1*), and heme oxygenase 1 (*Hmox1*) were prevalently upregulated by riebeckite asbestos treatment, although we cannot reliably tie it up with the higher iron content in this mineral, for instance, it can be just the part of an acute response response, such as inhibiting the pro-inflammatory HMGB1 molecules [78]. However, transcriptional upregulation of both ferritin and heme oxygenase 1 have been previously implicated in asbestos-related pathology [79, 80]. *Acod1* is a gene, which is upregulated in the macrophages upon a plethora of stimuli, and is coregulated together by TNF- α and IFN-gamma [81]. Complement factor B (*Cfb*) was significantly upregulated in all groups, while its competitor – complement factor H (*Cfh*) – downregulated. Downregulation of the *Cfh* can be related to the excessive NF κ B and IL-1 β signaling (through the microRNAs), seen across the samples. While it might be beneficial for the initial innate responses, sustained *Cfh* deficits may eventually result in pathogenic complement activation, chronic inflammation and autoimmunity, all seen in human asbestos exposures [82]. The role of *Cfh* in lung diseases is still not clear, however some lung cancers upregulate *Cfh* expression to avoid cell lysis through the complement activation [83]. A set of *Ifit* genes (*ifit3*, *ifit1b*, *ifit2*) was uniquely upregulated only in tremolite asbestos-exposed cells. This is peculiar, since those are mostly induced by type I (IFN- α/β) and type III (IFN- λ s) interferons whereas type II IFN (IFN- γ) is much less potent [84]. *Ifit* proteins lack enzymatic activity but bind a variety of RNAs and other macromolecules, contributing to the viral defense. Both KEGG pathway and GO BP analysis also revealed several highly enriched virus response pathways in tremolite asbestos samples. Type I interferons play an important role in lung homeostasis and can downplay the immune-mediated pathology [85], which is important for chronic conditions, such as asbestosis, and was already confirmed for silicosis [86]. KEGG analysis revealed that both asbestiform and non-asbestiform riebeckite EMPs induced *JAK-STAT*, *PPAR*, and *PI3K-Akt signaling pathways*, which may signify the close intracellular response patterns to that particular mineral – something we have not observed in tremolite-treated cells. Nevertheless, riebeckite asbestos had 41 enriched pathways, not present in respective CFs, including those related to NF- κ B and MAPK signaling, cell adhesion

molecules, as well as phagosome/lysosome function. GO BP also revealed more sophisticated transcriptional response to riebeckite asbestos, compared to CF. The fact that *NOD-like receptor signaling* KEGG pathway was enriched only in tremolite also requires further attestation by the functional assays, since we have observed the same in our mouse experiment at 7 days post-treatment with tremolite, but not riebeckite [87].

Figure 3.10. Summary of the alveolar macrophages' responses to asbestiform and non-asbestiform EMPS if compared at the critical fibers' surface area-equivalent doses.

Overall, magnitude and direction of the gene expression dysregulation was the highest in riebeckite asbestos-treated cells and followed the similar patterns for riebeckite asbestos and cleavage fragments but was quite divergent for tremolite polymorphs. Together with the cytotoxicity and cytokine secretion data, a pattern emerges, when if compared at the equivalent surface area of critical fibers, asbestiform and non-asbestiform EMP preparations show some similarity in how AMs respond to them in a short time-frame of 24 hours (Figure 3.10.). The similarity is more pronounced for the riebeckite mineral than for tremolite, which may have to do with the potential differences in surface reactivity. The observed AM response patterns align with the threshold paradigm outlined by Cox et al. [88]: individual cellular endpoints, such as membrane or

even DNA damage cannot serve as reliable predictors of an outcome. The notable differences in cell responses to riebeckite vs. tremolite mineral EMPs, including respective cleavage fragments might have to do with the variable iron content, presence of silanol groups, particle opsonization by specific proteins etc. It is more likely that only upon accumulation of different signals and the overcoming of activation threshold, the specific outcome can be observed. The combinations of signals may as well be different for different minerals: based on our results, growth habit might play bigger role for ones (tremolite) and be a less significant determinant for others (riebeckite).

Studies *in vitro* have intrinsic limitations and cannot address all the complexity of interactions among tissues and organs, and do not account for the modifying effects of deposition patterns and clearance by mucociliary actions as well as contribution from biopersistence. Specialists in the field all agree that "...what happens at the lung level is based on a population of fibers getting to the deep part of the lung and not based on one EMP interacting with one lung cell" [89]. Artificial approach to the sample preparation, although necessary for adequate comparisons, is not exactly representative of the typical human exposures. In *in vivo* settings, shorter EMPs, including non-asbestiform mineral analogues can be cleared more easily, but it does not mean they are less potent in causing cell damage in the sustained exposure scenario. Further studies focusing on the EMP-evoked free radical production and time-resolved cell death are necessary to complement the results of this study.

3.6. Conclusions

Our study is the first one that attempted to determine the toxicological profiles of comparable asbestiform and non-asbestiform EMPs populations *in vitro*, utilizing different dose metrics in a relevant alveolar macrophage model. We only partially confirmed the initial hypothesis. While equal mass-based doses resulted in markedly differential responses between asbestos and CF, at equal fiber surface area-based doses asbestos and corresponding CF had similar cytotoxic effects. However, when further comparing equivalent surface-area based treatments, there were distinct transcriptional reprogramming patterns and DNA damage, also specific for the mineral type, suggesting

that asbestiform habit itself may be an important determinant for some minerals, like tremolite. Altogether, the accumulated evidence points us in the direction of the selective approach to choosing relevant EMP parameters for hazard identification step of risk assessment. Further understanding the factors and mechanisms by which EMP exert toxicity may lead to the emerging of new paradigm, where aside from determining the number of EMPs per volume of air, other parameters, such as surface area of the “critical” fibers should be accounted for. Future studies that account for that selective approach may help improve preventive and therapeutic measures for an ultimate goal of the reduction of pulmonary disease caused by EMPs. The results of such studies may contribute towards development of sampling and analytical methods that closely measure the characteristics important for toxicity, development of appropriate recommendations for worker protection and determination of criteria that could be used to predict the potential risk associated with exposure to any particular type of EMP. Finally, the results may help fill in knowledge gaps beyond EMPs – exposure to other elongate particles, including inorganic and organic manufactured materials.

3.7. References

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Supplementary Materials

Supplementary Table 1. KEGG pathway enrichment analysis of the gene expression data from MPI cells treated with various elongate mineral particles (EMPs) at the doses equivalent for total critical fibers surface area					
term_id	term_name	-log ₁₀ (p-value) vs untreated cells			
		Riebeckite	Riebeckite CF	Tremolite	Tremolite CF
KEGG:04640	Hematopoietic cell lineage	6.146	4.680	2.738	5.549
KEGG:04060	Cytokine-cytokine receptor interaction	3.080	4.499	3.962	5.368
KEGG:04630	JAK-STAT signaling pathway	2.874	3.818	-	2.260
KEGG:04933	AGE-RAGE signaling pathway in diabetic complications	4.357	2.869	1.717	1.754
KEGG:03320	PPAR signaling pathway	3.222	2.762	-	1.556
KEGG:05200	Pathways in cancer	3.820	2.681	1.768	2.297
KEGG:05221	Acute myeloid leukemia	3.184	2.573	1.808	2.297
KEGG:04659	Th17 cell differentiation	2.297	2.385	2.012	1.721
KEGG:05321	Inflammatory bowel disease (IBD)	3.078	2.385	1.963	-
KEGG:04610	Complement and coagulation cascades	1.829	2.297	1.820	2.297
KEGG:04625	C-type lectin receptor signaling pathway	3.844	2.201	1.934	1.618
KEGG:04975	Fat digestion and absorption	2.317	2.049	1.378	-
KEGG:04380	Osteoclast differentiation	5.421	1.923	2.145	1.797
KEGG:05152	Tuberculosis	4.482	1.828	1.769	1.721
KEGG:05205	Proteoglycans in cancer	4.311	1.808	-	1.489
KEGG:04620	Toll-like receptor signaling pathway	1.702	1.659	1.754	-
KEGG:05140	Leishmaniasis	4.779	1.503	2.856	2.297
KEGG:05219	Bladder cancer	1.427	1.503	-	-
KEGG:05418	Fluid shear stress and atherosclerosis	2.697	1.449	2.210	-
KEGG:04151	PI3K-Akt signaling pathway	1.586	1.425	-	-
KEGG:05133	Pertussis	-	1.354	-	-
KEGG:04514	Cell adhesion molecules (CAMs)	3.321	-	1.619	1.618
KEGG:04145	Phagosome	2.519	-	1.462	2.072
KEGG:04371	Apelin signaling pathway	2.504	-	-	-

KEGG:05134	Legionellosis	2.504	-	1.963	1.704
KEGG:05323	Rheumatoid arthritis	2.350	-	2.923	2.003
KEGG:04072	Phospholipase D signaling pathway	2.185	-	-	-
KEGG:05230	Central carbon metabolism in cancer	2.155	-	-	-
KEGG:04510	Focal adhesion	2.115	-	-	-
KEGG:04064	NF-kappa B signaling pathway	2.115	-	1.963	2.003
KEGG:04810	Regulation of actin cytoskeleton	2.069	-	-	-
KEGG:04666	Fc gamma R-mediated phagocytosis	1.971	-	-	-
KEGG:00071	Fatty acid degradation	1.949	-	-	-
KEGG:04658	Th1 and Th2 cell differentiation	1.949	-	1.462	-
KEGG:05146	Amoebiasis	1.878	-	-	-
KEGG:05163	Human cytomegalovirus infection	1.829	-	1.454	-
KEGG:00052	Galactose metabolism	1.820	-	-	-
KEGG:04672	Intestinal immune network for IgA production	1.815	-	-	-
KEGG:05144	Malaria	1.807	-	2.103	-
KEGG:05332	Graft-versus-host disease	1.767	-	2.738	2.290
KEGG:05166	Human T-cell leukemia virus 1 infection	1.705	-	-	-
KEGG:05169	Epstein-Barr virus infection	1.704	-	6.740	-
KEGG:04650	Natural killer cell mediated cytotoxicity	1.669	-	-	-
KEGG:04929	GnRH secretion	1.669	-	-	-
KEGG:05143	African trypanosomiasis	1.644	-	-	-
KEGG:05150	Staphylococcus aureus infection	1.626	-	1.847	1.898
KEGG:01212	Fatty acid metabolism	1.586	-	-	-
KEGG:05225	Hepatocellular carcinoma	1.586	-	-	-
KEGG:04142	Lysosome	1.579	-	-	-
KEGG:04940	Type I diabetes mellitus	1.561	-	2.481	2.072
KEGG:04722	Neurotrophin signaling pathway	1.561	-	-	-
KEGG:04662	B cell receptor signaling pathway	1.556	-	-	-
KEGG:04010	MAPK signaling pathway	1.535	-	-	-
KEGG:04066	HIF-1 signaling pathway	1.453	-	-	-

KEGG:04668	TNF signaling pathway	1.453	-	1.547	1.618
KEGG:05223	Non-small cell lung cancer	1.453	-	-	-
KEGG:04270	Vascular smooth muscle contraction	1.453	-	-	-
KEGG:04216	Ferroptosis	1.427	-	-	-
KEGG:05206	MicroRNAs in cancer	1.387	-	-	-
KEGG:04670	Leukocyte transendothelial migration	1.376	-	-	-
KEGG:04920	Adipocytokine signaling pathway	1.309	-	-	1.898
KEGG:05213	Endometrial cancer	1.309	-	-	-
KEGG:04657	IL-17 signaling pathway	-	-	1.869	-
KEGG:05145	Toxoplasmosis	-	-	1.557	-
KEGG:05168	Herpes simplex virus 1 infection	-	-	1.963	-
KEGG:05162	Measles	-	-	4.450	-
KEGG:05160	Hepatitis C	-	-	3.458	-
KEGG:05330	Allograft rejection	-	-	1.597	-
KEGG:05164	Influenza A	-	-	4.450	-
KEGG:04623	Cytosolic DNA-sensing pathway	-	-	1.963	-
KEGG:04622	RIG-I-like receptor signaling pathway	-	-	2.226	-
KEGG:04621	NOD-like receptor signaling pathway	-	-	3.616	-
KEGG:05202	Transcriptional misregulation in cancer	-	-	1.462	1.708
KEGG:04061	Viral protein interaction with cytokine and cytokine receptor	-	-	1.754	-
KEGG:04115	p53 signaling pathway	-	-	1.754	-
KEGG:05416	Viral myocarditis	-	-	1.579	-
KEGG:04612	Antigen processing and presentation	-	-	1.511	-
KEGG:05167	Kaposi sarcoma-associated herpesvirus infection	-	-	1.462	-

Supplementary Table 2. Gene Ontology Biological Pathways enrichment analysis of the gene expression data from MPI cells treated with various elongate mineral particles (EMPs) at the doses equivalent for total critical fibers surface area

term_id	term_name	-log10(p-value)			
		Riebeckite	Riebeckite CF	Tremolite	Tremolite CF
GO:0002376	immune system process	18.136	15.270	26.955	14.758
GO:0016477	cell migration	15.859	14.077	9.964	11.244
GO:0048583	regulation of response to stimulus	16.298	14.077	9.964	12.436
GO:0051239	regulation of multicellular organismal process	13.996	13.920	12.453	10.381
GO:0032879	regulation of localization	14.558	13.871	5.539	7.334
GO:0009605	response to external stimulus	18.136	13.807	17.838	9.362
GO:0006952	defense response	15.241	13.795	22.707	10.639
GO:0051179	localization	17.796	13.252	5.134	7.978
GO:0051674	localization of cell	14.515	13.252	8.936	11.000
GO:0048870	cell motility	14.515	13.252	8.936	11.000
GO:0007166	cell surface receptor signaling pathway	13.707	13.192	9.698	8.935
GO:0070887	cellular response to chemical stimulus	17.371	13.024	12.435	10.931
GO:0051240	positive regulation of multicellular organismal process	12.350	12.938	10.141	7.316
GO:0030334	regulation of cell migration	12.784	12.884	8.936	7.834
GO:0006950	response to stress	16.660	12.746	18.120	11.235
GO:0034097	response to cytokine	17.134	12.374	18.572	9.728
GO:0048518	positive regulation of biological process	12.529	12.322	6.730	8.319
GO:0040011	locomotion	15.213	12.189	7.758	10.381
GO:2000145	regulation of cell motility	11.737	11.898	8.307	7.205
GO:0040012	regulation of locomotion	12.670	11.898	8.269	7.519
GO:0048646	anatomical structure formation involved in morphogenesis	11.737	11.898	6.763	8.722
GO:0006955	immune response	12.350	11.898	18.120	10.984
GO:0009611	response to wounding	13.964	11.869	5.863	7.205
GO:0048584	positive regulation of response to stimulus	10.893	11.626	8.632	8.935
GO:0035295	tube development	10.957	11.319	7.621	7.978

GO:0006954	inflammatory response	14.113	11.194	12.535	12.537
GO:0023051	regulation of signaling	12.586	11.165	5.061	8.537
GO:0010646	regulation of cell communication	12.736	11.002	5.117	8.598
GO:0048731	system development	12.010	10.962	6.976	8.537
GO:0051270	regulation of cellular component movement	10.883	10.526	7.709	6.510
GO:0035239	tube morphogenesis	10.737	10.448	7.075	7.978
GO:0001816	cytokine production	12.899	10.236	13.066	7.455
GO:0042060	wound healing	12.973	10.151	6.332	7.600
GO:0071310	cellular response to organic substance	12.133	10.149	10.842	7.849
GO:0002682	regulation of immune system process	12.338	10.139	12.453	8.722
GO:0007275	multicellular organism development	9.954	9.781	5.668	8.598
GO:0032103	positive regulation of response to external stimulus	10.584	9.770	9.351	8.238
GO:0006928	movement of cell or subcellular component	10.723	9.761	6.732	8.434
GO:0001525	angiogenesis	8.225	9.651	7.033	7.046
GO:0071345	cellular response to cytokine stimulus	14.262	9.651	13.397	7.664
GO:0050896	response to stimulus	9.211	9.586	8.724	7.666
GO:0009966	regulation of signal transduction	11.706	9.365	5.247	6.851
GO:0032101	regulation of response to external stimulus	9.587	9.306	7.909	6.984
GO:0048519	negative regulation of biological process	10.522	9.287	7.296	7.026
GO:0051707	response to other organism	12.322	8.863	18.120	5.933
GO:0098542	defense response to other organism	9.954	8.838	14.090	5.127
GO:0043207	response to external biotic stimulus	12.274	8.838	18.120	5.923
GO:0048522	positive regulation of cellular process	8.809	8.817	3.843	5.710
GO:0009888	tissue development	8.595	8.817	4.789	5.492
GO:0009607	response to biotic stimulus	12.419	8.817	17.746	5.681
GO:0010033	response to organic substance	11.792	8.816	12.818	7.399
GO:0042127	regulation of cell population proliferation	11.051	8.746	7.659	6.984
GO:0008283	cell population proliferation	9.608	8.746	6.877	7.858
GO:0001568	blood vessel development	8.259	8.741	7.587	8.424
GO:0032502	developmental process	9.338	8.717	3.359	6.714

GO:0072359	circulatory system development	9.449	8.664	6.779	7.978
GO:0002684	positive regulation of immune system process	8.585	8.560	12.099	7.858
GO:0048856	anatomical structure development	8.909	8.499	3.898	7.196
GO:0051049	regulation of transport	10.605	8.477	2.869	4.082
GO:0050663	cytokine secretion	10.255	8.449	7.857	7.617
GO:0015718	monocarboxylic acid transport	11.842	8.442	2.972	5.341
GO:0048514	blood vessel morphogenesis	7.959	8.408	6.730	7.952
GO:0043068	positive regulation of programmed cell death	7.854	8.408	3.546	4.402
GO:0010647	positive regulation of cell communication	7.559	8.408	4.551	5.097
GO:0007155	cell adhesion	11.051	8.357	8.938	8.332
GO:0023056	positive regulation of signaling	7.486	8.353	4.525	5.056
GO:0065008	regulation of biological quality	13.735	8.226	5.650	5.923
GO:0022610	biological adhesion	11.217	8.216	10.388	8.595
GO:0009653	anatomical structure morphogenesis	8.953	8.216	4.800	7.535
GO:0044419	interspecies interaction between organisms	11.737	8.198	16.579	5.417
GO:0001944	vasculature development	8.774	8.180	7.173	7.978
GO:0048513	animal organ development	8.846	8.166	6.814	5.661
GO:0010942	positive regulation of cell death	8.560	8.164	3.835	4.568
GO:0001817	regulation of cytokine production	11.807	8.161	11.881	7.091
GO:0043065	positive regulation of apoptotic process	7.559	8.072	3.160	4.030
GO:0051046	regulation of secretion	6.932	7.972	4.738	5.834
GO:2000026	regulation of multicellular organismal development	7.445	7.972	6.117	5.712
GO:0072358	cardiovascular system development	8.513	7.972	7.022	7.846
GO:0048523	negative regulation of cellular process	9.296	7.945	5.061	6.261
GO:0048869	cellular developmental process	7.559	7.938	2.898	4.932
GO:0035556	intracellular signal transduction	9.348	7.851	3.127	5.596
GO:0046942	carboxylic acid transport	12.770	7.834	2.897	5.406
GO:0030154	cell differentiation	7.576	7.787	2.928	4.824
GO:0015849	organic acid transport	12.670	7.745	2.869	5.354
GO:0051716	cellular response to stimulus	7.137	7.711	3.944	6.151

GO:0050707	regulation of cytokine secretion	9.127	7.702	7.173	7.843
GO:0030155	regulation of cell adhesion	8.491	7.565	7.441	5.976
GO:0080134	regulation of response to stress	7.777	7.551	7.481	7.859
GO:0030335	positive regulation of cell migration	7.296	7.262	4.074	3.471
GO:0051094	positive regulation of developmental process	6.552	7.201	2.931	2.767
GO:0045765	regulation of angiogenesis	5.155	7.147	3.889	5.354
GO:0023061	signal release	6.379	7.093	3.939	5.427
GO:0023052	signaling	6.477	7.079	2.514	5.605
GO:0002791	regulation of peptide secretion	7.716	7.051	4.485	5.710
GO:0050793	regulation of developmental process	6.693	7.029	4.317	5.912
GO:0050776	regulation of immune response	7.587	6.990	7.974	6.324
GO:0002790	peptide secretion	7.036	6.955	4.563	5.488
GO:0007154	cell communication	6.596	6.934	2.384	5.590
GO:0071692	protein localization to extracellular region	6.727	6.839	5.539	5.982
GO:0060429	epithelium development	6.526	6.821	3.043	4.404
GO:2000147	positive regulation of cell motility	6.776	6.799	3.855	3.213
GO:0042325	regulation of phosphorylation	7.363	6.751	2.633	5.933
GO:0009967	positive regulation of signal transduction	6.366	6.751	3.637	3.658
GO:0050715	positive regulation of cytokine secretion	8.268	6.750	7.476	6.936
GO:0090087	regulation of peptide transport	7.361	6.702	2.635	5.169
GO:0002252	immune effector process	6.657	6.660	15.659	4.750
GO:0045087	innate immune response	7.925	6.638	11.881	3.679
GO:1901342	regulation of vasculature development	6.381	6.629	4.404	6.227
GO:0015711	organic anion transport	11.566	6.621	2.319	4.981
GO:0019220	regulation of phosphate metabolic process	6.981	6.621	2.600	5.531
GO:0002526	acute inflammatory response	5.692	6.619	4.166	5.590
GO:0051174	regulation of phosphorus metabolic process	6.971	6.619	2.596	5.527
GO:0010941	regulation of cell death	6.481	6.586	2.936	4.798
GO:0009306	protein secretion	6.507	6.573	5.117	5.659
GO:0043067	regulation of programmed cell death	5.522	6.559	2.799	4.213

GO:0035592	establishment of protein localization to extracellular region	6.486	6.559	5.107	5.647
GO:0050708	regulation of protein secretion	7.017	6.552	5.005	5.858
GO:0040017	positive regulation of locomotion	6.455	6.525	3.702	3.049
GO:0046903	secretion	6.567	6.498	4.217	4.783
GO:0006468	protein phosphorylation	8.090	6.489	2.311	5.225
GO:1902531	regulation of intracellular signal transduction	8.358	6.489	3.939	5.222
GO:0016310	phosphorylation	7.762	6.489	2.057	5.056
GO:1903530	regulation of secretion by cell	5.936	6.488	3.809	5.127
GO:0008219	cell death	7.181	6.463	3.691	4.742
GO:0007165	signal transduction	5.736	6.408	2.258	4.575
GO:0051272	positive regulation of cellular component movement	6.327	6.408	3.638	2.978
GO:0001932	regulation of protein phosphorylation	7.128	6.397	2.982	5.681
GO:0001819	positive regulation of cytokine production	7.965	6.390	10.056	6.485
GO:0008284	positive regulation of cell population proliferation	6.396	6.351	4.800	3.137
GO:0022603	regulation of anatomical structure morphogenesis	5.273	6.321	2.658	4.761
GO:0042981	regulation of apoptotic process	5.289	6.234	2.656	4.082
GO:0051223	regulation of protein transport	6.708	6.228	2.916	5.230
GO:0051050	positive regulation of transport	8.778	6.200	3.411	4.921
GO:0019221	cytokine-mediated signaling pathway	7.021	6.142	7.974	4.981
GO:0006810	transport	9.678	6.142	2.385	2.464
GO:0007162	negative regulation of cell adhesion	4.900	6.125	4.525	2.856
GO:0001775	cell activation	8.268	6.083	4.048	4.091
GO:0050900	leukocyte migration	6.842	6.047	4.085	5.976
GO:0031349	positive regulation of defense response	7.963	6.018	8.357	7.240
GO:0097190	apoptotic signaling pathway	5.191	5.993	1.825	1.738
GO:0012501	programmed cell death	5.606	5.916	2.774	3.420
GO:0065009	regulation of molecular function	6.708	5.916	4.503	4.082
GO:0002274	myeloid leukocyte activation	7.168	5.857	4.539	5.328
GO:0070201	regulation of establishment of protein localization	6.245	5.793	2.744	4.935

