Transcriptomic Responses to Crystalline Silica Exposure

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Table of contents

1. Introduction

1.1. Exposure to crystalline silica: Silica, because of its abundance in earth's crust, presents numerous opportunities for human exposure. Between the two forms (amorphous and crystalline) of silica[1] present, crystalline silica assumes a major importance with respect to potential harmful human health effects. Even though incidental exposure to crystalline silica, mainly through dust, takes place routinely, occupational exposures are of major concern from a health effects stand point. Any occupation that involves the movement of earth's surface, especially mining, construction, silica milling, stone cutting, and sandblasting is considered as a major source for exposure to crystalline silica. Although small quantities of crystalline silica may enter human body orally, the major route for occupational exposure to crystalline silica is through inhalation. It has been estimated that approximately 2 million workers in the USA and millions more worldwide are occupationally exposed to crystalline silica annually (Sanderson, 2006). In several cases, occupational exposure to crystalline silica takes place at levels much higher than the National Institute for Occupational Safety and Health (NIOSH) Recommended Exposure Level (REL) of 0.05 mg/m^3 (Linch *et al.*, 1998).

1.2. Health effects of crystalline silica exposure: A large number of *in vitro* cell culture (Ding *et al.*, 1999; Gwinn *et al.*, 2009) and *in vivo* animal (Porter *et al.*, 2002) (Porter *et al.*, 2001; Porter *et al.*, 2004) studies conducted in the past have demonstrated the toxicity potential of crystalline silica. It has been reported that occupational exposure to crystalline silica is associated with the development of autoimmune diseases, rheumatoid arthritis, chronic renal diseases, and lupus (NIOSH, 2002). Based on overwhelming evidence, the International Agency for Research on Cancer (IARC) has classified crystalline silica as a type I human carcinogen (IARC, 1997; Steenland and Sanderson, 2001). However,

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[1] Based on the bonding geometry of SiO_2, five different crystalline silica structures have been characterized – quartz, cristobalite, coesite, tridymite, and stishovite (Wycoff, 1963). In this chapter, the term silica refers crystalline silica in general.

irreversible but preventable interstitial lung disease characterized by alveolar proteinosis and diffuse fibrosis resulting in progressively restrictive lung function and death (Castranova and Vallyathan, 2000). Epidemiologic studies have demonstrated that workers have a significant risk of developing chronic silicosis when they are exposed to respirable crystalline silica over a working lifetime at the current Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL), the Mine Safety and Health Administration (MSHA) PEL, or the NIOSH recommended exposure limit (REL) (Hnizdo and Sluis-Cremer, 1993; Steenland and Brown, 1995; Kreiss and Zhen, 1996). The number of cases of silicosis and silicosis related diseases in the USA is currently unknown. However, approximately 200 deaths were attributed to silicosis in 2005 in the USA (NIOSH, 2006). Currently, silicosis is diagnosed by chest x-ray and pulmonary function tests. Both of these tests, unfortunately, detect silicosis based on structural and/or functional impairment of lungs most likely representing an advanced stage of the disease. As such, the current diagnostic tools may not be helpful to prevent death associated with this incurable disease. On the other hand, the preventable nature of silicosis strongly supports the argument to develop tests sensitive enough to predict onset of the disease at an early preventable stage. In fact NIOSH has recommended developing highly sensitive and non-invasive or minimally invasive test to detect silicosis at an early, preventable stage (NIOSH, 2002).

1.3. Transcriptome – a sensitive and mechanistically relevant target for toxicity: Transcriptome refers to the entire set of transcripts or mRNAs present in a cell or an organism. Transcriptomics, also referred to as expression profiling, is the determination of the expression levels of all of the transcripts or mRNAs present in a cell or an organism at a given time by employing techniques such as DNA microarray, next generation RNA sequencing, subtractive hybridization, differential display, serial analysis of gene expression etc. Transcriptome, unlike genome, is highly dynamic in nature and often responds sensitively to exposure of a cell or an organism to a toxic agent. Therefore, expression profiling of the entire transcriptome in a cell or an organism following its exposure to a toxic agent

under controlled experimental conditions may be considered as a sensitive indicator of toxicity resulting from exposure to the toxic agent. The differentially expressed genes and/or their products, following appropriate validation, may be employed as biomarkers for exposure and toxicity of the agent being investigated. Similarly, diligent bioinformatics analysis of the significantly differentially expressed transcripts detected in a cell or an organism in response to its exposure to a toxic agent has been shown to provide valuable insight into the mechanism(s) underlying the toxicity of the agent. Therefore, global gene expression profiling, as supported by the results of several studies (Waring *et al.*, 2001; Hamadeh *et al.*, 2002; Amin *et al.*, 2004; Heinloth *et al.*, 2004), is often considered as a sensitive and mechanistically relevant approach to detect and understand toxicity associated with exposure to toxic agents.

2. Transcriptomics responses to crystalline silica exposure: Crystalline silica particles, following their entry into the lungs, are phagocytosed by the alveolar macrophages (AMs) for elimination. In the absence of efficient elimination from the lungs, such as under conditions of excessive crystalline silica exposure, interaction with the inhaled silica particles activates the AMs resulting in AM death and release of the silica particles and various signaling molecules within the lungs. The crystalline silica particles as well as the signaling molecules released into the lungs may interact with the alveolar epithelium to initiate a cascade of pulmonary and extra-pulmonary events. The net result is the recruitment of inflammatory cells into the lungs, release of toxic reactive oxygen and nitrogen species, the induction of inflammation, DNA damage, and fibrosis leading to the development of diseases, for example, silicosis and cancer. Normal functioning of cells/tissues/organs is regulated by the expression of a large number of genes that are organized as specific biological functions, pathways, and networks. The crystalline silica particles as well as the various signaling molecules released from activated AMs may cause alterations in the expression of one or several genes to result in the functional disruption of the corresponding biological functions, pathways and networks that are vital for normal cell/tissue/organ

function. In addition, the transcriptome may be affected as a secondary effect of the pulmonary toxicity resulting from the inhaled crystalline silica particles. Therefore, global gene expression profiling of biological samples that are exposed to crystalline silica and functional analysis of the genes differentially expressed in response to the exposure may provide valuable information with respect to the toxicity potential of silica as well as the underlying molecular mechanisms of crystalline silica-induced pulmonary and extra-pulmonary toxicity.

2.1. Transcriptomics changes in response to crystalline silica exposure correspond to silica-induced toxicity: A relationship between crystalline silica-induced toxicity and global gene expression changes taking place in biological samples has been investigated using cell culture and animal models. It has been fairly well established that crystalline silica, compared to amorphous silica, is more biologically active and pathogenic (Warheit *et al.*, 1995; Johnston *et al.*, 2000; Fubini *et al.*, 2001). Whether global gene expression changes would account for the reported differences in the biological activity and pathogenicity reported between amorphous and crystalline silica was investigated in human bronchial epithelial cell line (BEAS2B) and primary human bronchial epithelial cells (NHBE) (Perkins *et al.*, 2012). The effect of crystalline silica on the transcriptome, compared with amorphous silica, was more profound in both of the cell types. More number of genes was significantly differentially expressed in both the cell types exposed to crystalline silica compared with amorphous silica. Furthermore, the fold changes in gene expressions were significantly higher in the cells exposed to crystalline silica compared with those exposed to amorphous silica. Therefore, the effect of silica particles (amorphous and crystalline) on the transcriptome appeared to be related to their biological activities and pathogenic potentials. In a study reported by Sellamuthu *et al.* (2011b), human lung epithelial cells, A549, were treated with Min-U-Sil-5 crystalline silica at final concentrations of 15, 30, 60, 120, and 240 $\mu g/cm^2$ cultured area for 6-hours or 60 $\mu g/cm^2$ cultured area for time intervals of 2-, 6-, and 24-hours. At the end of the silica exposure period, cytotoxicity was determined by assaying lactate dehydrogenase (LDH)

activity in the cell culture medium. Simultaneously, total RNA was isolated from the control and silica treated cell culture samples to determine global gene expression profiles using Human HT-12_v3_Beadchip Arrays (Illumina, Inc, San Diego, CA). The crystalline silica-induced cytotoxicity and the number of significantly differentially expressed genes (SDEGs)[2] in the cells correlated very well (Figure 1 A-D) and exhibited correlation coefficients (r^2 values) of 0.89 and 0.98, respectively, for the silica concentration-response and time-course study. Bioinformatics analysis of the SDEGs in the A549 cells identified the biological functions that were significantly enriched in response to crystalline silica-induced cytotoxicity. As presented in Figure 2A and 2B, the biological functions significantly enriched in response to silica exposure in the A549 cells were highly relevant to the already established mechanisms of crystalline silica-induced pulmonary toxicity. It is worth mentioning that the enrichment of the biological functions, represented by the number of SDEGs belonging to each of the biological function categories, in the silica exposed A549 cells exhibited a similar quantitative response to the concentration and duration of crystalline silica exposure as was seen in the case of the crystalline silica-induced cytotoxicity. A similar positive relationship has been reported between cytotoxicity and the number of SDEGs detected in human bronchial epithelial cells, BEAS 2B, treated with increasing concentrations of crystalline silica (Perkins *et al.*, 2012).

Further evidence to support the existence of a relationship between crystalline silica-induced pulmonary toxicity and global gene expression changes is obtained from the results of animal experiments. In a study conducted in our laboratory, rats were exposed to Min-U-Sil-5 crystalline silica by inhalation (15 mg/m^3, 6 hours/day, 5 days). Pulmonary damage and global gene expression profiles were determined in the lungs at post-silica exposure time intervals of 0, 1, 2, 4, 8, 16, and 32 weeks (Sellamuthu *et al.,* 2011a; 2011b; 2012a; 2012b). Determination of pulmonary damage was performed

[2] Genes whose expressions are significantly different between control and toxicant exposed samples are considered as SDEGs. The criteria commonly employed to select significantly differentially expressed genes include a fold change in expression, p value [either simple or False Discovery Rate (FDR) p value] or a combination of the two.

on the basis of LDH activity and concentrations of albumin, total protein and the pro-inflammatory cytokine, macrophage chemoattractant protein 1 (MCP1), in the bronchoalveolar lavage fluid (BALF). Global gene expression profiles were determined in the lungs by employing RatRef-12V1.0 Expression BeadChip Arrays (Illumina, Inc, San Diego, CA). A steady progression of pulmonary toxicity was seen during the post-silica exposure time intervals in the crystalline silica exposed rats on the basis of histological changes in the lungs compared to the corresponding time-matched controls (Figure 3). A very strong positive correlation was seen between the progression of silica-induced pulmonary toxicity and the number of SDEGs detected in the lungs of the rats (Figure 4 and Table 1). Functional analysis of the SDEGs in the lungs of the silica exposed rats identified the biological functions that were significantly enriched in response to crystalline silica-induced pulmonary toxicity. The biological functions that were significantly enriched in the lungs of the silica exposed rats were mechanistically relevant to the already known pulmonary effects of crystalline silica exposure (Figure 5). In addition, the number of SDEGs present in each of the significantly enriched biological functions steadily increased similar to the progression of pulmonary toxicity seen in the rats during the post-silica exposure time intervals. The strong correlation between crystalline silica-induced toxicity and the number of SDEGs as well as the enrichment of biological functions seen in the cell culture as well as animal tissue samples suggested the potential application of transcriptomics as a relevant approach to detect and study crystalline silica-induced toxicity.

2.2. Crystalline silica and cancer: One of the human disease research areas that has benefited immensely by the advancements in genomics, especially transcriptomics, is cancer research. Cancer, in general, is considered to be the outcome of an imbalance in cell division regulated by the abnormal gene expression patterns. In general, oncogenes promote and tumor suppressor genes suppress cell growth and division. Loss of control of cell division, either by the activation of oncogenes and/or the inactivation of tumor suppressor genes may facilitate uncontrolled cell division resulting in cancer.

There is overwhelming evidence in the literature, which include those obtained from *in vitro* cell culture studies, *in vivo* animal studies, and epidemiologic studies, supporting the carcinogenic potential of crystalline silica. Initial evidence of neoplastic transformation induced by crystalline silica was obtained in cultures of Syrian hamster embryo cells (Hesterberg and Barrett, 1984). Subsequently, Saffiotti and Ahmed (Saffiotti and Ahmed, 1995) demonstrated that crystalline silica is capable to morphologically transform the mouse embryo cell line BALB/3T3/A31-1-1. However, the report that crystalline silica is not carcinogenic in hamsters and mice (Saffiotti, 2005) questions the relevance of the cell transformation data obtained in the cell lines developed in these species with respect to the carcinogenic potential of crystalline silica. In a study conducted by Williams et al. (1996) (Williams *et al.*, 1996) crystalline silica was able to transform fetal rat lung epithelial cells (FRLE), retaining markers of alveolar type II pneumocytes. Similarly, Saffiotti (1998) (Saffiotti, 1998) demonstrated crystalline silica induced cell transformation in AE6 cell lines developed from rat primary cultures of alveolar type II pneumocytes. In both these studies, involving rat lung cell lines, the transformed cells were tumorigenic in immune-deficient mouse confirming the carcinogenic potential of crystalline silica. Intratracheal administration of crystalline silica resulted in adenocarcinomas, epidermoid carcinomas, undifferenated carcinomas, mixed carcinomas, and adenomas in the lungs of F344 rats (Saffiotti, 1996), further confirming the carcinogenic potential of crystalline silica. There is also substantial epidemiologic evidence for human carcinogenesis by crystalline silica (IARC, 1997).

In spite of the identification of crystalline silica as an animal and human carcinogen (IARC, 1997) and the unequivocal role played by the transcriptome in cancer (Stadler and Come, 2009; Abba *et al.*, 2010), the involvement of gene expression changes, if any, in crystalline silica-induced cancer is not well understood. Therefore, expression profiling of the whole genome in cells transformed with crystalline silica or tumor samples developed in response to crystalline silica exposure in animals and/or humans may provide valuable insight into the mechanisms underlying cancer resulting from exposure to

crystalline silica. Similarly, the expression levels of these genes, following appropriate validation, may be employed as biomarkers for detecting crystalline silica-induced cancer. To date neither transcriptomics studies using either cells transformed with crystalline silica or tumor samples developed in animals and/or humans in response to their exposure to crystalline silica have been reported.

