TOXICOLOGICAL SCIENCES **122(2)**, 253–264 (2011)
doi:10.1093/toxsci/kfr125
Advance Access publication May 19, 2011

Blood Gene Expression Profiling Detects Silica Exposure and Toxicity

Rajendran Sellamuthu,* Christina Umbright,* Jenny R. Roberts,† Rebecca Chapman,† Shih-Houng Young,†, Diana Richardson,* Howard Leonard,† Walter McKinney,† Bean Chen,† David Frazer,† Shengqiao Li,‡ Michael Kashon,‡ and Pius Joseph*,[1]

*Toxicology and Molecular Biology Branch, †Pathology and Physiology Research Branch, and ‡Biostatistics and Epidemiology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505

[1]To whom correspondence should be addressed at MS 3014, Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, Morgantown, WV 26505. Fax: (304) 285-5708. E-mail: pcj5@cdc.gov.

Received February 23, 2011; accepted May 12, 2011

Blood gene expression profiling was investigated as a minimally invasive surrogate approach to detect silica exposure and resulting pulmonary toxicity. Rats were exposed by inhalation to crystalline silica (15 mg/m^3, 6 h/day, 5 days), and pulmonary damage and blood gene expression profiles were determined after latency periods (0–16 weeks). Silica exposure resulted in pulmonary toxicity as evidenced by histological and biochemical changes in the lungs. The number of significantly differentially expressed genes in the blood, identified by microarray analysis, correlated with the severity of silica-induced pulmonary toxicity. Functional analysis of the differentially expressed genes identified activation of inflammatory response as the major biological signal. Induction of pulmonary inflammation, as suggested by the blood gene expression data, was supported by significant increases in the number of macrophages and infiltrating neutrophils as well as the activity of pro-inflammatory chemokines observed in the lungs of the silica-exposed rats. A gene expression signature developed using the blood gene expression data predicted the exposure of rats to lower, minimally toxic and nontoxic concentrations of silica. Taken together, our findings suggest the potential application of peripheral blood gene expression profiling as a minimally invasive surrogate approach to detect pulmonary toxicity induced by silica in the rat. However, further research is required to determine the potential application of our findings specifically to monitor human exposure to silica and the resulting pulmonary effects.

Key Words: **silica; pulmonary toxicity; blood; gene expression.**

Occupational exposure to respirable crystalline silica is a major health threat to millions of workers in the United States and elsewhere (Sanderson, 1986). Repeated exposure to crystalline silica such as those taking place in sandblasting, silica milling, surface mining, and tunneling is associated with the development of autoimmune diseases, rheumatoid arthritis, chronic renal diseases, lupus, and cancer (Parks *et al.*, 1999; Steenland *et al.*, 2001). The major health effect of occupational exposure to crystalline silica, however, is the development of silicosis. Silicosis is an irreversible, but preventable, interstitial lung disease characterized by alveolar proteinosis and diffuse fibrosis, resulting in progressively restrictive lung function (Castranova and Vallyathan, 2000). Currently, chest x-ray and pulmonary function tests are employed to help detect silicosis. Both of these techniques, unfortunately, depend on the clinical manifestations of the disease and, therefore, may not predict or detect silicosis subclinically. Because silicosis is an irreversible and life-threatening pulmonary condition, from an intervention or prevention standpoint, any exposure to crystalline silica should be carefully monitored and documented. More importantly, practical and sensitive techniques capable of predicting the silicosis outcome well before the appearance of clinical symptoms of the disease should be developed and employed. In fact, the National Institute for Occupational Safety and Health (NIOSH) has recommended developing noninvasive or minimally invasive and sensitive techniques capable of predicting or detecting silicosis subclinically (NIOSH, 2002).