GO:0050789	regulation of biological process	5.005	5.766	3.726	4.014
GO:0006915	apoptotic process	5.581	5.757	2.710	3.401
GO:0051234	establishment of localization	9.408	5.743	2.080	2.083
GO:0000165	MAPK cascade	6.732	5.730	4.016	4.190
GO:0002793	positive regulation of peptide secretion	6.737	5.728	5.117	5.463
GO:0050679	positive regulation of epithelial cell proliferation	4.939	5.717	5.542	4.400
GO:1903036	positive regulation of response to wounding	5.167	5.698	2.306	3.152
GO:0071702	organic substance transport	8.909	5.678	1.708	2.857
GO:0051222	positive regulation of protein transport	6.441	5.643	4.044	5.122
GO:0065007	biological regulation	4.856	5.640	3.434	3.526
GO:0002237	response to molecule of bacterial origin	10.230	5.640	8.405	5.324
GO:0006796	phosphate-containing compound metabolic process	7.575	5.588	1.882	4.460
GO:0032880	regulation of protein localization	5.606	5.588	1.722	3.548
GO:0050714	positive regulation of protein secretion	6.315	5.521	5.751	5.548
GO:0051047	positive regulation of secretion	6.299	5.464	3.652	4.460
GO:0098754	detoxification	6.544	5.464	2.004	1.952
GO:0051241	negative regulation of multicellular organismal process	5.581	5.450	5.625	4.837
GO:0023014	signal transduction by protein phosphorylation	6.399	5.446	3.856	4.001
GO:0006793	phosphorus metabolic process	7.371	5.434	2.001	4.351
GO:0043408	regulation of MAPK cascade	5.952	5.395	2.898	3.972
GO:0050729	positive regulation of inflammatory response	8.222	5.349	6.573	9.158
GO:1904951	positive regulation of establishment of protein localization	6.081	5.339	3.873	4.897
GO:0031347	regulation of defense response	6.473	5.272	7.313	5.636
GO:0090303	positive regulation of wound healing	4.985	5.270	2.852	3.986
GO:0140352	export from cell	5.553	5.269	2.936	4.014
GO:0007267	cell-cell signaling	4.627	5.246	2.491	4.434
GO:0010648	negative regulation of cell communication	6.930	5.238	3.835	5.755
GO:0023057	negative regulation of signaling	6.872	5.194	3.813	5.719
GO:0032940	secretion by cell	5.295	5.168	2.905	4.056

GO:0044093	positive regulation of molecular function	4.999	5.051	1.570	3.065
GO:0002697	regulation of immune effector process	5.155	4.972	7.587	4.147
GO:0097529	myeloid leukocyte migration	6.375	4.913	4.051	4.900
GO:0009987	cellular process	6.215	4.905	2.503	2.382
GO:0009636	response to toxic substance	5.972	4.868	1.509	1.997
GO:0061041	regulation of wound healing	6.473	4.864	2.905	3.424
GO:0051247	positive regulation of protein metabolic process	5.049	4.812	1.409	5.169
GO:0061061	muscle structure development	3.379	4.802	2.654	2.703
GO:0045937	positive regulation of phosphate metabolic process	4.927	4.787	1.588	5.206
GO:0010562	positive regulation of phosphorus metabolic process	4.927	4.787	1.588	5.206
GO:1903034	regulation of response to wounding	5.930	4.736	2.280	2.577
GO:0006820	anion transport	9.463	4.729	1.673	3.517
GO:0030595	leukocyte chemotaxis	4.008	4.708	2.769	3.517
GO:0050794	regulation of cellular process	3.642	4.691	2.610	3.227
GO:0009617	response to bacterium	7.886	4.687	10.842	5.026
GO:0050790	regulation of catalytic activity	5.292	4.680	3.701	3.012
GO:1903532	positive regulation of secretion by cell	5.812	4.671	3.750	4.222
GO:0042327	positive regulation of phosphorylation	4.729	4.657	1.633	5.492
GO:0045859	regulation of protein kinase activity	4.939	4.649	3.691	3.587
GO:0097530	granulocyte migration	4.323	4.603	3.426	3.911
GO:0043085	positive regulation of catalytic activity	4.513	4.599	-	2.533
GO:0051246	regulation of protein metabolic process	4.416	4.558	1.517	3.719
GO:0007596	blood coagulation	6.445	4.529	2.445	3.408
GO:0032496	response to lipopolysaccharide	8.585	4.517	8.731	5.565
GO:0031399	regulation of protein modification process	5.105	4.509	2.081	4.082
GO:0001934	positive regulation of protein phosphorylation	5.077	4.495	1.678	5.047
GO:0007599	hemostasis	6.367	4.464	2.420	3.359
GO:0098609	cell-cell adhesion	7.213	4.450	4.749	5.277
GO:0009968	negative regulation of signal transduction	5.350	4.450	3.318	4.571
GO:0050817	coagulation	6.327	4.435	2.406	3.337

GO:0032270	positive regulation of cellular protein metabolic process	4.722	4.434	1.358	4.997
GO:0009893	positive regulation of metabolic process	5.868	4.430	2.565	3.909
GO:0072001	renal system development	4.655	4.419	3.039	3.468
GO:0006935	chemotaxis	5.606	4.395	1.546	2.318
GO:0051048	negative regulation of secretion	4.405	4.395	3.638	5.354
GO:0042592	homeostatic process	5.577	4.372	4.520	2.532
GO:1901700	response to oxygen-containing compound	8.313	4.370	5.251	4.578
GO:0050678	regulation of epithelial cell proliferation	3.177	4.352	4.509	3.732
GO:0042330	taxis	6.327	4.352	1.819	2.297
GO:0048871	multicellular organismal homeostasis	6.202	4.328	5.005	3.078
GO:0022407	regulation of cell-cell adhesion	5.316	4.318	4.906	4.001
GO:1990266	neutrophil migration	4.222	4.296	3.336	3.969
GO:0001822	kidney development	4.517	4.196	2.710	3.141
GO:0015908	fatty acid transport	4.397	4.190	1.463	2.722
GO:0045321	leukocyte activation	5.581	4.180	3.825	3.035
GO:0010604	positive regulation of macromolecule metabolic process	5.574	4.147	2.440	3.834
GO:0045597	positive regulation of cell differentiation	3.920	4.126	2.289	1.479
GO:0045766	positive regulation of angiogenesis	3.683	4.124	1.360	2.560
GO:0032501	multicellular organismal process	3.124	4.082	2.715	3.734
GO:0032757	positive regulation of interleukin-8 production	8.493	4.065	4.074	3.492
GO:1904018	positive regulation of vasculature development	4.517	4.051	1.548	2.702
GO:0043549	regulation of kinase activity	4.659	4.041	3.271	4.117
GO:0032268	regulation of cellular protein metabolic process	3.374	4.039	1.476	3.164
GO:0048585	negative regulation of response to stimulus	5.581	3.991	3.530	4.425
GO:0050878	regulation of body fluid levels	5.781	3.991	4.026	5.377
GO:0071705	nitrogen compound transport	5.156	3.991	1.597	2.097
GO:0050673	epithelial cell proliferation	3.060	3.987	4.652	3.749
GO:0001655	urogenital system development	4.441	3.973	2.999	3.300
GO:1901701	cellular response to oxygen-containing compound	7.011	3.946	4.358	3.514
GO:0030856	regulation of epithelial cell differentiation	4.244	3.915	4.051	5.923

GO:0048002	antigen processing and presentation of peptide antigen	5.168	3.903	2.601	2.074
GO:0044281	small molecule metabolic process	6.319	3.832	2.574	2.957
GO:0045860	positive regulation of protein kinase activity	3.716	3.796	1.579	2.611
GO:0050818	regulation of coagulation	4.093	3.785	2.182	2.273
GO:1902533	positive regulation of intracellular signal transduction	4.849	3.763	2.420	2.964
GO:0051338	regulation of transferase activity	4.349	3.760	2.884	3.888
GO:0042221	response to chemical	4.973	3.719	6.106	3.210
GO:0048729	tissue morphogenesis	3.427	3.689	-	2.259
GO:0006801	superoxide metabolic process	4.900	3.678	3.251	1.414
GO:0030593	neutrophil chemotaxis	3.741	3.614	2.391	2.394
GO:0060055	angiogenesis involved in wound healing	3.060	3.614	1.745	2.317
GO:0002009	morphogenesis of an epithelium	2.816	3.596	-	1.682
GO:0061042	vascular wound healing	3.106	3.574	-	1.918
GO:2001235	positive regulation of apoptotic signaling pathway	2.371	3.574	1.368	-
GO:0071900	regulation of protein serine/threonine kinase activity	5.047	3.573	3.106	2.712
GO:0007159	leukocyte cell-cell adhesion	6.014	3.571	3.850	3.693
GO:0060326	cell chemotaxis	3.489	3.571	2.631	3.037
GO:0071219	cellular response to molecule of bacterial origin	7.965	3.559	8.385	4.804
GO:0043436	oxoacid metabolic process	4.967	3.559	-	1.926
GO:0090184	positive regulation of kidney development	2.817	3.536	-	-
GO:0006953	acute-phase response	3.563	3.536	4.364	3.771
GO:0042326	negative regulation of phosphorylation	3.854	3.533	3.082	3.526
GO:1901564	organonitrogen compound metabolic process	4.692	3.524	1.525	1.926
GO:2001233	regulation of apoptotic signaling pathway	3.775	3.512	2.138	2.297
GO:0045630	positive regulation of T-helper 2 cell differentiation	1.936	3.509	1.633	-
GO:0050730	regulation of peptidyl-tyrosine phosphorylation	4.488	3.471	-	2.665
GO:0097191	extrinsic apoptotic signaling pathway	3.802	3.459	5.076	2.819
GO:0071621	granulocyte chemotaxis	3.430	3.459	2.574	1.952
GO:0008285	negative regulation of cell population proliferation	5.610	3.427	4.476	4.837
GO:0002831	regulation of response to biotic stimulus	4.278	3.419	2.346	1.603

GO:0001933	negative regulation of protein phosphorylation	3.915	3.408	3.059	3.615
GO:0018212	peptidyl-tyrosine modification	3.755	3.407	-	2.090
GO:1902622	regulation of neutrophil migration	3.421	3.407	2.427	2.735
GO:0050727	regulation of inflammatory response	6.029	3.407	6.274	6.307
GO:0010876	lipid localization	6.756	3.397	2.634	4.058
GO:0050778	positive regulation of immune response	4.176	3.369	5.276	4.001
GO:0033036	macromolecule localization	5.119	3.363	1.769	2.398
GO:1990748	cellular detoxification	4.684	3.363	2.259	-
GO:0045595	regulation of cell differentiation	3.632	3.363	2.883	2.575
GO:0002520	immune system development	4.655	3.333	5.209	2.033
GO:1902624	positive regulation of neutrophil migration	3.467	3.303	2.883	2.425
GO:0001906	cell killing	2.526	3.294	2.642	2.392
GO:0006082	organic acid metabolic process	4.600	3.294	-	1.808
GO:0015909	long-chain fatty acid transport	3.843	3.290	-	1.560
GO:0033029	regulation of neutrophil apoptotic process	2.882	3.251	1.546	1.331
GO:0060054	positive regulation of epithelial cell proliferation involved in wound healing	2.882	3.251	4.244	3.904
GO:0002685	regulation of leukocyte migration	3.671	3.246	2.331	2.050
GO:0030097	hemopoiesis	4.024	3.228	4.888	1.885
GO:0030193	regulation of blood coagulation	3.578	3.215	1.623	1.769
GO:0071222	cellular response to lipopolysaccharide	7.061	3.215	8.565	4.935
GO:0071396	cellular response to lipid	4.961	3.195	6.171	3.854
GO:1900046	regulation of hemostasis	3.529	3.171	1.605	1.748
GO:0060562	epithelial tube morphogenesis	3.319	3.164	-	-
GO:0090322	regulation of superoxide metabolic process	4.166	3.157	2.787	-
GO:0043405	regulation of MAP kinase activity	4.365	3.156	2.217	1.815
GO:0072006	nephron development	3.477	3.155	-	1.541
GO:0043410	positive regulation of MAPK cascade	3.319	3.150	1.623	2.210
GO:0007167	enzyme linked receptor protein signaling pathway	3.374	3.140	-	1.843
GO:1903531	negative regulation of secretion by cell	3.001	3.131	2.027	3.659

GO:0097237	cellular response to toxic substance	4.356	3.126	2.135	-
GO:0006869	lipid transport	5.581	3.126	1.964	2.969
GO:0031401	positive regulation of protein modification process	3.903	3.120	1.308	4.463
GO:0042116	macrophage activation	3.287	3.093	1.855	2.469
GO:0002700	regulation of production of molecular mediator of immune response	3.534	3.086	4.340	1.781
GO:0010628	positive regulation of gene expression	4.983	3.061	3.923	1.953
GO:0071216	cellular response to biotic stimulus	8.162	3.061	7.725	4.283
GO:0018108	peptidyl-tyrosine phosphorylation	3.447	3.061	-	1.811
GO:0031663	lipopolysaccharide-mediated signaling pathway	5.407	3.058	3.939	3.337
GO:0048534	hematopoietic or lymphoid organ development	4.564	3.056	4.888	1.876
GO:0071902	positive regulation of protein serine/threonine kinase activity	3.778	3.055	1.756	2.097
GO:0032677	regulation of interleukin-8 production	6.635	3.055	4.174	2.706
GO:0002699	positive regulation of immune effector process	3.744	3.055	4.689	2.535
GO:1900048	positive regulation of hemostasis	2.506	3.039	1.516	1.970
GO:0010563	negative regulation of phosphorus metabolic process	3.604	3.039	2.515	2.696
GO:0007399	nervous system development	3.887	3.039	-	2.317
GO:0002819	regulation of adaptive immune response	4.551	3.039	3.637	1.809
GO:0045936	negative regulation of phosphate metabolic process	3.604	3.039	2.515	2.696
GO:0030194	positive regulation of blood coagulation	2.506	3.039	1.516	1.970
GO:2000484	positive regulation of interleukin-8 secretion	7.218	3.031	-	2.612
GO:0002830	positive regulation of type 2 immune response	1.742	3.031	1.958	-
GO:0030855	epithelial cell differentiation	3.803	3.031	3.032	3.986
GO:0045785	positive regulation of cell adhesion	5.155	2.980	3.781	3.434
GO:0006865	amino acid transport	4.878	2.979	2.009	1.876
GO:0002688	regulation of leukocyte chemotaxis	2.071	2.977	1.527	-
GO:0022408	negative regulation of cell-cell adhesion	3.124	2.971	2.232	1.614
GO:0022008	neurogenesis	2.886	2.925	-	2.046
GO:0009887	animal organ morphogenesis	3.305	2.925	2.693	2.036

GO:0002521	leukocyte differentiation	3.043	2.915	4.142	2.053
GO:0051347	positive regulation of transferase activity	2.796	2.909	-	2.706
GO:0042886	amide transport	3.330	2.909	1.421	1.826
GO:0033674	positive regulation of kinase activity	2.731	2.905	-	2.717
GO:0006629	lipid metabolic process	6.459	2.902	1.944	3.695
GO:0019222	regulation of metabolic process	2.882	2.895	1.944	1.445
GO:0050820	positive regulation of coagulation	2.352	2.876	1.450	1.892
GO:0090023	positive regulation of neutrophil chemotaxis	2.352	2.876	1.450	-
GO:0001781	neutrophil apoptotic process	2.508	2.863	1.409	-
GO:0019752	carboxylic acid metabolic process	4.647	2.844	-	1.907
GO:0044092	negative regulation of molecular function	2.512	2.837	2.586	2.074
GO:0034113	heterotypic cell-cell adhesion	4.195	2.807	2.852	3.986
GO:0030217	T cell differentiation	2.640	2.805	2.760	1.586
GO:0001818	negative regulation of cytokine production	4.329	2.805	4.453	2.368
GO:0002478	antigen processing and presentation of exogenous peptide antigen	3.297	2.805	-	-
GO:0007369	gastrulation	2.526	2.805	1.669	2.913
GO:0001889	liver development	2.614	2.805	3.631	3.994
GO:0032637	interleukin-8 production	6.187	2.803	3.939	2.521
GO:1905039	carboxylic acid transmembrane transport	6.399	2.802	-	-
GO:1903825	organic acid transmembrane transport	6.399	2.802	-	-
GO:0072073	kidney epithelium development	3.166	2.796	-	-
GO:0044706	multi-multicellular organism process	2.255	2.768	-	2.779
GO:0002687	positive regulation of leukocyte migration	2.665	2.768	2.731	2.041
GO:0002456	T cell mediated immunity	2.746	2.741	3.093	1.876
GO:0050921	positive regulation of chemotaxis	1.835	2.741	-	-
GO:0072593	reactive oxygen species metabolic process	7.486	2.740	5.185	2.905
GO:0061008	hepaticobiliary system development	2.539	2.734	3.570	3.925
GO:0071624	positive regulation of granulocyte chemotaxis	2.227	2.730	1.398	-
GO:0090183	regulation of kidney development	2.080	2.720	-	-

GO:0002833	positive regulation of response to biotic stimulus	3.578	2.720	2.150	-
GO:0001935	endothelial cell proliferation	2.617	2.720	1.640	2.534
GO:0040007	growth	2.263	2.720	1.650	2.599
GO:0051345	positive regulation of hydrolase activity	3.223	2.719	-	1.389
GO:0002703	regulation of leukocyte mediated immunity	2.631	2.717	4.791	1.774
GO:2000379	positive regulation of reactive oxygen species metabolic process	5.924	2.716	2.838	2.785
GO:0033993	response to lipid	4.798	2.716	5.547	3.436
GO:0051051	negative regulation of transport	5.087	2.712	3.092	2.575
GO:0002367	cytokine production involved in immune response	2.955	2.706	4.053	-
GO:0008625	extrinsic apoptotic signaling pathway via death domain receptors	3.095	2.691	3.013	1.823
GO:0030858	positive regulation of epithelial cell differentiation	2.617	2.681	-	2.229
GO:0110110	positive regulation of animal organ morphogenesis	2.386	2.671	-	-
GO:0048659	smooth muscle cell proliferation	2.470	2.671	2.881	1.309
GO:0002828	regulation of type 2 immune response	-	2.667	1.368	-
GO:0009628	response to abiotic stimulus	4.683	2.625	2.073	2.086
GO:0006873	cellular ion homeostasis	2.419	2.623	1.934	-
GO:0150076	neuroinflammatory response	1.936	2.612	-	1.787
GO:0001936	regulation of endothelial cell proliferation	2.607	2.612	1.858	2.299
GO:0002690	positive regulation of leukocyte chemotaxis	1.471	2.602	1.368	-
GO:0070120	ciliary neurotrophic factor-mediated signaling pathway	-	2.602	-	-
GO:0050731	positive regulation of peptidyl-tyrosine phosphorylation	3.120	2.602	-	2.722
GO:0071221	cellular response to bacterial lipopeptide	3.843	2.602	-	1.653
GO:0071220	cellular response to bacterial lipoprotein	3.843	2.602	-	1.653
GO:0070339	response to bacterial lipopeptide	3.843	2.602	-	1.653
GO:0035879	plasma membrane lactate transport	5.796	2.602	-	1.653
GO:0010035	response to inorganic substance	3.427	2.596	2.786	1.958
GO:0051093	negative regulation of developmental process	3.655	2.589	3.354	3.049

GO:0002702	positive regulation of production of molecular mediator of immune response	2.292	2.575	1.882	-
GO:0006469	negative regulation of protein kinase activity	2.206	2.571	2.687	2.368
GO:0006957	complement activation, alternative pathway	2.227	2.570	3.572	3.166
GO:1902287	semaphorin-plexin signaling pathway involved in axon guidance	2.227	2.570	-	-
GO:0048771	tissue remodeling	3.072	2.562	2.471	1.399
GO:0061900	glial cell activation	1.936	2.555	-	2.127
GO:2000377	regulation of reactive oxygen species metabolic process	7.247	2.541	4.209	2.687
GO:0051173	positive regulation of nitrogen compound metabolic process	2.957	2.524	-	2.112
GO:0015833	peptide transport	2.838	2.524	1.346	1.601
GO:0048468	cell development	3.039	2.520	-	1.963
GO:0097435	supramolecular fiber organization	2.510	2.484	1.862	2.480
GO:0032611	interleukin-1 beta production	2.206	2.479	2.423	2.446
GO:0050772	positive regulation of axonogenesis	1.766	2.479	-	-
GO:0030003	cellular cation homeostasis	2.321	2.470	2.009	1.329
GO:0002792	negative regulation of peptide secretion	2.340	2.458	1.997	1.865
GO:0007565	female pregnancy	1.780	2.456	-	2.280
GO:0030098	lymphocyte differentiation	2.578	2.456	3.259	1.554
GO:0009790	embryo development	2.219	2.450	1.417	1.910
GO:0001780	neutrophil homeostasis	1.398	2.450	-	-
GO:0022409	positive regulation of cell-cell adhesion	3.088	2.447	2.792	1.972
GO:0015031	protein transport	2.673	2.443	1.476	1.603
GO:0038094	Fc-gamma receptor signaling pathway	2.106	2.441	-	1.918
GO:1902285	semaphorin-plexin signaling pathway involved in neuron projection guidance	3.106	2.441	-	-
GO:0045628	regulation of T-helper 2 cell differentiation	1.324	2.441	-	-

GO:0002822	regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	3.049	2.436	3.287	1.978
GO:0002437	inflammatory response to antigenic stimulus	3.605	2.435	2.573	3.552
GO:0060255	regulation of macromolecule metabolic process	2.151	2.388	2.111	1.324
GO:0009719	response to endogenous stimulus	2.751	2.369	2.275	1.722
GO:0048017	inositol lipid-mediated signaling	2.660	2.369	-	-
GO:0090022	regulation of neutrophil chemotaxis	2.549	2.353	-	-
GO:0032835	glomerulus development	2.275	2.349	-	1.999
GO:0032493	response to bacterial lipoprotein	3.433	2.348	-	1.523
GO:0032680	regulation of tumor necrosis factor production	3.086	2.348	3.643	1.806
GO:0007178	transmembrane receptor protein serine/threonine kinase signaling pathway	2.315	2.343	3.287	1.909
GO:0150078	positive regulation of neuroinflammatory response	-	2.317	-	1.853
GO:0034616	response to laminar fluid shear stress	1.993	2.317	2.157	2.913
GO:0009914	hormone transport	1.672	2.305	-	2.372
GO:0061564	axon development	2.123	2.304	-	-
GO:1903555	regulation of tumor necrosis factor superfamily cytokine production	3.034	2.303	3.609	1.780
GO:0019884	antigen processing and presentation of exogenous antigen	2.662	2.295	-	-
GO:0032928	regulation of superoxide anion generation	2.662	2.295	-	-
GO:0031113	regulation of microtubule polymerization	2.979	2.295	1.525	-
GO:0031325	positive regulation of cellular metabolic process	2.771	2.292	-	2.022
GO:0010952	positive regulation of peptidase activity	2.461	2.290	-	-
GO:0071560	cellular response to transforming growth factor beta stimulus	-	2.288	2.294	2.015
GO:0048660	regulation of smooth muscle cell proliferation	2.165	2.287	2.396	-
GO:0006811	ion transport	5.655	2.287	-	-
GO:0002718	regulation of cytokine production involved in immune response	2.204	2.280	4.051	-

GO:0002064	epithelial cell development	3.391	2.260	2.019	3.079
GO:0001706	endoderm formation	2.290	2.253	-	2.430
GO:0008630	intrinsic apoptotic signaling pathway in response to DNA damage	2.939	2.248	1.702	-
GO:0032963	collagen metabolic process	-	2.248	3.633	-
GO:0034109	homotypic cell-cell adhesion	4.021	2.244	-	1.929
GO:0032640	tumor necrosis factor production	2.946	2.244	3.548	1.741
GO:0008152	metabolic process	3.349	2.239	1.465	-
GO:0010575	positive regulation of vascular endothelial growth factor production	-	2.228	2.406	-
GO:0009892	negative regulation of metabolic process	2.372	2.228	2.023	-
GO:2000482	regulation of interleukin-8 secretion	5.473	2.228	-	2.015
GO:0051336	regulation of hydrolase activity	2.235	2.228	-	-
GO:0051017	actin filament bundle assembly	1.444	2.226	2.893	2.668
GO:0048661	positive regulation of smooth muscle cell proliferation	1.952	2.225	2.259	-
GO:0007517	muscle organ development	-	2.225	-	1.733
GO:0006875	cellular metal ion homeostasis	1.811	2.225	2.057	-
GO:0071559	response to transforming growth factor beta	-	2.222	2.255	1.968
GO:2000106	regulation of leukocyte apoptotic process	2.031	2.221	-	-
GO:0090594	inflammatory response to wounding	1.913	2.221	-	-
GO:0071622	regulation of granulocyte chemotaxis	2.247	2.220	1.480	-
GO:0071704	organic substance metabolic process	3.149	2.219	1.461	-
GO:0043407	negative regulation of MAP kinase activity	2.668	2.217	1.751	1.352
GO:0001909	leukocyte mediated cytotoxicity	1.507	2.211	1.420	-
GO:0045444	fat cell differentiation	4.195	2.205	1.977	-
GO:0033673	negative regulation of kinase activity	1.850	2.205	2.420	2.074
GO:0002218	activation of innate immune response	-	2.205	-	-
GO:0001938	positive regulation of endothelial cell proliferation	1.996	2.195	-	-
GO:0002366	leukocyte activation involved in immune response	3.128	2.195	2.603	1.815
GO:0071706	tumor necrosis factor superfamily cytokine production	2.864	2.195	3.483	1.704