Nevertheless, the involvement of a large number of genes has been implicated in crystalline silica-induced cancer based on the associations seen between differentially expressed genes and crystalline silica exposure and/or toxicity in cell culture and animal tissue samples. Enhanced cell proliferation is characteristic of a carcinogenic response; and, therefore, overexpression of genes regulating the cellular process of proliferation has been considered for their involvement in cancer. Activator protein 1(AP1) is a transcription factor consisting of homo or heterodimers of the fos and jun genes and a definite role for AP1, through transcriptional activation of its downstream target genes, in cellular proliferation has been established (Ryseck *et al.*, 1988). The significant overexpression of AP1 and its downstream target genes has been reported in cells and lung samples in response to their exposures to crystalline silica (Ding *et al.*, 1999; Sellamuthu *et al.*, 2011a; Perkins *et al.*, 2012). The Kruppel-like factors (KLF) are a group of transcription factors whose involvement in cellular proliferation and cancer has been recognized for some time (Ghaleb and Yang, 2008; Mori *et al.*, 2009; Nakamura *et al.*, 2009) Several KLF genes were significantly overexpressed in crystalline silica exposed A549 human lung epithelial cells (Table 2). Expression of the tumor suppressor gene, p53, was significantly lower in lungs of mice exposed to crystalline silica (Ishihara *et al.*, 2002). However, in spite of the major role played by p53 in cancer in general, the report that crystalline silica is not carcinogenic in mouse (Saffiotti, 2005) questions the role of p53 in silica-induced carcinogenesis. The involvement of oxidative stress, inflammation, DNA damage, and fibrosis in carcinogenesis has been well established. As presented in Tables 2, 3 and 6 and described in the corresponding sections elsewhere in this chapter, several genes involved in oxidative stress, DNA damage, inflammation, and

fibrosis were significantly differentially expressed in cells and animal tissues in response to crystalline silica exposure and/or toxicity. Additional transcriptomics studies, as mentioned above, by employing crystalline silica transformed cells and/or tumors developed in animals and/or in humans in response to their exposure to crystalline silica are required to determine precisely the involvement of transcriptional changes of specific genes in crystalline silica induced cancer. Furthermore, whole transcriptome studies may potentially explain the species differences seen among rats, mice, and hamsters with respect to their responses to crystalline silica-induced lung cancer.

2.3. Crystalline silica and oxidative stress: The finding that silicotic lungs are in a state of oxidative stress (Vallyathan and Shi, 1997) has led to the belief that oxidant-mediated lung damage may play a role in the development of toxicity and diseases associated with crystalline silica exposure. The observations that freshly fractured crystalline silica is more toxic to cells (Ding *et al.*, 1999; Gwinn *et al.*, 2009) and animals (Vallyathan *et al.*, 1995) compared to aged crystalline silica further support for the involvement of oxidative stress, through the generation of reactive oxygen species (ROS), in crystalline silica toxicity. Generation of toxic ROS taking place in biological samples in response to their exposure to crystalline silica may be the direct effect of silica particles (Vallyathan *et al.*, 1995) and/or it may be mediated by silica particles indirectly through cellular processes (Vallyathan *et al.*, 1992). It is known that crystalline silica-induced oxidative stress rises even after termination of silica exposure and lungs have cleared most of the deposited silica (Fubini and Hubbard, 2003; Rimal *et al.*, 2005).

Transcriptome analysis has provided support for the generation of ROS in biological samples in response to crystalline silica exposure. A significant and crystalline silica concentration-dependent overexpression of several oxidative stress responsive genes belonging to the nuclear factor kappa B (NFκB) and AP-1 family has been observed in the A549 cells (Table 2). Superoxide anion, a reactive oxygen species generated in response to crystalline silica exposure, undergoes dismutation catalyzed by

superoxide dismutase to generate hydrogen peroxide (H_2O_2) (Liochev and Fridovich, 2007). H_2O_2 is also generated during the metabolism of spermine catalyzed by spermine oxidase (Murray-Stewart *et al.*, 2008). The toxic and reactive H_2O_2 generated is detoxified, predominantly by catalase, in order to prevent its interaction with intracellular target(s) potentially leading to toxicity. The net result of the significant and crystalline silica concentration-dependent overexpression of SOD and SMOX with simultaneous decrease in the expression of catalase, as noticed in the crystalline silica exposed A549 cells (Table 2), is the cellular accumulation of toxic and reactive H_2O_2 contributing to crystalline silica-induced oxidative stress and toxicity. A significant overexpression of the SOD gene has also been reported in human bronchial epithelial cell line (BEAS2B) in response to their exposure to crystalline silica (Perkins *et al.*, 2012).

In laboratory animals, inhaled crystalline silica particles interact with AMs and alveolar epithelium to result in the generation of ROS (Porter *et al.*, 2002). Several genes involved in the generation of ROS as well as in the cellular response to oxidative stress, viz: SOD2, HMOX1, MT1A, NCF1, LCN2, ARG1, LPO, and NOXO1, were significantly overexpressed in the lungs of the crystalline silica-exposed rats suggesting the involvement of oxidative stress in the induction of pulmonary toxicity (Table 3). The NOXO1 gene codes a protein that is an activator of the superoxide-generating gene NOX1 (Banfi *et al.*, 2003), and its significant overexpression in the crystalline silica exposed rat lungs may, therefore, imply the generation of superoxide anion capable of resulting in oxidative stress. As seen in the A549 cells exposed to crystalline silica, the significant overexpression of SOD2 with no corresponding increase in the expression of H_2O_2 detoxifying genes, catalase and glutathione peroxidase/reductase (Gaetani *et al.*, 1994), seen in the rat lungs should favor the excessive generation and tissue accumulation of reactive and toxic H_2O_2, contributing to oxidative stress and pulmonary toxicity in the crystalline silica exposed rat lungs (Sellamuthu *et al.*, 2012b). This argument

is further supported by the significant overexpression LPO, an H_2O_2-responsive gene (Davies *et al.*, 2008), seen in the crystalline silica-exposed rat lungs (Langley *et al.*, 2011; Sellamuthu *et al.*, 2012b).

2.4. Crystalline silica and apoptosis: Crystalline silica particles, following their entry into the respiratory system, are engulfed by AMs for their clearance from the system and detoxification. The interaction between crystalline silica particles and AM may result in a cascade of cellular events resulting in the activation of AMs culminating in their death and the release of silica particles into the lungs. Apoptosis plays a major role in crystalline silica-induced death of the activated AMs and neutrophils and in turn may contribute significantly to the pulmonary effects of crystalline silica particles. The observation that apoptosis-deficient $FasL^{-/-}$ *gld* mice did not develop silicosis (Borges *et al.*, 2002) suggests the crucial role played by apoptosis in the adverse health effects, especially silicosis, associated with exposure to crystalline silica.

Induction of apoptosis, in response to crystalline silica exposure, has been demonstrated in *in vitro* cell culture (Hamilton *et al.*, 2000) and *in vivo* animal (Srivastava *et al.*, 2002) models. The involvement of reactive oxygen and nitrogen species (Srivastava *et al.*, 2002; Santarelli *et al.*, 2004) as well as scavenger receptors (Hamilton *et al.*, 2000; Fubini and Hubbard, 2003) in silica-induced apoptosis has been demonstrated. Srivastava *et al.* (2002) have demonstrated the involvement of interleukin 1beta (IL1β) and nitric oxide (NO) in crystalline silica-induced apoptosis. Crystalline silica-induced apoptosis in the IC-21 macrophage cell line was inhibited by anti-IL1β antibody and the NO synthase (NOS) inhibitor, N(G)-nitro-L-arginine-methyl ester, suggesting the release of IL1β-mediated NO in silica-induced apoptosis. These findings obtained from cell culture experiments were further supported by the results of animal studies (Srivastava *et al.*, 2002). Exposure of IL1β and inducible NOS (iNOS) knockout mice to crystalline silica resulted in significantly reduced apoptosis, inflammation and silicotic lesions compared to the wild type mice. These results, in addition to demonstrating an

association between apoptosis and inflammation, supported a potential role for IL1β-dependent NO-mediated apoptosis in silicosis.

An important role for apoptosis in crystalline silica-induced autoimmune diseases has been suggested based on the results of a study conducted in the autoimmune-prone New Zealand Mixed (NZM) mouse (Brown *et al.*, 2005). Significant DNA fragmentation noticed in the bone marrow-derived macrophages of the crystalline silica administered NZM mice suggested apoptosis induction. Increased levels of anti-histone autoantibodies, high proteinuria, and glomerulonephritis seen in the NZM mice suggested the autoimmune effects of crystalline silica. Rottlerin, a protein kinase C (PKC) delta inhibitor blocked crystalline silica-induced apoptosis and autoimmune effects in the mice. These results, in addition to demonstrating the involvement of PKC in crystalline silica-induced apoptosis, suggested the involvement of apoptosis in the development of autoimmune diseases in response to crystalline silica exposure.

The studies conducted in our laboratory (Sellamuthu *et al.*, 2011a; Sellamuthu *et al.*, 2012a; Sellamuthu *et al.*, 2012b), by employing A549 cells and rats, suggested the involvement of several genes in crystalline silica-induced apoptosis (Tables 2 and 3). In addition, bioinformatics analysis of the SDEGs in the crystalline silica exposed A549 cells and rat lungs provided insights into the mechanisms potentially underlying silica-induced apoptosis. DNA damage plays an important role in apoptosis. The ability of silica to interact with DNA and, therefore, to result in DNA damage has been previously demonstrated (Mao *et al.*, 1994)). Even though it has been fairly well established that crystalline silica exposure results in apoptosis indirectly through the generation of ROS (Santarelli *et al.*, 2004), the apoptotic implication of direct DNA damage induced by crystalline silica, if any, is not well understood. GADD genes are a family of genes that are found significantly overexpressed in cells undergoing apoptosis (Hollander *et al.*, 1997). GADD proteins interact with a diverse array of proteins to facilitate apoptosis. For example, *GADD45α*, a *p53*-regulated gene, interacts with other *p53*-regulated genes that

play an important role in apoptosis (Sarkar *et al.*, 2002). The significant and crystalline silica concentration-dependent overexpression of *GADD34* and *GADD 45α* – two important members of the *GADD* family of genes, seen in the crystalline silica exposed A549 cells (Table 2) suggests their involvement by yet to be identified mechanism(s) in crystalline silica induced apoptosis. EGR1 is a transcription factor that is characterized by rapid and transient up-regulation of expression in response to stress (Yu *et al.*, 2007). Expression of the EGR1 mRNA was significantly up-regulated in response to crystalline silica exposure and toxicity in A549 cells (Table 2). EGR1, similar to the GADD family of genes, plays a prominent role in apoptosis through its interaction with the p53 family of genes (Yu *et al.*, 2007).

2.5. Crystalline silica and DNA damage: Direct interaction between crystalline silica and DNA resulting in structural changes in the DNA phosphate backbone has been demonstrated (Mao *et al.*, 1994). Similarly, the ability of crystalline silica to result in DNA strand break has been reported (Shi *et al.*, 1994). Several lines of evidence suggest a major role played by ROS in the DNA damage caused by crystalline silica. Chemical etching of freshly fractured silica particles with hydrofluoric acid resulted in diminished DNA damage mainly because of the removal of metal ion impurities and reactive centers created by fracturing (Daniel *et al.*, 1993). The observations that crystalline silica-induced DNA damage is significantly lower in the absence of molecular oxygen and that catalase and scavengers of ROS are capable of blocking crystalline silica-induced DNA damage further support the involvement of ROS in crystalline silica-induced DNA damage. The DNA damage resulting from exposure to crystalline silica may contribute to apoptosis and carcinogenesis, attributed to play significant roles in the pulmonary toxicity induced by crystalline silica.

Results of transcriptomics analysis further support the DNA damage resulting from exposure of cells to crystalline silica. In a study conducted by Gwinn *et al.* (2009), human bronchial epithelial cells (BEAS-2B) and lung cancer cells (H460 and H1299) were exposed to crystalline silica and expression

levels of several genes involved in DNA damage signaling pathways were determined. The genes whose expression levels were significantly up-regulated in response to crystalline silica exposure in the cells included ataxia telangiectasia mutated (ATM), sestrin 1(SESN1), mouse double minute 2 (MDM2), cell division cycle 25 homolog A (CDC25), B-cell lymphoma 6 (BCL6), BCL-2 associated X gene (BAX), proliferating cell nuclear antigen (PCNA), GADD45, excision repair cross-complementing 3 (ERCC3), and RAD23A. Similarly, Sellamuthu *et al.* (2011b) detected significant overexpression of GADD45α and GADD34 in A549 cells in response to silica exposure (Table 2).

2.6. Crystalline silica and pulmonary fibrosis: Pulmonary fibrosis is a major component of silicosis (Ng and Chan, 1991), a serious health outcome of exposure to respirable crystalline silica. Crystalline silica-induced fibrosis includes the release of fibrogenic factors from alveolar macrophages, proliferation of the fibroblasts and increased production of collagen by pulmonary fibroblasts. Positive collagen staining of lung tissues with Masson's trichrome stain and increased levels of hydroxyproline, a component of collagen, in the lungs are often considered markers for pulmonary fibrosis. Inhalation exposure of rats to crystalline silica for one week (15 mg/m^3, 6 hours/day, 5 days) resulted in pulmonary fibrosis that was detectable histologically by Masson's trichrome stain at the 32-week post-silica exposure time interval (Sellamuthu *et al.*, 2012a). A further progression of pulmonary fibrosis was seen at the 44-week post-silica exposure time interval in the same rat model (Sellamuthu *et al.,* 2013). Crystalline silica-induced pulmonary fibrosis has also been reported by other investigators (Porter *et al.*, 2001).

Global gene expression profiling and bioinformatics analysis of the gene expression data identified significant differential expression of several genes involved in tissue remodeling and fibrosis in the lungs of the crystalline silica exposed rats as compared to the time-matched control rats (Table 3). Many of the fibrosis-related genes whose expression levels were significantly different following inhalation exposure to crystalline silica in our rat model (Table 3) were also significantly differentially

expressed in the lungs of acute and chronic rat silicosis models developed by Langley *et al.* (2011) as well as in human fibrotic diseases (Nau *et al.*, 1997; Pardo *et al.*, 2005). Matrix metlloproteinases (MMPs) are a family of proteins that participate in many homeostatic biological processes as well as in pathological processes including fibrotic lung diseases (Nagase and Woessner, 1999). MMPs have been implicated in airway remodeling and granuloma formation because of their involvement in extracellular matrix degradation (Scabilloni *et al.*, 2005). Of the *MMPs* that were significantly overexpressed in the crystalline silica exposed rat lungs, *MMP12* overexpression was most significant (Table 3). A definite role for *MMP12* in the induction of pulmonary fibrosis has been demonstrated previously in mice carrying a targeted deletion of the *MMP12* gene (Matute-Bello *et al.*, 2007). Osteopontin, one of the key components of extracellular matrix, mediates the migration, adhesion, and proliferation of fibroblasts culminating in pulmonary fibrosis (Takahashi *et al.*, 2001). The profibrotic gene *SPP1,* which codes osteopontin protein, was significantly overexpressed in the crystalline silica exposed rat lungs, especially at the late post-silica exposure time intervals (Table 3). A definite role for SPP1 in fibrosis is suggested based on decreased expression levels of collagen type 1in $SPP1^{-/-}$ mice (Berman *et al.*, 2004). The *ARG1* gene which was significantly and progressively overexpressed in the silica exposed lung samples (Table 3) has been found associated with bleomycin-induced pulmonary fibrosis in mice (Endo *et al.*, 2003). The significant overexpression of the profibrotic chemokines *CCl2* (Mercer *et al.*, 2009) and *CCl7* (Moore and Hogaboam, 2008) observed in the crystalline silica-exposed rat lungs (Table 3; Langely *et al.,* 2011) may indicate their involvement in silica-induced pulmonary fibrosis in the rats. This view is further supported by the significant overexpression of these chemokines in tuberculosis, a human fibrotic disease (Nau *et al.*, 1997). The involvement of RETNLA in the induction of pulmonary fibrosis by promoting the differentiation of myoblasts that mediate collagen deposition has been suggested (Liu *et al.*, 2004). The *RETNLA* gene was highly overexpressed in the silica exposed rat lungs (Table 3). Since a definite relationship is known to exist between unresolved pulmonary inflammation

and fibrosis (Reynolds, 2005), it is reasonable to assume that the significant overexpression of the pro-inflammatory genes presented in Table 3 is facilitating the unresolved pulmonary inflammation observed in the rat lungs contributing to crystalline silica-induced pulmonary fibrosis. The magnitude of overexpression for all these genes that are known to be involved in tissue remodeling and fibrosis steadily increased in parallel with the progression of crystalline silica-induced pulmonary toxicity in the rats suggesting their role in crystalline silica-induced pulmonary fibrosis and toxicity.