Traditionally, blood has been used as a surrogate tissue to detect toxicity or disease conditions in target organs that are not easily accessible for human experimental or epidemiological studies or clinical evaluations. Global gene expression profiling of peripheral blood has been employed successfully as a surrogate diagnostic approach to detect disease conditions affecting organs that are difficult to access (Glatt *et al.*, 2005; Scherzer *et al.*, 2007). There has also been limited effort recently to employ peripheral blood gene expression profiling to detect target organ toxicity induced by exposure of animals to toxic chemicals. The superior sensitivity of peripheral blood gene expression profiling, compared with the traditional biochemical, hematological, and histological toxicity markers, to detect hepatotoxicity has been demonstrated in the rat (Bushel *et al.*, 2007). In a subsequent study, Lobenhofer *et al.*

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the NIOSH.

(2008) demonstrated the classification of histopathological differences induced by hepatotoxic chemicals in the rat, likely reflecting differences in mechanisms of cell-specific toxicity, using blood transcriptomics data. Recently, we have reported the superior sensitivity of blood gene expression markers, compared with established biochemical markers of target organ toxicity, to detect hepatotoxicity and neurotoxicity induced by model chemicals in the rat (Umbright *et al.*, 2010).

Whether peripheral blood gene expression profiling may be used to detect and study pulmonary toxicity induced by toxic agents, including silica, is not known. Therefore, in compliance with the recommendation of NIOSH, we initiated a research project to investigate whether peripheral blood gene expression profiles may be employed as minimally invasive surrogate biomarkers to detect subclinical silicosis. As a prelude to this project, a proof-of-concept study was undertaken using rat as the experimental model to determine whether silica exposure and pulmonary damage induced by inhalation of crystalline silica could be detected by peripheral blood gene expression profiling. The results of our study demonstrated the potential application of peripheral blood gene expression profiling as a minimally invasive and efficient surrogate approach to detect silica exposure and study pulmonary toxicity induced by silica. In the future, this approach may lead to the development of biomarkers for subclinical silicosis in humans.

MATERIALS AND METHODS

Exposure of Rats to Silica Aerosol

Approximately 3 months old, pathogen-free male Fisher 344 rats (CDF strain) were purchased from Charles River Laboratories (Wilmington, MA) and used in this study. The entire study was conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International–approved animal facility (NIOSH, Morgantown, WV) following a protocol approved by the Institutional Animal Care and Use Committee. The rats, upon arrival, were allowed to acclimate to the animal facility for 10 days prior to their use in the study. Silica aerosol for inhalation exposure of the rats was generated from a bulk supply of Min-U-Sil 5 silica (U.S. Silica, Berkeley Springs, WV). An automated computer-controlled exposure system was used to deliver precise concentrations of uniformly dispersed airborne silica particles. The design of the silica aerosol exposure system was based on a modified version of a previously developed system (McKinney *et al.*, 2009). Because the silica powder is sticky and tends to form agglomerates, a Venturi disperser was installed downstream of the generator to break up the agglomerates. A diagram of the silica exposure system is shown in Supplementary figure 1. The mass median aerodynamic diameter of the airborne silica particles within the exposure chamber was 1.6 μm with a geometric standard deviation of 1.6 (Supplementary fig. 2). This size distribution is within the respirable range recommended for rats. For this study, target levels of temperature (22.2–25.6°C), humidity (40%–60%), and silica concentration (15 ± 1 mg/m^3) in the exposure chamber were monitored and controlled continuously by the exposure system. Rats were exposed to crystalline silica at a concentration of 15 mg/m^3, 6 h/day for five consecutive days. Rats exposed simultaneously to filtered air served as the controls. Throughout the period of the experiment, the rats were maintained on a 12-h light-dark schedule with free access to food and tap water except during the 6-h silica exposure period. Eight rats each from the control and silica-exposed groups were sacrificed at postexposure time intervals of 0, 1, 2, 4, 8, and 16 weeks for the determination of pulmonary toxicity and blood gene expression profile. Calculation of each of the postexposure time interval started from 16 h following termination of silica exposure on the fifth day.