GO:0001659	temperature homeostasis	4.900	2.195	2.864	2.112
GO:0019538	protein metabolic process	2.376	2.192	-	-
GO:0033002	muscle cell proliferation	1.835	2.192	2.897	-
GO:0071901	negative regulation of protein serine/threonine kinase activity	2.242	2.192	2.439	1.853
GO:0019882	antigen processing and presentation	4.590	2.181	1.656	-
GO:0006898	receptor-mediated endocytosis	3.408	2.177	-	1.931
GO:0061572	actin filament bundle organization	1.403	2.177	2.852	2.611
GO:0050709	negative regulation of protein secretion	2.126	2.177	2.102	1.969
GO:0071499	cellular response to laminar fluid shear stress	1.936	2.166	1.633	2.606
GO:0046883	regulation of hormone secretion	1.692	2.166	-	2.296
GO:0010533	regulation of activation of Janus kinase activity	1.936	2.166	-	1.414
GO:0042092	type 2 immune response	-	2.161	-	-
GO:0002263	cell activation involved in immune response	3.070	2.152	2.570	1.792
GO:0098771	inorganic ion homeostasis	2.278	2.146	1.789	-
GO:0002449	lymphocyte mediated immunity	1.483	2.134	2.550	-
GO:0050920	regulation of chemotaxis	2.151	2.107	-	-
GO:0072080	nephron tubule development	2.386	2.107	-	-
GO:0002709	regulation of T cell mediated immunity	1.516	2.107	2.562	-
GO:0050865	regulation of cell activation	3.498	2.107	2.255	1.997
GO:0048699	generation of neurons	2.206	2.107	-	1.602
GO:0048608	reproductive structure development	1.961	2.106	2.613	1.929
GO:0072009	nephron epithelium development	2.257	2.105	-	-
GO:0044255	cellular lipid metabolic process	4.381	2.104	-	1.823
GO:2000448	positive regulation of macrophage migration inhibitory factor signaling pathway	1.952	2.093	-	-
GO:0045404	positive regulation of interleukin-4 biosynthetic process	1.952	2.093	-	-
GO:0072573	tolerance induction to lipopolysaccharide	1.952	2.093	2.630	2.392
GO:0038112	interleukin-8-mediated signaling pathway	1.952	2.093	2.630	2.392
GO:0071938	vitamin A transport	1.952	2.093	-	-

GO:0071939	vitamin A import	1.952	2.093	-	-
GO:0048878	chemical homeostasis	2.874	2.087	2.232	1.550
GO:0045582	positive regulation of T cell differentiation	1.899	2.086	1.921	-
GO:0002706	regulation of lymphocyte mediated immunity	1.339	2.084	2.768	-
GO:0043030	regulation of macrophage activation	-	2.072	1.409	2.263
GO:0002443	leukocyte mediated immunity	1.933	2.066	3.873	1.346
GO:0043406	positive regulation of MAP kinase activity	2.821	2.063	1.316	-
GO:0061458	reproductive system development	1.921	2.055	2.573	1.900
GO:0031099	regeneration	2.076	2.045	-	1.479
GO:0043409	negative regulation of MAPK cascade	2.662	2.045	2.221	2.491
GO:0045064	T-helper 2 cell differentiation	-	2.045	-	-
GO:0030950	establishment or maintenance of actin cytoskeleton polarity	-	2.045	-	-
GO:0032305	positive regulation of icosanoid secretion	2.575	2.045	1.958	2.612
GO:0048598	embryonic morphogenesis	1.627	2.039	-	1.791
GO:0050801	ion homeostasis	2.446	2.035	1.701	1.399
GO:0001503	ossification	1.443	2.035	3.158	1.501
GO:0008104	protein localization	3.387	2.023	-	1.331
GO:0055080	cation homeostasis	2.201	2.018	1.862	-
GO:0045862	positive regulation of proteolysis	2.230	2.015	-	1.376
GO:0007179	transforming growth factor beta receptor signaling pathway	-	2.015	1.525	1.439
GO:0001704	formation of primary germ layer	2.152	2.012	-	2.052
GO:0061043	regulation of vascular wound healing	1.783	2.010	-	1.331
GO:0035873	lactate transmembrane transport	4.264	2.010	-	1.331
GO:0042590	antigen processing and presentation of exogenous peptide antigen via MHC class I	1.783	2.010	-	-
GO:2000698	positive regulation of epithelial cell differentiation involved in kidney development	1.783	2.010	-	1.331
GO:0002246	wound healing involved in inflammatory response	1.783	2.010	1.546	1.331

GO:0015727	lactate transport	4.264	2.010	-	1.331
GO:0061326	renal tubule development	2.261	2.010	-	-
GO:0071276	cellular response to cadmium ion	-	2.000	-	-
GO:0048015	phosphatidylinositol-mediated signaling	2.317	2.000	-	-
GO:0031649	heat generation	2.288	2.000	2.227	1.855
GO:0072606	interleukin-8 secretion	4.927	2.000	-	1.855
GO:0032612	interleukin-1 production	2.576	1.994	3.336	2.608
GO:0072503	cellular divalent inorganic cation homeostasis	1.588	1.992	1.370	-
GO:0051224	negative regulation of protein transport	1.770	1.989	2.358	2.074
GO:0035987	endodermal cell differentiation	2.110	1.986	-	2.015
GO:0071495	cellular response to endogenous stimulus	1.846	1.969	-	-
GO:0050710	negative regulation of cytokine secretion	1.961	1.969	1.977	2.163
GO:0010038	response to metal ion	2.367	1.969	1.546	1.449
GO:0034111	negative regulation of homotypic cell-cell adhesion	-	1.968	-	-
GO:0031664	regulation of lipopolysaccharide-mediated signaling pathway	3.426	1.968	-	1.610
GO:0019233	sensory perception of pain	1.613	1.958	-	-
GO:1903039	positive regulation of leukocyte cell-cell adhesion	2.803	1.956	2.331	-
GO:0060627	regulation of vesicle-mediated transport	3.404	1.956	1.605	1.551
GO:0045184	establishment of protein localization	2.550	1.953	-	1.309
GO:0030574	collagen catabolic process	-	1.944	2.185	-
GO:0045429	positive regulation of nitric oxide biosynthetic process	2.690	1.944	2.427	2.735
GO:0051146	striated muscle cell differentiation	-	1.942	-	-
GO:1904950	negative regulation of establishment of protein localization	1.707	1.921	2.306	2.023
GO:2001056	positive regulation of cysteine-type endopeptidase activity	1.590	1.914	-	-
GO:0016042	lipid catabolic process	1.981	1.913	-	-
GO:0050764	regulation of phagocytosis	4.387	1.905	2.391	3.776
GO:1904407	positive regulation of nitric oxide metabolic process	2.640	1.905	2.401	2.706

GO:1903793	positive regulation of anion transport	3.720	1.905	1.316	2.097
GO:1901652	response to peptide	2.954	1.905	1.599	-
GO:0002673	regulation of acute inflammatory response	2.014	1.905	-	2.706
GO:0032623	interleukin-2 production	1.904	1.905	1.316	-
GO:0002720	positive regulation of cytokine production involved in immune response	1.493	1.905	2.401	-
GO:0032635	interleukin-6 production	3.532	1.905	5.001	1.791
GO:0007568	aging	3.157	1.905	1.887	2.577
GO:0050819	negative regulation of coagulation	-	1.905	-	1.329
GO:0002523	leukocyte migration involved in inflammatory response	-	1.899	-	-
GO:0002902	regulation of B cell apoptotic process	2.366	1.899	-	-
GO:0048384	retinoic acid receptor signaling pathway	2.164	1.899	2.143	1.781
GO:0062197	cellular response to chemical stress	3.510	1.899	1.527	1.504
GO:0060099	regulation of phagocytosis, engulfment	1.606	1.899	-	2.456
GO:0071498	cellular response to fluid shear stress	1.606	1.899	1.850	2.456
GO:0072112	glomerular visceral epithelial cell differentiation	1.606	1.899	-	1.554
GO:0043032	positive regulation of macrophage activation	-	1.899	1.368	1.781
GO:0038093	Fc receptor signaling pathway	1.606	1.899	-	1.554
GO:0061318	renal filtration cell differentiation	1.606	1.899	-	1.554
GO:0033032	regulation of myeloid cell apoptotic process	2.164	1.899	1.368	-
GO:1904894	positive regulation of receptor signaling pathway via STAT	1.693	1.892	-	2.382
GO:0014065	phosphatidylinositol 3-kinase signaling	1.991	1.889	-	-
GO:0051128	regulation of cellular component organization	3.070	1.886	-	3.090
GO:0007015	actin filament organization	1.470	1.880	1.633	2.459
GO:2001057	reactive nitrogen species metabolic process	4.678	1.867	3.572	2.217
GO:0001776	leukocyte homeostasis	2.563	1.867	-	-
GO:0002285	lymphocyte activation involved in immune response	2.411	1.867	2.080	1.476
GO:0019216	regulation of lipid metabolic process	2.625	1.862	-	-
GO:0031400	negative regulation of protein modification process	2.845	1.858	2.284	2.086
GO:0040008	regulation of growth	1.426	1.858	1.400	2.280

GO:0071407	cellular response to organic cyclic compound	2.337	1.858	2.284	1.566
GO:0051130	positive regulation of cellular component organization	3.229	1.855	1.511	1.722
GO:0030036	actin cytoskeleton organization	3.456	1.851	1.640	2.915
GO:0042832	defense response to protozoan	2.821	1.850	4.011	1.745
GO:0010574	regulation of vascular endothelial growth factor production	-	1.850	2.102	-
GO:0003158	endothelium development	2.371	1.848	1.974	3.065
GO:0051101	regulation of DNA binding	-	1.848	-	-
GO:0055065	metal ion homeostasis	1.764	1.846	1.921	-
GO:0006631	fatty acid metabolic process	2.798	1.846	-	1.365
GO:0010950	positive regulation of endopeptidase activity	1.767	1.846	-	-
GO:0010605	negative regulation of macromolecule metabolic process	1.819	1.844	1.977	-
GO:0010243	response to organonitrogen compound	3.403	1.835	-	-
GO:0010043	response to zinc ion	2.536	1.835	-	-
GO:1905153	regulation of membrane invagination	1.544	1.830	-	2.386
GO:0072311	glomerular epithelial cell differentiation	1.544	1.830	-	1.512
GO:1903037	regulation of leukocyte cell-cell adhesion	3.713	1.824	2.294	1.554
GO:0000302	response to reactive oxygen species	1.818	1.824	2.884	1.688
GO:0071248	cellular response to metal ion	1.986	1.824	-	-
GO:0042110	T cell activation	3.357	1.824	2.232	-
GO:0043627	response to estrogen	1.701	1.821	2.096	-
GO:0097755	positive regulation of blood vessel diameter	2.897	1.821	-	-
GO:0045428	regulation of nitric oxide biosynthetic process	5.029	1.821	3.321	2.706
GO:0120162	positive regulation of cold-induced thermogenesis	3.870	1.821	1.510	-
GO:0072507	divalent inorganic cation homeostasis	1.420	1.818	-	-
GO:0030182	neuron differentiation	1.882	1.817	-	1.686
GO:0046631	alpha-beta T cell activation	2.617	1.816	1.640	-
GO:0071354	cellular response to interleukin-6	-	1.808	1.311	-
GO:0034110	regulation of homotypic cell-cell adhesion	2.043	1.808	-	-
GO:0034405	response to fluid shear stress	1.462	1.808	2.066	2.473

GO:0002920	regulation of humoral immune response	2.043	1.808	2.922	2.473
GO:0071347	cellular response to interleukin-1	2.975	1.805	2.289	-
GO:0032787	monocarboxylic acid metabolic process	2.336	1.804	-	-
GO:0010718	positive regulation of epithelial to mesenchymal transition	1.403	1.803	2.298	1.868
GO:0051480	regulation of cytosolic calcium ion concentration	-	1.799	1.440	-
GO:0043069	negative regulation of programmed cell death	1.734	1.799	1.750	1.751
GO:0046879	hormone secretion	1.324	1.795	-	2.059
GO:0032147	activation of protein kinase activity	1.710	1.795	1.337	1.414
GO:0150063	visual system development	-	1.795	1.964	2.163
GO:0051348	negative regulation of transferase activity	1.462	1.795	2.102	1.775
GO:0045621	positive regulation of lymphocyte differentiation	1.589	1.784	1.702	-
GO:1901222	regulation of NIK/NF-kappaB signaling	1.974	1.784	-	-
GO:0046546	development of primary male sexual characteristics	1.779	1.782	2.118	2.498
GO:1901698	response to nitrogen compound	3.046	1.782	-	-
GO:1901724	positive regulation of cell proliferation involved in kidney development	1.561	1.773	-	-
GO:0030952	establishment or maintenance of cytoskeleton polarity	-	1.771	-	-
GO:2000193	positive regulation of fatty acid transport	2.201	1.771	1.745	2.317
GO:0150105	protein localization to cell-cell junction	1.486	1.771	-	1.460
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	2.201	1.771	-	-
GO:0050702	interleukin-1 beta secretion	-	1.770	-	-
GO:0033028	myeloid cell apoptotic process	1.986	1.766	-	-
GO:0002292	T cell differentiation involved in immune response	2.236	1.766	1.819	-
GO:0010573	vascular endothelial growth factor production	-	1.766	2.026	-
GO:0050866	negative regulation of cell activation	3.049	1.750	-	-
GO:0035691	macrophage migration inhibitory factor signaling pathway	1.606	1.749	-	-
GO:0043066	negative regulation of apoptotic process	1.710	1.749	1.600	1.628

GO:2000768	positive regulation of nephron tubule epithelial cell differentiation	1.606	1.749	-	-
GO:0031110	regulation of microtubule polymerization or depolymerization	2.061	1.749	-	-
GO:0048880	sensory system development	-	1.749	1.921	2.109
GO:0070494	regulation of thrombin-activated receptor signaling pathway	1.606	1.749	-	2.015
GO:0042231	interleukin-13 biosynthetic process	1.606	1.749	-	-
GO:0042097	interleukin-4 biosynthetic process	1.606	1.749	-	-
GO:0010124	phenylacetate catabolic process	1.606	1.749	-	-
GO:0098758	response to interleukin-8	3.157	1.749	2.264	2.015
GO:0098759	cellular response to interleukin-8	3.157	1.749	2.264	2.015
GO:0046865	terpenoid transport	1.606	1.749	-	-
GO:0046864	isoprenoid transport	1.606	1.749	-	-
GO:0002752	cell surface pattern recognition receptor signaling pathway	1.606	1.749	-	-
GO:0003331	positive regulation of extracellular matrix constituent secretion	1.606	1.749	-	-
GO:1904035	regulation of epithelial cell apoptotic process	2.564	1.749	2.034	2.722
GO:0090101	negative regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	1.545	1.749	1.672	-
GO:2000446	regulation of macrophage migration inhibitory factor signaling pathway	1.606	1.749	-	-
GO:0033030	negative regulation of neutrophil apoptotic process	1.606	1.749	2.264	-
GO:0045402	regulation of interleukin-4 biosynthetic process	1.606	1.749	-	-
GO:2000295	regulation of hydrogen peroxide catabolic process	1.606	1.749	-	2.015
GO:0034633	retinol transport	1.606	1.749	-	-
GO:1904444	regulation of establishment of Sertoli cell barrier	1.606	1.749	-	2.015
GO:0070358	actin polymerization-dependent cell motility	1.606	1.749	-	2.015
GO:0070495	negative regulation of thrombin-activated receptor signaling pathway	1.606	1.749	-	2.015

GO:0072139	glomerular parietal epithelial cell differentiation	1.606	1.749	-	-
GO:0071725	response to triacyl bacterial lipopeptide	3.157	1.749	-	-
GO:0071727	cellular response to triacyl bacterial lipopeptide	3.157	1.749	-	-
GO:0007492	endoderm development	1.606	1.736	-	1.550
GO:0050892	intestinal absorption	1.398	1.732	-	-
GO:0001562	response to protozoan	2.614	1.732	3.831	1.639
GO:0006638	neutral lipid metabolic process	1.459	1.728	-	-
GO:0048568	embryonic organ development	1.658	1.725	-	-
GO:0032675	regulation of interleukin-6 production	3.387	1.723	4.545	1.929
GO:2001236	regulation of extrinsic apoptotic signaling pathway	2.465	1.723	3.186	2.425
GO:0032303	regulation of icosanoid secretion	2.950	1.722	1.701	2.250
GO:0042509	regulation of tyrosine phosphorylation of STAT protein	-	1.722	1.772	1.929
GO:0014068	positive regulation of phosphatidylinositol 3-kinase signaling	1.676	1.722	-	-
GO:0002704	negative regulation of leukocyte mediated immunity	1.780	1.712	2.218	-
GO:0015807	L-amino acid transport	3.291	1.688	-	-
GO:0070555	response to interleukin-1	3.059	1.686	1.844	1.339
GO:0071526	semaphorin-plexin signaling pathway	1.897	1.685	-	-
GO:0072182	regulation of nephron tubule epithelial cell differentiation	1.470	1.682	-	-
GO:0045088	regulation of innate immune response	1.913	1.682	1.320	-
GO:0045086	positive regulation of interleukin-2 biosynthetic process	1.470	1.682	1.346	-
GO:0051923	sulfation	1.470	1.682	-	-
GO:0002883	regulation of hypersensitivity	1.470	1.682	-	2.074
GO:0002253	activation of immune response	1.583	1.682	-	2.049
GO:0031392	regulation of prostaglandin biosynthetic process	1.470	1.682	-	-
GO:0001774	microglial cell activation	1.743	1.678	-	1.769
GO:0048589	developmental growth	1.890	1.678	-	1.425
GO:0002269	leukocyte activation involved in inflammatory response	1.743	1.678	-	1.769
GO:0031175	neuron projection development	1.699	1.676	-	-
GO:0055082	cellular chemical homeostasis	1.835	1.674	1.368	-

GO:0016525	negative regulation of angiogenesis	-	1.672	1.356	1.781
GO:0071887	leukocyte apoptotic process	1.470	1.668	-	-
GO:0050766	positive regulation of phagocytosis	2.631	1.665	3.106	4.082
GO:0072010	glomerular epithelium development	1.398	1.664	-	1.376
GO:0045624	positive regulation of T-helper cell differentiation	-	1.664	-	-
GO:0032753	positive regulation of interleukin-4 production	2.846	1.664	-	-
GO:0032616	interleukin-13 production	-	1.664	-	-
GO:0006925	inflammatory cell apoptotic process	1.398	1.664	-	-
GO:0097193	intrinsic apoptotic signaling pathway	2.116	1.659	-	-
GO:0031331	positive regulation of cellular catabolic process	1.402	1.658	-	-
GO:0008360	regulation of cell shape	2.361	1.657	-	-
GO:0030029	actin filament-based process	3.408	1.657	1.743	2.530
GO:2001238	positive regulation of extrinsic apoptotic signaling pathway	-	1.650	2.157	-
GO:0042531	positive regulation of tyrosine phosphorylation of STAT protein	-	1.650	1.480	2.392
GO:0042554	superoxide anion generation	2.488	1.650	-	-
GO:0042267	natural killer cell mediated cytotoxicity	-	1.650	-	-
GO:1901571	fatty acid derivative transport	2.247	1.650	-	1.741
GO:0071715	icosanoid transport	2.247	1.650	-	1.741
GO:0052547	regulation of peptidase activity	-	1.647	-	-
GO:0006809	nitric oxide biosynthetic process	4.567	1.642	3.906	2.491
GO:0007260	tyrosine phosphorylation of STAT protein	-	1.642	1.710	1.867
GO:0033554	cellular response to stress	2.505	1.632	1.302	1.597
GO:0060541	respiratory system development	-	1.622	1.624	2.163
GO:1905954	positive regulation of lipid localization	4.081	1.621	4.823	4.014
GO:0035710	CD4-positive, alpha-beta T cell activation	1.493	1.621	2.537	-
GO:0032722	positive regulation of chemokine production	4.267	1.615	2.883	-
GO:0014070	response to organic cyclic compound	2.714	1.613	2.790	2.086
GO:0031589	cell-substrate adhesion	1.882	1.610	1.523	1.815

GO:0001894	tissue homeostasis	1.399	1.609	-	-
GO:0070741	response to interleukin-6	-	1.609	-	-
GO:0015812	gamma-aminobutyric acid transport	1.344	1.607	-	-
GO:1903708	positive regulation of hemopoiesis	1.723	1.605	3.397	-
GO:2000181	negative regulation of blood vessel morphogenesis	-	1.600	1.771	2.724
GO:0050713	negative regulation of interleukin-1 beta secretion	-	1.594	-	-
GO:0001660	fever generation	1.399	1.594	3.572	3.166
GO:0016264	gap junction assembly	2.227	1.594	2.307	-
GO:0072160	nephron tubule epithelial cell differentiation	1.399	1.594	-	-
GO:2001279	regulation of unsaturated fatty acid biosynthetic process	1.399	1.594	-	-
GO:0002683	negative regulation of immune system process	6.278	1.594	3.289	-
GO:0031324	negative regulation of cellular metabolic process	2.206	1.593	1.743	1.338
GO:0038061	NIK/NF-kappaB signaling	2.609	1.591	-	-
GO:0002250	adaptive immune response	3.094	1.589	1.844	-
GO:0050732	negative regulation of peptidyl-tyrosine phosphorylation	-	1.587	-	-
GO:0036211	protein modification process	2.340	1.576	-	1.551
GO:0032755	positive regulation of interleukin-6 production	2.292	1.576	3.939	1.929
GO:1902903	regulation of supramolecular fiber organization	2.915	1.576	1.638	1.876
GO:0002262	myeloid cell homeostasis	-	1.576	1.943	-
GO:0006575	cellular modified amino acid metabolic process	2.249	1.576	-	-
GO:0006464	cellular protein modification process	2.340	1.576	-	1.551
GO:0003382	epithelial cell morphogenesis	1.755	1.573	-	-
GO:0007043	cell-cell junction assembly	1.722	1.573	1.547	1.562
GO:0044070	regulation of anion transport	3.617	1.573	1.547	2.608
GO:0007584	response to nutrient	2.481	1.566	-	1.909
GO:0043367	CD4-positive, alpha-beta T cell differentiation	1.521	1.564	-	-
GO:0015850	organic hydroxy compound transport	4.461	1.561	-	2.207
GO:1902532	negative regulation of intracellular signal transduction	2.321	1.561	2.345	1.978
GO:2000116	regulation of cysteine-type endopeptidase activity	-	1.560	-	-
GO:0001654	eye development	-	1.559	1.630	1.864

GO:0050873	brown fat cell differentiation	3.384	1.559	-	-
GO:0002228	natural killer cell mediated immunity	-	1.559	-	-
GO:0001843	neural tube closure	1.431	1.559	-	-
GO:0048872	homeostasis of number of cells	2.288	1.559	2.768	-
GO:0002221	pattern recognition receptor signaling pathway	3.821	1.552	7.441	3.294
GO:0070371	ERK1 and ERK2 cascade	2.669	1.552	2.218	1.444
GO:0002694	regulation of leukocyte activation	2.681	1.550	2.210	1.501
GO:0009896	positive regulation of catabolic process	1.399	1.546	-	-
GO:0034612	response to tumor necrosis factor	1.952	1.539	3.907	1.978
GO:0050870	positive regulation of T cell activation	1.734	1.538	1.633	-
GO:0045601	regulation of endothelial cell differentiation	2.315	1.538	1.831	3.964
GO:0098869	cellular oxidant detoxification	2.233	1.538	1.326	-
GO:0002369	T cell cytokine production	-	1.538	2.622	-
GO:0060606	tube closure	1.409	1.538	-	-
GO:0031109	microtubule polymerization or depolymerization	2.071	1.537	-	-
GO:0009991	response to extracellular stimulus	2.201	1.536	-	1.771
GO:0001794	type IIa hypersensitivity	1.403	1.532	-	1.812
GO:0071409	cellular response to cycloheximide	1.403	1.532	-	-
GO:0071724	response to diacyl bacterial lipopeptide	2.658	1.532	-	1.812
GO:0071726	cellular response to diacyl bacterial lipopeptide	2.658	1.532	-	1.812
GO:0055085	transmembrane transport	3.108	1.532	-	-
GO:1901529	positive regulation of anion channel activity	1.403	1.532	-	1.812
GO:0002894	positive regulation of type II hypersensitivity	1.403	1.532	-	1.812
GO:0000320	re-entry into mitotic cell cycle	1.403	1.532	-	-
GO:0010536	positive regulation of activation of Janus kinase activity	1.403	1.532	-	-
GO:0001798	positive regulation of type IIa hypersensitivity	1.403	1.532	-	1.812
GO:0038165	oncostatin-M-mediated signaling pathway	-	1.532	-	-
GO:0001796	regulation of type IIa hypersensitivity	1.403	1.532	-	1.812
GO:0044789	modulation by host of viral release from host cell	1.403	1.532	-	-
GO:0060548	negative regulation of cell death	1.790	1.532	1.545	1.868