An interesting finding of the gene expression data, with respect to crystalline silica-induced pulmonary fibrosis, was the superior sensitivity of the changes in the expression levels of the marker genes of fibrosis compared with a conventional approach, Masson's trichrome staining of lung tissue. The findings by Porter *et al.* (2001) have suggested a slightly superior sensitivity of Masson's trichrome staining as an indicator for crystalline silica-induced pulmonary fibrosis compared with a biochemical estimation of hydroxyproline in the rat lung samples. In our rat model, the earliest indication of crystalline silica induced pulmonary fibrosis, as detectable by positive trichrome staining of lung tissues, was detected at the 32-week post-silica exposure time interval (Sellamuthu *et al.,* 2012a). However, as presented in Table 3, significant overexpression of several genes involved in tissue remodeling and fibrosis were detectable in the lungs of crystalline silica exposed rats as early as one week following termination of crystalline silica exposure. Overexpression of these genes steadily increased during the post-silica exposure time intervals. The post-silica exposure time interval with the highest overexpression of the fibrosis-related genes was detected matched with the onset of fibrosis, as revealed by the results of Masson's trichrome staining of the lung tissues in the rats (Sellamuthu *et al.,* 2012a).

2.7. Crystalline silica and inflammation: Crystalline silica exposure results in the induction of inflammation (Chen *et al.*, 1999; Fubini and Hubbard, 2003; Porter *et al.*, 2004), and a central role for inflammation in the pulmonary effects associated with silica exposure has been established (Castranova, 2004). Pulmonary inflammation has been suggested as a major factor contributing to silicosis, a

devastating health effect associated with exposure to respirable crystalline silica. Significant increases in AMs and polymorphonuclear leukocytes (PMNs) and pro-inflammatory chemokines, MCP1 and macrophage inflammatory protein-2 (MIP2), in the lung samples of rats following their inhalation exposure to crystalline silica are strong indicators of silica induced pulmonary inflammation (Sellamuthu *et al.,* 2011a; Sellamuthu *et al.,* 2012a). Crystalline silica particles, following their entry into the lungs, are taken up by AMs, primarily for their elimination from the lungs. In this process, the silica particles may activate AMs resulting in their death and release of the silica particles along with various signaling molecules. The crystalline silica particles as well as the released signaling molecules, in turn, interact with additional AMs and alveolar epithelial cells, further resulting in AM activation and secretion of additional signaling molecules. Many of the signaling molecules thus released in response to the crystalline silica-mediated activation of AMs and epithelial cells are inflammatory in nature and may result in the recruitment of additional inflammatory cells (i.e. neutrophils, macrophages, lymphocytes, etc) into the lungs resulting in pulmonary inflammation. The significant increase in the number of neutrophils seen in the blood of crystalline silica exposed rats may suggest the release of the inflammatory mediators generated in the lungs into systemic circulation and the induction of systemic inflammation (Sellamuthu *et al.,* 2011a; Sellamuthu *et al.,* 2012a). The crystalline silica-induced pulmonary inflammation in the rats, similar to the trend exhibited by the various parameters of crystalline silica-induced pulmonary toxicity, exhibited a steady progression during the post-exposure time intervals analyzed (Sellamuthu *et al.,* 2011a; Sellamuthu *et al.,* 2012a). These findings strongly support the previous suggestion that inflammation plays a central role in crystalline silica toxicity and the associated adverse health effects.

Microarray analysis of global gene expression profiles in the lungs of the crystalline silica exposed rats supported the induction and progression of pulmonary inflammation and toxicity seen in the crystalline silica exposed rats. Inflammatory response, inflammatory diseases, and cellular

movement were three of the top ranking IPA biological functions identified as being significantly enriched by crystalline silica exposure in the rat lungs (Figure 5; Sellamuthu *et al.,* 2012b). In addition, multiple canonical pathways and molecular networks involved in the induction of inflammation were significantly and progressively enriched during the silica post-exposure time intervals (Figure 5 and 6). Interestingly, the number of inflammation related biological functions, pathways, and networks that were significantly affected by crystalline silica exposure in the lungs also steadily increased (Figure 5) along with the progression of crystalline silica-induced pulmonary toxicity in the rats (Figure 3) suggesting a possible relationship between crystalline silica-induced differential expression of genes involved in inflammation and the toxicity progression seen in the rat lungs.

Bioinformatics analysis of the SDEGs provided insights into the molecular mechanisms underlying the progression of crystalline silica-induced pulmonary inflammation and toxicity in the rats. Several inflammatory response genes that encode inflammatory cytokines/chemokines were significantly overexpressed in crystalline silica exposed rat lungs, and the magnitude of their overexpression steadily increased during the post-exposure time intervals (Table 3) along with the progression of pulmonary toxicity induced by crystalline silica in the rats (Figure 3). Many of these pro-inflammatory cytokines/chemokines function as chemoattractants and recruit inflammatory cells, especially PMNs, into the lungs (Olson and Ley, 2002) in response to pulmonary damage. This may, therefore, account, at least in part, for the significant increase in the number of PMNs detected in the lungs resulting in the induction and progression of pulmonary inflammation and toxicity in the crystalline silica exposed rats (Sellamuthu *et al.,* 2011a; Sellamuthu *et al.,* 2012a). In addition to the genes encoding inflammatory cytokines/chemokines, significant overexpression of other genes known to play prominent roles in the induction of inflammation such as *S100A8* (Ryckman *et al.*, 2003), *RETNLA* (Holcomb *et al.*, 2000), *TREM1* and *TREM2* (Ford and McVicar, 2009), *LCN2* (Zhang *et al.*, 2008), *CHI3Ll* (Eurich *et al.*, 2009), *SPP1* (Sabo-Attwood *et al.*, 2011), and several members of the

complement system (Li *et al.*, 2007) and acute phase response (Whicher *et al.*, 1999) were found in the crystalline silica exposed rat lungs (Table 3). It is noteworthy that overexpression of these inflammatory response genes steadily increased along with the progression of crystalline silica-induced pulmonary inflammation and toxicity in the rats during the post-exposure time intervals analyzed, further supporting their involvement in the progression of pulmonary inflammation and toxicity.

Lipoxins play an important role in the resolution of pulmonary inflammation (Chan and Moore, 2010). Lipoxins are the products of arachidonic acid metabolism catalyzed by 15-lipoxygenase (Alox15) (Kronke *et al.*, 2009). An anti-inflammatory role has been attributed to lipoxins mainly because of their ability to inhibit chemotaxis, adhere and transmigrate neutrophils, and antagonize the pro-inflammatory effects of leukotriens (Colgan *et al.*, 1993; Scalia *et al.*, 1997; Godson and Brady, 2000). *Alox-15* expression was significantly lower in the lungs of the silica exposed rats compared with the time-matched controls (Table 3). Therefore, it is reasonable to assume that, in addition to the significant overexpression of the multiple pro-inflammatory genes, the significant down-regulation of *Alox-15* gene expression may have contributed to the unresolved pulmonary inflammation in the crystalline silica exposed rats. Measurement of lipoxins in the crystalline silica exposed rats, would be beneficial to further establish the role of lipoxins in the resolution of crystalline silica-induced pulmonary inflammation.

The role of aryl hydrocarbon receptor (AhR) in the pulmonary effects of crystalline silica, especially in inflammation and fibrosis, was investigated recently (Beamer *et al.*, 2012). AhR plays a major role in the immune system and is best known as the receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Activation of AhR by TCDD results in a multitude of toxic endpoints, including, profound immunosuppression (Marshall and Kerkvliet, 2010). $AhR^{-/-}$ mice were hypersensitive to crystalline silica-induced inflammation and had increased levels of inflammatory cytokines and chemokines in their BALF. Furthermore, macrophages derived from $AhR^{-/-}$ mice secreted enhanced

amounts of cytokines and chemokines in response to crystalline silica exposure compared to the wild-type C57Bl/6 mice. Analysis of gene expression in macrophages revealed that $AhR^{-/-}$ mice exhibited increased levels of pro-IL-1β, IL-6, and BcL-2 and decreased levels of STAT2, STAT5A and serpin B2 (Pai-2) in response to crystalline silica exposure. Based on their findings, Beamer and colleagues (2012) have concluded that AhR functions as a negative regulator of crystalline silica-induced inflammation. The authors, furthermore, reported that AhR was not involved in the fibrogenic response induced by crystalline silica in mice.

Additional support for the important role proposed for inflammation in the pulmonary effects associated with crystalline silica exposure is provided by the results of global gene expression profiling and bioinformatics analysis conducted in *in vitro* cell culture experiments. Bioinformatics analysis of the gene expression profile of the crystalline silica exposed A549 cells, in addition to supporting the involvement of inflammation in the pulmonary effects of crystalline silica, provided insights into the molecular mechanisms underlying crystalline silica-induced inflammation. Inflammation response ranked very high among the functional categories of genes whose expression levels were significantly affected by crystalline silica exposure in the A549 cells (Sellamuthu *et al.,* 2011b). Transcripts for a number of pro-inflammatory interleukins, in agreement with the results of previous studies (Rao *et al.*, 2004; Herseth *et al.*, 2008), and several members of the *CXCL* family of pro-inflammatory cytokines were significantly overexpressed in the crystalline silica-exposed A549 cells; and their overexpression levels, as in the case of crystalline silica-induced cytotoxicity, were dependent on the concentration and duration of crystalline silica exposure (Sellamuthu *et al.,* 2011b). A definite role for the *CXCL* family of pro-inflammatory cytokines as mediators of crystalline silica induced pulmonary inflammation has been demonstrated in rats (Borm and Driscoll, 1996) and mice (Chao *et al.*, 2001). In summary, the results of the transcriptomics studies conducted in *in vitro* cell culture and *in vivo* animal models of crystalline silica toxicity, in addition to supporting the prominent role played by inflammation in crystalline silica-

induced pulmonary toxicity, have provided molecular insights into the mechanisms underlying the initiation and progression of silica-induced pulmonary inflammation and toxicity.

2.8. Novel mechanisms of crystalline silica-induced pulmonary toxicity: A unique feature of global gene expression profiling is its potential to screen all the cellular targets and processes for their potential involvement in response of a cell or an organism to exposure to a toxic agent. Therefore, whole genome expression profiling may facilitate the identification of novel target(s) and mechanism(s) of toxicity that may not be achieved by conventional toxicity studies. The involvement of the novel gene target(s) and/or mechanism(s) in the toxicity of the agent being investigated can be further studied and confirmed by employing cell culture and/or animal models that are transgenic for the novel target gene(s) identified.

The transcriptomic studies conducted in the recent past by employing RNA isolated from cells and animal tissues that are exposed to crystalline silica, in addition to confirming many of the previously identified targets and mechanisms of crystalline silica toxicity, have facilitated the identification of several novel toxicity targets and mechanisms potentially involved in crystalline silica toxicity. As presented in Table 4, significant overexpression of several members of the solute carrier (SLC) family of genes was found in the lungs of the crystalline silica exposed rats at all post-exposure time intervals. In parallel with the progression of pulmonary toxicity noticed in the crystalline silica exposed rats (Figure 3), all of the SLC genes listed in Table 4 exhibited a steady increase in their overexpression levels in the lungs of the silica exposed rats compared with the corresponding time-matched controls suggesting their potential involvement in the initiation as well as the progression of crystalline silica-induced pulmonary toxicity.

The SLC gene that was most significantly overexpressed in the lungs of the crystalline silica exposed rats was *SLC26A4,* and several lines of evidence suggest a potential role for this gene in

crystalline silica-induced pulmonary toxicity. The *SLC26A4* gene codes the protein pendrin, which is responsible for excessive mucus production by airway epithelial cells (Nakao *et al.*, 2008). A relationship is known to exist between excessive mucus production by airway epithelial cells and morbidity and mortality from certain respiratory diseases (Rogers, 2004; Rose and Voynow, 2006). The steady increase in the overexpression of the *SLC26A4* gene noticed in the crystalline silica exposed rats may also account, at least in part, for the progression of pulmonary inflammation noticed in them. It has been reported previously that forced overexpression of the *SLC26A4* gene, by yet to be identified mechanism(s), resulted in the activation of the CXCl1 and CXCl2 chemoattractants and facilitated the infiltration of neutrophils into lungs resulting in the induction of pulmonary inflammation (Nakao *et al.*, 2008). In this regard, it is important to note that both the *CXCl1* and *CXCl2* genes were significantly and progressively overexpressed and a significant increase in the number of infiltrating PMNs and induction of inflammation occurred in rats in response to their exposure to crystalline silica (Sellamuthu *et al.*, 2011b; Sellamuthu *et al.*, 2012a; Sellamuthu *et al.*, 2012b). Collectively, the findings of our study and those reported previously (Nakao *et al.*, 2008) may suggest the involvement of the *SLC26A4* gene in crystalline silica-induced pulmonary inflammation and toxicity in the rats. Similar to our finding, a significant overexpression of the SLC26A4 transcript has been reported in acute and chronic rat silicosis models (Langley *et al.*, 2011). It is encouraging to know that a transgenic mouse model for the SLC26A4 gene is available (Lu *et al.*, 2011), and future investigations by employing this mouse model may facilitate the understanding of and confirmation of the role of this gene in crystalline silica-induced pulmonary toxicity.