In a separate experiment, rats were exposed to air (controls) or lower concentrations of silica (1 or 2 mg/m^3, 6 h/day, 5 days) and sacrificed 16 h following termination of the last day of silica exposure to determine pulmonary toxicity and a blood gene expression profile. The 16-h postexposure time interval selected to sacrifice these rats corresponded to the 0-week postexposure time interval of the rats, which were exposed to the higher silica concentration (15 mg/m^3, 6 h/day, 5 days).

Determination of Pulmonary Toxicity

At the above specified postexposure time intervals, the control and silica-exposed rats were euthanized with an ip injection of $\geq$ 100 mg sodium pentobarbital/kg body weight (Fort Dodge Animal Health, Fort Dodge, IA). Blood collected directly from the abdominal aorta was transferred to Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) containing EDTA as anticoagulant and further processed to determine a global gene expression profile and hematological parameters as described in corresponding sections below.

The right lung of the control and silica-exposed rats were clamped off and bronchoalveolar lavage (BAL) was performed in the left lung as previously described (Antonini and Roberts, 2007). The cellular and acellular fractions of the bronchoalveolar lavage fluid (BALF) collected from the left lung were used for further biochemical analysis of lung injury. The lower lobe of the right lung was inflated and preserved in 10% neutral-buffered formalin for histopathological analysis of lung injury.

Biochemical Parameters of Pulmonary Toxicity

Albumin and total protein contents, indices of alveolar epithelial integrity and lactate dehydrogenase (LDH) activity, a general cytotoxicity indicator, were determined in the acellular fraction of BALF obtained from the rats. The measurements were performed with a COBRAS MIRA autoanalyzer (Roche Diagnostic Systems, Mont Clair, NJ) as described previously (Antonini and Roberts, 2007; Porter *et al.*, 2001).

Determination of Silica-Induced Pulmonary Inflammation

Levels of inflammatory cytokines, monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-2 (MIP-2) were assayed in the acellular fraction of BALF. Protein concentrations of the selected cytokines were determined using ELISA kits (Invitrogen, Carlsbad, CA) and a Spectramax 250 plate spectrophotometer using Softmax Pro 2.6 software (Molecular Devices, Sunnyvale, CA). The cellular fraction of BALF was resuspended in 1 ml of PBS buffer, and the total number of cells (alveolar macrophages [AM] and polymorphonuclear leukocytes [PMNs]) was determined using a Coulter Multisizer II and Accu Comp software (Coulter Electronics, Hialeah, FL). BAL cells (5×10^4) were spun onto microscopic slides using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, UK) and stained with a Leukostat stain (Fisher Scientific, Pittsburgh, PA) to differentiate AM and PMN. Two hundred cells were counted per rat, and PMN percentages were multiplied back across the total cell count to obtain total AM and PMN numbers.

Histopathology of Lungs

The diaphragmatic lobes of the right lung from the control and silica-exposed rats were fixed in 10% neutral-buffered formaldehyde solution, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin. The slides were examined by board-certified pathologists (Experimental Pathology Laboratory, Inc., Sterling, VA) for histological changes.

Hematology

The total and differential white blood cell counts of the blood samples obtained from the control and silica-exposed rats were performed by the previously described flow cytometer procedure (Erdely *et al.*, 2011) using rat antibodies purchased from BD PharMingen, (San Diego, CA). The leukocytes

were separated by side scattering and forward scattering into three gates: lymphocytes, monocytes, and neutrophils. After collecting 3500 counting beads, the data were exported for further analysis using FlowJo software (Treestar, Costa Mesa, CA).

Gene Expression Profiling of Blood Samples

Preparation of globin messenger RNA-depleted total RNA. Total RNA was isolated from the blood samples using the Mouse Ribopure Blood Isolation Kit (Ambion, Inc., Austin, TX) following the protocol provided by the kit manufacturer. The total RNA isolated was digested with RNase-free DNase (Qiagen, Inc., Valencia, CA) and further purified using RNeasy Mini Kit (Qiagen, Inc.) following the recommendations of the supplier. Globin messenger RNA (mRNA), present in abundance in the blood RNA samples, was depleted using the Globinclear—Mouse/Rat Globin mRNA Removal Kit (Ambion Inc.). The integrity of the globin-depleted RNA samples thus obtained was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantitated by UV spectrophotometry. Only RNA samples exhibiting an RIN $\geq$ 8.0 were used in gene expression analysis.