GO:0002892	regulation of type II hypersensitivity	1.403	1.532	-	1.812
GO:0044791	positive regulation by host of viral release from host cell	1.403	1.532	-	-
GO:0048861	leukemia inhibitory factor signaling pathway	-	1.532	-	-
GO:0045917	positive regulation of complement activation	1.403	1.532	2.036	1.812
GO:0007354	zygotic determination of anterior/posterior axis, embryo	1.403	1.532	-	-
GO:0002445	type II hypersensitivity	1.403	1.532	-	1.812
GO:0033031	positive regulation of neutrophil apoptotic process	-	1.532	-	-
GO:0032929	negative regulation of superoxide anion generation	1.403	1.532	-	-
GO:0002886	regulation of myeloid leukocyte mediated immunity	2.080	1.532	2.052	1.639
GO:1904714	regulation of chaperone-mediated autophagy	1.403	1.532	-	1.812
GO:0014902	myotube differentiation	-	1.530	-	-
GO:0046209	nitric oxide metabolic process	4.980	1.530	3.730	2.356
GO:0002821	positive regulation of adaptive immune response	2.315	1.530	2.225	-
GO:0046785	microtubule polymerization	2.391	1.530	-	-
GO:1904892	regulation of receptor signaling pathway via STAT	-	1.529	1.441	1.769
GO:0006968	cellular defense response	1.324	1.528	-	-
GO:1903980	positive regulation of microglial cell activation	-	1.528	-	1.918
GO:1903961	positive regulation of anion transmembrane transport	1.324	1.528	-	-
GO:0002524	hypersensitivity	1.324	1.528	-	1.918
GO:2000347	positive regulation of hepatocyte proliferation	2.106	1.528	2.232	1.918
GO:0007423	sensory organ development	-	1.525	-	-
GO:0034114	regulation of heterotypic cell-cell adhesion	-	1.523	3.426	2.952
GO:0001783	B cell apoptotic process	1.844	1.523	-	-
GO:0034762	regulation of transmembrane transport	1.644	1.517	-	-
GO:1902107	positive regulation of leukocyte differentiation	1.471	1.515	1.877	-
GO:0010508	positive regulation of autophagy	1.315	1.514	-	-
GO:0046427	positive regulation of receptor signaling pathway via JAK-STAT	-	1.509	-	1.864
GO:0032890	regulation of organic acid transport	3.529	1.507	1.605	2.324
GO:0010468	regulation of gene expression	1.889	1.502	2.133	-

GO:0048666	neuron development	1.511	1.501	-	-
GO:0007204	positive regulation of cytosolic calcium ion concentration	-	1.494	1.437	-
GO:0017015	regulation of transforming growth factor beta receptor signaling pathway	1.362	1.486	-	1.348
GO:0030278	regulation of ossification	-	1.486	2.210	-
GO:1903428	positive regulation of reactive oxygen species biosynthetic process	2.571	1.485	2.001	2.195
GO:0010812	negative regulation of cell-substrate adhesion	-	1.485	2.001	1.587
GO:0050701	interleukin-1 secretion	1.524	1.485	1.370	1.587
GO:0034614	cellular response to reactive oxygen species	-	1.484	1.859	-
GO:1903169	regulation of calcium ion transmembrane transport	-	1.484	-	-
GO:0002460	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	2.040	1.481	2.060	-
GO:0051154	negative regulation of striated muscle cell differentiation	-	1.478	-	-
GO:0010829	negative regulation of glucose transmembrane transport	2.506	1.472	-	-
GO:0046649	lymphocyte activation	2.332	1.468	1.600	-
GO:0001890	placenta development	-	1.468	-	-
GO:0050767	regulation of neurogenesis	2.236	1.468	-	-
GO:0048145	regulation of fibroblast proliferation	1.343	1.468	-	-
GO:0006641	triglyceride metabolic process	1.693	1.468	-	-
GO:0032269	negative regulation of cellular protein metabolic process	1.337	1.464	1.607	-
GO:0009612	response to mechanical stimulus	3.335	1.462	2.656	1.528
GO:0007010	cytoskeleton organization	3.049	1.462	-	2.022
GO:0032642	regulation of chemokine production	3.430	1.460	2.182	-
GO:0030512	negative regulation of transforming growth factor beta receptor signaling pathway	1.493	1.458	1.353	1.560
GO:0043010	camera-type eye development	-	1.456	1.597	1.909
GO:0045216	cell-cell junction organization	1.411	1.455	-	-
GO:0007409	axonogenesis	-	1.454	-	-

GO:0048732	gland development	1.766	1.453	3.704	3.190
GO:0048308	organelle inheritance	-	1.453	-	-
GO:0048313	Golgi inheritance	-	1.453	-	-
GO:0032736	positive regulation of interleukin-13 production	-	1.453	-	-
GO:0002468	dendritic cell antigen processing and presentation	-	1.453	-	-
GO:0042976	activation of Janus kinase activity	-	1.453	-	-
GO:0010817	regulation of hormone levels	1.913	1.453	-	1.335
GO:0030336	negative regulation of cell migration	2.564	1.453	2.947	2.963
GO:0050777	negative regulation of immune response	3.578	1.453	2.694	-
GO:0051098	regulation of binding	-	1.453	1.882	-
GO:0030162	regulation of proteolysis	-	1.453	-	-
GO:1903844	regulation of cellular response to transforming growth factor beta stimulus	1.324	1.453	-	1.329
GO:0035850	epithelial cell differentiation involved in kidney development	1.602	1.450	-	1.396
GO:0046460	neutral lipid biosynthetic process	-	1.450	-	-
GO:0046463	acylglycerol biosynthetic process	-	1.450	-	-
GO:0042692	muscle cell differentiation	-	1.445	-	-
GO:0071241	cellular response to inorganic substance	1.525	1.442	-	-
GO:0008584	male gonad development	1.470	1.438	1.633	1.999
GO:0035456	response to interferon-beta	1.936	1.436	5.302	1.541
GO:0002438	acute inflammatory response to antigenic stimulus	1.734	1.434	-	1.929
GO:2000108	positive regulation of leukocyte apoptotic process	-	1.434	-	-
GO:0014020	primary neural tube formation	1.302	1.434	-	-
GO:0048144	fibroblast proliferation	1.302	1.434	-	-
GO:0051085	chaperone cofactor-dependent protein refolding	-	1.434	-	-
GO:0043280	positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	-	1.428	-	-
GO:0051172	negative regulation of nitrogen compound metabolic process	1.644	1.424	1.563	-

GO:1901343	negative regulation of vasculature development	-	1.423	1.623	2.498
GO:0009725	response to hormone	1.627	1.421	-	-
GO:1904427	positive regulation of calcium ion transmembrane transport	-	1.420	-	-
GO:0043086	negative regulation of catalytic activity	1.516	1.419	2.099	1.760
GO:0007157	heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules	1.562	1.418	-	1.369
GO:0030195	negative regulation of blood coagulation	-	1.418	-	-
GO:0010259	multicellular organism aging	-	1.418	-	2.015
GO:2000241	regulation of reproductive process	1.992	1.418	1.368	2.577
GO:0046661	male sex differentiation	1.386	1.418	1.796	2.074
GO:0010720	positive regulation of cell development	1.427	1.413	-	-
GO:1903845	negative regulation of cellular response to transforming growth factor beta stimulus	1.442	1.412	1.316	1.523
GO:0061448	connective tissue development	1.368	1.412	2.017	-
GO:0001667	ameboidal-type cell migration	3.091	1.408	1.641	1.809
GO:0060284	regulation of cell development	2.236	1.403	-	-
GO:0030279	negative regulation of ossification	1.347	1.398	-	-
GO:0050867	positive regulation of cell activation	1.606	1.390	1.771	1.304
GO:1902235	regulation of endoplasmic reticulum stress-induced intrinsic apoptotic signaling pathway	-	1.390	-	-
GO:0002695	negative regulation of leukocyte activation	3.176	1.390	-	-
GO:0042119	neutrophil activation	2.352	1.390	2.265	2.735
GO:1901722	regulation of cell proliferation involved in kidney development	-	1.390	-	-
GO:0042178	xenobiotic catabolic process	1.913	1.390	-	-
GO:0002861	regulation of inflammatory response to antigenic stimulus	1.676	1.390	-	1.892
GO:0032673	regulation of interleukin-4 production	2.352	1.390	-	-
GO:1902042	negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	1.676	1.390	1.450	1.892

GO:0050872	white fat cell differentiation	-	1.390	-	-
GO:0015802	basic amino acid transport	-	1.390	-	-
GO:0042537	benzene-containing compound metabolic process	1.676	1.390	-	-
GO:0045137	development of primary sexual characteristics	1.940	1.390	2.265	1.924
GO:0002863	positive regulation of inflammatory response to antigenic stimulus	1.913	1.390	-	1.787
GO:0071294	cellular response to zinc ion	1.913	1.390	-	-
GO:0032720	negative regulation of tumor necrosis factor production	2.391	1.390	1.899	-
GO:1900047	negative regulation of hemostasis	-	1.390	-	-
GO:0043277	apoptotic cell clearance	1.525	1.390	2.427	1.978
GO:0010830	regulation of myotube differentiation	-	1.390	-	-
GO:0032892	positive regulation of organic acid transport	2.690	1.390	-	1.340
GO:0002220	innate immune response activating cell surface receptor signaling pathway	-	1.387	-	-
GO:0007264	small GTPase mediated signal transduction	-	1.387	-	-
GO:0006931	substrate-dependent cell migration, cell attachment to substrate	-	1.387	-	-
GO:0019087	transformation of host cell by virus	-	1.387	-	-
GO:0031328	positive regulation of cellular biosynthetic process	1.740	1.387	-	-
GO:0002904	positive regulation of B cell apoptotic process	-	1.387	-	-
GO:1903596	regulation of gap junction assembly	2.340	1.387	1.870	-
GO:0046898	response to cycloheximide	-	1.387	-	-
GO:0061073	ciliary body morphogenesis	-	1.387	-	-
GO:0035470	positive regulation of vascular wound healing	-	1.387	-	-
GO:0010138	pyrimidine ribonucleotide salvage	-	1.387	-	-
GO:0001905	activation of membrane attack complex	-	1.387	1.870	1.653
GO:0008655	pyrimidine-containing compound salvage	-	1.387	-	-
GO:0008643	carbohydrate transport	2.081	1.387	-	-
GO:0043433	negative regulation of DNA-binding transcription factor activity	-	1.387	-	-

GO:0120161	regulation of cold-induced thermogenesis	3.716	1.387	1.409	-
GO:0051387	negative regulation of neurotrophin TRK receptor signaling pathway	-	1.387	-	-
GO:0099039	sphingolipid translocation	-	1.387	-	1.653
GO:2000468	regulation of peroxidase activity	-	1.387	-	1.653
GO:0006639	acylglycerol metabolic process	-	1.387	-	-
GO:0010827	regulation of glucose transmembrane transport	2.161	1.387	-	-
GO:0060161	positive regulation of dopamine receptor signaling pathway	-	1.387	-	-
GO:0006477	protein sulfation	-	1.387	-	1.653
GO:0044206	UMP salvage	-	1.387	-	-
GO:0042117	monocyte activation	-	1.387	-	-
GO:0002440	production of molecular mediator of immune response	1.909	1.387	2.707	-
GO:0032262	pyrimidine nucleotide salvage	-	1.387	-	-
GO:0106106	cold-induced thermogenesis	3.716	1.387	1.409	-
GO:0032602	chemokine production	3.250	1.387	2.096	-
GO:0031667	response to nutrient levels	1.882	1.386	-	1.731
GO:0090092	regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	1.451	1.386	2.095	1.446
GO:0010629	negative regulation of gene expression	1.688	1.386	1.772	-
GO:0051960	regulation of nervous system development	2.643	1.379	-	-
GO:0034341	response to interferon-gamma	4.461	1.379	4.545	1.929
GO:0010770	positive regulation of cell morphogenesis involved in differentiation	-	1.377	-	-
GO:0048146	positive regulation of fibroblast proliferation	1.399	1.376	-	-
GO:0045089	positive regulation of innate immune response	-	1.375	-	-
GO:1901653	cellular response to peptide	1.561	1.375	-	-
GO:0050769	positive regulation of neurogenesis	1.359	1.375	-	-
GO:0019725	cellular homeostasis	1.516	1.374	-	-

GO:1902041	regulation of extrinsic apoptotic signaling pathway via death domain receptors	1.493	1.371	1.656	1.950
GO:0140353	lipid export from cell	1.493	1.371	1.656	1.329
GO:0002675	positive regulation of acute inflammatory response	2.288	1.364	1.420	2.695
GO:0007507	heart development	-	1.364	-	-
GO:0098656	anion transmembrane transport	3.096	1.363	-	-
GO:0009891	positive regulation of biosynthetic process	1.782	1.361	-	-
GO:0000902	cell morphogenesis	2.029	1.359	-	-
GO:0043149	stress fiber assembly	-	1.357	2.859	1.716
GO:0030038	contractile actin filament bundle assembly	-	1.357	2.859	1.716
GO:0045446	endothelial cell differentiation	1.936	1.357	1.672	3.519
GO:1903556	negative regulation of tumor necrosis factor superfamily cytokine production	2.315	1.352	1.862	-
GO:0033627	cell adhesion mediated by integrin	3.550	1.352	-	1.454
GO:0001885	endothelial cell development	2.315	1.352	1.862	2.706
GO:0010832	negative regulation of myotube differentiation	-	1.342	-	-
GO:0048711	positive regulation of astrocyte differentiation	-	1.342	-	-
GO:0030449	regulation of complement activation	-	1.342	2.019	1.729
GO:0071285	cellular response to lithium ion	1.818	1.342	2.019	1.729
GO:1901550	regulation of endothelial cell development	2.681	1.342	-	2.706
GO:1903140	regulation of establishment of endothelial barrier	2.681	1.342	-	2.706
GO:0062099	negative regulation of programmed necrotic cell death	-	1.342	2.019	-
GO:0070102	interleukin-6-mediated signaling pathway	-	1.342	-	-
GO:1905155	positive regulation of membrane invagination	-	1.342	-	1.729
GO:0060100	positive regulation of phagocytosis, engulfment	-	1.342	-	1.729
GO:0002864	regulation of acute inflammatory response to antigenic stimulus	-	1.342	-	1.729
GO:0002922	positive regulation of humoral immune response	1.818	1.342	3.092	2.706
GO:0032231	regulation of actin filament bundle assembly	-	1.341	2.224	1.697
GO:0007160	cell-matrix adhesion	1.755	1.337	-	-

GO:1902105	regulation of leukocyte differentiation	1.493	1.335	-	-
GO:0051248	negative regulation of protein metabolic process	1.525	1.335	1.398	-
GO:0048708	astrocyte differentiation	-	1.333	-	-
GO:0051962	positive regulation of nervous system development	1.544	1.333	-	-
GO:0071417	cellular response to organonitrogen compound	1.933	1.333	-	-
GO:0046330	positive regulation of JNK cascade	1.757	1.333	1.368	1.339
GO:0031645	negative regulation of nervous system process	1.589	1.330	2.185	2.630
GO:0002724	regulation of T cell cytokine production	-	1.330	2.185	-
GO:0050869	negative regulation of B cell activation	2.227	1.330	-	-
GO:0043372	positive regulation of CD4-positive, alpha-beta T cell differentiation	-	1.330	-	-
GO:0031116	positive regulation of microtubule polymerization	2.227	1.330	1.398	-
GO:0002825	regulation of T-helper 1 type immune response	2.227	1.330	1.398	-
GO:0014047	glutamate secretion	-	1.330	-	-
GO:2000146	negative regulation of cell motility	2.318	1.327	2.788	2.746
GO:0002286	T cell activation involved in immune response	2.315	1.326	1.641	-
GO:0007417	central nervous system development	1.761	1.326	-	-
GO:0010632	regulation of epithelial cell migration	-	1.321	1.606	1.765
GO:0043412	macromolecule modification	1.952	1.318	-	-
GO:0034599	cellular response to oxidative stress	1.990	1.316	-	-
GO:0051056	regulation of small GTPase mediated signal transduction	-	1.316	-	-
GO:0032309	icosanoid secretion	1.936	1.316	-	-
GO:0006970	response to osmotic stress	1.606	1.316	-	-
GO:0090207	regulation of triglyceride metabolic process	1.429	1.316	-	-
GO:0048812	neuron projection morphogenesis	-	1.316	-	-
GO:1902904	negative regulation of supramolecular fiber organization	-	1.312	-	1.439
GO:0090407	organophosphate biosynthetic process	1.699	1.309	-	-
GO:1901699	cellular response to nitrogen compound	1.573	1.305	-	-
GO:1903426	regulation of reactive oxygen species biosynthetic process	4.340	-	2.368	1.826
GO:0003013	circulatory system process	3.661	-	2.081	1.504

GO:0002224	toll-like receptor signaling pathway	3.610	-	5.302	2.957
GO:1903409	reactive oxygen species biosynthetic process	3.579	-	2.645	1.550
GO:1990845	adaptive thermogenesis	3.269	-	-	-
GO:0008015	blood circulation	3.259	-	2.186	1.331
GO:0003018	vascular process in circulatory system	3.120	-	1.570	-
GO:2000351	regulation of endothelial cell apoptotic process	2.921	-	1.505	2.430
GO:0032970	regulation of actin filament-based process	2.892	-	1.699	1.864
GO:0035296	regulation of tube diameter	2.874	-	-	-
GO:0097746	regulation of blood vessel diameter	2.874	-	-	-
GO:0035150	regulation of tube size	2.845	-	-	-
GO:1905952	regulation of lipid localization	2.838	-	2.852	2.097
GO:0006979	response to oxidative stress	2.826	-	1.732	1.537
GO:0043270	positive regulation of ion transport	2.785	-	-	-
GO:0030168	platelet activation	2.746	-	-	-
GO:0042311	vasodilation	2.746	-	1.311	-
GO:0071346	cellular response to interferon-gamma	2.714	-	2.456	-
GO:0072577	endothelial cell apoptotic process	2.705	-	1.421	2.297
GO:0051250	negative regulation of lymphocyte activation	2.689	-	-	-
GO:0031666	positive regulation of lipopolysaccharide-mediated signaling pathway	2.672	-	-	-
GO:0019915	lipid storage	2.668	-	2.749	2.163
GO:0031334	positive regulation of protein-containing complex assembly	2.668	-	-	-
GO:1902475	L-alpha-amino acid transmembrane transport	2.662	-	-	-
GO:0032370	positive regulation of lipid transport	2.631	-	3.106	2.521
GO:0008217	regulation of blood pressure	2.630	-	1.546	1.389
GO:1901570	fatty acid derivative biosynthetic process	2.617	-	-	-
GO:0007249	I-kappaB kinase/NF-kappaB signaling	2.584	-	2.221	1.931
GO:0045730	respiratory burst	2.581	-	-	-
GO:0045410	positive regulation of interleukin-6 biosynthetic process	2.575	-	-	-

GO:0006066	alcohol metabolic process	2.566	-	1.566	1.873
GO:0042089	cytokine biosynthetic process	2.548	-	2.068	-
GO:0070527	platelet aggregation	2.536	-	-	-
GO:0071260	cellular response to mechanical stimulus	2.511	-	-	-
GO:0043254	regulation of protein-containing complex assembly	2.511	-	1.537	-
GO:1902563	regulation of neutrophil activation	2.508	-	1.409	2.184
GO:0044539	long-chain fatty acid import into cell	2.471	-	-	-
GO:0045019	negative regulation of nitric oxide biosynthetic process	2.471	-	-	-
GO:0140354	lipid import into cell	2.471	-	-	-
GO:0001976	nervous system process involved in regulation of systemic arterial blood pressure	2.471	-	-	-
GO:1904406	negative regulation of nitric oxide metabolic process	2.471	-	-	-
GO:0042035	regulation of cytokine biosynthetic process	2.433	-	-	-
GO:0072604	interleukin-6 secretion	2.433	-	3.983	-
GO:0045600	positive regulation of fat cell differentiation	2.391	-	-	-
GO:2000378	negative regulation of reactive oxygen species metabolic process	2.391	-	-	-
GO:0045123	cellular extravasation	2.351	-	-	2.734
GO:0002396	MHC protein complex assembly	2.340	-	1.870	-
GO:0042107	cytokine metabolic process	2.319	-	1.942	-
GO:0045408	regulation of interleukin-6 biosynthetic process	2.288	-	-	-
GO:0050670	regulation of lymphocyte proliferation	2.277	-	-	-
GO:0051493	regulation of cytoskeleton organization	2.259	-	-	1.665
GO:0042226	interleukin-6 biosynthetic process	2.227	-	-	-
GO:0045080	positive regulation of chemokine biosynthetic process	2.227	-	-	-
GO:0032944	regulation of mononuclear cell proliferation	2.226	-	-	-
GO:0032368	regulation of lipid transport	2.222	-	1.880	1.380
GO:0042108	positive regulation of cytokine biosynthetic process	2.204	-	-	-
GO:0003333	amino acid transmembrane transport	2.196	-	-	-
GO:0090066	regulation of anatomical structure size	2.176	-	-	-

GO:0032355	response to estradiol	2.175	-	1.633	-
GO:0002260	lymphocyte homeostasis	2.165	-	-	-
GO:2000191	regulation of fatty acid transport	2.164	-	1.368	1.781
GO:0032956	regulation of actin cytoskeleton organization	2.164	-	1.398	1.963
GO:0032649	regulation of interferon-gamma production	2.148	-	3.039	-
GO:0040013	negative regulation of locomotion	2.135	-	2.439	2.372
GO:0001676	long-chain fatty acid metabolic process	2.115	-	-	-
GO:0006837	serotonin transport	2.115	-	-	1.414
GO:2000343	positive regulation of chemokine (C-X-C motif) ligand 2 production	2.110	-	-	-
GO:1903706	regulation of hemopoiesis	2.104	-	2.098	-
GO:0104004	cellular response to environmental stimulus	2.103	-	-	-
GO:0001782	B cell homeostasis	2.103	-	-	-
GO:0071214	cellular response to abiotic stimulus	2.103	-	-	-
GO:0060322	head development	2.085	-	-	-
GO:1901568	fatty acid derivative metabolic process	2.081	-	-	-
GO:0032091	negative regulation of protein binding	2.057	-	-	-
GO:0016192	vesicle-mediated transport	2.057	-	-	-
GO:0005975	carbohydrate metabolic process	2.057	-	-	-
GO:0034330	cell junction organization	2.057	-	-	1.660
GO:0045598	regulation of fat cell differentiation	2.045	-	1.321	-
GO:0015749	monosaccharide transmembrane transport	2.044	-	-	-
GO:0016043	cellular component organization	2.043	-	-	1.960
GO:0032271	regulation of protein polymerization	2.041	-	-	-
GO:0051955	regulation of amino acid transport	2.014	-	-	1.329
GO:0042088	T-helper 1 type immune response	2.014	-	-	-
GO:0031650	regulation of heat generation	1.993	-	-	-
GO:2000778	positive regulation of interleukin-6 secretion	1.986	-	4.977	1.669
GO:0001773	myeloid dendritic cell activation	1.986	-	-	-
GO:0051953	negative regulation of amine transport	1.986	-	-	1.669

GO:0051180	vitamin transport	1.986	-	-	-
GO:0070663	regulation of leukocyte proliferation	1.968	-	-	-
GO:0034219	carbohydrate transmembrane transport	1.968	-	-	-
GO:2000353	positive regulation of endothelial cell apoptotic process	1.963	-	-	1.335
GO:0034121	regulation of toll-like receptor signaling pathway	1.961	-	1.977	-
GO:0060935	cardiac fibroblast cell differentiation	1.952	-	-	-
GO:0042495	detection of triacyl bacterial lipopeptide	1.952	-	-	-
GO:0001579	medium-chain fatty acid transport	1.952	-	-	-
GO:2000334	positive regulation of blood microparticle formation	1.952	-	2.630	2.392
GO:1903038	negative regulation of leukocyte cell-cell adhesion	1.952	-	-	-
GO:0030329	prenylcysteine metabolic process	1.952	-	-	-
GO:0030328	prenylcysteine catabolic process	1.952	-	-	-
GO:0031930	mitochondria-nucleus signaling pathway	1.952	-	-	-
GO:0045017	glycerolipid biosynthetic process	1.952	-	-	-
GO:0060940	epithelial to mesenchymal transition involved in cardiac fibroblast development	1.952	-	-	-
GO:0072564	blood microparticle formation	1.952	-	2.630	2.392
GO:0060939	epicardium-derived cardiac fibroblast cell development	1.952	-	-	-
GO:0060938	epicardium-derived cardiac fibroblast cell differentiation	1.952	-	-	-
GO:0060936	cardiac fibroblast cell development	1.952	-	-	-
GO:2000332	regulation of blood microparticle formation	1.952	-	2.630	2.392
GO:2000417	negative regulation of eosinophil migration	1.952	-	-	2.392
GO:1901615	organic hydroxy compound metabolic process	1.950	-	1.450	1.779
GO:0036230	granulocyte activation	1.941	-	1.993	2.382
GO:0031112	positive regulation of microtubule polymerization or depolymerization	1.941	-	-	-
GO:1901552	positive regulation of endothelial cell development	1.936	-	-	1.414
GO:1904464	regulation of matrix metalloproteinase secretion	1.936	-	-	1.414
GO:1903142	positive regulation of establishment of endothelial barrier	1.936	-	-	1.414
GO:0014812	muscle cell migration	1.936	-	-	-