3. Prediction of silica exposure/toxicity

3.1. Blood transcriptomics and crystalline silica-induced pulmonary toxicity: It has been well recognized that silicosis, a significant pulmonary health effect associated with crystalline silica exposure in humans, is an incurable, but preventable, fatal disease. The incurable but preventable nature of

silicosis makes it absolutely necessary to detect silicosis pre-clinically, i.e. prior to the appearance of clinical symptoms that represent the irreversible stage of the disease. Currently, silicosis is detected by chest x-ray and pulmonary function tests. Both techniques detect silicosis based on structural and/or functional impairment of lungs that are associated with an advanced and, therefore, irreversible stage of the disease. In consideration of the health risks, especially silicosis, and the large number of workers who are occupationally exposed to potentially toxic levels of crystalline silica in the USA and elsewhere, NIOSH has recommended developing highly sensitive and practical (non-invasive or minimally invasive) techniques capable of detecting silicosis pre-clinically (NIOSH, 2002). Transcriptomic studies conducted in the past several years have demonstrated the earlier appearance of gene expression changes in target organs as markers of toxicity compared to traditional histological and biochemical toxicity markers, suggesting their superior sensitivity as markers of target organ toxicity (Heinloth *et al.*, 2004; Luhe *et al.*, 2005; McBurney *et al.*, 2009; McBurney *et al.*, 2012). It is not ethical or practically possible to obtain lung samples to determine either exposure to crystalline silica or a person's probability of developing silicosis. A major limitation in employing lung gene expression profiling as a practical approach in monitoring workers routinely for their occupational exposure to crystalline silica and the potential health effects is the inability to routinely collect lung tissue samples from workers potentially exposed to crystalline silica. Surrogate biospecimens that can be obtained by non-invasive or minimally invasive techniques are essential for routine monitoring of occupational exposure to toxic agents and the resulting health effects. Since small quantities of blood required for gene expression profiling can be safely obtained by a minimally invasive approach from humans, blood may be a suitable surrogate biospecimen to predict/detect silicosis pre-clinically provided gene expression changes taking place in the blood, in response to crystalline silica exposure/toxicity, reflect crystalline silica's effects in lungs, the primary target organ.

Recently a research project was undertaken in our laboratory in compliance with the NIOSH recommendation to develop highly sensitive and practical biomarkers to predict/detect silicosis pre-clinically. It has been reported previously that rats, similar to humans, develop silicosis following exposure to crystalline silica (Porter *et al.*, 2001). For this reason, a rat silicosis model was employed to investigate the potential application of blood gene expression profiling as a highly sensitive and practical surrogate approach to detect and/or to predict crystalline silica exposure and the resulting pulmonary toxicity. Details regarding the study design can be found in our recent publications (Sellamuthu *et al.*, 2011a; Sellamuthu *et al.*, 2011b; Sellamuthu *et al.*, 2012a; Sellamuthu *et al.*, 2012b). Stated briefly, approximately 3-months old, healthy, male Fischer 344 rats (CDF strain) were exposed to filtered air (control) or an aerosol consisting of respirable crystalline silica particles (15 mg/m^3, 6 hours/day for 5 days). Following exposure, groups of control and silica exposed rats were sacrificed at post-exposure time intervals of 0, 1, 2, 4, 8, 16, 32 and 44 weeks. BALF, including the cells within it, lungs, and blood were collected from the rats to determine the effects of pulmonary exposure to crystalline silica as well as to determine global gene expression profiles in the blood. Specifically, we investigated (1) whether global gene expression changes in the blood reflected crystalline silica-induced pulmonary toxicity, (2) whether bioinformatics analysis of the differentially expressed genes in the blood of the crystalline silica exposed rats could provide insights into the mechanisms underlying the pulmonary toxicity induced by crystalline silica exposure, and (3) whether exposure to a sub-toxic concentration of crystalline silica could be detected or predicted using a blood gene expression signature.

3.2. Transcriptomics changes in the blood reflected crystalline silica-induced pulmonary toxicity in rats: As presented in our original publications (Sellamuthu *et al.*, 2011b; Sellamuthu *et al.*, 2012a; Sellamuthu *et al.*, 2012b), inhalation exposure of rats to crystalline silica resulted in pulmonary toxicity as evidenced from increased LDH activity and albumin and protein contents in their BALF compared with corresponding time-matched control rats. Lung histological changes such as the accumulation of

inflammatory cells, appearance of type II pneumocyte hyperplasia, and fibrosis, further supported the silica induced pulmonary toxicity in the rats (Figure 3). Induction of pulmonary inflammation in the silica exposed rats compared with the control rats was evidenced from an increase in the number of neutrophils and the level of MCP1 protein in their BALF. The crystalline silica-induced pulmonary toxicity in the crystalline silica exposed rats steadily progressed during the post-exposure time intervals as evidenced from the various pulmonary toxicity parameters determined in the rats. Pulmonary fibrosis, characteristic of silicosis, was detectable in the rats at late post-exposure time intervals of 32 and 44 weeks (Sellamuthu *et al.*, 2012a; Sellamuthu, 2013).

Global gene expression profiles in the blood samples collected from the control and crystalline silica exposed rats were determined using RatRef-12 V1.0 Expression BeadChip Arrays (Illumina, Inc., San Diego, CA) as described in detail in our original publications (Sellamuthu *et al.*, 2011b; Sellamuthu *et al.*, 2012a). The number of SDEGs in the blood samples of the crystalline silica exposed rats, compared with the corresponding time-matched control rat samples, was determined at each post-silica exposure time interval. As presented in Table 5 the number of SDEGs in the surrogate tissue, blood, correlated well with markers of pulmonary toxicity (BALF LDH activity) and inflammation (BALF PMN count and MCP1 level). The strong correlation seen between the pulmonary toxicity and inflammation parameters and the number of SDEGs in the blood of the silica exposed rats suggested that the blood gene expression changes, in fact, were indicative of the silica-induced pulmonary toxicity. Furthermore, these results, in agreement with several previous publications (Bushel *et al.*, 2007; Lobenhofer *et al.*, 2008; Huang *et al.*, 2010; Umbright *et al.*, 2010), confirmed the potential value of global gene expression changes in the surrogate biospecimen, blood, as an indicator of target organ toxicity. However, slightly better correlation noticed between pulmonary toxicity markers and the number of SDEGs in the blood (Table 5), compared to that of the lungs (Table 1), suggested that the gene expression changes taking place in blood, the surrogate tissue, may be better indicators of target

organ toxicity than the gene expression changes taking place in the target organ itself. A similar superior sensitivity of blood gene expression changes, compared with liver gene expression changes, as markers of hepatotoxicity has been reported previously by Lobenhofer *et al.* (2008).

3.3. Bioinformatics analysis of blood transcriptome provided molecular insights into the mechanisms of crystalline silica-induced pulmonary toxicity: Bioinformatics analysis of the SDEGs in the blood of crystalline silica exposed rats (Sellamuthu *et al.*, 2011b; Sellamuthu *et al.*, 2012a), in addition to supporting the previously recognized toxicity and health effects of crystalline silica, provided insights into the molecular mechanisms underlying crystalline silica-induced pulmonary toxicity. A remarkable similarity was noticed with respect to the IPA biological functions, canonical pathways, and molecular networks that were significantly enriched in the lungs (target organ) and blood (surrogate tissue) (Figure 6A and 6B). The top 8 IPA biological functions that were significantly enriched in the target organ, lungs, were also significantly enriched in the surrogate tissue, blood, of the crystalline silica exposed rats (Figure 5A). Most of the IPA biological function categories that were significantly enriched in the blood of the silica exposed rats, viz. respiratory diseases, cell to cell signaling and interaction, immune cell trafficking, cellular movement, cancer, and inflammatory response were functions that are known to be associated with toxicity and negative health effects of crystalline silica exposure. Comparable similarities in the canonical pathways and molecular networks that were significantly enriched in the lungs and the blood of the rats in response to the crystalline silica-induced pulmonary toxicity were also seen (Figure 6B). It has been very well established that induction of inflammation plays a central role in the pulmonary effects of crystalline silica exposure in animal models (Castranova, 2004). In fact, the majority of the biological functions, molecular networks and canonical pathways that were significantly enriched in the blood of the rats in response to crystalline silica-induced pulmonary toxicity were those involved in an inflammatory response (Sellamuthu *et al.*, 2011b; Sellamuthu *et al.*, 2012a). Given these results, bioinformatics analysis of the differentially

expressed genes in the blood appears to be a toxicologically relevant surrogate approach to gain insights into the mechanisms of crystalline silica-induced pulmonary toxicity. A partial list of the genes that were significantly differentially expressed in the blood of the crystalline silica exposed rats compared to the corresponding, time matched control rats that were involved in biological functions highly relevant to crystalline silica-induced pulmonary toxicity are presented in Table 6. The functional significance of the genes listed in Table 6 is discussed below to demonstrate their relevance to the pulmonary toxicity induced by crystalline silica exposure.

Inhaled crystalline silica particles, either directly or due to their interaction with AMs, result in the generation of ROS and RNS that play a significant role in crystalline silica induced pulmonary damage. Functional analysis of the SDEGs in the blood of the crystalline silica exposed rats supported the existing evidence for the involvement of ROS and RNS in the crystalline silica-induced pulmonary toxicity. Compared with the corresponding time matched control rats, significant differential expression of several genes that code for enzymes that are involved in the generation and/or detoxification of ROS and RNS was noticed in the blood of the crystalline silica exposed rats. The NOX family of NADPH oxidases (NCF1, NCF2, CYBA and CYBB), NOS2, XDH, and SOD2 were significantly overexpressed, whereas the expression of catalase was significantly down-regulated. The NOX family of NADPH oxidases is involved in the generation of toxic superoxide anion. Similarly, through its involvement in purine metabolism, XDH plays a significant role in the generation of ROS. Significant SOD overexpression along with down-regulated catalase expression, as noticed in the blood of the crystalline silica exposed rats, may facilitate excess production and accumulation of toxic hydrogen peroxide. As a result, hydrogen peroxide may induce oxidative stress-mediated pulmonary toxicity. NOS2, primarily responsible for the generation of RNS, may also facilitate the interaction of superoxide anion with nitric oxide to form toxic peroxynitrite. The significant overexpression of oxidative stress responsive genes

such as Nrf2, jun-B, and fos, as observed in the blood of the silica exposed rats, further supported the induction of oxidative stress in the crystalline silica exposed rats.

The significant induction of pulmonary inflammation seen in our rat model is in agreement with the previously suggested central role for inflammation in crystalline silica-induced pulmonary toxicity (Castranova, 2004). A significant increase in the number of AMs and infiltrating neutrophils as well as an increased level of the pro-inflammatory cytokine, MCP-1, in the BALF suggested the induction of pulmonary inflammation in the rat model employed in our silica study. Microarray analysis of the blood gene expression profile and further functional analysis of the differentially expressed genes in the silica exposed rats provided evidence for the induction of inflammation and insight into the various molecular events that are relevant to crystalline silica-induced pulmonary inflammation and damage. It has been well documented that, following inhalation exposure, crystalline silica causes cellular damage resulting in necrosis and death of AMs. This is often associated with the release of intracellular molecules, referred to commonly as endogenous danger signals or alarmins, that play a significant role in the inflammatory response. The transcripts for two such alarmins, S100A8 and S100A9, were significantly overexpressed in the blood of the crystalline silica exposed rats during the post-exposure time intervals (Table 6). The alarmins are recognized by innate immune receptors such as the pattern recognition receptors (PRRs) that are involved in the induction of inflammation. Transcripts for the PRRs, viz. NLRP3, CLEC4E, TLR2, and FPR1, were significantly overexpressed in the blood of the crystalline silica exposed rats (Table 6) suggesting their potential activation and involvement in the crystalline silica induced pulmonary inflammation. In addition to the PRRs, transcripts for other receptors of the alarmins-mediated signaling pathway such as TREM1, P2RX4 and CD44 were also significantly overexpressed in the blood of the crystalline silica exposed rats (Table 6) suggesting their potential involvement in crystalline silica induced inflammatory response. Transcript for MyD88, an adaptor molecule for TLR, and CASP1, an adaptor molecule for the NLRP3 inflammasome complex, were also

significantly overexpressed in the blood of the crystalline silica exposed rats (Table 6). A definite role for the NLRP3 inflammasome complex in silicosis and asbestosis has been demonstrated previously. The net result of the activation of the PRRs and other inflammatory response receptors along with their respective pathways is the release of pro-inflammatory cytokines and chemokines resulting in the induction of inflammation. The transcript for IL1β, the pro-inflammatory cytokine that plays a major role in crystalline silica-induced pulmonary inflammation and damage (Srivastava *et al.*, 2002), was significantly overexpressed in the blood of the crystalline silica exposed rats (Table 6). Thus, as expected and in agreement with the results of previous studies (Castranova, 2004) and as suggested by the blood gene expression data of our studies (Sellamuthu *et al.,* 2011a; Sellamuthu *et al.,* 2012a), the pulmonary damage induced by crystalline silica in the rats was associated with significant induction of inflammation.

Silicosis, one of the most devastating health outcomes of crystalline silica exposure, is characterized by the development of fibrosis resulting in progressively restrictive pulmonary function. Type II cell hyperplasia seen in the lungs of the silica exposed rats at the later post-exposure time intervals (Figure 3) may be considered an early indicator of fibrosis. Thickening of the alveolar septa and positive staining of the lung sections with Masson's trichrome stain further suggested the induction of pulmonary fibrosis at late post-silica exposure time intervals of 32- and 44-weeks in our rat model (Sellamuthu *et al.*, 2012a; Sellamuthu, 2013). Functional analysis of the differentially expressed genes in the blood of the crystalline silica exposed rats indicated the activation of several cellular processes relevant to type II pneumocyte hyperplasia and fibrosis. Hyperplasia is the result of rapid cell proliferation, and the involvement of the redox sensitive transcription factor, AP1, in cell proliferation has been very well documented (Canettieri *et al.*, 2009). The individual constituents of the AP1 element, fos and jun, were significantly overexpressed in the blood samples of the crystalline silica exposed rats at the late crystalline silica post-exposure time intervals. Many tissue remodeling and fibrosis related

genes, for example, CCl17, CCR2, Fas, MMP8, MMP9, and MyD88, were significantly overexpressed in the blood of the crystalline silica exposed rats (Table 6), especially at the late post-exposure time intervals.