Microarray analysis of gene expression profile. The global gene expression profile of the RNA samples purified from the blood samples of the control and silica-exposed rats was determined using RatRef-12 V1.0 Expression BeadChip array (Illumina, Inc., San Diego, CA). All microarray experiments were performed to comply with Minimal Information About a Microarray Experiment protocols. Biotin-labeled complementary RNA was generated from 375 ng globin mRNA-depleted RNA samples each by employing the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc.). Chip hybridizations, washing, Cy3-streptavidin staining, and scanning of the chips on the BeadStation 500 platform (Illumina, Inc.) were performed following the protocols provided by the manufacturer.

Metrics files from the bead scanner were checked to ensure that all samples fluoresced at comparable levels before samples were loaded into Beadstudio (Framework version 3.0.19.0) Gene Expression module v.3.0.14. Housekeeping, hybridization control, stringency, and negative control genes were checked for proper chip detection. BeadArray expression data were then exported with mean fluorescent intensity across like beads and bead variance estimates into flat files for subsequent analysis.

Illumina BeadArray expression data were analyzed in Bioconductor using the "lumi" and "limma" packages. Bioconductor is a project for the analysis and comprehension of genomic data and operates in R, a statistical computing environment (Ihaka and Gentleman, 1996). The lumi Bioconductor package was specifically developed to process Illumina microarrays and covers data input, quality control, variance stabilization, normalization, and gene annotation (Gentleman *et al.*, 2004). Normalized data were then analyzed using the limma package in R. In short, limma fits a linear model for each gene, generates group means of expression, and calculates p values and log fold changes that are converted to standard fold changes. The raw p values were corrected for false discovery rate (FDR) using the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995). Only genes with FDR p value $\leq$ 0.05 compared with the controls were considered as significantly differentially expressed and used as input for subsequent bioinformatic analysis using the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). IPA software is designed to map the biological relationship of the uploaded genes and classify them into categories according to published literature in the database.

Blood gene expression markers to detect/predict silica exposure and pulmonary toxicity. Whether a peripheral blood gene expression profile may be employed to develop a predictive molecular signature to detect exposure of rats to silica and the resulting pulmonary damage was investigated. The gene expression data obtained from the control rats and those exposed to silica at 15 mg/m^3, 6 h/day for 5 days (0-week postexposure group), were used as the training set to develop the predictive signature. Application of a test filter (alpha = 0.05) to the total number of genes detected on the chip using RNA from the training set identified 1115 genes. Thirty-five genes were selected from this group of genes using a correlation-based feature subset selection algorithm (Hall, 1999). A further reduction of the 35 gene signature was performed by ranking using Relief methodology (Kira and Rendel, 1992; Kononeko, 1994) and the top seven genes were selected as the predictor signature genes. The predictor signature thus identified was tested by using linear discriminant analysis (Duda, 2000) in the data obtained from a test set of rats that were exposed to lower concentrations of silica (1 or 2 mg/m^3, 6 h/day, 5 days).

Statistical analysis of the data. Statistical significance of the data (LDH, albumin, protein, PMNs, AMs, MIP-2, and MCP-1 in the BALF and neutrophils in the blood samples) presented as mean ± SE was analyzed by Student's "t"-test (comparison was done between the silica-exposed and the time-matched control group of rats). The level of statistical significance was set at $p < 0.05$.

RESULTS

BALF Parameters of Pulmonary Toxicity

With the exception of the 2-week postexposure time interval, the BALF parameters of pulmonary toxicity, viz, LDH activity and total protein and albumin content, were significantly ($p < 0.05$) elevated in the silica-exposed (15 mg/m^3, 6 h/day, 5 days) rats compared with the time-matched controls at all postexposure time intervals analyzed (Figs. 1A–C). In general, the time course of these parameters following the 5 days of silica exposure exhibited a biphasic or U-shaped response in the rats.