GO:1990773	matrix metalloproteinase secretion	1.936	-	-	1.414
GO:0019637	organophosphate metabolic process	1.930	-	-	-
GO:0002827	positive regulation of T-helper 1 type immune response	1.913	-	1.581	-
GO:0001820	serotonin secretion	1.913	-	-	-
GO:0045073	regulation of chemokine biosynthetic process	1.913	-	-	-
GO:0042033	chemokine biosynthetic process	1.913	-	-	-
GO:0046634	regulation of alpha-beta T cell activation	1.899	-	1.382	-
GO:0061028	establishment of endothelial barrier	1.899	-	-	1.868
GO:0032633	interleukin-4 production	1.897	-	-	-
GO:0055076	transition metal ion homeostasis	1.890	-	-	-
GO:0030100	regulation of endocytosis	1.857	-	-	1.538
GO:0051090	regulation of DNA-binding transcription factor activity	1.856	-	2.271	-
GO:0033622	integrin activation	1.844	-	-	-
GO:0044087	regulation of cellular component biogenesis	1.842	-	1.825	1.818
GO:0007229	integrin-mediated signaling pathway	1.840	-	-	-
GO:0050868	negative regulation of T cell activation	1.833	-	-	-
GO:0051271	negative regulation of cellular component movement	1.825	-	2.420	2.348
GO:2001234	negative regulation of apoptotic signaling pathway	1.822	-	1.343	1.522
GO:0051044	positive regulation of membrane protein ectodomain proteolysis	1.818	-	-	-
GO:0046651	lymphocyte proliferation	1.817	-	-	-
GO:0060324	face development	1.814	-	-	-
GO:0043618	regulation of transcription from RNA polymerase II promoter in response to stress	1.814	-	-	-
GO:0050672	negative regulation of lymphocyte proliferation	1.811	-	-	-
GO:0071229	cellular response to acid chemical	1.811	-	-	1.707
GO:0043900	regulation of multi-organism process	1.811	-	2.182	-
GO:0043269	regulation of ion transport	1.807	-	-	-
GO:0045596	negative regulation of cell differentiation	1.797	-	1.437	-
GO:0043647	inositol phosphate metabolic process	1.796	-	-	-

GO:0009056	catabolic process	1.787	-	-	-
GO:0070542	response to fatty acid	1.786	-	-	1.398
GO:0032609	interferon-gamma production	1.784	-	2.731	-
GO:1904019	epithelial cell apoptotic process	1.784	-	1.590	2.086
GO:0097368	establishment of Sertoli cell barrier	1.783	-	-	1.331
GO:0060586	multicellular organismal iron ion homeostasis	1.783	-	2.762	1.331
GO:0032945	negative regulation of mononuclear cell proliferation	1.783	-	-	-
GO:0002035	brain renin-angiotensin system	1.783	-	-	-
GO:0110095	cellular detoxification of aldehyde	1.783	-	-	-
GO:0044089	positive regulation of cellular component biogenesis	1.783	-	-	-
GO:0006644	phospholipid metabolic process	1.782	-	-	-
GO:0032943	mononuclear cell proliferation	1.780	-	-	-
GO:0032653	regulation of interleukin-10 production	1.780	-	1.525	-
GO:0050863	regulation of T cell activation	1.767	-	-	-
GO:0031623	receptor internalization	1.766	-	-	-
GO:0070372	regulation of ERK1 and ERK2 cascade	1.766	-	1.977	-
GO:0030099	myeloid cell differentiation	1.766	-	2.604	1.408
GO:0015804	neutral amino acid transport	1.755	-	-	-
GO:0071840	cellular component organization or biogenesis	1.752	-	-	1.950
GO:0008610	lipid biosynthetic process	1.743	-	-	-
GO:0033623	regulation of integrin activation	1.742	-	-	-
GO:0035461	vitamin transmembrane transport	1.742	-	-	-
GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	1.742	-	-	-
GO:0008406	gonad development	1.734	-	1.890	1.628
GO:0072575	epithelial cell proliferation involved in liver morphogenesis	1.734	-	1.480	1.929
GO:0072574	hepatocyte proliferation	1.734	-	1.480	1.929
GO:0089718	amino acid import across plasma membrane	1.734	-	-	-
GO:0055072	iron ion homeostasis	1.732	-	1.525	-

GO:1904659	glucose transmembrane transport	1.722	-	-	-
GO:0051403	stress-activated MAPK cascade	1.721	-	-	-
GO:0046456	icosanoid biosynthetic process	1.710	-	-	-
GO:0098657	import into cell	1.707	-	-	-
GO:0008645	hexose transmembrane transport	1.699	-	-	-
GO:0071356	cellular response to tumor necrosis factor	1.699	-	3.726	1.864
GO:0110053	regulation of actin filament organization	1.696	-	1.328	1.411
GO:0031348	negative regulation of defense response	1.688	-	-	-
GO:0045893	positive regulation of transcription, DNA-templated	1.681	-	-	-
GO:0045807	positive regulation of endocytosis	1.676	-	1.527	1.536
GO:0002824	positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	1.675	-	2.307	-
GO:0043620	regulation of DNA-templated transcription in response to stress	1.672	-	-	-
GO:0042398	cellular modified amino acid biosynthetic process	1.667	-	-	-
GO:0060644	mammary gland epithelial cell differentiation	1.667	-	-	-
GO:0045986	negative regulation of smooth muscle contraction	1.667	-	-	-
GO:0032516	positive regulation of phosphoprotein phosphatase activity	1.667	-	-	1.610
GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	1.667	-	-	-
GO:0033631	cell-cell adhesion mediated by integrin	1.667	-	-	-
GO:0072567	chemokine (C-X-C motif) ligand 2 production	1.657	-	-	-
GO:2000341	regulation of chemokine (C-X-C motif) ligand 2 production	1.657	-	-	-
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	1.657	-	-	-
GO:0006914	autophagy	1.655	-	-	-
GO:0061919	process utilizing autophagic mechanism	1.655	-	-	-
GO:0007254	JNK cascade	1.649	-	-	-

GO:0070664	negative regulation of leukocyte proliferation	1.649	-	-	-
GO:0045453	bone resorption	1.646	-	-	-
GO:0032613	interleukin-10 production	1.636	-	2.102	-
GO:0001649	osteoblast differentiation	1.631	-	2.503	1.414
GO:0006897	endocytosis	1.627	-	-	-
GO:0001763	morphogenesis of a branching structure	1.627	-	-	-
GO:0007548	sex differentiation	1.626	-	2.261	1.864
GO:0000038	very long-chain fatty acid metabolic process	1.624	-	-	-
GO:0072576	liver morphogenesis	1.624	-	1.420	1.855
GO:0001666	response to hypoxia	1.613	-	-	-
GO:0019751	polyol metabolic process	1.609	-	-	-
GO:0046474	glycerophospholipid biosynthetic process	1.606	-	-	-
GO:0046890	regulation of lipid biosynthetic process	1.606	-	-	-
GO:0050728	negative regulation of inflammatory response	1.606	-	-	-
GO:0070340	detection of bacterial lipopeptide	1.606	-	-	-
GO:1904466	positive regulation of matrix metalloproteinase secretion	1.606	-	-	-
GO:0099178	regulation of retrograde trans-synaptic signaling by endocannabinoid	1.606	-	-	-
GO:1904705	regulation of vascular associated smooth muscle cell proliferation	1.606	-	-	-
GO:1904996	positive regulation of leukocyte adhesion to vascular endothelial cell	1.606	-	-	1.554
GO:1990874	vascular associated smooth muscle cell proliferation	1.606	-	-	-
GO:0060559	positive regulation of calcidiol 1-monooxygenase activity	1.606	-	2.264	2.015
GO:0060557	positive regulation of vitamin D biosynthetic process	1.606	-	2.264	2.015
GO:0051956	negative regulation of amino acid transport	1.606	-	1.850	1.554
GO:0045664	regulation of neuron differentiation	1.606	-	-	-
GO:1903598	positive regulation of gap junction assembly	1.606	-	-	-
GO:1901329	regulation of odontoblast differentiation	1.606	-	-	-
GO:0030327	prenylated protein catabolic process	1.606	-	-	-

GO:0006690	icosanoid metabolic process	1.606	-	-	-
GO:0033033	negative regulation of myeloid cell apoptotic process	1.606	-	1.850	-
GO:0043506	regulation of JUN kinase activity	1.606	-	-	-
GO:0045082	positive regulation of interleukin-10 biosynthetic process	1.606	-	-	-
GO:0002397	MHC class I protein complex assembly	1.606	-	-	-
GO:0002086	diaphragm contraction	1.606	-	-	-
GO:0001966	thigmotaxis	1.606	-	-	-
GO:0002755	MyD88-dependent toll-like receptor signaling pathway	1.606	-	-	-
GO:0046718	viral entry into host cell	1.602	-	2.358	-
GO:0002686	negative regulation of leukocyte migration	1.602	-	-	-
GO:0019058	viral life cycle	1.597	-	5.831	-
GO:0043087	regulation of GTPase activity	1.595	-	-	-
GO:0045932	negative regulation of muscle contraction	1.589	-	-	-
GO:0032891	negative regulation of organic acid transport	1.589	-	2.185	1.812
GO:0045824	negative regulation of innate immune response	1.585	-	1.409	-
GO:0002698	negative regulation of immune effector process	1.585	-	1.974	-
GO:0031032	actomyosin structure organization	1.584	-	2.442	2.177
GO:0045637	regulation of myeloid cell differentiation	1.583	-	1.755	-
GO:0044283	small molecule biosynthetic process	1.583	-	-	-
GO:0034329	cell junction assembly	1.571	-	-	-
GO:0032874	positive regulation of stress-activated MAPK cascade	1.569	-	-	1.405
GO:2001242	regulation of intrinsic apoptotic signaling pathway	1.569	-	-	-
GO:0031098	stress-activated protein kinase signaling cascade	1.563	-	-	-
GO:0002820	negative regulation of adaptive immune response	1.562	-	-	-
GO:0032958	inositol phosphate biosynthetic process	1.562	-	-	-
GO:1902579	multi-organism localization	1.561	-	1.409	-
GO:0110096	cellular response to aldehyde	1.561	-	-	-
GO:0044766	multi-organism transport	1.561	-	1.409	-
GO:0090237	regulation of arachidonic acid secretion	1.561	-	-	-
GO:0071223	cellular response to lipoteichoic acid	1.561	-	1.409	-

GO:0070391	response to lipoteichoic acid	1.561	-	1.409	-
GO:0046794	transport of virus	1.561	-	1.409	-
GO:0071398	cellular response to fatty acid	1.554	-	-	-
GO:0051447	negative regulation of meiotic cell cycle	1.544	-	-	1.512
GO:0032495	response to muramyl dipeptide	1.544	-	2.769	1.512
GO:0006656	phosphatidylcholine biosynthetic process	1.544	-	-	-
GO:1905898	positive regulation of response to endoplasmic reticulum stress	1.543	-	-	-
GO:0051235	maintenance of location	1.543	-	1.469	-
GO:0002691	regulation of cellular extravasation	1.543	-	-	-
GO:1902905	positive regulation of supramolecular fiber organization	1.538	-	-	-
GO:0070304	positive regulation of stress-activated protein kinase signaling cascade	1.535	-	-	1.380
GO:0008277	regulation of G protein-coupled receptor signaling pathway	1.525	-	-	-
GO:0032768	regulation of monooxygenase activity	1.525	-	1.684	-
GO:0033003	regulation of mast cell activation	1.525	-	-	-
GO:0043300	regulation of leukocyte degranulation	1.525	-	1.684	-
GO:0046328	regulation of JNK cascade	1.525	-	-	-
GO:0032922	circadian regulation of gene expression	1.524	-	-	-
GO:0030888	regulation of B cell proliferation	1.524	-	-	-
GO:0042093	T-helper cell differentiation	1.524	-	-	-
GO:0046486	glycerolipid metabolic process	1.521	-	-	-
GO:0051100	negative regulation of binding	1.520	-	1.463	-
GO:0042310	vasoconstriction	1.516	-	-	-
GO:0044267	cellular protein metabolic process	1.515	-	-	-
GO:0034220	ion transmembrane transport	1.511	-	-	-
GO:0032273	positive regulation of protein polymerization	1.507	-	-	-
GO:1903827	regulation of cellular protein localization	1.499	-	-	-
GO:0043304	regulation of mast cell degranulation	1.496	-	-	-

GO:0046640	regulation of alpha-beta T cell proliferation	1.496	-	2.102	-
GO:0009595	detection of biotic stimulus	1.496	-	-	-
GO:1901224	positive regulation of NIK/NF-kappaB signaling	1.494	-	-	-
GO:0072088	nephron epithelium morphogenesis	1.494	-	-	-
GO:0044409	entry into host	1.494	-	2.264	-
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	1.494	-	-	-
GO:0002294	CD4-positive, alpha-beta T cell differentiation involved in immune response	1.493	-	-	-
GO:2001237	negative regulation of extrinsic apoptotic signaling pathway	1.493	-	1.382	1.462
GO:0070661	leukocyte proliferation	1.493	-	-	-
GO:2000345	regulation of hepatocyte proliferation	1.486	-	1.745	1.460
GO:0090276	regulation of peptide hormone secretion	1.486	-	-	1.855
GO:0046641	positive regulation of alpha-beta T cell proliferation	1.486	-	-	-
GO:0010743	regulation of macrophage derived foam cell differentiation	1.486	-	1.745	-
GO:0046325	negative regulation of glucose import	1.486	-	-	-
GO:0140112	extracellular vesicle biogenesis	1.486	-	-	-
GO:0050755	chemokine metabolic process	1.486	-	-	-
GO:0051258	protein polymerization	1.483	-	-	-
GO:0036293	response to decreased oxygen levels	1.483	-	-	-
GO:0060993	kidney morphogenesis	1.471	-	-	-
GO:0002532	production of molecular mediator involved in inflammatory response	1.471	-	1.623	-
GO:0097278	complement-dependent cytotoxicity	1.470	-	1.346	-
GO:0044764	multi-organism cellular process	1.470	-	1.346	-
GO:2000121	regulation of removal of superoxide radicals	1.470	-	2.406	-
GO:0010958	regulation of amino acid import across plasma membrane	1.470	-	-	-

GO:0002293	alpha-beta T cell differentiation involved in immune response	1.470	-	-	-
GO:0014911	positive regulation of smooth muscle cell migration	1.470	-	-	-
GO:1903789	regulation of amino acid transmembrane transport	1.470	-	-	-
GO:0015800	acidic amino acid transport	1.470	-	-	1.541
GO:0032725	positive regulation of granulocyte macrophage colony-stimulating factor production	1.470	-	2.406	-
GO:0032308	positive regulation of prostaglandin secretion	1.470	-	1.346	-
GO:0090557	establishment of endothelial intestinal barrier	1.470	-	-	-
GO:1903508	positive regulation of nucleic acid-templated transcription	1.468	-	-	-
GO:0032872	regulation of stress-activated MAPK cascade	1.463	-	-	-
GO:1902680	positive regulation of RNA biosynthetic process	1.463	-	-	-
GO:0043090	amino acid import	1.462	-	-	-
GO:0010543	regulation of platelet activation	1.462	-	-	-
GO:0097242	amyloid-beta clearance	1.462	-	1.311	-
GO:0050832	defense response to fungus	1.462	-	-	-
GO:0050999	regulation of nitric-oxide synthase activity	1.462	-	2.066	-
GO:0033006	regulation of mast cell activation involved in immune response	1.462	-	-	-
GO:0051701	interaction with host	1.459	-	1.870	-
GO:0014909	smooth muscle cell migration	1.455	-	-	-
GO:0072028	nephron morphogenesis	1.451	-	-	-
GO:0046635	positive regulation of alpha-beta T cell activation	1.442	-	-	-
GO:0002287	alpha-beta T cell activation involved in immune response	1.442	-	-	-
GO:0080135	regulation of cellular response to stress	1.442	-	-	1.504
GO:2000810	regulation of bicellular tight junction assembly	1.432	-	1.701	2.250
GO:0051043	regulation of membrane protein ectodomain proteolysis	1.432	-	-	-
GO:0032930	positive regulation of superoxide anion generation	1.432	-	-	-
GO:0060252	positive regulation of glial cell proliferation	1.432	-	-	-
GO:0061912	selective autophagy	1.429	-	-	-

GO:0060612	adipose tissue development	1.429	-	-	-
GO:0070302	regulation of stress-activated protein kinase signaling cascade	1.423	-	-	-
GO:0030509	BMP signaling pathway	1.420	-	-	-
GO:1903427	negative regulation of reactive oxygen species biosynthetic process	1.418	-	-	-
GO:1903959	regulation of anion transmembrane transport	1.418	-	-	-
GO:0045576	mast cell activation	1.412	-	-	-
GO:0007610	behavior	1.410	-	-	1.351
GO:0000187	activation of MAPK activity	1.409	-	-	-
GO:1990830	cellular response to leukemia inhibitory factor	1.409	-	-	-
GO:0003073	regulation of systemic arterial blood pressure	1.409	-	-	-
GO:1990823	response to leukemia inhibitory factor	1.409	-	-	-
GO:0044057	regulation of system process	1.408	-	-	-
GO:0034395	regulation of transcription from RNA polymerase II promoter in response to iron	1.403	-	2.036	-
GO:0002501	peptide antigen assembly with MHC protein complex	1.403	-	-	-
GO:0002351	serotonin production involved in inflammatory response	1.403	-	-	-
GO:0002442	serotonin secretion involved in inflammatory response	1.403	-	-	-
GO:0071881	adenylate cyclase-inhibiting adrenergic receptor signaling pathway	1.403	-	-	-
GO:0002159	desmosome assembly	1.403	-	-	-
GO:0007412	axon target recognition	1.403	-	-	-
GO:0070836	caveola assembly	1.403	-	-	-
GO:0006663	platelet activating factor biosynthetic process	1.403	-	-	-
GO:0006659	phosphatidylserine biosynthetic process	1.403	-	-	-
GO:0006636	unsaturated fatty acid biosynthetic process	1.403	-	-	-
GO:0003011	involuntary skeletal muscle contraction	1.403	-	-	-
GO:0002906	negative regulation of mature B cell apoptotic process	1.403	-	-	-
GO:0002905	regulation of mature B cell apoptotic process	1.403	-	-	-

GO:1903613	regulation of protein tyrosine phosphatase activity	1.403	-	-	-
GO:1904612	response to 2,3,7,8-tetrachlorodibenzodioxine	1.403	-	-	-
GO:0051896	regulation of protein kinase B signaling	1.403	-	-	-
GO:0002901	mature B cell apoptotic process	1.403	-	-	-
GO:0001922	B-1 B cell homeostasis	1.403	-	-	-
GO:0001712	ectodermal cell fate commitment	1.403	-	-	-
GO:0019932	second-messenger-mediated signaling	1.403	-	-	-
GO:0018197	peptidyl-aspartic acid modification	1.403	-	-	-
GO:0043456	regulation of pentose-phosphate shunt	1.403	-	-	-
GO:0016259	selenocysteine metabolic process	1.403	-	-	-
GO:0032632	interleukin-3 production	1.403	-	-	-
GO:0000904	cell morphogenesis involved in differentiation	1.403	-	-	-
GO:0042494	detection of bacterial lipoprotein	1.403	-	-	-
GO:0075509	endocytosis involved in viral entry into host cell	1.403	-	-	-
GO:0019065	receptor-mediated endocytosis of virus by host cell	1.403	-	-	-
GO:0002764	immune response-regulating signaling pathway	1.402	-	-	-
GO:0052126	movement in host environment	1.401	-	1.838	-
GO:0002573	myeloid leukocyte differentiation	1.401	-	1.413	-
GO:0001657	ureteric bud development	1.401	-	-	-
GO:0002016	regulation of blood volume by renin-angiotensin	1.399	-	-	-
GO:0046324	regulation of glucose import	1.399	-	-	-
GO:0002903	negative regulation of B cell apoptotic process	1.399	-	-	-
GO:0090209	negative regulation of triglyceride metabolic process	1.399	-	-	-
GO:0072216	positive regulation of metanephros development	1.399	-	-	-
GO:0032490	detection of molecule of bacterial origin	1.399	-	-	-
GO:0031652	positive regulation of heat generation	1.399	-	-	-
GO:0022604	regulation of cell morphogenesis	1.399	-	-	-
GO:0010737	protein kinase A signaling	1.398	-	-	-
GO:0033630	positive regulation of cell adhesion mediated by integrin	1.398	-	-	-
GO:0070498	interleukin-1-mediated signaling pathway	1.398	-	-	-

GO:0010884	positive regulation of lipid storage	1.398	-	1.656	1.376
GO:0019430	removal of superoxide radicals	1.398	-	1.656	-
GO:0014048	regulation of glutamate secretion	1.398	-	-	1.376
GO:0045780	positive regulation of bone resorption	1.398	-	-	-
GO:0009069	serine family amino acid metabolic process	1.398	-	-	-
GO:0002693	positive regulation of cellular extravasation	1.398	-	-	1.376
GO:0010042	response to manganese ion	1.398	-	-	-
GO:0046852	positive regulation of bone remodeling	1.398	-	-	-
GO:1901566	organonitrogen compound biosynthetic process	1.397	-	-	-
GO:0051897	positive regulation of protein kinase B signaling	1.390	-	-	-
GO:0007420	brain development	1.389	-	-	-
GO:0072163	mesonephric epithelium development	1.386	-	-	-
GO:0045619	regulation of lymphocyte differentiation	1.386	-	1.368	-
GO:0072164	mesonephric tubule development	1.386	-	-	-
GO:0046849	bone remodeling	1.386	-	-	-
GO:0009620	response to fungus	1.383	-	-	-
GO:0006766	vitamin metabolic process	1.377	-	-	1.454
GO:1900180	regulation of protein localization to nucleus	1.365	-	-	-
GO:0097756	negative regulation of blood vessel diameter	1.362	-	-	-
GO:0046916	cellular transition metal ion homeostasis	1.362	-	-	-
GO:0032733	positive regulation of interleukin-10 production	1.356	-	-	-
GO:0030225	macrophage differentiation	1.356	-	-	1.603
GO:0097421	liver regeneration	1.356	-	-	-
GO:0061138	morphogenesis of a branching epithelium	1.356	-	-	-
GO:0044403	symbiotic process	1.356	-	6.106	-
GO:1904037	positive regulation of epithelial cell apoptotic process	1.356	-	-	-
GO:0051354	negative regulation of oxidoreductase activity	1.356	-	-	-
GO:0009314	response to radiation	1.355	-	-	-
GO:0090130	tissue migration	1.353	-	1.810	1.815
GO:0043542	endothelial cell migration	1.350	-	2.231	2.392

GO:0050905	neuromuscular process	1.349	-	1.527	-
GO:0043299	leukocyte degranulation	1.348	-	-	-
GO:0022612	gland morphogenesis	1.348	-	1.771	-
GO:1904994	regulation of leukocyte adhesion to vascular endothelial cell	1.344	-	-	1.335
GO:0051156	glucose 6-phosphate metabolic process	1.344	-	-	-
GO:0071450	cellular response to oxygen radical	1.344	-	1.621	-
GO:0002577	regulation of antigen processing and presentation	1.344	-	-	-
GO:0042908	xenobiotic transport	1.344	-	-	1.335
GO:0090330	regulation of platelet aggregation	1.344	-	-	-
GO:0071451	cellular response to superoxide	1.344	-	1.621	-
GO:0006909	phagocytosis	1.343	-	-	1.616
GO:0120036	plasma membrane bounded cell projection organization	1.331	-	-	-
GO:0009890	negative regulation of biosynthetic process	1.329	-	-	-
GO:0033043	regulation of organelle organization	1.324	-	-	1.399
GO:0062207	regulation of pattern recognition receptor signaling pathway	1.324	-	2.749	-
GO:1904478	regulation of intestinal absorption	1.324	-	-	-
GO:0001892	embryonic placenta development	1.324	-	-	-
GO:0061097	regulation of protein tyrosine kinase activity	1.324	-	-	-
GO:0050805	negative regulation of synaptic transmission	1.324	-	-	1.627
GO:0032645	regulation of granulocyte macrophage colony-stimulating factor production	1.324	-	2.232	-
GO:0032868	response to insulin	1.324	-	-	-
GO:0032604	granulocyte macrophage colony-stimulating factor production	1.324	-	2.232	-
GO:0045416	positive regulation of interleukin-8 biosynthetic process	1.324	-	-	-
GO:0032306	regulation of prostaglandin secretion	1.324	-	-	-
GO:0035589	G protein-coupled purinergic nucleotide receptor signaling pathway	1.324	-	-	-