3.4. Blood gene expression signature predicted exposure of rats to a sub-toxic concentration of crystalline silica: Typically occupational exposure to crystalline silica among workers takes place at very low concentrations over a prolonged period of time. In addition, there is a latency period between occupational exposure to crystalline silica and the onset of pulmonary diseases, especially silicosis and cancer, among exposed workers. Therefore, the adverse health effects associated with crystalline silica exposure may not be detectable immediately. Similar to any other adverse health effect, the key to preventing silicosis and other health effects associated with occupational exposure to crystalline silica is early detection of the exposure and/or the associated potential health effects. This is because the preventable stage in silicosis, if there is one, would be the very early stage where the pulmonary effects may still be reversible and, therefore, preventable by the application of appropriate preventative and therapeutic approaches. The observation that blood gene expression changes are superior in sensitivity to biochemical, hematological, and histological changes as markers of target organ toxicity (Bushel *et al.*, 2007; Lobenhofer *et al.*, 2008; Umbright *et al.*, 2010) prompted us to investigate whether exposure to crystalline silica at a very low sub-toxic concentration (concentration that does not result in pulmonary toxicity detectable by conventional toxicity detection methods such as biochemical and histological changes) could be detected by employing a blood gene expression signature. Blood gene expression data obtained from rats exposed to crystalline silica at 15 mg/m^3, 6 hours/day for 5 days (0-week post-exposure time interval) or filtered air (control) were used as the training set data to develop gene expression signatures for crystalline silica exposure and/or toxicity. One of the blood gene expression signatures consisting of 7 genes (Sellamuthu *et al.*, 2011b) was tested in rats that were exposed to lower concentrations of crystalline silica (1 or 2 mg/m^3, 6 hours/day, 5 days). Rats exposed to crystalline silica

at 2 mg/m^3, 6 hours/day for 5 days resulted in mild pulmonary toxicity as evidenced from the observation of a slight, but statistically significant, elevation in BALF parameters of pulmonary toxicity (LDH activity, albumin and protein content). In contrast, rats exposed to crystalline silica at 1 mg/m^3, 6 hours/day for 5 days did not result in any detectable pulmonary toxicity as evidenced from normal LDH activity and protein and albumin contents in their BALF (Sellamuthu *et al.*, 2011b). The predictive blood gene expression signature developed for crystalline silica exposure and toxicity correctly identified seven out of 8 rats (87.5%) that were exposed to crystalline silica at 2 mg/m^3, 6 hours/day for 5 days (Sellamuthu *et al.*, 2011b). Six out of eight rats (75%) that were exposed to crystalline silica at 1 mg/m^3, 6 hours/day, 5 days and did not result in any detectable pulmonary toxicity were correctly identified as crystalline silica exposed rats by the predictive blood gene expression signature (Sellamuthu *et al.*, 2011b). These results, therefore, demonstrated the potential application of blood gene expression profiling to detect/predict crystalline silica exposure and the resulting pulmonary toxicity in the rats. A similar study reported earlier by Bushel *et al.* (2007) developed a blood gene expression signature for hepatotoxicity in rats that was able to correctly predicting the exposure of a group of rats that were administered a sub-toxic oral dose of acetaminophen. Taken together, these studies show promising use of blood transcriptomics to predict toxic exposures.

3.5. Blood transcriptomics to monitor human exposure to crystalline silica: The ability of the predictive blood gene expression signature to detect crystalline silica exposure in the absence of pulmonary toxicity detectable by traditional approaches (biochemical and histological toxicity markers) in the rat model employed in our studies raises the question whether a similar approach can be employed to monitor workers for occupational exposure to crystalline silica that might eventually result in silicosis and/or other serious health effects. For this to happen, the blood gene expression signature(s) for crystalline silica exposure, in addition to being highly sensitive should be specific to the pulmonary effects associated with crystalline silica exposure. Whether the blood gene expression signature

developed in our rat model is specific to crystalline silica exposure and resulting pulmonary toxicity has not been investigated any further. However, it is important to mention that a significant specificity has been demonstrated between target organ toxicity and blood gene expression profile, especially, in the case of agents that cause hepatotoxicity (Miyamoto *et al.*, 2008; Wetmore *et al.*, 2010). In addition, the blood gene expression signature reported by Bushel *et al.* (2007) for hepatotoxicity prediction in rats is quite different from the signature that we have identified for crystalline silica-induced pulmonary toxicity in spite of the predominance of inflammatory response genes in both signatures. Since most of the genes constituting the crystalline silica-responsive blood gene expression signature developed in our study are involved in an inflammatory response, it is reasonable to question whether a similar gene expression profile may be noticed with any exposure that involves induction of inflammation. Recently, Charlesworth *et al.* (2010) have identified 342 genes that are significantly differentially expressed in the blood samples obtained from a population consisting of 297 current smokers. It has been fairly well established that inflammation plays a major role in many of the pulmonary effects associated with cigarette smoking (Bhalla *et al.*, 2009). In spite of the central role played by inflammation in the pulmonary effects of crystalline silica and cigarette smoke, none of the genes constituting the crystalline silica-responsive blood gene expression signature identified in our study were differentially expressed in the blood of cigarette smokers (Charlesworth *et al.*, 2010). Similarly, none of the blood signature genes identified for the crystalline silica-induced pulmonary toxicity were found differentially expressed in blood under conditions of inflammation induced by diesel exhaust particles (Peretz *et al.*, 2007) or endotoxin (Calvano *et al.*, 2005). LDH3 isoenzyme which is considered as an indicator of pulmonary damage (Drent *et al.*, 1996) is the protein product of LDHC, a member of the blood gene expression signature identified in our rat model. Significant elevation in the blood level of LDH3 has been found associated with the pulmonary damage observed in miners who are exposed to crystalline silica (Cobben *et al.*, 1997; Kuempel *et al.*, 2003). The available evidence, therefore, suggest that it may be possible to

develop discriminatory blood gene expression signatures that are specific to the pulmonary effects associated with crystalline silica exposure. This will, of course, require additional investigations. These investigations can be done using appropriate animal models similar to the rat model employed in our study. The validated markers capable of detecting pre-clinical silicosis should eventually be tested in a population of workers who are occupationally exposed to crystalline silica and, therefore, are at risk of developing silicosis. The relevance of such an approach is justified by the observations that (1) human orthologs of the hepatotoxicity signature genes developed in a rat hepatotoxicity model were able to correctly identify the hepatotoxicity resulting from the ingestion of acetaminophen in individuals (Bushel *et al.*, 2007); and (2) mechanistically relevant gene expression changes were detectable in the blood of individuals who had received a bolus of acetaminophen that did not result in biochemical and clinical changes indicative of liver toxicity (Fannin *et al.*, 2010). Thus additional investigations involving animal and human studies to determine the potential application of peripheral blood gene expression profiling as a practical approach to monitor human exposure to crystalline silica and the possible detection of pre-clinical silicosis are required. The outcome of these investigations is expected to have a major implication in the prevention/intervention of silicosis.

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FIGURE LEGENDS

Figure 1. Cytotoxicity and differential gene expression profile in crystalline silica exposed A549 cells. Exponentially growing human lung epithelial cells (A549) were treated with Min-U-Sil 5 crystalline silica particles at concentrations of 15, 30, 60, 120, and 240 μg/cm^2 for 6-hours (A and C) or 60 μg/cm^2 for time intervals of 2-, 6-, or 24-hours (B and D). Cytotoxicity and global gene expression profile were determined by LDH assay and microarray analysis, respectively, as described in our original publication (Sellamuthu *et al.,* 2011b). Cytotoxicity and the number of SDEGs (FDR p value <0.05) were calculated with respect to the corresponding control. * Statistically significant, $p<0.05$ (n = 5). Reproduced with permission from Inhalation Toxicology, 23(14):927-937(2011).

Figure 2. Effect of crystalline silica concentration and duration of exposure on the major biological functions perturbed by crystalline silica exposure in A549 cells. Exponentially growing human lung epithelial cells (A549) were treated with Min-U-Sil 5 crystalline silica particles at concentrations of 15, 30, 60, 120, and 240 μg/cm^2 for 6-hours (A) or 60 μg/cm^2 for time intervals of 2-, 6-, or 24-hours (B). Global gene expression profiles in the cells were determined by microarray analysis and the genes significantly differentially expressed in response to crystalline silica exposure were subjected to bioinformatics analysis as described in detail in our original publication (Sellamuthu *et al.,* 2011b). The number of SDEGs involved in the top eight ranking IPA biological processes that were significantly enriched in response to crystalline silica exposure are presented. Data represents the mean of five independent experiments. Reproduced with permission from Inhalation Toxicology, 23(14):927-937 (2011).

Figure 3. Progression of lung damage in crystalline silica exposed rats. Rats were exposed to Min-U-Sil 5 crystalline silica (15 mg/m^3, 6 hours/day, 5 days) or air (control) as described in detail in our original publications (Sellamuthu *et al.,* 2011a; Sellamuthu *et al.,* 2012a). Lung sections prepared at the post-silica exposure time intervals of 0, 1, 2, 4, 8, 16, and 32 weeks were stained with hematoxylin and

eosin. Only photomicrographs of lung sections of 8, 16, and 32 weeks post-silica exposure time intervals are presented and those of earlier time intervals can be found in our original publication (Sellamuthu et al., 2011a). Arrows indicate AMs in alveolar space (8 weeks) and type II pneumocyte hyperplasia (16- and 32-weeks). Magnification = 20x. Reproduced with permissions from Toxicological Sciences, 122(2): 253-264 (2011) and Inhalation Toxicology, 24(9):570-579 (2012).

Figure 4. Differential gene expression profile in the lungs of crystalline silica exposed rats. Rats were exposed to crystalline silica (15 mg/m^3, 6 hours/day, 5 days) and the number of SDEGs (>1.5-fold change and <0.01 FDR p value) was determined by microarray analysis as described in detail in our original publications (Sellamuthu et al., 20112a and 2012b). The number of genes significantly differentially expressed (total), overexpressed (up) and under expressed (down) in the silica exposed rat lungs compared with the corresponding time-matched controls are presented for the post-exposure time intervals presented on the X-axis. Data represents the mean of eight crystalline silica exposed rats compared with four corresponding time-matched control rats per time point. Reproduced with permission from Journal of Applied Toxicology, DOI 10.1002/jat.2733 (2012) and Inhalation Toxicology, 24(9):570-579 (2012).

Figure 5. Enrichment of top ranking IPA biological functions in crystalline silica exposed rat lungs. Rats were exposed to Min-U-Sil 5 crystalline silica (15 mg/m^3, 6 hours/day, 5 days) and the number of SDEGs in their lungs was determined by microarray analysis as described in detail in our original publications (Sellamuthu *et al.,* 20112a; Sellamuthu *et al.,* 2012b). The microarray data was subjected to bioinformatics analysis and the number of SDEGs in the crystalline silica exposed rat lungs belonging to the six top ranking IPA biological functions at each of the post-exposure time interval is presented. Data represents the group mean of eight crystalline silica exposed and four time-matched control rats per time point. Modified with permission from Journal of Applied Toxicology, DOI 10.1002/jat.2733 (2012) and Inhalation Toxicology, 24(9):570-579 (2012).

Figure 6. Enrichment of IPA biological functions and canonical pathways in the lungs and blood of crystalline silica exposed rats. Rats were exposed to Min-U-Sil 5 crystalline silica (15 mg/m^3, 6 hours/day, 5 days) and the number of SDEGs at 32-weeks post-silica exposure time interval was determined by microarray analysis as described in detail in our original publications (Sellamuthu *et al.*, 2012a). Bioinformatics analysis of the SDEGs identified in the silica exposed rat lungs and blood was done using IPA software. The top eight significantly enriched IPA biological functions (A) and canonical pathways (B) in the lungs and blood of the silica exposed rats are presented to demonstrate the similarity in gene expression profile between lungs and blood of the silica exposed rats. Data represents the mean of six rats per group. Reproduced with permission from Inhalation Toxicology, 24(9):570-579 (2012).

Fig. 1

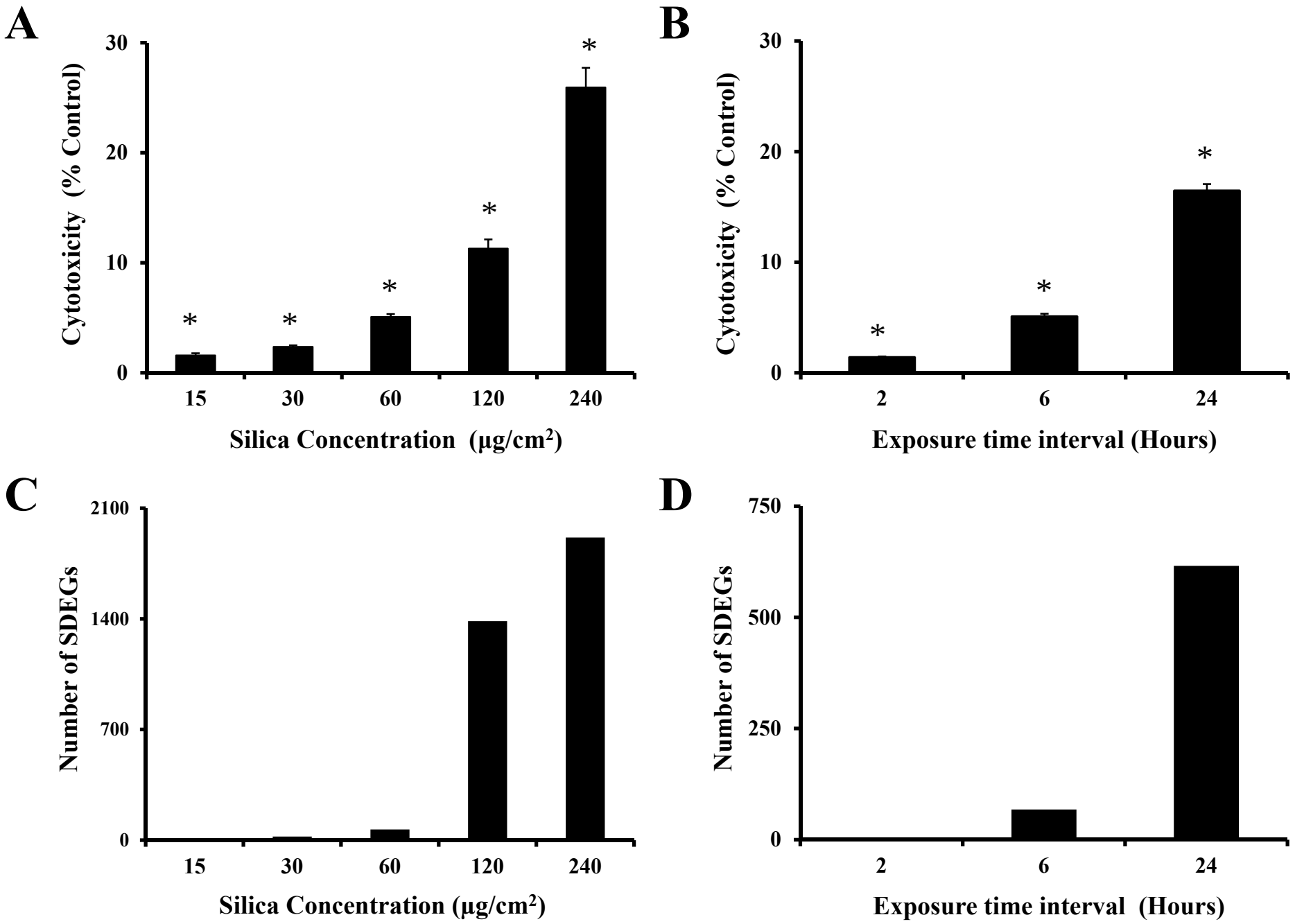
A
Cytotoxicity (% Control)
30
20
10
0
*
15
30
60
120
240
Silica Concentration (µg/cm²)
B
Cytotoxicity (% Control)
30
20
10
0
*
2
6
24
Exposure time interval (Hours)
C
Number of SDEGs
2100
1400
700
0
15
30
60
120
240
Silica Concentration (µg/cm²)
D
Number of SDEGs
750
500
250
0
2
6
24
Exposure time interval (Hours)