Lung Histopathology

Histological changes in the rat lungs, indicative of silica-induced pulmonary damage, exhibited a similar trend as that of the BALF parameters of pulmonary toxicity (Fig. 2). Acute inflammatory response, as evidenced from the accumulation of PMNs around small- and medium-sized airways, was noticed in all the silica-exposed rats at the 0-week postexposure time interval (Fig. 2, left panel). No significant histological change, indicating pulmonary toxicity, was noticed in the lungs of the silica-exposed rats during the intermediate postexposure time intervals of 1, 2, and 4 weeks. A further progression in histological changes of the lungs was noticed during the late postexposure time intervals of 8 and 16 weeks (Fig. 2, middle and right panels). In addition to infiltration of PMNs, the lungs of these rats exhibited a significant increase in the number of AMs both in the alveolar space and alveolar septa. The histological changes observed in the lungs of the silica-exposed rats were more significant at 16 weeks and consisted mainly of type II pneumocyte hyperplasia in addition to the infiltration of PMNs and accumulation of AMs.

Hematology

Compared with the control rats, a statistically significant ($p < 0.05$) increase was noticed in the percentage of neutrophils in the silica-exposed rats at postexposure time intervals of 2, 4, 8, and 16 weeks (Fig. 3). All other hematological parameters

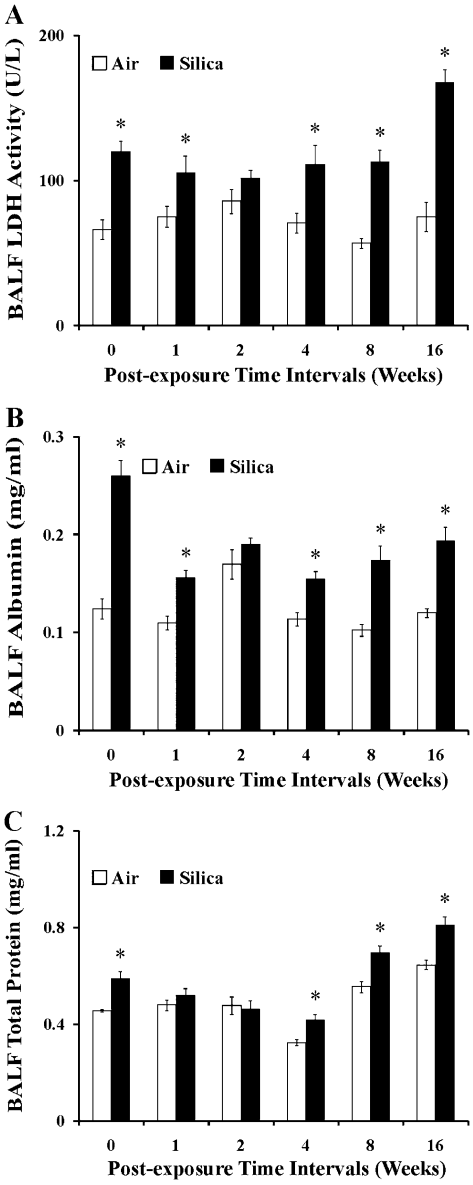

FIG. 1. BALF parameters of pulmonary damage in control and silica-exposed rats. Rats were exposed to silica (15 mg/m^3, 6 h/day, 5 days) or air (control). BALF parameters of pulmonary damage, viz, LDH (A), albumin (B), and total protein (C) were determined at the indicated postexposure time intervals. Values represent mean ± SE of eight rats per group. *Statistically significant ($p < 0.05$) compared with the time-matched control rats.

were comparable in the silica-exposed rats compared with the time-matched control rats at all postexposure time intervals analyzed (data not presented).