GO:0045835	negative regulation of meiotic nuclear division	1.324	-	-	1.918
GO:0019432	triglyceride biosynthetic process	1.321	-	-	-
GO:0010039	response to iron ion	1.321	-	-	-
GO:0046633	alpha-beta T cell proliferation	1.321	-	1.921	-
GO:0046394	carboxylic acid biosynthetic process	1.313	-	-	-
GO:0006812	cation transport	1.307	-	-	-
GO:0001823	mesonephros development	1.302	-	-	-
GO:0046136	positive regulation of vitamin metabolic process	-	-	1.870	1.653
GO:0060558	regulation of calcidiol 1-monooxygenase activity	-	-	1.633	1.414
GO:0031622	positive regulation of fever generation	-	-	1.546	1.331
GO:0031620	regulation of fever generation	-	-	1.469	-
GO:0060556	regulation of vitamin D biosynthetic process	-	-	1.469	-
GO:0042368	vitamin D biosynthetic process	-	-	1.346	-
GO:0034116	positive regulation of heterotypic cell-cell adhesion	-	-	2.157	1.853
GO:0038034	signal transduction in absence of ligand	-	-	2.922	1.769
GO:0097192	extrinsic apoptotic signaling pathway in absence of ligand	-	-	2.922	1.769
GO:0009110	vitamin biosynthetic process	-	-	1.701	1.414
GO:0051341	regulation of oxidoreductase activity	-	-	2.406	-
GO:0060445	branching involved in salivary gland morphogenesis	-	-	1.656	1.376
GO:0010893	positive regulation of steroid biosynthetic process	-	-	2.456	1.304
GO:0032770	positive regulation of monooxygenase activity	-	-	1.450	-
GO:0045940	positive regulation of steroid metabolic process	-	-	2.102	-
GO:0002708	positive regulation of lymphocyte mediated immunity	-	-	1.870	-
GO:0006775	fat-soluble vitamin metabolic process	-	-	1.311	-
GO:1903792	negative regulation of anion transport	-	-	1.862	1.512
GO:0030866	cortical actin cytoskeleton organization	-	-	-	-
GO:0007431	salivary gland development	-	-	-	1.480
GO:0002705	positive regulation of leukocyte mediated immunity	-	-	2.406	-
GO:0006959	humoral immune response	-	-	1.469	1.425
GO:0000122	negative regulation of transcription by RNA polymerase II	-	-	1.525	1.650

GO:0051353	positive regulation of oxidoreductase activity	-	-	1.463	-
GO:0045840	positive regulation of mitotic nuclear division	-	-	1.421	-
GO:0042742	defense response to bacterium	-	-	2.765	1.513
GO:0002696	positive regulation of leukocyte activation	-	-	1.548	-
GO:0002711	positive regulation of T cell mediated immunity	-	-	1.772	-
GO:0070427	nucleotide-binding oligomerization domain containing 1 signaling pathway	-	-	1.870	1.653
GO:0002678	positive regulation of chronic inflammatory response	-	-	1.870	-
GO:2000556	positive regulation of T-helper 1 cell cytokine production	-	-	1.739	-
GO:0030198	extracellular matrix organization	-	-	1.437	1.548
GO:0010717	regulation of epithelial to mesenchymal transition	-	-	1.493	-
GO:2000554	regulation of T-helper 1 cell cytokine production	-	-	1.739	-
GO:0043062	extracellular structure organization	-	-	1.420	1.528
GO:0032760	positive regulation of tumor necrosis factor production	-	-	1.899	-
GO:0070487	monocyte aggregation	-	-	1.633	1.414
GO:2001182	regulation of interleukin-12 secretion	-	-	1.633	-
GO:0031665	negative regulation of lipopolysaccharide-mediated signaling pathway	-	-	1.633	1.414
GO:0071695	anatomical structure maturation	-	-	1.549	2.509
GO:0021700	developmental maturation	-	-	-	2.259
GO:0010594	regulation of endothelial cell migration	-	-	1.796	2.074
GO:0045602	negative regulation of endothelial cell differentiation	-	-	-	1.918
GO:0010631	epithelial cell migration	-	-	1.882	1.897
GO:0048469	cell maturation	-	-	-	1.895
GO:0050901	leukocyte tethering or rolling	-	-	1.450	1.892
GO:0030212	hyaluronan metabolic process	-	-	2.265	1.892
GO:0090132	epithelium migration	-	-	1.862	1.869
GO:0030857	negative regulation of epithelial cell differentiation	-	-	2.298	1.868
GO:0070306	lens fiber cell differentiation	-	-	-	1.855
GO:0042551	neuron maturation	-	-	-	1.791

GO:0042493	response to drug	-	-	-	1.765
GO:0110020	regulation of actomyosin structure organization	-	-	1.702	1.748
GO:0030072	peptide hormone secretion	-	-	-	1.719
GO:1904467	regulation of tumor necrosis factor secretion	-	-	2.066	1.709
GO:0030324	lung development	-	-	-	1.706
GO:0044106	cellular amine metabolic process	-	-	-	1.685
GO:0030323	respiratory tube development	-	-	-	1.667
GO:0050711	negative regulation of interleukin-1 secretion	-	-	-	1.665
GO:0010310	regulation of hydrogen peroxide metabolic process	-	-	-	1.665
GO:0051129	negative regulation of cellular component organization	-	-	-	1.664
GO:0070291	N-acyl ethanolamine metabolic process	-	-	-	1.653
GO:0010810	regulation of cell-substrate adhesion	-	-	1.437	1.650
GO:1990774	tumor necrosis factor secretion	-	-	1.993	1.639
GO:0045786	negative regulation of cell cycle	-	-	1.496	1.623
GO:0007566	embryo implantation	-	-	-	1.612
GO:0048644	muscle organ morphogenesis	-	-	-	1.564
GO:1904029	regulation of cyclin-dependent protein kinase activity	-	-	1.997	1.532
GO:2000416	regulation of eosinophil migration	-	-	-	1.523
GO:0097696	receptor signaling pathway via STAT	-	-	-	1.523
GO:0090166	Golgi disassembly	-	-	-	1.523
GO:0060059	embryonic retina morphogenesis in camera-type eye	-	-	-	1.523
GO:0043305	negative regulation of mast cell degranulation	-	-	-	1.523
GO:1903510	mucopolysaccharide metabolic process	-	-	1.316	1.523
GO:0006145	purine nucleobase catabolic process	-	-	-	1.523
GO:0050830	defense response to Gram-positive bacterium	-	-	3.262	1.523
GO:0034201	response to oleic acid	-	-	-	1.523
GO:0061756	leukocyte adhesion to vascular endothelial cell	-	-	-	1.512
GO:0032692	negative regulation of interleukin-1 production	-	-	-	1.512
GO:2000242	negative regulation of reproductive process	-	-	-	1.502
GO:0009308	amine metabolic process	-	-	-	1.502

GO:0010470	regulation of gastrulation	-	-	1.831	1.480
GO:0051492	regulation of stress fiber assembly	-	-	1.921	1.462
GO:0050691	regulation of defense response to virus by host	-	-	1.797	1.449
GO:0001955	blood vessel maturation	-	-	1.633	1.414
GO:1905939	regulation of gonad development	-	-	-	1.414
GO:1902396	protein localization to bicellular tight junction	-	-	-	1.414
GO:1904469	positive regulation of tumor necrosis factor secretion	-	-	1.701	1.414
GO:0097305	response to alcohol	-	-	-	1.402
GO:0046425	regulation of receptor signaling pathway via JAK-STAT	-	-	-	1.393
GO:0021756	striatum development	-	-	-	1.376
GO:0032026	response to magnesium ion	-	-	-	1.376
GO:0051494	negative regulation of cytoskeleton organization	-	-	-	1.356
GO:0043535	regulation of blood vessel endothelial cell migration	-	-	-	1.348
GO:0001937	negative regulation of endothelial cell proliferation	-	-	1.684	1.340
GO:0043491	protein kinase B signaling	-	-	-	1.335
GO:1903978	regulation of microglial cell activation	-	-	-	1.335
GO:0008340	determination of adult lifespan	-	-	-	1.335
GO:0033601	positive regulation of mammary gland epithelial cell proliferation	-	-	1.546	1.331
GO:0002885	positive regulation of hypersensitivity	-	-	-	1.331
GO:0033007	negative regulation of mast cell activation involved in immune response	-	-	-	1.331
GO:0000820	regulation of glutamine family amino acid metabolic process	-	-	-	1.331
GO:0046113	nucleobase catabolic process	-	-	-	1.331
GO:0006108	malate metabolic process	-	-	-	1.331
GO:0060426	lung vasculature development	-	-	-	1.331
GO:0035234	ectopic germ cell programmed cell death	-	-	1.546	1.331
GO:0000050	urea cycle	-	-	1.546	1.331

GO:0010727	negative regulation of hydrogen peroxide metabolic process	-	-	1.546	1.331
GO:2000389	regulation of neutrophil extravasation	-	-	-	1.331
GO:2000772	regulation of cellular senescence	-	-	-	1.329
GO:1901031	regulation of response to reactive oxygen species	-	-	2.401	1.329
GO:0071230	cellular response to amino acid stimulus	-	-	-	1.305
GO:0010458	exit from mitosis	-	-	-	1.304
GO:0010226	response to lithium ion	-	-	1.581	1.304
GO:0009615	response to virus	-	-	13.377	-
GO:0051607	defense response to virus	-	-	10.421	-
GO:0045071	negative regulation of viral genome replication	-	-	6.730	-
GO:0045069	regulation of viral genome replication	-	-	5.754	-
GO:1903901	negative regulation of viral life cycle	-	-	5.389	-
GO:0019079	viral genome replication	-	-	5.062	-
GO:0016032	viral process	-	-	4.800	-
GO:0048525	negative regulation of viral process	-	-	4.648	-
GO:0060700	regulation of ribonuclease activity	-	-	4.509	-
GO:0035455	response to interferon-alpha	-	-	4.219	-
GO:1903900	regulation of viral life cycle	-	-	4.174	-
GO:0043903	regulation of symbiotic process	-	-	3.651	-
GO:0072608	interleukin-10 secretion	-	-	3.572	-
GO:0032069	regulation of nuclease activity	-	-	3.352	-
GO:0050792	regulation of viral process	-	-	3.340	-
GO:0035457	cellular response to interferon-alpha	-	-	3.194	-
GO:0034340	response to type I interferon	-	-	3.106	-
GO:0098586	cellular response to virus	-	-	3.106	-
GO:0035458	cellular response to interferon-beta	-	-	3.065	-
GO:0034138	toll-like receptor 3 signaling pathway	-	-	2.911	-
GO:2000562	negative regulation of CD4-positive, alpha-beta T cell proliferation	-	-	2.909	-

GO:0002753	cytoplasmic pattern recognition receptor signaling pathway	-	-	2.656	-
GO:2000561	regulation of CD4-positive, alpha-beta T cell proliferation	-	-	2.630	-
GO:0060337	type I interferon signaling pathway	-	-	2.543	-
GO:2001179	regulation of interleukin-10 secretion	-	-	2.507	-
GO:0071357	cellular response to type I interferon	-	-	2.461	-
GO:0046642	negative regulation of alpha-beta T cell proliferation	-	-	2.406	-
GO:0002676	regulation of chronic inflammatory response	-	-	2.406	-
GO:0035739	CD4-positive, alpha-beta T cell proliferation	-	-	2.406	-
GO:0045648	positive regulation of erythrocyte differentiation	-	-	2.358	-
GO:0006357	regulation of transcription by RNA polymerase II	-	-	2.307	-
GO:0007050	cell cycle arrest	-	-	2.294	-
GO:1900245	positive regulation of MDA-5 signaling pathway	-	-	2.264	-
GO:0060546	negative regulation of necroptotic process	-	-	2.157	-
GO:0050688	regulation of defense response to virus	-	-	2.157	-
GO:0030213	hyaluronan biosynthetic process	-	-	2.157	-
GO:0060340	positive regulation of type I interferon-mediated signaling pathway	-	-	2.157	-
GO:0070266	necroptotic process	-	-	2.143	-
GO:0006366	transcription by RNA polymerase II	-	-	2.124	-
GO:0070098	chemokine-mediated signaling pathway	-	-	2.096	-
GO:0010712	regulation of collagen metabolic process	-	-	2.078	-
GO:2000514	regulation of CD4-positive, alpha-beta T cell activation	-	-	2.052	-
GO:0044406	adhesion of symbiont to host	-	-	2.019	-
GO:0010875	positive regulation of cholesterol efflux	-	-	1.958	-
GO:0062208	positive regulation of pattern recognition receptor signaling pathway	-	-	1.958	-
GO:0002719	negative regulation of cytokine production involved in immune response	-	-	1.893	-
GO:1990869	cellular response to chemokine	-	-	1.882	-

GO:1903557	positive regulation of tumor necrosis factor superfamily cytokine production	-	-	1.882	-
GO:1990868	response to chemokine	-	-	1.882	-
GO:0098900	regulation of action potential	-	-	1.879	-
GO:0045667	regulation of osteoblast differentiation	-	-	1.870	-
GO:0045185	maintenance of protein location	-	-	1.870	-
GO:0007219	Notch signaling pathway	-	-	1.870	-
GO:0060544	regulation of necroptotic process	-	-	1.850	-
GO:0032924	activin receptor signaling pathway	-	-	1.831	-
GO:0045639	positive regulation of myeloid cell differentiation	-	-	1.800	-
GO:0002726	positive regulation of T cell cytokine production	-	-	1.796	-
GO:0060547	negative regulation of necrotic cell death	-	-	1.796	-
GO:0035634	response to stilbenoid	-	-	1.796	-
GO:0097300	programmed necrotic cell death	-	-	1.769	-
GO:0036072	direct ossification	-	-	1.739	-
GO:0039533	regulation of MDA-5 signaling pathway	-	-	1.739	-
GO:0001957	intramembranous ossification	-	-	1.739	-
GO:0032652	regulation of interleukin-1 production	-	-	1.718	-
GO:0030522	intracellular receptor signaling pathway	-	-	1.703	-
GO:0002544	chronic inflammatory response	-	-	1.701	-
GO:0039529	RIG-I signaling pathway	-	-	1.701	-
GO:0035743	CD4-positive, alpha-beta T cell cytokine production	-	-	1.656	-
GO:0062098	regulation of programmed necrotic cell death	-	-	1.656	-
GO:0032727	positive regulation of interferon-alpha production	-	-	1.656	-
GO:2001181	positive regulation of interleukin-10 secretion	-	-	1.633	-
GO:0039530	MDA-5 signaling pathway	-	-	1.633	-
GO:0043122	regulation of I-kappaB kinase/NF-kappaB signaling	-	-	1.633	-
GO:0045646	regulation of erythrocyte differentiation	-	-	1.612	-
GO:0090077	foam cell differentiation	-	-	1.581	-
GO:0010742	macrophage derived foam cell differentiation	-	-	1.581	-

GO:0032606	type I interferon production	-	-	1.568	-
GO:0032481	positive regulation of type I interferon production	-	-	1.563	-
GO:0070373	negative regulation of ERK1 and ERK2 cascade	-	-	1.549	-
GO:0060343	trabecula formation	-	-	1.546	-
GO:0042542	response to hydrogen peroxide	-	-	1.546	-
GO:0030214	hyaluronan catabolic process	-	-	1.546	-
GO:0034139	regulation of toll-like receptor 3 signaling pathway	-	-	1.546	-
GO:0071493	cellular response to UV-B	-	-	1.546	-
GO:0032965	regulation of collagen biosynthetic process	-	-	1.546	-
GO:0051099	positive regulation of binding	-	-	1.546	-
GO:0039528	cytoplasmic pattern recognition receptor signaling pathway in response to virus	-	-	1.546	-
GO:0007589	body fluid secretion	-	-	1.527	-
GO:0032655	regulation of interleukin-12 production	-	-	1.525	-
GO:0045668	negative regulation of osteoblast differentiation	-	-	1.525	-
GO:0000305	response to oxygen radical	-	-	1.516	-
GO:0000303	response to superoxide	-	-	1.516	-
GO:0032376	positive regulation of cholesterol transport	-	-	1.516	-
GO:0032373	positive regulation of sterol transport	-	-	1.516	-
GO:0032715	negative regulation of interleukin-6 production	-	-	1.505	-
GO:0010883	regulation of lipid storage	-	-	1.505	-
GO:0000079	regulation of cyclin-dependent protein serine/threonine kinase activity	-	-	1.493	-
GO:0045662	negative regulation of myoblast differentiation	-	-	1.480	-
GO:2000515	negative regulation of CD4-positive, alpha-beta T cell activation	-	-	1.480	-
GO:0061082	myeloid leukocyte cytokine production	-	-	1.480	-
GO:0060338	regulation of type I interferon-mediated signaling pathway	-	-	1.480	-
GO:0032647	regulation of interferon-alpha production	-	-	1.480	-

GO:0045927	positive regulation of growth	-	-	1.476	-
GO:0014050	negative regulation of glutamate secretion	-	-	1.469	-
GO:2000172	regulation of branching morphogenesis of a nerve	-	-	1.469	-
GO:0019627	urea metabolic process	-	-	1.469	-
GO:0032615	interleukin-12 production	-	-	1.463	-
GO:0060760	positive regulation of response to cytokine stimulus	-	-	1.463	-
GO:0002701	negative regulation of production of molecular mediator of immune response	-	-	1.450	-
GO:0002832	negative regulation of response to biotic stimulus	-	-	1.449	-
GO:0001501	skeletal system development	-	-	1.445	-
GO:0060759	regulation of response to cytokine stimulus	-	-	1.442	-
GO:0051726	regulation of cell cycle	-	-	1.437	-
GO:0070265	necrotic cell death	-	-	1.421	-
GO:0006081	cellular aldehyde metabolic process	-	-	1.421	-
GO:0034101	erythrocyte homeostasis	-	-	1.420	-
GO:1900407	regulation of cellular response to oxidative stress	-	-	1.420	-
GO:0051497	negative regulation of stress fiber assembly	-	-	1.420	-
GO:0032607	interferon-alpha production	-	-	1.420	-
GO:0070848	response to growth factor	-	-	1.417	-
GO:0000082	G1/S transition of mitotic cell cycle	-	-	1.413	-
GO:1902741	positive regulation of interferon-alpha secretion	-	-	1.409	-
GO:0072641	type I interferon secretion	-	-	1.409	-
GO:1902739	regulation of interferon-alpha secretion	-	-	1.409	-
GO:1902023	L-arginine transport	-	-	1.409	-
GO:0072642	interferon-alpha secretion	-	-	1.409	-
GO:0071941	nitrogen cycle metabolic process	-	-	1.409	-
GO:1900246	positive regulation of RIG-I signaling pathway	-	-	1.409	-
GO:0072610	interleukin-12 secretion	-	-	1.409	-
GO:0035744	T-helper 1 cell cytokine production	-	-	1.409	-
GO:0070673	response to interleukin-18	-	-	1.409	-

GO:0045661	regulation of myoblast differentiation	-	-	1.409	-
GO:1904030	negative regulation of cyclin-dependent protein kinase activity	-	-	1.398	-
GO:0002444	myeloid leukocyte mediated immunity	-	-	1.398	-
GO:0032689	negative regulation of interferon-gamma production	-	-	1.398	-
GO:0033598	mammary gland epithelial cell proliferation	-	-	1.398	-
GO:0032964	collagen biosynthetic process	-	-	1.370	-
GO:0001779	natural killer cell differentiation	-	-	1.368	-
GO:0010714	positive regulation of collagen metabolic process	-	-	1.368	-
GO:0006879	cellular iron ion homeostasis	-	-	1.353	-
GO:0043301	negative regulation of leukocyte degranulation	-	-	1.346	-
GO:0032926	negative regulation of activin receptor signaling pathway	-	-	1.346	-
GO:0070431	nucleotide-binding oligomerization domain containing 2 signaling pathway	-	-	1.346	-
GO:1905097	regulation of guanyl-nucleotide exchange factor activity	-	-	1.346	-
GO:0051384	response to glucocorticoid	-	-	1.342	-
GO:0043393	regulation of protein binding	-	-	1.333	-
GO:0043170	macromolecule metabolic process	-	-	1.320	-

Chapter 4. Elongate mineral particles of different growth habit elicit differential pulmonary immune cell responses in A/J mice

4.1. Abstract

In this study we focused on the long-term effects of EMP exposure, including 3- and 12-months post-exposure time points. Neoplastic manifestations at 1 year were much more frequent in asbestos-treated animals, while cleavage fragments did not have significantly increased number of neoplastic lesions. Histopathology revealed hyperplasia, mild alveolar septal fibrosis and immune cell infiltration, however, no extensive lung fibrosis in asbestiform EMP groups. Cleavage fragments provoked very little histological changes and were mostly found inside the alveoli lumens. DNA damage response was higher in asbestiform EMPs with the foci of γ H2AX staining found in the vicinity of deposited fibers, in AMs, epithelial and lymphoid cells, while CF did not induce significant DNA double strand breaks. Increased ARG1 staining in association with elevated IL-3, IL-4, and IL-5 cytokines levels characterize the immune response to asbestos particles as leaning towards TH2-type. Flow cytometry revealed more drastic changes in the immune cell populations caused by asbestiform EMPs at the same mass doses compared to CF. There was relative depletion of resident AMs, expressing high Siglec-F surface marker, and negative for CD11b molecule, observed in all asbestos-treated groups at 3 months, and persisting at 1-year time point, but mean fluorescence intensity of CD11b on AM surface was higher than control at 12 months in all treated groups, including CF. In the asbestos-treated animals depletion of resident AMs could have exacerbated the pathologic process. In conclusion, exposure to asbestiform and non-asbestiform EMPs led to discrete pulmonary immune responses at 3- and 12-months post-exposure, leading to specific long-term fibroproliferative and carcinogenic outcomes. Asbestos induced a distinct immune response pattern, and a much stronger response than cleavage fragments at mass- and surface area-equivalent doses. The studied cleavage fragments were not as potent as asbestos in driving the fibroproliferation and cancer, most likely due to the lack of resident AM depletion.

4.2. Introduction

Although various studies have suggested that fiber dimensions, surface properties, and physical durability are important criteria for the EMP fibrogenicity and carcinogenicity [1], the essential knowledge of the significance and role of different features/characteristics that define the relative risk of developing specific long-term outcomes is still missing. This is due, at least in part, to the lack of comprehensive studies that assess the adverse effects of asbestiform and non-asbestiform EMPs in relation to their physical, chemical, morphological and surface characteristics as well as their bioavailability and biopersistence. Thus, evaluating/quantifying inflammatory and pulmonary outcomes upon exposure to different asbestos/asbestiform fibers as a function of time using animal models is critical for understanding the detailed mechanisms underlying mineral fiber-induced toxicity. Such studies will also provide a unique opportunity to uncover the time dependent cellular and molecular changes associated with fiber-mediated fibro- and carcinogenesis.

In humans fibrosis induced by asbestos is characterized by the presence of so-called asbestos bodies within the fibrous tissue, and fewer myofibroblasts foci or bronchial wall fibrosis [2]. Deposition of asbestiform EMPs causes alveolar macrophage and epithelial cell apoptosis, recruitment of inflammatory mononuclear cells with subsequent M2 polarization of macrophages, as well as T-lymphocyte activation that leads to the increase in pro-fibrotic cytokines secretion [3, 4]. Carcinogenicity of asbestos is probably its most worrying property. Cancer itself is a complex pathologic manifestation that takes a long time to develop from the time of the initial insult. Often this involves a sequence of gene-environment interactions in a progressive fashion and requires dysfunction of multiple systems (e.g., DNA repair, apoptosis, immune function etc.); Although it is not easy to recapitulate human asbestos-induced lung disease in rodents, first murine asbestosis models were proposed in 1980s [5, 6]. They utilized BALB/C mice and chrysotile asbestos administered via inhalation or instillation. A bolus administration of asbestiform EMPs leads to central pattern fibrosis and quite often uneven distribution of the lesions between lungs. Inhalation allows more uniform distribution pattern and is physiological, at the same time being cumbersome, time- and effort-consuming and

posing significant health hazard to research personnel. Additionally, it requires huge amounts of test material and it is quite difficult to adequately assess the delivered dose. To account for the pros and cons of both methods, we utilized repeated pharyngeal aspiration model, where the total dose is delivered throughout the 3 weeks, twice per week, with 2-3 days breaks. This allows some time for clearance as to not overwhelm the clearance mechanisms, which can happen in bolus dose scenarios.

Animal models are indispensable for studying carcinogenic effects of asbestos/asbestiform fibers. US Environmental Protection Agency (EPA) in its OPPTS 870.8355 Health Effects Test Guidelines recommends using the rat model in the asbestos toxicity studies if the endpoints include fibrosis, lung cancer and mesothelioma. However, for our purposes of primarily assessing the immune responses in relation to fibrosis and lung cancer, but not mesothelioma, we utilized mice of the A/J strain, which is a common and scientifically accepted cancer prone animal model for testing lung carcinogens. Another consideration was the 10-times larger required amount of EMP preparations for rat exposures. We wanted to assess the immune cells population dynamic, presence/absence of specific cell subsets, such as T_{regs} , and polarization pattern (ARG1 vs iNOS in macrophages). Based on the accumulated knowledge and previous studies utilizing mouse model of fibrous nanomaterials pharyngeal aspiration [7], we produced the following hypothesis: after initial stimulus and innate immune response (usually inflammatory), local immune polarization patterns emerge, accompanied by increased proliferative activity of pulmonary cells (hyperplasia). Hyperplastic outcomes in this case may be predicted not solely on structure, biopersistence and chemical composition of particles, but on the function and composition of local immune responses, which undergoes disparate changes in various type of exposures following the cellular and humoral (cytokines and chemokines) pathways. This may result in the resolution of inflammation and/or specific adverse outcomes, such as fibrosis/cancer. Towards this goal, we employed flow-cytometric analysis of lung immune cell populations, cytokine profiling, histopathology and immunohistochemistry studies to characterize subchronic (3 months) and long term (1 year) pulmonary effects associated with repeated exposure to the studied EMPs using repeated pharyngeal aspiration method. A dose, containing comparable amounts of critical fibers ($>5 \mu\text{m}$ length and $>1:3$ aspect ratio) was selected

(see Chapter 2). Further, we estimated a total number of fibers per gram lung dry weight along with their respective size distributions to determine the relevance of selected dose to human exposures.