Fig. 2

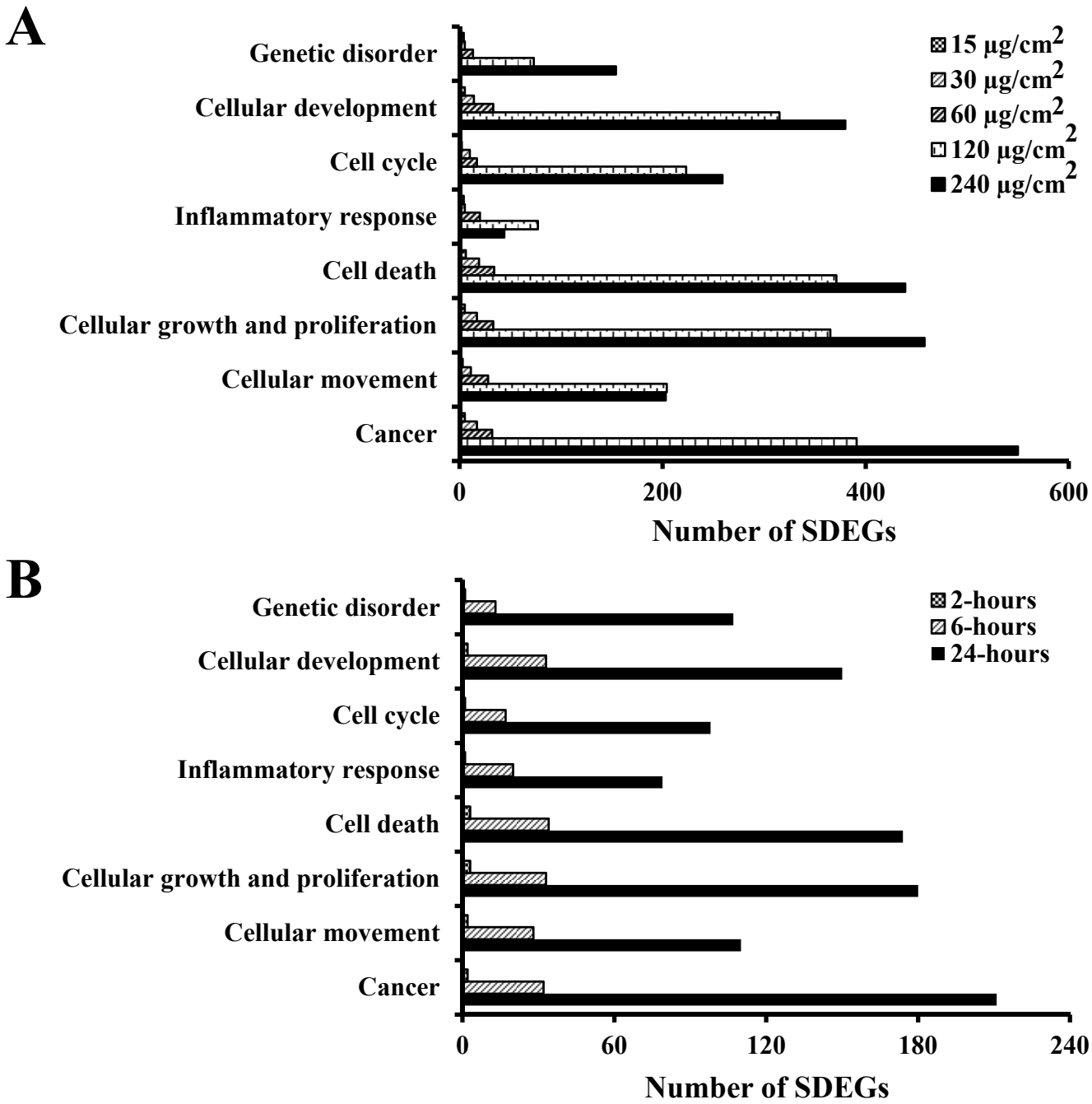
A
Genetic disorder
Cellular development
Cell cycle
Inflammatory response
Cell death
Cellular growth and proliferation
Cellular movement
Cancer
15 µg/cm2
30 µg/cm2
60 µg/cm2
120 µg/cm2
240 µg/cm2
0
200
400
600
Number of SDEGs
B
Genetic disorder
Cellular development
Cell cycle
Inflammatory response
Cell death
Cellular growth and proliferation
Cellular movement
Cancer
2-hours
6-hours
24-hours
0
60
120
180
240
Number of SDEGs

Fig. 3

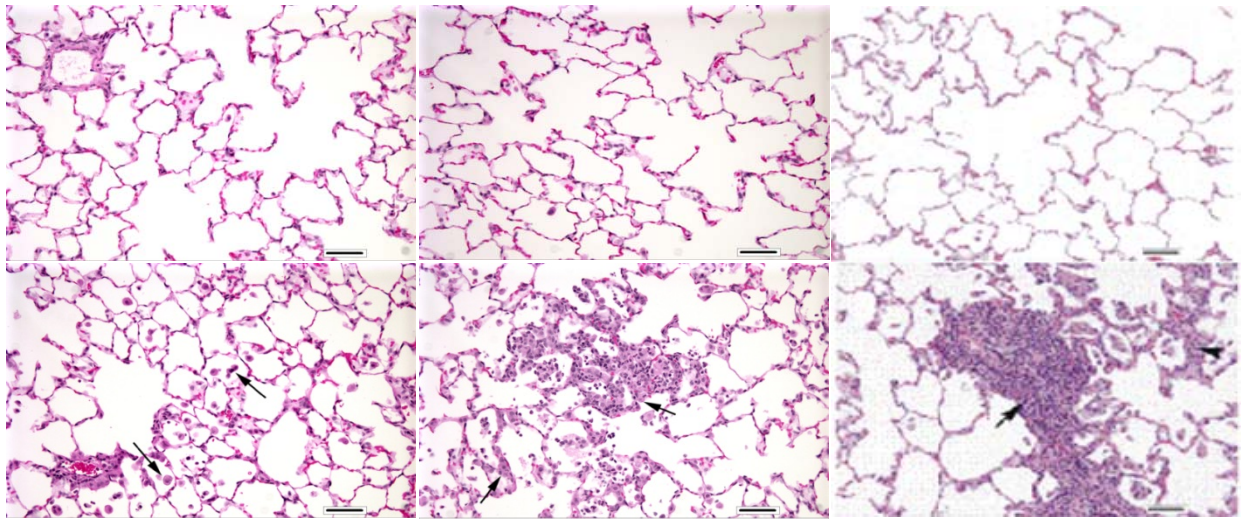

Fig. 4

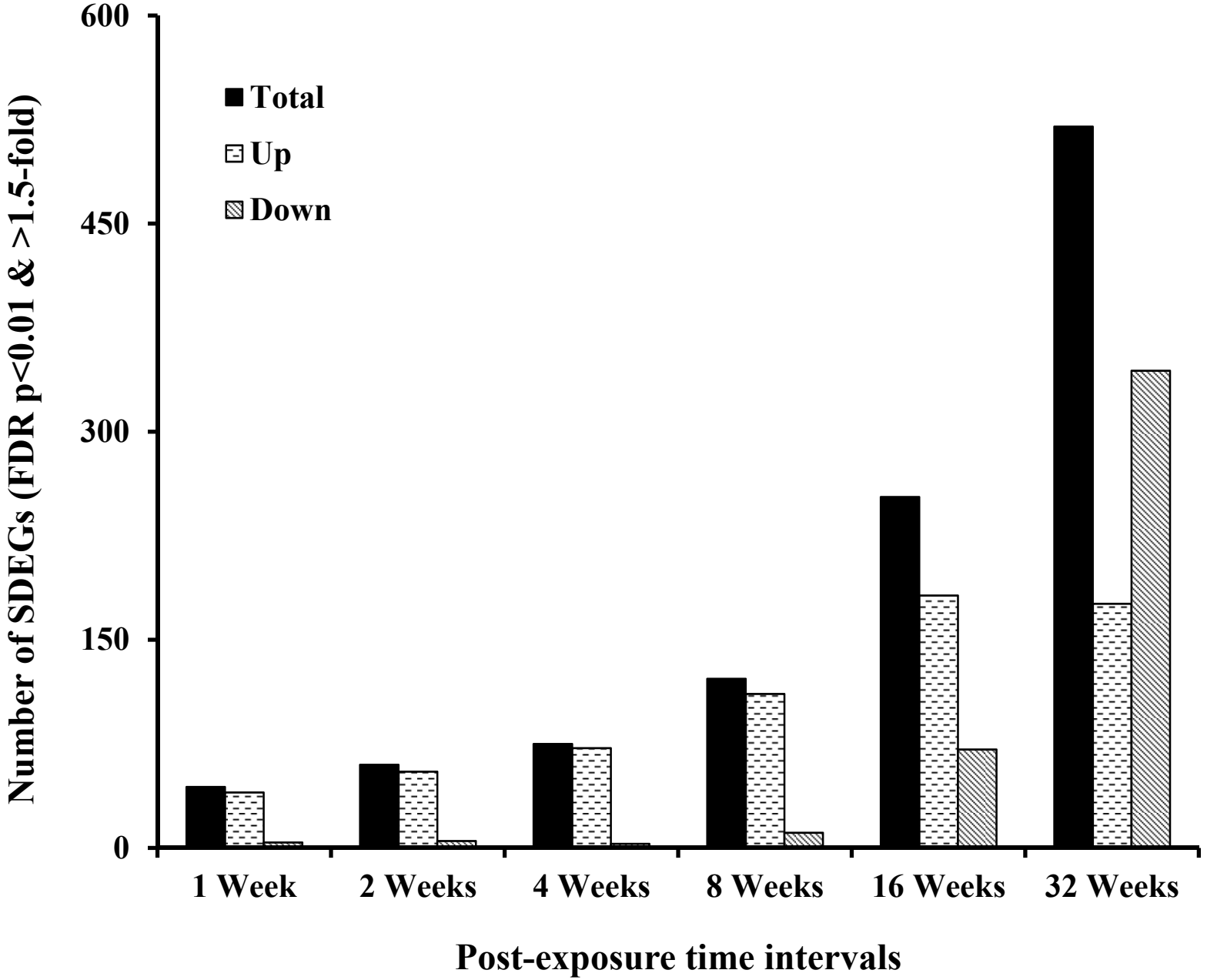
600
450
300
150
0
Total
Up
Down
Number of SDEGs (FDR p<0.01 & >1.5-fold)
1 Week
2 Weeks
4 Weeks
8 Weeks
16 Weeks
32 Weeks
Post-exposure time intervals

Fig. 5

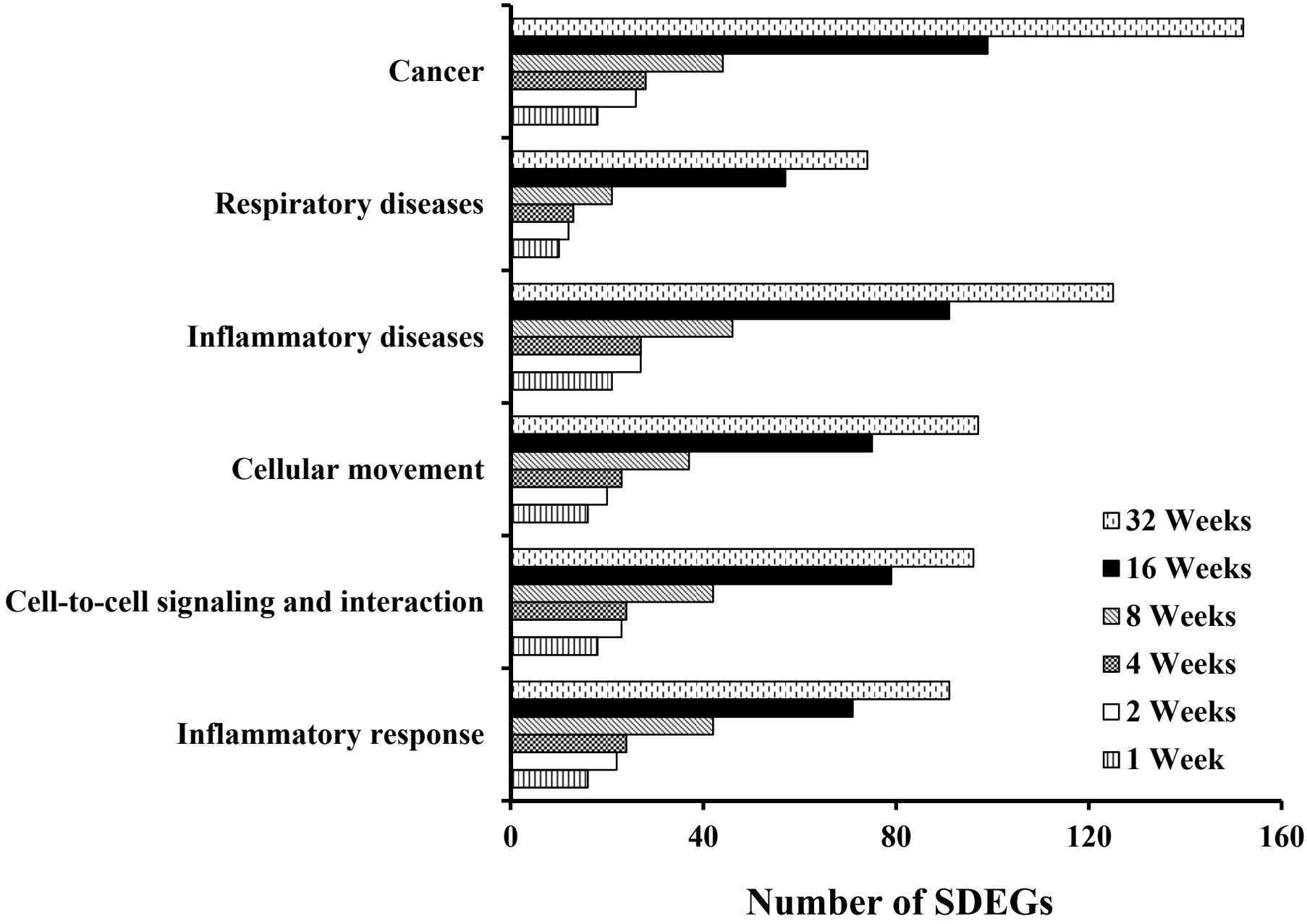
Cancer
Respiratory diseases
Inflammatory diseases
Cellular movement
Cell-to-cell signaling and interaction
Inflammatory response
32 Weeks
16 Weeks
8 Weeks
4 Weeks
2 Weeks
1 Week
0
40
80
120
160
Number of SDEGs

Table 1. Correlation co-efficients (r^2 values) for the relationship between crystalline silica-induced pulmonary toxicity (BALF LDH, PMN, and MCP-1) and the number of SDEGs in the lungs of the rats. The toxicity measurements and the number of SDEGs in the lungs of the silica exposed rats at the post-exposure time intervals of 0, 1, 2, 4, 8, 16, and 32 weeks following a one week exposure to crystalline silica (15 mg/m^3, 6 hours/day) were done as described in our original publications (Sellamuthu *et al.,* 2012; Sellamuthu *et al.,* 2012b). Modified with permission from Journal of Applied Toxicology, DOI 10.1002/jat.2733 (2012).