4.3. Methods

4.3.1. Animals

Specific pathogen-free A/J mice (female, 7-8-wk-old) were obtained from Jackson Laboratories (Bar Harbor, ME). A/J strain is a commonly used strain in lung cancer research. These animals develop spontaneous lung tumors and also in response to carcinogens. The number of mice requested was based on statistical requirement to determine the biological effects of different EMPs at both short-term (n=6) as well as long-term (n=8) post exposure time points. Statistical power analysis was performed to estimate the minimal animal group size required for each goal. While six animals per treatment group will be sufficient for carrying out the short-term study (3 months post exposure), a total of at least 8 mice per group was requested for 1-year post exposure time point. This is to account for animal loss during the course of the study due to aging and other factors such as the high potency of A/J mice in developing spontaneous tumors and associated complications. Moreover, the request for an increase in 20% of mice was further justified by previous studies which explored the survival rate of A/J mice exposed to asbestos particles and reported loss of ~10-15% (depending on dose) of mice starting 300 days post exposure to crocidolite asbestos [8].

4.3.2. Animal treatment

A total of 196 mice were assigned to 2 sets, 14 groups in each, with n=6 for 3 months and n=8 for 1-year time points. Six different suspensions of materials: high and low dose of riebeckite asbestos (Rasb), riebeckite cleavage fragments (RCF), high and low dose of tremolite asbestos (TAsb), and tremolite cleavage fragments (TCF) were used to perform repeated pharyngeal aspiration exposures (twice a week for 3 weeks), while the corresponding control mice were administered sterile Ca²⁺ and Mg²⁺ free PBS (50µl) vehicle (Figure 4.1.). Animals were administered mass-equivalent doses of 120 µg/mouse for all EMPs. This dose has been chosen to be consistent across the various materials

and allow for the cross-comparison with the other studies utilizing the same dose of asbestos. Two additional groups were given critical fiber total surface area-equivalent doses of riebeckite (30 $\mu\text{g}/\text{mouse}$) and tremolite (10 $\mu\text{g}/\text{mouse}$). Given that occupational limits are based on “counts”, number-based dosing was considered as well, however, the resulting doses would have been quite close to the total surface area-equivalent ones, thus the results would not be dramatically different. Pharyngeal aspiration provides good distribution and particle deposition in the lungs, including alveolar space, and corresponds to the inhalation studies results [9, 10]. Number of fibers per gram dry weight of the lungs was compared with Helsinki criterion (see Chapter 2). Mice were euthanized with an overdose of ≥ 100 mg/kg of pentobarbital, and deep anesthesia was confirmed when the mouse no longer responded to a toe pinch. Exsanguination by cutting the posterior vena cava performed to make sure the animal does not wake up.

Figure 4.1. Experimental design of the animal study

4.3.3. Hematological analysis

We assessed the blood cellular composition and other the physiological parameters using ProCyte-Dx Hematology Analyzer and found slight decrease in red blood cell count as well as hemoglobin content in the treated group, however no significant differences in immune cell differentials or other parameters were observed (Supplementary table 2).

4.3.4. Cytokine content in the lung homogenates

23 cytokines were measured in the mouse lung homogenates and plasma using Bio-Plex Pro Mouse Cytokine 23-plex assay on a Bio-Plex 200 Reader (Bio-Rad Laboratories, Hercules, CA) following manufacturer’s directions, with three technical replicates allocated for each sample. Prior to measurements, 1 ml of PBS was added, and homogenates were centrifuged at 2500 g. Cytokine quantities was normalized for the total

protein measured in respective homogenates. This assay detects the levels of IL-1 α , -1 β , -2, -3, -4, -5, -6, -9, -10, -12(p40), -12(p70), -13, -17, Eotaxin, G-CSF, GM-CSF, IFN- γ , KC, MCP-1 (MCAF), MIP-1 α , MIP-1 β , RANTES and TNF- α in the samples.

4.3.5. Flow Cytometric analysis of the immune cell subsets:

Half of the mice from each group had their left lungs excised and placed in PBS for immediate single-cell suspensions preparation. To prepare single-cell suspensions, large airways were dissected from the peripheral lung tissue; the latter was cut into small pieces, transferred into C-tubes (Miltenyi Biotec Inc., Auburn, CA), and processed in digestion buffer (Miltenyi Biotec Inc., Auburn, CA) using GentleMACS dissociator (Miltenyi Biotec Inc., Auburn, CA), according to the manufacturer's instructions. Single-cell suspension will be obtained by passing through 40- μ m nylon mesh. The remaining red blood cells will be lysed using the red cell lysis buffer (Sigma Aldrich). Single-cell suspensions from spleens and lungs will be washed and resuspended in FACS buffer (Rockland Immunochemicals Inc., Limerick, PA). Fc-block antibody will be used to reduce non-specific binding. Cells were then stained with fluorochrome-conjugated antibodies versus CD45 (Clone: 30-F11, BD), Siglec F (Clone: E50-2440, BD Horizon™), MHC Class II, CD11b, Ly6G, Ly6C, CD11c (Clone: N418, eBioscience™), CD64 (Clone: X54-5/7.1, Biolegend) and CD24 (Clone: M1/69, BD) (all obtained from Fischer Scientific, Pittsburgh, PA). Samples were run on Amnis FlowSight imaging flow cytometer (Luminex Corp., Austin, TX) and analyzed using IDEAS 6.2 software to distinguish specific immune cells populations in the lung: alveolar macrophages, T- and B-lymphocytes, incoming inflammatory and regulatory monocytes, neutrophils and eosinophils). Additionally, evaluating the mean fluorescent intensity (MFI) of selected markers allowed elucidating the activation state and unique phenotypic profiles of the different cell types. Gating strategy is shown in Supplementary figure.

4.3.6. Histopathological analysis of mouse lung tissue

Half of the mice per group per time point had their lungs inflated with buffered formalin and embedded in paraffin blocks. Lung tissue sections were stained by H&E and Masson's Trichrome. The slides were analyzed for a presence of fibroproliferative and neoplastic lesions. and lymphohistiocytic aggregates, specifically, parenchymal,

peribronchial, perivascular, and subpleural aggregates. Assessment was performed by board-certified pathologist. Sample identification was coded to ensure blinded evaluation.

4.3.7. Immunohistochemistry staining of the lung tissues:

Additional formalin-fixed paraffin-embedded lung tissue sections have been further stained with antibodies against CD4, CD8, CD20, CD68, FOXP3, Arg1, iNOS, and pH2AX. The benefits of using IHC included visualization of individual cells and using additional parameters, such as morphological differences and compartmental translocation for the analysis of cell populations.

4.3.8. Statistical Analyses

Treatment-related differences in various outcomes were evaluated using two-way ANOVA, followed by pair wise comparison using the Student-Newman-Keuls tests. Results are presented as mean $\pm$ SEM and those p values <0.05 considered statistically significant. We used SigmaPlot 12.5 and R statistical package (<http://www.r-project.org>).

4.4. Results

4.4.1. Cytokine production in the lungs

Concentrations of 23 cytokines and chemokines present in the lung homogenates of mice exposed to various EMPs are shown in Table 4.1. There were no statistically significant changes in the levels of IL-1b in all groups and time points. At 3-months' time point levels of IL-9 were significantly increased in all treatment groups except 30 μ g RAsb compared to controls. IL-3 and IL-4 were several-fold increased in tremolite-treated animals both at 3 and 12 months. GM-CSF was increased compared to control in Tremolite-treated animals only (both asbestos and CF); in Riebeckite groups it was also increased, but with no statistical significance. Chemokines – KC, MCP-1, MIP-1a, MIP-1b were increased in several treatment groups at 3 months, while at 12 month we only observed MIP-1a in TAsb and RAsb as well as KC in Tasb. At 12 months 120 μ g riebeckite asbestos only had increased IL-13, IL-17 and Eotaxin (each $p < 0.05$ vs. control). Lastly, TAsb group had increased G-CSF, KC, and IL-12p70 at 12 months ($p < 0.05$ vs. control), while both TAsb and Rasb had increased IL-12p40. IL-2 was increased in CF-treated animals at 12 months compared to controls.

Table 4.1. Detailed cytokine concentrations in the mouse lungs at 3- and 12-months post exposure, pg/mg protein. RCF - Riebeckite cleavage fragments, RAsb - Riebeckite Asbestos, TCF - Tremolite cleavage fragments, TAsb - Tremolite Asbestos. Values, showing significant difference ($p < 0.05$) compared to respective controls have a red font color.

3 Months																								
Value type	Treatment	IL-1a	IL-1b	IL-2	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12(p40)	IL-12(p70)	IL-13	IL-17	Eotaxin	G-CSF	GM-CSF	IFN-g	KC	MCP-1	MIP-1a	MIP-1b	RANTES	TNF-a
Mean	Control	1.56	0.542	1.084	0.416	0.282	0.746	0.532	1.9	1.787	29.604	7.126	20.992	0.516	117.645	4.057	1.754	2.26	4.681	19.25	1.7	4.824	93.07	5.679
	RAsb 120 µg	1.102	0.456	1.339	0.925	0.785	0.661	0.697	2.924	2.548	46.218	16.539	20.591	0.655	158.01	13.188	2.639	3.358	7.964	15.616	3.909	5.233	153.962	7.122
	RCF 120 µg	1.281	0.619	1.365	0.555	0.437	0.756	0.949	3.265	2.768	34.035	13.53	25.228	0.775	237.559	6.175	2.333	3.337	9.088	18.224	4.175	8.238	222.045	8.073
	RAsb 30 µg	0.481	0.388	1.429	0.978	0.89	0.667	0.576	2.486	2.459	23.087	15.8	19.895	0.824	131.961	11.777	2.448	3.111	4.508	8.527	1.402	5.22	136.256	6.777
	TAsb 120 µg	0.903	0.481	1.365	1.514	1.338	0.796	0.645	3.141	3.151	41.479	19.545	26.348	0.815	160.537	14.794	2.652	4.008	10.076	14.604	5.51	6.573	133.866	8.279
	TCF 120 µg	0.607	0.38	1.243	1.427	1.243	0.681	0.628	2.764	2.589	26.008	19.712	18.097	0.732	112.183	18.018	2.401	3.108	4.506	9.566	2.321	5.323	141.346	7.786
Standard Error of Mean (SEM)	TAsb 10 µg	0.675	0.425	1.588	1.587	1.15	0.705	0.719	3.047	2.639	35.89	18.917	18.844	0.839	156.674	12.072	3.378	3.319	6.356	9.047	1.756	7.285	163.062	8.545
	Control	0.0791	0.0825	0.111	0.0271	0.0307	0.178	0.0212	0.07	0.285	2.197	1.239	4.057	0.158	12.028	0.432	0.107	0.364	0.538	1.583	0.114	0.125	10.959	0.697
	RAsb 120 µg	0.0779	0.0673	0.228	0.474	0.408	0.0879	0.0301	0.178	0.237	4.791	7.443	2.253	0.0701	9.896	6.458	0.287	0.402	0.0579	1.183	0.0822	0.243	1.774	1.019
	RCF 120 µg	0.178	0.0847	0.177	0.346	0.272	0.0648	0.129	0.279	0.0804	5.355	2.413	2.7	0.0759	47.464	1.884	0.158	0.287	1.426	2.584	0.268	1.008	52.9	0.818
	RAsb 30 µg	0.0583	0.0643	0.298	0.374	0.305	0.0645	0.0847	0.28	0.311	1.213	6.956	2.333	0.117	13.424	5.396	0.129	0.43	0.223	1.661	0.178	0.0389	19.393	0.831
	TAsb 120 µg	0.151	0.0579	0.231	0.285	0.274	0.139	0.057	0.0796	0.153	5.192	2.574	1.813	0.0962	27.882	4.475	0.11	0.356	0.258	2.929	0.389	0.517	7.093	0.312
Standard Error of Mean (SEM)	TCF 120 µg	0.0248	0.047	0.0684	0.183	0.102	0.126	0.0373	0.111	0.0512	3.219	1.951	1.183	0.0722	14.473	2.799	0.0561	0.109	0.368	0.145	0.166	0.176	14.557	0.359
	TAsb 10 µg	0.0519	0.021	0.154	0.323	0.384	0.148	0.0862	0.28	0.417	7.933	4.822	1.604	0.0755	21.733	1.112	0.3	0.316	0.645	0.357	0.249	0.657	23.929	0.192

12 Months																								
Value type	Treatment	IL-1a	IL-1b	IL-2	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12(p40)	IL-12(p70)	IL-13	IL-17	Eotaxin	G-CSF	GM-CSF	IFN-g	KC	MCP-1	MIP-1a	MIP-1b	RANTES	TNF-a
Mean	Control	1.436	0.644	3.284	1.455	1.882	1.559	3.154	7.564	2.702	18.689	11.902	20.41	1.453	189.121	4.896	3.651	3.237	8.957	19.519	3.426	2.426	96.469	14.993
	RAsb 120 µg	2.079	0.906	4.286	0.559	0.799	2.339	3.711	6.867	3.251	31.53	15.12	27.103	2.07	295.301	4.058	3.726	3.781	11.076	25.173	4.723	2.742	91.646	20.203
	RCF 120 µg	1.502	0.923	5.634	1.052	1.419	2.156	2.546	6.85	4.012	21.878	9.808	27.381	1.84	199.085	4.537	3.488	3.814	9.475	19.919	2.665	2.202	83.241	22.447
	RAsb 30 µg	2.31	0.9	3.425	2.636	2.636	1.357	3.576	7.884	3.172	21.205	11.646	24.16	1.183	260.065	6.193	3.361	3.515	12.31	21.125	6.884	3.033	123.857	15.449
	TAsb 120 µg	2.154	0.595	3.861	3.816	4.659	1.599	2.944	11.998	3.311	27.814	22.569	17.009	1.477	279.314	15.435	5.432	4.583	13.684	16.532	6.611	2.089	81.232	17.562
	TCF 120 µg	2.335	0.925	5.176	2.046	2.728	2.144	2.866	10.88	3.266	24.07	17.96	27.682	2.234	314.902	7.423	4.486	4.633	9.075	17.689	5.737	3.917	147.197	25.571
Standard Error of Mean (SEM)	TAsb 10 µg	2.065	0.849	3.345	1.013	1.2	1.502	3.875	5.919	2.604	20.648	7.486	25.106	1.128	265.464	3.474	2.689	2.78	7.87	20.109	6.06	2.455	119.434	13.277
	Control	0.0564	0.0449	0.213	0.325	0.38	0.138	0.159	0.345	0.169	1.871	0.69	1.321	0.17	6.703	0.422	0.291	0.148	0.363	1.393	0.295	0.251	6.712	0.987
	RAsb 120 µg	0.0897	0.0143	0.733	0.0321	0.098	0.129	0.265	0.348	0.0983	0.879	0.0907	1.104	0.128	34.537	0.381	0.127	0.162	0.43	1.238	0.735	0.494	8.639	1.863
	RCF 120 µg	0.131	0.203	0.848	0.162	0.202	0.359	0.212	0.906	0.417	2.969	0.867	5.129	0.371	43.399	0.725	0.589	0.65	1.134	3.169	0.312	0.282	11.852	2.435
	RAsb 30 µg	0.495	0.0735	0.378	0.853	0.819	0.174	0.52	1.279	0.23	1.552	1.665	3.535	0.152	25.577	1.682	0.459	0.462	2.515	2.988	2.035	0.953	24.603	1.567
	TAsb 120 µg	0.174	0.0306	0.118	0.455	1.111	0.0302	0.258	1.991	0.403	0.462	4.388	1.233	0.118	1.077	6.669	1.345	0.8	2.048	1.772	0.672	0.127	2.952	0.318
Standard Error of Mean (SEM)	TCF 120 µg	0.392	0.119	0.889	0.715	0.957	0.289	0.425	1.51	0.248	0.975	3.719	4.796	0.258	66.434	2.186	0.443	0.581	1.328	2.465	0.428	1.181	10.595	4.046
	TAsb 10 µg	0.274	0.261	0.328	0.267	0.307	0.395	0.83	0.206	0.656	2.524	1.638	5.549	0.226	30.327	0.591	0.461	0.631	0.88	3.59	1.051	0.652	32.301	2.146

4.4.2. Lung histopathology

At both time-points microscopic sections in the control group revealed normal histology of conductive and respiratory airways. Exposure of A/J mice to the studied EMPs led to appearance of chronic inflammatory aggregates in parenchymal, peribronchial, perivascular, and subpleural locations (Table 4.2.). All aggregates were composed of various sized mononuclear cells (lymphocytes and histiocytes). No polymorphonuclear cells (neutrophils, basophils, eosinophils) were appreciated. In Control animals these lymphohistiocytic aggregates (LHA) were rare, and either parenchymal or subpleural (see Table 4.2). In Riebeckite and Tremolite groups LHA were present in all cases in different locations. Further evaluation of cellular composition of these aggregates was achieved by immunohistochemical analysis. Some infiltrates in asbestos-treated animals contained ferruginous bodies.

Table 4.2. Lymphohistiocytic aggregates findings in the mouse lungs at 1-year post-exposure time point. CF- cleavage fragments, LD – low dose.

	Total	Neg	Subpleural	Parenchymal	Peribronch	Perivascu
Control	20	17	2	1	0	0
Riebeckite asbestos	15	7	5	1	6	1
Riebeckite CF	14	6	3	1	3	1
Tremolite asbestos	16	4	2	5	3	2
Tremolite CF	14	6	0	4	4	1
Riebeckite asbestos LD	8	6	0	0	2	0
Tremolite asbestos LD	8	3	1	1	3	0

The slides were also analyzed for a presence of fibroproliferative and neoplastic pulmonary lesions, such as adenomatous hyperplasia, adenoma, and adenocarcinoma (Table 4.3.). Adenomatous hyperplasia sites were characterized by epithelialization of alveolar walls by rounded low cuboidal cells with uniform, variably atypical nuclei, scant cytoplasm, and minimal mitotic figures. They were small lesions, ranging 0.1-0.2 mm in size (Figure 4.2. A). The margins were irregular and indiscrete but there was no compression of adjacent parenchyma and no obliteration of alveolar spaces. Adenomas were typically spherical in appearance, ranging 0.3 – 1 mm in size, and exhibited a mild

to moderate loss of normal architecture by the proliferating cells (Figure 4.2, B). Adenoma cells were generally well differentiated but had some pleomorphism. contained a central round to oval nucleus and a moderate amount of eosinophilic stained cytoplasm and distinct cell margins. Compression of adjacent lung parenchyma was present in varying degrees. Adenocarcinomas were ranging in size from 0.5 to 5 mm and demonstrated a complete loss of alveolar structure (Figure 4.2, C and D.). Compression of adjacent lung parenchyma and invasion into adjacent parenchyma and bronchioles occurred frequently. The cells had varying degrees of differentiation and formed papillary, solid, and mixed architectural patterns.

Figure 4.2. **A** – Adenomatous hyperplasia. **B** – Adenoma. **C and D** – Adenocarcinoma. All slides have H&E staining.

Carcinoma cells were pleomorphic, showed nuclear hyperchromasia and had varying degrees of nuclear crowding and mitotic figures. Altogether, in control group (n=20) 19 cases were negative for neoplasia, and one case showed small adenocarcinoma (0.5 mm). In 120 µg RAsb group (n=15) 9 cases were negative for neoplasia, 3 cases showed adenoma, 3 cases showed adenocarcinoma (1 mm, 5 mm, and 5 mm). In 30 µg RAsb group (n=8) one case was positive for adenomatous hyperplasia. In riebeckite CF group (n=15) 12 cases were negative for neoplasia, 1 case showed adenoma, 1 case showed

adenocarcinoma (1 mm). In 120 µg TAsb 1-year post-exposure group (n=16) 7 cases were negative for neoplasia, 7 cases showed adenomatous hyperplasia, 1 case showed adenoma, and 1 case showed adenocarcinoma (5 mm). In 10 µg TAsb (n=8) at 1 year – 5 cases were negative for neoplasia, 2 showed adenomatous hyperplasia, 1 showed adenoma. In tremolite CF-exposed animals at 1-year time-point (n=14) 12 cases were negative for neoplasia, and 2 cases showed adenocarcinoma (3 mm, 2.5 mm). Altogether, the incidence of spontaneous carcinogenesis in our A/J mice by 1 year was low (1 of 20 cases – 5%).

Table 4.3. Neoplastic lesions in the mouse lungs at 1-year post-exposure time point. CF- cleavage fragments. LD - low dose.

	Total	Negative	Adenomatous hyperplasia	Adenoma	Adenocarcinoma
Control	20	19	0	0	1
Riebeckite asbestos	15	9	0	3	3
Riebeckite CF	14	12	0	1	1
Tremolite asbestos	16	7	7	1	1
Tremolite CF	14	11	1	0	2
Riebeckite asbestos LD	8	5	2	1	0
Tremolite asbestos LD	8	7	1	0	0

Hyperplastic sites in asbestos-treated animals were characterized by the presence of mild fibrosis, including as seen on the slides stained with Masson trichrome (Fig.4.3.). Cleavage fragments, both riebeckite and tremolite did not cause detectable fibroproliferation (Fig.4.4.).

Figure 4.3. A –Multifocal adenomatous hyperplasia with mild fibrosis (blue staining). Tremolite asbestos-treated animal. **B** –Focal type II pneumocyte hyperplasia and mild alveolar septal fibrosis (blue staining). Riebeckite asbestos-treated animal. Both slides stained with Masson trichrome.

Figure 4.4. Tremolite CF-treated animal. Arrow points at the singular cleavage fragment with no fibrosis. Masson trichrome staining.

4.4.3. Lung immunohistochemistry

Intense ARG1 staining was revealed in macrophages that phagocytosed or surrounded asbestos fibers, but not cleavage fragments (Figure 4.5.). AMs in tumor-bearing animals also exhibited increased staining. No marked iNOS protein expression was observed in macrophages in any of the slides, except the one containing large adenocarcinoma.

Figure 4.5. Macrophages containing riebeckite and tremolite asbestos fibers were stained positive for ARG1 (A); CF-treated animals did not exhibit much staining (B).

Foci of γ H2AX staining were found in asbestos-treated animals, but not cleavage fragments (Figures 4.6).

Figure 4.6. **A** – Riebeckite asbestos-treated animal. Arrows point at γ H2AX staining. **B** – Tremolite asbestos-treated animal. Arrows point at γ H2AX staining. **C** – Riebeckite CF-treated animal. Arrow points at a singular CF. No γ H2AX staining to be found. **D** – Tremolite CF-treated animal. Arrow points at a singular CF. No γ H2AX staining to be found.

4.4.4. Flow Cytometric analysis of the immune cell subsets

The most prominent change included depletion of alveolar macrophage populations in comparison to other non-lymphocytic cells at both time points (Figure 4.6.A). This relative depletion could be seen across all asbestos groups, but not cleavage fragments. At the same time, number of AMs expressing CD11b on the cell surface increased in treated groups, in asbestos-treated animals only at 3 months, and in all treated groups at 12 months (Figure 4.7.B).

Figure 4.7. A – Alveolar Macrophage population in the mouse lungs, as a % of non-lymphocytic cell, at 3- and 12-months' time points. **B** – Mean Fluorescence Intensity of CD11b surface marker in gated Alveolar Macrophages at 3- and 12-months' time points.

RA – Riebeckite asbestos, RALD – Riebeckite asbestos low dose, RC – Riebeckite cleavage fragments, TA – Tremolite asbestos, TALD – Tremolite asbestos low dose, TC – Tremolite cleavage fragments.

Relative number of eosinophils went up for riebeckite asbestos-treated groups at 3 months and persisted in the 120 μ g RAsb group until 1 year (Figure 4.8). Neutrophils, at the same time were elevated at 3 months in all groups except 10 μ g TAsb, but dropped down by 12 months post exposure, even below control values (Figure 4.9).

Figure 4.8 Eosinophil populations in the mouse lungs, as a % of non-lymphocytic cell, at 3- and 12-months' time points. RA – Riebeckite asbestos, RALD – Riebeckite asbestos low dose, RC – Riebeckite cleavage fragments, TA – Tremolite asbestos, TALD – Tremolite asbestos low dose, TC – Tremolite cleavage fragments.

Figure 4.9. Neutrophils populations in the mouse lungs, as a % of non-lymphocytic cell, at 3- and 12-months' time points. RA – Riebeckite asbestos, RALD – Riebeckite asbestos low dose, RC – Riebeckite cleavage fragments, TA – Tremolite asbestos, TALD – Tremolite asbestos low dose, TC – Tremolite cleavage fragments.