	BALF LDH	**BALF PMN**	**BALF MCP-1**
Lung SDEGs	0.776	0.879	0.927

Table 2. Differentially expressed genes in A549 cells exposed to crystalline silica. Exponentially growing human lung epithelial cells (A549) were treated with Min-U-Sil 5 crystalline silica particles at concentrations of 15, 30, 60, 120, and 240 μg/cm^2 for 6-hours or 60 μg/cm^2 for time intervals of 2-, 6-, or 24-hours. Global gene expression profiles in the cells were determined by microarray analysis and the genes significantly differentially expressed in response to silica exposure were subjected to bioinformatics analysis as described in detail in our original publication (Sellamuthu *et al.,* 2011b). A partial list of the SDEGs involved in IPA biological processes and canonical pathways that are relevant to crystalline silica toxicity and their fold changes in expressions compared to the control cells are presented. Reproduced with permission from Inhalation Toxicology, 23(14):927-937 (2011).

Gene		Crystalline silica (μg/cm^2)					Time interval (hrs)		
		15	**30**	**60**	**120**	**240**	**2**	**6**	**24**
Reactive Oxygen Species									
SOD2	*Superoxide dismutase 2*	1.08	1.11	1.21*	1.98*	2.50*	-1.05	1.21*	4.52*
CAT	*Catalase*	1.02	-1.05	-1.07	-1.16*	-1.14*	-1.08	-1.07	-1.24
SMOX	*Spermine oxidase*	1.33	1.39*	1.55*	2.99*	3.52*	1.33	1.55*	2.55*
CYR61	*Cysteine-rich angiogenic inducer 61*	1.12	1.59*	1.90*	2.47*	1.57*	2.11*	1.90*	1.55*
Antioxidant/ oxidative stress									
NFE2L2	*Nuclear factor erythroid derived 2 like 2*	1.03	1.05	1.15	1.83*	1.61*	1.28*	1.15	-1.05
NFKB1	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1*	-1.04	1.18	1.31*	2.00*	2.03*	1.11	1.31*	1.50*
NFKB2	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2*	1.04	1.02	1.05	1.12*	1.11*	1.05	1.05	1.09*
NFKBIZ	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta*	1.15	1.21	1.23	2.51*	3.31*	1.18	1.23	2.86*
DUSP1	*Dual specificity phosphatase 1*	1.24	1.22*	1.49*	2.47*	3.00*	1.56	1.49*	1.55*
DUSP5	*Dual specificity phosphatase 5*	1.92*	1.79*	2.48*	5.74*	8.02*	2.84*	2.48*	2.98*
FOS	*v-fos FBJ murine osteosarcoma viral oncogene homolog*	1.24	1.36	1.42	3.93*	20.34*	2.06*	1.42	1.24*
JUNB	*jun B proto-oncogene*	1.02	-1.00	1.07	1.46*	1.72*	1.20	1.07	-1.03
c-JUN	*jun oncogene*	1.27	1.46	1.87*	5.46*	10.64*	2.17*	1.87*	2.40*
STC1	*Stanniocalcin 1*	1.63*	1.75*	2.37*	4.67*	5.40*	1.90*	2.37*	2.75*

STC2	*Stanniocalcin 2*	1.22	1.31*	1.55*	2.06*	2.03*	1.02	1.55*	3.35*
Inflammation									
IRF1	*Interferon regulatory factor 1*	1.10	1.06	1.14	1.54*	1.33*	-1.04	1.54*	1.15
RELA	*v-rel reticuloendotheliosis viral oncogene homolog A*	1.02	1.06	1.11*	1.22*	1.12*	1.05	1.11*	1.13*
RELB	*v-rel reticuloendotheliosis viral oncogene homolog B*	1.20*	1.24*	1.51*	1.92*	1.73*	-1.01	1.51*	1.92*
IL1A	*Interleukin 1 alpha*	1.05	1.02	1.05	1.86*	2.62*	ND	1.05	1.22*
IL1B	*Interleukin 1 beta*	ND	-1.00	1.02	1.28*	1.45*	ND	1.28*	1.6*
IL6	*Interleukin 6*	1.12	1.24	1.58*	6.17*	8.51*	1.43*	1.58*	6.14*
IL8	*Interleukin 8*	3.16*	3.84*	7.13*	35.72*	41.60	9.77*	7.13*	18.21*
IL11	*Interleukin 11*	1.08	1.23	1.47	3.75*	4.92*	1.89*	1.47	1.49*
IRAK2	*Interleukin-1 receptor-associated kinase 2*	1.63*	1.77*	1.89*	5.56*	7.15*	1.66*	1.89*	3.54*
PTGS2	*Prostaglandin-endoperoxide synthase 2*	1.51	1.55*	2.21*	11.13*	15.09*	2.38*	2.21*	3.91*
CCL2/ MCP1	*Chemokine (C-C motif) ligand 2*	1.68	1.77*	1.67*	3.30*	3.57*	1.38	1.67*	3.23*
CCL20/ MIP3α	*Chemokine (C-C motif) ligand 20*	1.06	1.06	1.23*	3.20*	4.16*	1.13	1.23*	2.39*
CXCL1/ GRO1	*Chemokine (C-X-C motif) ligand 1*	ND	-1.00	1.04	1.41*	1.50*	ND	1.04	1.60*
CXCL2/ MIP-2	*Chemokine (C-X-C motif) ligand 2*	1.24	1.12	1.29	3.81*	3.58*	1.38	1.29	3.23*
CXCL5	*Chemokine (C-X-C motif) ligand 5*	1.24	1.17	1.57*	2.70*	4.16*	1.13	1.57*	2.39*
CXCL8/ IL8	*Interleukin 8*	3.16*	3.84*	7.13*	35.72*	41.60	9.77*	7.13*	18.21*
PLAU	*Plasminogen activator urokinase*	1.23	1.44	1.75*	2.44*	1.83*	1.48*	1.75*	1.53*
ITGA2	*Integrin alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)*	-1.05	1.11	1.26	1.74*	1.57*	1.03	1.26	1.81*
MMP10	*Matrix metallopeptidase 10*	ND	1.03	1.04	1.87*	3.82*	ND	1.04	1.71*
CEBPB	*CCAAT/enhancer binding protein*	1.13	1.23*	1.42*	2.78*	3.62*	1.86*	1.42*	2.08*
Apoptosis									
NFE2L2	*Nuclear factor erythroid derived 2 like 2*	1.03	1.05	1.15	1.83*	1.61*	1.28*	1.15	-1.05
NFKB1	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1*	-1.04	1.18	1.31*	2.00*	2.03*	1.11	1.31*	1.50*
NFKB2	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)*	1.04	1.02	1.05	1.12*	1.11*	1.05	1.05	1.09*
NFKBIA	*Nuclear factor of kappa light polypeptide gene enhancer in*	1.10	1.32*	1.45*	2.93*	4.48*	1.35	1.45*	1.94*

	B-cells inhibitor alpha								
NFKBIZ	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta*	1.15	1.21	1.23	2.51*	3.31*	1.18	1.23	2.86*
GDF15	*Growth differentiation factor 15*	1.65*	1.50*	1.75*	5.33*	9.68*	1.74	1.75*	2.38*
GADD45α	*Growth arrest and DNA-damage-inducible 45 alpha*	1.19	1.46*	1.62*	3.08*	3.81*	1.59*	1.62*	1.79*
GADD34	*Growth arrest and DNA-damage-inducible 34*	1.86*	2.10*	2.82*	5.88*	10.93*	2.72*	2.82*	3.56*
EGR1	*Early Growth Response 1*	1.91*	2.05*	2.51*	13.65*	36.34*	6.46*	2.51*	2.49*
BIRC3	*Baculoviral IAP repeat-containing 3*	1.27	1.58*	2.01*	5.78*	8.51*	1.59*	2.01*	2.43*
c-FOS	*v-fos FBJ murine osteosarcoma viral oncogene homolog*	1.24	1.36	1.42	3.93*	20.34*	2.06*	1.42	1.24*
FOSB	*FBJ murine osteosarcoma viral oncogene homolog B*	3.58*	3.87*	4.30*	11.64*	34.73*	9.83*	4.30*	3.74*
FOSL1	*FOS-like antigen 1*	1.10	1.31*	1.63*	2.93*	2.33*	1.39*	1.63*	1.44*
CYR61	*Cysteine-rich angiogenic inducer 61*	1.12	1.59*	1.90*	2.47*	1.57*	2.11*	1.90*	1.55*
JUND	*jun D proto-oncogene*	-1.03	1.10	1.20	2.26*	3.26*	1.17	1.20	-1.01
OKL-38	*Pregnancy-induced growth inhibitor*	-1.18	1.10	1.16	1.79*	2.49*	1.32	1.16	-1.16
Cancer									
EFNA1	*Ephrin-A1*	1.31	1.32	1.45	2.72*	2.93*	1.10	1.45	1.88*
MYC	*v-myc myelocytomatosis viral oncogene homolog (avian)*	1.14	1.22	1.32	1.69*	2.16*	1.23	1.32	1.16
CEBPB	*CCAAT/enhancer binding protein*	1.13	1.23*	1.42*	2.78*	3.62*	1.86*	1.42*	2.08*
TRIB1	*Tribbles homolog 1 (Drosophila)*	1.13	1.09	1.22	2.57*	3.90*	1.34	1.22	1.55*
PTGS2	*Prostaglandin-endoperoxide synthase 2*	1.51	1.55*	2.21*	11.13*	15.09*	2.38*	2.21*	3.91*
ZFP36	*Zinc finger protein 36*	1.36	1.44	1.58*	6.43*	10.65*	2.16*	1.58*	1.10
KLF2	*Kruppel-like factor 2*	1.33	1.67	1.87*	2.38*	1.90*	1.88*	1.87*	1.18*
KLF6	*Kruppel-like factor 6*	1.38*	1.61*	1.97*	5.14*	6.84*	2.03*	1.97*	2.38*
KLF11	*Kruppel-like factor 11*	1.08	1.13	1.21	1.60*	1.65*	1.14	1.21	1.58*
KLF13	*Kruppel-like factor 13*	1.14	1.22	1.38*	1.71*	1.32*	1.01	1.38*	1.15
ETS1	*v-ets erythroblastosis virus E26 oncogene homolog 1*	1.36	1.44*	1.77*	3.50*	3.58*	1.35*	1.77*	2.06*
DDIT3	*DNA-damage-inducible transcript 3*	1.36*	1.48*	1.63*	3.11*	4.15*	1.25	1.63*	2.17*
FGF2	*Fibroblast growth factor 2*	1.00	1.00	1.10	1.25*	1.27*	1.07	1.10	1.07
Cellular Growth and Proliferation									

EGR1	*Early Growth Response 1*	1.91*	2.05*	2.51*	13.65*	36.34*	6.46*	2.51*	2.49*
RELB	*v-rel reticuloendotheliosis viral oncogene homolog B*	1.20*	1.24*	1.51*	1.92*	1.73*	-1.01	1.51*	1.92*
IL8	*Interleukin 8*	3.16*	3.84*	7.13*	35.72*	41.60*	9.77*	7.13*	18.21*
DUSP5	*Dual specificity phosphatase 5*	1.92*	1.79*	2.48*	5.74*	8.02*	2.84*	2.48*	2.98*
FST	*Follistatin*	1.41*	1.62*	2.20*	4.57*	4.60*	1.25	2.20*	2.48*
ETS1	*v-ets erythroblastosis virus E26 oncogene homolog 1*	1.36	1.44*	1.77*	3.50*	3.58*	1.35*	1.77*	2.06*
Cell Cycle									
EGR1	*Early Growth Response 1*	1.91*	2.05*	2.51*	13.65*	36.34*	6.46*	2.51*	2.49*
ETS1	*v-ets erythroblastosis virus E26 oncogene homolog 1*	1.36	1.44*	1.77*	3.50*	3.58*	1.35*	1.77*	2.06*
STC1	*Stanniocalcin 1*	1.63*	1.75*	2.37*	4.67*	5.40*	1.90*	2.37*	2.75*
c-JUN	*jun oncogene*	1.27	1.46	1.87*	5.46*	10.64*	2.17*	1.87*	2.40*
NFKBIA	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha*	1.10	1.32*	1.45*	2.93*	4.48*	1.35	1.45*	1.94*
PTGS2	*Prostaglandin-endoperoxide synthase 2*	1.51	1.55*	2.21*	11.13*	15.09*	2.38*	2.21*	3.91*
Cellular Development									
CSF2	*Colony stimulating factor 2 (granulocyte-macrophage)*	ND	1.07	1.13	2.48*	3.81*	1.08	1.13	2.04*
CEBPB	*CCAAT/enhancer binding protein (C/EBP)*	1.13	1.23*	1.42*	2.78*	3.62*	1.86*	1.42*	2.08*
EGR1	*Early Growth Response 1*	1.91*	2.05*	2.51*	13.65*	36.34*	6.46*	2.51*	2.49*
FOSL1	*FOS-like antigen 1*	1.10	1.31*	1.63*	2.93*	2.33*	1.39*	1.63*	1.44*
c-JUN	*jun oncogene*	1.27	1.46	1.87*	5.46*	10.64*	2.17*	1.87*	2.40*
FOXO1	*Forkhead box O1*	1.17	1.21*	1.41*	2.06*	1.76*	1.16	1.41*	1.33*

Data represent the fold change in expression of the individual genes and are mean of five independent microarray experiments. Some of the genes are listed under multiple categories since they are involved in multiple functions.

*statistically significant (FDR p value < 0.05) change in expression compared to the corresponding control

ND- Gene expression not detected

Table 3. Fold change in expression of a selected list of significantly differentially expressed genes in the lungs of crystalline silica exposed rats. Rats were exposed to Min-U-Sil 5 crystalline silica (15 mg/m^3, 6 hours/day, 5 days) and the number of SDEGs in their lungs was determined by microarray analysis as described in detail in our original publications (Sellamuthu et al., 20112a and 2012b). The microarray data was subjected to bioinformatics analysis and the fold changes in expressions of a selected list of genes belonging to IPA biological processes that are relevant to mechanism(s) of crystalline silica-induced pulmonary toxicity are presented for each of the post-silica exposure time intervals. Data represents the group mean of eight crystalline silica exposed and four time-matched control rats per time point. Reproduced with permission from Journal of Applied Toxicology, DOI 10.1002/jat.2733 (2012) and Inhalation Toxicology, 24(9):570-579 (2012).

Gene	Fold change in expression (Post-exposure time intervals)					
	1 Week	2 Weeks	4 Weeks	8 Weeks	16 Weeks	32 Weeks
Antioxidants and Oxidative stress						
Superoxide dismutase 2 (SOD2)	1.76*	1.85*	1.98*	2.47*	2.44*	1.53*
Heme oxygenase 1 (HMOX1)	1.40*	1.39*	1.46*	1.58*	2.05*	1.63*
Metallothionein 1a (MT1A)	1.81*	1.77*	1.65*	2.11*	2.44*	2.03*
NADPH oxidase organizer 1 (NOXO1)	2.54*	2.07*	2.50*	3.24*	3.16*	1.70*
Lipocalin 2 (LCN2)	3.25*	3.34*	3.58*	5.53*	5.96*	4.20*
Arginase 1(ARG1)	1.59*	1.29*	1.38*	1.93*	2.28*	1.55*
Lactoperoxidase (LPO)	1.09*	1.02	1.03	1.23*	2.09*	4.08*
Cancer						
Lipocalin 2 (LCN2)	3.25*	3.34*	3.54*	5.53*	5.96*	4.20*
Chitinase 3-like 1 (CHI3L1)	1.89*	2.15*	2.17*	2.75*	3.34*	2.95*

Secreted phosphoprotein 1 (SPP1)	1.34	1.01	1.12	1.85*	6.27*	8.61*
Inflammation						
Chemokine (C-C motif) ligand 2 (CCl2)	2.36*	1.99*	2.30*	4.28*	6.22*	2.68*
Chemokine (C-C motif) ligand 3 (CCl3)	1.84*	1.53*	1.58*	2.24*	2.25*	1.35*
Chemokine (C-C motif) ligand 4 (CCl4)	1.21*	1.12	1.23*	1.50*	1.50*	1.24*
Chemokine (C-C motif) ligand 7 (CCl7)	1.39*	1.29*	1.35*	2.37*	4.32*	1.96*
Chemokine (C-X-C motif) ligand 1 (CXCl1)	3.05*	2.26*	2.76*	3.20*	2.71*	ND
Chemokine (C-X-C motif) ligand 2 (CXCl2)	1.46*	1.12	1.22*	1.57*	1.42*	1.15*
Chemokine (C-X-C motif) ligand 5 (CXCl5)	2.52*	2.06*	3.39*	4.32*	3.96*	1.69*
Chemokine (C-X-C motif) ligand 9 (CXCl9)	1.09	1.29*	1.31*	2.08*	2.91*	3.17*
Chemokine (C-X-C motif) ligand 10 (CXCl10)	1.00	1.05	1.04	1.04	1.52*	1.59*
Chemokine (C-X-C motif) ligand 11 (CXCl11)	1.15	1.04	1.05	1.40*	2.63*	2.61*
Interleukin 1 beta (IL1β)	1.21*	1.16	1.22*	1.48*	1.73*	1.92*
Interleukin 1 receptor antagonist, transcript variant 2 (IL1R2)	1.45*	1.21*	1.21*	2.27*	2.80*	1.82*
Resistin like alpha (RETNLA)	2.46*	2.52*	2.42*	3.57*	8.46*	11.04*
S100 calcium binding protein A8 (S100A8)	1.26*	1.16	1.42*	2.50*	3.59*	2.94*
Triggering receptor expressed on myeloid cells 1 (TREM1)	1.30*	1.08	1.10	1.46*	1.72*	1.09
Triggering receptor expressed on myeloid cells 2 (TREM2)	1.50*	1.28*	1.27	1.63*	1.64*	1.78*
Lipocalin 2 (LCN2)	3.24*	3.34*	3.58*	5.53*	5.96*	4.20*
Chitinase 3-like 1 (CHI3L1)	1.89*	2.15*	2.17*	2.75*	3.34*	2.95*
Secreted phosphoprotein 1 (SPP1)	1.34	1.01	1.12	1.85*	6.27*	8.61*
Arachidonate 15-lipoxygenase (ALOX15)	-1.44*	-1.40*	-1.30*	-1.89*	-1.71*	-1.50*
Tissue Remodeling/Fibrosis						
Matrix metallopeptidase 8 (MMP8)	1.09	1.13*	1.22*	1.33*	1.50*	1.13*
Matrix metallopeptidase 12 (MMP12)	3.78*	3.49*	3.87*	4.34*	4.96*	4.33*
Secreted phosphoprotein 1 (SPP1)	1.34	1.01	1.12	1.85*	6.27*	8.61*
Haptoglobin (HP)	1.49	1.56*	1.78*	2.11*	2.42*	2.51*
Arginase 1 (ARG1)	1.59*	1.29*	1.38*	1.93*	2.28*	1.55*
Chemokine (C-X-C motif) ligand 9 (CXCL9)	1.09	1.29*	1.31*	2.08*	2.91*	3.17*
Chemokine (C-C motif) ligand 2 (CCl2)	2.36*	1.99*	2.30*	4.28*	6.22*	2.68*

Chemokine (C-C motif) ligand 7 (CCl7)	1.39*	1.29*	1.35*	2.37*	4.32*	1.96*
Complement component 2 (C2)	1.21*	1.02	1.05	1.19*	1.5*	1.77*
Complement component 3 (C3)	2.50*	2.14*	2.26*	2.88*	3.02*	ND
Complement component 4 binding protein, alpha (C4BPA)	2.14*	2.19*	2.23*	2.90*	3.55*	3.07*
Complement component 5 (C5)	1.19*	1.24*	1.31*	1.533*	1.62*	ND
Complement factor B (CFB)	1.32*	1.18*	1.22*	1.37*	1.5*	1.18*
Complement factor 1 (CFI)	1.28	1.47*	1.65*	2.09*	4.18*	4.88*

Data represents the group mean of the silica exposed rats (n=8) compared with the corresponding time-matched controls (n=4) and is obtained from the microarray analysis results. Some of the genes are listed under more than one category since bioinformatics analysis identified their involvement in multiple categories.

*Statistically significantly different (FDR $p < 0.01$) compared to the time-matched control samples.

Table 4. Differential expressions of solute carrier (SLC) family of genes in rat lungs. Rats were exposed to Min-U-Sil 5 crystalline silica (15 mg/m^3, 6 hours/day, 5 days) and the expressions of the SLC genes listed in the table were determined by microarray analysis as described in detail in our original publication (Sellamuthu *et al.*, 2012b). Data represents the group mean of eight crystalline silica exposed and four time-matched control rats per time point. Reproduced with permission from Journal of Applied Toxicology, DOI 10.1002/jat.2733 (2012).

Gene	Fold change in expression (Post-exposure time intervals)					
	1 Week	2 Week	4 Weeks	8 Weeks	16 Weeks	32 Weeks
Solute carrier family 26, member 4 (SLC26A4)	4.46*	3.67*	4.10*	6.51*	9.93*	6.69*
Solute carrier family 13, member 2 (SLC13A2)	1.74*	1.51*	1.72*	2.21*	2.50*	1.67*
Solute carrier family 7, member 7 (SLC7A7)	1.50*	1.43*	1.48*	1.86*	2.33*	3.13*
Solute carrier family 16, member 3 (SLC16A3)	1.43*	1.23*	1.22*	1.72*	2.02*	2.04*
Solute carrier family 16, member 11 (SLC16A11)	1.43*	1.58*	1.59*	1.81*	1.88*	1.89*

Table 5. Correlation co-efficients (r^2 values) for the relationship between crystalline silica-induced pulmonary toxicity (BALF LDH, PMN, and MCP-1) and the number of SDEGs in the blood of the rats. The toxicity measurements and the number of SDEGs in the blood of the crystalline silica exposed rats at post-exposure time intervals of 0, 1, 2, 4, 8, 16, and 32 weeks following their one week exposure to crystalline silica (15 mg/m^3, 6 hours/day, 5 days) were done as described in our original publications (Sellamuthu *et al.,* 2011a; Sellamuthu *et al.,* 2012a). Modified with permission from Toxicological Sciences 122(2): 253-264(2011) and Inhalation Toxicology, 24(9):570-579 (2012).

	BALF LDH	**BALF PMN**	**BALF MCP-1**
Blood SDEGs	0.831	0.923	0.958

Table 6. Fold change in expression of a selected list of significantly differentially expressed genes in the blood of crystalline silica exposed rats. Rats were exposed to Min-U-Sil 5 crystalline silica (15 mg/m^3, 6 hours/day, 5 days) and the number of SDEGs in their blood samples was determined by microarray analysis as described in detail in our original publications (Sellamuthu *et al.,* 2011a; Sellamuthu *et al.,* 2012a). The microarray data was subjected to bioinformatics analysis and the fold changes in expressions of a selected list of genes belonging to IPA biological processes that are relevant to mechanism(s) of crystalline silica-induced pulmonary toxicity are presented for each of the post-silica exposure time intervals. Data represents the group mean of eight crystalline silica exposed and four time-matched control rats per time point. Reproduced with permission from Toxicological Sciences 122(2): 253-264(2011) and Inhalation Toxicology, 24(9):570-579 (2012).

Gene		Post exposure time intervals (weeks)						
		0	**1**	**2**	**4**	**8**	**16**	**32**
Reactive Oxygen Species								
CYBA	Cytochrome b-245, alpha polypeptide	Up	Up*	Up*	Dn	Up	Up	Up*
NCF1	Neutrophil cytosolic factor 1	Up*	Up*	Up	Dn	Up	Up	Up*
NCF2	Neutrophil cytosolic factor 2	Up	Up*	Dn	Up	Up*	Up*	Up*
SOD2	Superoxide dismutase 2, mitochondrial	Up*	Dn	Dn	Dn	Up	Up	Dn*
CAT	Catalase	Dn	Dn*	Dn*	Up	Dn	Dn	Up*
XDH	Xanthine dehydrogenase	Up*	Dn	Dn	Dn	Up*	Up	ND
NOS2	Nitric oxide synthase, inducible (NOS type II) nitric oxide synthase 2, inducible	Up*	Dn	Up	Up	Up*	Up*	Up*
Antioxidant								
NFE2L2	Nuclear factor, erythroid derived 2, like 2	Up*	Up	Up	Dn	Up*	Up*	Up*
FOS	FBJ osteosarcoma oncogene	Up	Up	Up	Dn	Up*	Up*	Up*
JUNB	jun B proto-oncogene	Up	Up	Dn	Up	Up*	Up*	Up*
Danger Signals or Alarmins								
S100A8	S100 calcium binding protein A8	Up*	Up*	Up*	Up	Up*	Up*	Up*

S100A9	S100 calcium binding protein A9	Up	Up	Up	Dn	Up*	Up*	Dn*
Inflammation								
NLRP3	NLR family, pyrin domain containing 3	Up*	Up	Up	Up	Up*	Up*	Up*
TLR2	Toll-like receptor 2	Up*	Up*	Up	Up	Up*	Up*	Up*
CLEC4E	C-type lectin domain family 4, member e	Up*	Up*	Up*	Dn	Up*	Up*	Up*
FPR1	Formyl peptide receptor 1	Up*	Up*	Up	Up	Up*	Up*	Up*
TLR13	Similar to toll-like receptor 13	Up*	Up	Up	Dn	Up*	Up*	ND
TREM1	Triggering receptor expressed on myeloid cells 1	Up*	Up*	Up*	Dn	Up	Up*	Up*
CD44	Cd44 molecule	Up*	Up*	Up*	Dn	Up*	Up*	Up*
P2RX4	Purinergic receptor P2X, ligand-gated ion channel 4	Up*	Up	Up	Up	Up*	Up*	Dn
MYD88	Myeloid differentiation primary response gene 88	Up*	Up	Up	Up	Up*	Up*	Up*
CASP1	Caspase 1	Up*	Up	Up	Dn	Up	Up	Dn*
IL1β	Interleukin 1 beta	Up*	Up	Up*	Up	Up*	Up*	Up*
PTAFR	Platelet-activating factor receptor	Up*	Up*	Up	Up	Up*	Up*	Up*
CXCR2	Interleukin 8 receptor, beta	Up*	Up	Up	Up	Up*	Up*	Up*
LGALS3	Lectin, galactoside-binding, soluble, 3	Up*	Up*	Up*	Up	Up*	Up*	Up*
ILR17A	Interleukin 17 receptor A	Up*	Up*	Up*	Dn	Up*	Up*	Up*
MMP8	Matrix metallopeptidase 8	Up*	Up*	Up	Up*	Up*	Up*	Up*
MMP9	Matrix metallopeptidase 9	Up	Up*	Up*	Up	Up*	Up*	Up*
ALOX5AP	Arachidonate 5-lipoxygenase activating protein	Up*	Up*	Up*	Up	Up*	Up*	Up*
Fibrosis/Tissue remodeling								
ALOX5	Arachidonate 5-lipoxygenase	Dn	Up	Up	Up	Up	Up*	Up*
CCR2	chemokine (C-C motif) receptor 2	Up	Dn	Dn	Up	Dn	Up*	Up*
CCL17	Chemokine (C-C motif) ligand 17	Up	Up	Up	Dn	Up	Up*	Up*
FAS	Fas (TNF receptor superfamily, member 6)	Up	Up	Up	Dn	Up	Up*	Up*
FOS	FBJ osteosarcoma oncogene	Up	Up	Up	Dn	Up*	Up*	Up*
JUNB	jun B proto-oncogene	Up	Up	Dn	Up	Up*	Up*	Up*
MMP8	Matrix metallopeptidase 8	Up*	Up*	Up	Up*	Up*	Up*	Up*
MMP9	Matrix metallopeptidase 9	Up	Up*	Up*	Up	Up*	Up*	Up*