At 3 months we were able to detect significant influx of monocytes in all 120 µg asbestos- and CF-treated groups, with the biggest and statistically significant contribution of regulatory monocytes (CD11b+, Ly6C-) (Figure 4.10).

Figure 4.10. Inflammatory (iMono) and regulatory (rMono) monocyte populations in the mouse lungs, as a % of non-lymphocytic cell, at 3- and 12-months' time points. RA – Riebeckite asbestos, RALD – Riebeckite asbestos low dose, RC – Riebeckite cleavage fragments, TA – Tremolite asbestos, TALD – Tremolite asbestos low dose, TC – Tremolite cleavage fragments.

Figure 4.11. B- and T-Cell populations in the mouse lungs, as a % of lymphocytes in the appropriate gate, at 3- and 12-months' time points. RA – Riebeckite asbestos, RALD – Riebeckite asbestos low dose, RC – Riebeckite cleavage fragments, TA –

Tremolite asbestos, TALD – Tremolite asbestos low dose, TC – Tremolite cleavage fragments.

In the lymphoid cell populations, major finding included relative increase of the T-cells and decrease in B-cells in asbestos-treated groups. This pattern persisted until 1 year, with RC-treated animals also exhibiting similar responses (Figure 4.11).

4.5. Discussion

Verified studies established a structure-activity relationship paradigm for long and thin particles with high aspect ratio. Several other factors, including chemistry, shape, surface characteristics as well as duration, frequency of exposures and individual susceptibility are also implicated in pathogenicity. Substantial evidence exists, that alterations of the immune system functions play important role in the development of asbestos pathology. For instance, lung cancer and mesothelioma following pulmonary exposure to all types of asbestos, are directly associated with immunosuppression [11]. Autoimmunity is also an issue – the impairment of phagocytic function, constant recruitment of neutrophils and cellular damage can lead to an increased secretion of host-derived antigens, that drive the autoantibodies production upon exposure to asbestos [12]. Since carcinogenesis is a lengthy process, it is quite hard to model it in short-living animals, such as most rodents. For instance in asbestos-exposed humans, decades are needed for developing lung cancer or mesothelioma [13, 14]. The pattern of cancer progression in susceptible A/J mice follows specific path: hyperplasia to adenoma to adenocarcinoma [15]. Lung tumorigenesis is under complex genetic control involving the pulmonary adenoma susceptibility 1 (*Pas1*) locus and *Kras* [16]. In humans, *Kras* mutations are common in lung adenocarcinoma [17]. A/J mice were used previously in the studies of tobacco smoke-induced and welding fumes-induced carcinogenesis [18, 19]. More so, in most cases, both human and A/J mouse lung tumors originate from atypical hyperplastic lesions in the periphery of the lung [20]. A/J mice have higher basal cells count in the large airways – thus repair after initial injury is more effective. However, it may also contribute to the non-genotoxic tumor progression mechanisms.

Our preliminary *in vivo* study comparing acute pulmonary effects of several asbestiform EMPs, including tremolite and riebeckite asbestos, revealed differential

innate inflammatory responses. For instance, it was found that tremolite elicited inflammatory responses with polymorphonuclear leukocytes (PMN) influx peaking at day 1 and eosinophil response at day 7 post-exposure. In contrast, exposure to riebeckite asbestos fibers induced a "delayed" inflammatory response with maximal influx of PMNs and eosinophils occurring on day 7 post exposure [21]. Current study focused on the long-term effects of EMP exposure, including 3- and 12-months post-exposure time points.

Neoplastic manifestations were evaluated at 1 year after exposure. A/J mice are prone to developing spontaneous lung tumors, however the deaths caused by tumors were usually seen after 1 year in previous studies [8, 22]. Histopathological examination of lung tumors revealed riebeckite asbestos as having a prominent oncogenic potential, affecting 6 of 15 animals (40%). Proneoplastic potential was also high in tremolite asbestos, affecting 9 of 16 animals (56%), although majority of the lesions were of lower grade compared to riebeckite. Riebeckite and Tremolite cleavage fragments did not have significantly increased number of neoplastic lesions. Only one mouse, exposed to tremolite asbestos developed mesothelial reaction. In asbestos-treated animals we could still clearly see EMP-laden macrophages 1 year post-exposure. Areas of EMP deposition were characterized by cell hyperplasia, mild alveolar septal fibrosis and immune cell infiltration, however, no extensive lung fibrosis was observed. This is probably a characteristic of A/J mice, which have been shown to be resistant to bleomycin-induced lung fibrosis as well [23, 24]. Several incidents of squamous metaplasia were observed, which is one of the outcomes triggered by EMPs that is hypothesized to give rise to lung cancer [25]. Cleavage fragments provoked very little histological changes and were mostly found inside the alveoli with occasional AM next to it.

Immunohistochemistry allowed locating and assessing some of the changes, including specific immune cell subsets, such as ARG1 and iNOS-positive macrophages, B-lymphocytes, CD4, CD8 T-cells and T_{regs}.. Acute asbestos exposure was previously shown to upregulate iNOS [26], but we did not observe increase in iNOS (NOX2) macrophage staining via IHC in any group, which, together with no increase in other accompanying proinflammatory markers, such as TNF-alpha probably means that by 1 year no active inflammation is present. Myeloid Arginase 1 was shown to be responsible

for arginine depletion related immune suppression in cancer, and we have seen increased staining in macrophages containing asbestos EMPs as well as in macrophage of animals with lung tumors. Number of T-cells, positive for FOXP3 marker was evidently higher in the asbestos-treated groups, often inside the lymphohistiocytic infiltrates and near the visible asbestos fibers.

There is an indication that asbestos-initiated chronic oxidative stress contributes to carcinogenesis and fibrosis by promoting oxidative DNA damage and regulating redox signaling pathways [27-31]. We used anti γ H2AX antibody to assess the potential for increased DNA double strand breaks occurrence in the exposed animals and Foci of γ H2AX staining were found in the vicinity of asbestos EMPs, in AMs, epithelial and lymphoid cells, while cleavage fragments did not induce significant DNA double strand breaks *in vivo* as shown by pH2AX staining. It has to be noted that cells do not have to be phagocytosing or be in contact with asbestos fibers capable of promoting the redox reactions. DNA damage can occur through several mechanisms other than direct reactive surface-biomolecule interaction, i.e. through the oxidized lipid derivatives, arachidonic acid pathways or self-produced ROS and reactive nitrogen species [32].

By performing complete cytokine panel assessments in the lungs of the exposed animals, we were able to infer some specific immune response polarization patterns. Increased ARG1 staining in association with elevated IL-3, IL-4, and IL-5 cytokines levels characterize the immune response to asbestos particles as T_H2 -type. One of the interesting observations included CF-treated, but not asbestos-treated animals showing the highest IL-2 levels and relative increase in T-lymphocyte populations at 12 months compared to control. IL-9 was elevated in all but one treatment groups. There are speculations that IL-9 has antifibrotic action and may shift the response away from T_H2 [33]. However, more recent studies argue that IL-9 is probably directly implicated in the pathogenesis of fibrosis and T_H2 -type inflammation [34, 35]. FOXP3⁺ T_{reg} cells could be recruited to control inflammatory and effector T-cells-induced fibrotic responses. Persistent accumulation of immunosuppressive T_{reg} cells in the lungs may contribute to pulmonary fibrosis by stimulating fibroblasts through the secretion of platelet-derived growth factor (PDGF)-B via a TGF- β autocrine signaling pathway [36]. At the same time,

T_{reg} accumulation is what leads to the inflammation resolve. The balanced response thus is critical for positive outcome. Different asbestos fibers with different morphology and chemistry have been shown to activate NLRP-3 inflammasome through different mechanisms [37-40]. This inflammasome participates in initial inflammatory response and the chronic inflammatory conditions arising in fiber exposure settings, including driving of the IL-17 responses [41], implicated in amphibole asbestos pathogenicity [42] and specifically in the associated reduction in antitumor immunity [43]. We have not observed increased IL-1 β production in treated animals, but IL-17 was significantly higher in riebeckite asbestos-treated animals at 12 months.

To supplement the IHC findings and cytokine data, imaging flow cytometry was used to analyze single cell suspensions from enzymatically digested mouse lungs. Imaging FC is a relatively new method, which requires slightly different approach to sample preparation, data acquisition, fluorescence compensations, compared to the non-imaging flow cytometers, and requires special software for data analysis. However, it allows to clearly distinguish between different gated events, and observe the actual fibers present, on their own or in the cells. In our study asbestos EMPs caused more drastic changes in the pulmonary immune cell populations in mice at the same mass doses compared to cleavage fragments. There was relative depletion of resident AMs, expressing high Siglec-F surface marker, and negative for CD11b molecule, observed in all asbestos-treated groups at 3 months, and persisting at 1-year time point. In all treated groups MFI of CD11b on AM surface was higher than control, due to the incoming monocytes giving origin to new AMs (CD11b^{High}, Siglec-F^{Low}), and upregulation of CD11b by the resident AMs, with two distinct populations distinguishable on scatter plots at 12 months. AM death through necrosis/necrosis/apoptosis is implicated hugely in asbestosis progression [44]. Accordingly, changes in the population of AMs, which have a long turnover rate in mice [45] may lead to recruitment of phagocytes that actively participate in clearance mechanisms, proinflammatory and pro/antifibrotic signaling, contributing to the polarization of immune response. The CD11b marker was shown to characterize different stages of pulmonary inflammation [46, 47], and is proposed as a target for therapeutical interventions [48, 49]. However, in humans CD11b expression on AMs is not dichotomic, but follows rather a continuous distribution. This complicates the process

of CD11b MFI evaluation and comparisons in humans, but it is possible to reliably compare CD11b marker expression pattern using a reference neutrophils expression values [48].

Although it has previously been noticed that in humans asbestos exposure increases the number of macrophages in the lungs [50]. this relative increase, however, may turn to depletion with time if resolution of inflammation is not achieved. AM depletion can enhance antigen-presenting function of resident phagocytes (parenchymal dendritic cells) and their migration, leading to re-activation of the T-cells [51]. Relative increase in T-cells seen in almost all groups should be regarded with caution as well, since they are the major players in immune response polarization, contributing to fibrosis, autoimmunity and/or resolution [52]. There was no statistically significant increase in PMN content detected by FC. Histopathology also did not reveal significant PMN presence. However, even if the overall number of PMNs did not change much, Kamp et al. (1994) have shown that PMNs are facilitating epithelial cell apoptosis, promoting the lung injury through, while AMs have protective role [53], thus changes in relative abundances may also have long-term implications. Eosinophils count was higher in riebeckite asbestos-treated animals, something previously observed in animal models and humans exposed to asbestos [54, 55]. By 12 months there was also increased eotaxin production in the mouse lungs. Eosinophils are known to facilitate lymphocytes-driven T_H2 -type response. Altogether, we could see that in all treated groups resident AM population was supplemented with incoming CD11b positive cells. Apparently, this was more “successful” in CF-treated animals, but not in the asbestos-treated and the depletion of resident AMs only exacerbated the pathologic process, accompanied by the influx of eosinophils, increase of T_H2 type cytokines and T_{reg} cells. While changes were more severe at 3 months, by 12 months the whole alternative activation process was only partially alleviated, with the response shifted towards T_H2 -type. This comes to no surprise and correspond to the historical data.

4.6. Conclusions

Exposure to asbestiform and non-asbestiform EMPs led to discrete pulmonary immune responses at 3- and 12-months post-exposure, leading to specific long-term

fibroproliferative and carcinogenic outcomes. Of all tested articles, riebeckite and tremolite asbestos exposures were indicative of a chronic inflammation and possible T_H2- or T_H17-type polarization. Asbestos induced a distinct immune response pattern, and a much stronger response than cleavage fragments at mass- and surface area-equivalent doses, including the evidence for continuing DNA damage as elucidated by γ -H2AX foci findings. The studied cleavage fragments were not as potent as asbestos in driving the fibroproliferation and cancer, most likely due to the lack of resident AM depletion. The results of this study call for further investigations of the immune cell population dynamics and contributions to the expression development of long-term carcinogenic and non-carcinogenic outcomes following exposures to various EMPs. Such approaches can aid in providing mechanistic insights into the human exposure scenario and mechanisms involved in potential human disease following inhalation exposures to EMP.

4.7. References

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Chapter 5. Conclusions and Future Directions

5.1 - Conclusions

Despite decades of research, both epidemiological and toxicological, debates regarding the potential adverse health effects of elongate mineral particles are still going on. While asbestos ban in most of the countries, and overall trend in exposure reduction instill hope, still existing and newly found cases of occupational and incidental exposures to EMPs raise new concerns. Unsolved terminological discrepancies between material and natural scientists, regulators and research agencies add to the confusion and complicate the matter further, while constant litigation battles mean that a lot of biased stakeholders are present. Asbestiform EMPs are undeniably responsible for fibrosis and cancer development in exposed humans [1]. Much less is known about non-asbestiform EMPs, such as amphibole cleavage fragments, especially if they conform to the “critical” dimensions (i.e. longer than 5 μm). Some evidence for pulmonary fibrosis and extrapulmonary pathology exists, while no significant risk of mesothelioma or lung cancer is shown by current epidemiological evidence for several types of cleavage fragments (tremolite, anthophyllite, talc) [2]. It is still not feasible to name one particle characteristic the most important determinant. The importance of length was undisputed for a long time, but plenty of studies support the notion that asbestiform EMPs of all lengths can induce pathology in humans [3-5]. Particle surface area and chemical composition are also implicated in the disparity of toxicological responses[1, 6-8].

This study aimed to provide an insight into one of the knowledge gaps currently existing in the field of fiber toxicology – specifics of the immunological component of pulmonary response to inhaled EMPs of asbestiform and non-asbestiform morphologies. The principal findings from this work confirm that alveolar macrophages play a major role in responding to EMPs, and, following exposure, pulmonary immune landscape undergoes changes, that are dependent on the nature of particles in question. Both crystal growth habit and mineral composition (or type) are implicated in the outcomes.

I would like to first describe this study, highlighting strengths and limitations in the process. It was of paramount importance to obtain the test articles suitable for adequately

addressing the knowledge gap. We chose riebeckite and tremolite amphiboles because of their similar dimensional characteristics and at the same time different chemical composition; riebeckite containing significant portion of Fe molecules in the crystal lattice. Bulk EMP samples used for the studies were all procured from the reliable sources and are relevant for the real-life exposure scenarios. The harvesting procedure, that incorporated intermittent PCM measurements during all the important steps, allowed gathering the required amounts of EMPs with the lengths of more than 5 μm (Chapter 2). EMP samples were then characterized using TEM and EDS and dimensional parameters calculated to assist in doses determination for toxicological studies. The cleavage fragments harvesting/characterization approach, while being unique and providing significant benefits to toxicologists, still does not guarantee that obtained EMP populations contain more than 60% of “critical” fibers. [9]. Serpentine variety of asbestos – chrysotile – has not been evaluated in this study. Overall, artificial approach to the sample preparation is not exactly representative of the typical human exposures, but it is necessary for adequate toxicological comparisons.

Alveolar macrophages are one of the most important mediators of all immune responses in the lungs. Therefore, a significant part of the dissertation (Chapters 3 and 4) was dedicated to study macrophage-related changes. In our *in vitro* model (alveolar macrophage-like Max Plank Institute cells), equal mass-based doses resulted in markedly differential responses between asbestos and cleavage fragments, however, at equal fiber surface area-based doses asbestos and corresponding CF had similarities in cytotoxic effects. When we looked into the changes in gene expression, distinct transcriptional reprogramming patterns and DNA damage was observed, specific not only for the habit, but also for the mineral type, leading us to conclude that asbestiform habit itself may be an important determinant for some minerals, like tremolite. Of course, studies *in vitro*, do not account for the modifying effects of deposition patterns and clearance by mucociliary actions as well as contribution from biopersistence. “...what happens at the lung level is based on a population of fibers getting to the deep part of the lung and not based on one EMP interacting with one lung cell” [10].

In our animal model we wanted to evaluate the myeloid lineage cellular composition and humoral component of the immune response in the mouse lungs upon exposure to the tested EMPs, in relation to the histopathological changes. All EMPs were still present in the mouse lungs after 1 year, even cleavage fragments. Asbestiform EMPs caused hyperplasia, mild alveolar septal fibrosis, inflammatory infiltrates, but no extensive lung fibrosis, which is probably a characteristic of A/J mouse model. We had several reasonings for looking at the diverse immune cell population dynamic in the lungs: for instance, recruited mononuclear phagocytes actively participate in clearance mechanisms, proinflammatory and pro/antifibrotic signaling, contributing to the polarization of immune response. Asbestiform EMPs still caused more cytotoxicity in the *in vitro* model, and more drastic changes in the pulmonary immune cell populations in mice at the same mass doses compared to cleavage fragments. Consistent changes included alveolar macrophage depletion and eosinophilia. Increased apoptosis was observed in the asbestos-treated cells. There was clear depletion of resident AMs (expressing high Siglec-F, and negative for CD11b) observed in all treated groups at 3 months and persisting until 12 months in asbestos-treated animals. In all groups incoming monocytes (most likely) gave origin to new AMs (CD11b⁺, Siglec F^{low}). In cleavage fragments group AMs were “successfully” substituted by incoming CD11b positive cells and supplemented the population of resident AMs. This never happened in the asbestos-treated groups, only partially alleviated by 12 months it was, and accompanied by the influx of eosinophils, increase of T_H2 type cytokines, and T_{reg} cells. Asbestos is a complete carcinogen, combining initiating and promoting properties [11]. In this study we wanted to assess the oncogenic potential of asbestiform and non-asbestiform EMPs in a lung-cancer prone mouse model. Proneoplastic properties of asbestos were evident from the histopathology observations, while non-asbestiform cleavage fragments did not lead to significant increase in neoplastic lesions, when compared at equal mass doses. While the long-term neoplastic outcomes were still evidenced in the non-asbestiform group, most likely their carcinogenic potential in real exposure scenario is negligible. Based on the combined *in vitro* and *in vivo* results, one of the plausible explanations for rather benign effects of cleavage fragments exposure could be the preservation of resident “homeostatic” pool of AMs in the lungs.

If approached from another metrics standpoint, such as surface area, we start seeing more similarities between asbestos and its non-asbestiform analogues in the *in vitro* settings. In animal model, however, due to its complexity and numerous confounding factors, no such conclusions can be drawn from our study. Still, it seems that the chemical composition, unique for different minerals, also plays major role in the outcomes. In our mouse model we demonstrated that even one year after exposure non-asbestiform EMPs are still present in the mouse lungs. It supplements the notion that biodurability is another common parameter, that needs to be accounted for both habits. In *in vivo* settings, shorter EMPs can be cleared more easily, but it does not mean they are less potent in causing damage in the sustained exposure scenario. Importantly, responses to asbestiform tremolite and asbestiform riebeckite were discreet both in the cell model and animal model, confirming yet again that commercial term “asbestos” should probably be avoided in EMP toxicity research except for the narrative reasons. It is only possible to incorporate a finite amount of research in the dissertation; thus, I have included the future directions section to address several questions existing before and arising in the course of the study.

5.2 - Future Directions and Questions

Future toxicity studies can follow two paths: one is to focus on a comprehensive assessment of the EMP-induced changes in the pulmonary (and extrapulmonary) immune contexture following exposure to a panel of premade well-characterized reference materials; second is to investigate in more detail individual components of the general immune response using only a couple of pre-designed test articles at a time, in relevant *in vitro* and *in vivo* models. Experimental methods that do not involve animals include using established immortalized cell cultures, 3d organoids, organs-on-chip, and precision-cut lung tissues sections from donor lungs unsuitable for transplantation. For instance, a comprehensive assessment of the EMP-induced changes in the cultures of the major lung-derived cell lines can be performed, with an emphasis on immunological responses, such as production of cytokines and M1/M2 polarization, oxidative stress, genotoxic outcomes, modes/mediators of cell death. While we as a biomedical research community try to minimize the use of animals, sometimes it is the only way to

obtained reliable and relevant toxicological data. Specific endpoints for *in vivo* studies may include inflammation, changes in the immune cell compositions, functional activity of macrophages, immune activation/suppression, and autoantibodies titers in the blood. Induced carcinogenesis model (Lewis lung carcinoma cells) can be used to assess pre-metastatic niches in exposed animals. Animal research involving transgenic animals relevant for EMP-related pathology may focus on the KO/KD models, such as OGG1 -/- [12, 13], HMOX1 -/- [14], and others. Studies that include inhibition of EMP recognition apparatus by macrophages (i.e. using SR-A inhibitor – fucoidan, or MARCO inhibitor – polyguanylic acid).

Question 1: What could be the new prospective treatment approaches for asbestos-induced lung pathology?

There are several implications for treatment strategies in human asbestos exposures coming from the research on EMP immunotoxicity. Knowledge of macrophage polarization patterns allows for targeting or antagonizing a subset, known to have a role in pathology. For example, identification and selective blockade of inflammatory CD11b^{pos} macrophages in the airways have been proposed for the treatment of acute lung exacerbations [15] therefore, one might also consider related. Treatment that deals with inflammasome pathways might be another viable strategy [16]. Inflammasome components and IL-1 β have the potential to be biomarkers in malignancies, including lung cancer and mesothelioma [17]. There are also reports of successful use of IL-1R agonist for slowing down the progression of silicosis [18], making a possibility to use it for the treatment of asbestosis. Th17 targeting is another therapeutic opportunity in asbestos-related pathology [19, 20]. Targeting CD11b positive cells have been considered as one of the approaches to deal with chronic inflammation, including following asbestos exposures [15, 21]. Recently, a new approach has been suggested, aimed at modulating the CD11b expression, rather than depletion of CD11b positive cells [22]. Whether it can be successfully used for asbestos-related pathology can be evaluated in the successive studies. A good example of another approach are studies on MARCO receptor-based therapeutics against pulmonary fibrosis [23, 24].

Question 2: How can we improve the particle characterization/harvesting approaches?

Manual measurements of fiber lengths and widths is labor-intensive; thus, a faster and accurate measurement method is necessary to determine particle size distribution of the separated fibers. Cho et al. showed the capability of an automatic counting of asbestos fibers (High-Throughput Microscopy), although the study focused on fiber counting rather reporting fiber length or diameter information [25]. In addition, differential interference contrast microscopy might able to reduce fiber counting and detection time by increasing the contrast [26]. Cossio et al. reported an unattended scanning electron microscope (SEM)-energy dispersive spectroscopy (EDS) asbestos analysis method and it showed high precision and reproducibility, which can reduce analytical time (maximum 2 hours/sample)[27].

Question 3: What methods can be used to track the EMP fate in the lungs?

One of the novel approaches is the investigation of the mechanisms of EMP bioprocessing in the mouse lungs utilizing the high-resolution analytical scanning transmission electron microscopy (HRTEM/STEM), electron energy loss spectroscopy (EELS) and energy dispersive spectroscopy (EDS). This method has not been previously implemented in the largescale toxicological studies, particularly not for the non-asbestiform EMPs. Paraffin-embedded lung tissue sections can be analyzed to investigate the mechanisms of EMP bioprocessing and its role in the overall toxicity, fibrogenicity and carcinogenicity as described in an article [28]. In our study the use of imaging flow cytometer allowed capturing some of the macrophages with phagocytized EMPs, as well as free fibers during the run (Figures 5.1. and 5.2.).

Figure 5.1. Images of the alveolar macrophages from exposed mice 1 year after exposure with phagocytized riebeckite asbestos fibers, obtained by imaging flow cytometry.

Figure 5.2. Images of the alveolar macrophage with tremolite cleavage fragment inside and free floating EMPs from exposed mouse 1 year after exposure, obtained by imaging flow cytometry.

Further experimentation with lung digestion procedure, cell sorting and EMP labeling techniques can yield a method for reliably quantifying the number and size distribution of the EMPs still present in the mouse lungs at different time points as well as the cell-fiber interaction peculiarities.

Question 5: How can bioinformatic approaches aid in deciphering EMP toxicity profiles?

Something that is currently missing in the field of EMP toxicology is an integrated bioinformatics approach. This approach, if implemented on a large scale, could help establishing correlations between the EMP characteristics and their immunological impact relevant to the progression into fibrosis and/or cancer. For example, utilizing RNA sequencing and epigenomic assessment of isolated (sorted) lung cells can give us a comprehensive picture of the 2-layered (transcriptome and epigenome) molecular responses. Add in global proteomics analysis using mass spectrometry, and one gets a plethora of data to mine for immunologically relevant parameters. Verification and validation of identified biomarkers in gene/protein expression/abundance profiles of patients with lung illnesses or in pulmonary human cells exposed to asbestos and other mineral fibers (such as found in Gene Expression Omnibus Datasets – <http://www.ncbi.nlm.nih.gov/geo/>) can be used to select the biomarkers with highest predictive potency.

In the end, comparison and validation of the short- and long-term assessments using major lung-derived cell lines and *in vivo* animal models will permit developing the

predictive models of EMP-induced immunotoxicity and immunity contribution to the disease progression, as well as tools that could facilitate prognostic risk assessments in humans. This together with the identified unique profiles of genes/proteins can aid in identification of disease-causing potential of EMPs based on their size, shape, growth habit and chemistry.

5.3. References

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