Blood Gene Expression Profile

Exposure of rats to silica resulted in significant differential expression (FDR $p \leq 0.05$) of a large number of genes in the blood of the rats at all postexposure time intervals analyzed. The number of differentially expressed genes in the blood of the silica-exposed rats at the various postexposure time

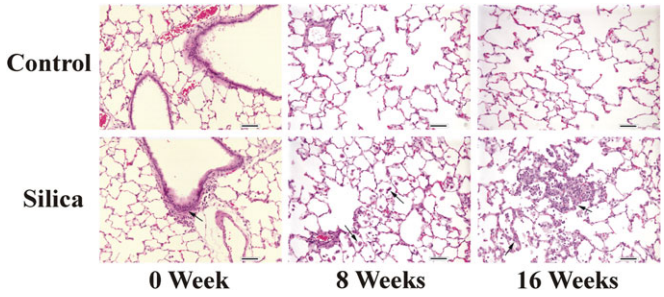

FIG. 2. Photomicrograph of the lung from control and silica-exposed rats. Rats were exposed to silica (15 mg/m^3, 6 h/day, 5 days) or air (control). Lung sections prepared at the indicated postexposure time intervals were stained with hematoxylin and eosin. Arrows indicate PMNs surrounding airways (left panel), AMs in alveolar space (middle panel), and type II pneumocyte hyperplasia (right panel). Magnification = ×20.

intervals followed the same U-shaped trend (Fig. 4) that was noticed in the case of indicators of pulmonary toxicity (BALF biochemical parameters and lung histology).

IPA analysis of the differentially expressed genes in the blood of the silica-exposed rats compared with the control rats identified several functional categories of genes such as inflammatory response, inflammatory diseases, cancer, respiratory diseases (lung damage), oxidative stress/free radical scavenging, energy production, and organismal injury and abnormalities (fibrosis) (Supplementary table 1). Activation of an inflammatory response was the most prominent biological signal identified in the blood of the silica-exposed rats. A selected list of genes belonging to cellular functions such as oxidative stress, inflammation, and fibrosis/tissue remodeling and their differential expression at each postexposure time interval is presented in Table 1, and the functional significance of their differential expression with respect to the silica-induced pulmonary damage is discussed in the "Discussion" section.

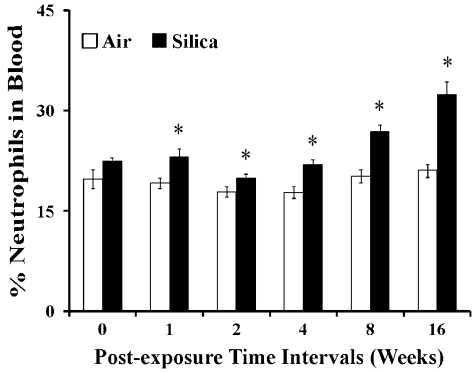

FIG. 3. Percent neutrophils in the peripheral blood of control and silica-exposed rats. Rats were exposed to silica (15 mg/m^3, 6 h/day, 5 days) or air (control). The number of neutrophils in the blood samples was determined at the indicated postexposure time intervals. Values represent mean ± SE of eight rats per group. *Statistically significant ($p < 0.05$) compared with the time-matched control rats.

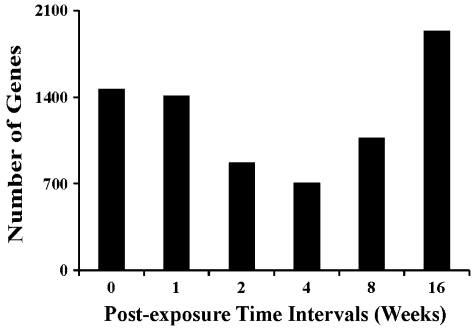

FIG. 4. Number of differentially expressed genes in the blood of the silica-exposed rats. Rats were exposed to silica (15 mg/m^3, 6 h/day, 5 days) or air (control). Global gene expression profile was determined in rats by microarray analysis, and the number of differentially expressed genes (FDR p value $\leq$ 0.05) in the silica-exposed rats compared with the controls at the indicated postexposure time intervals is presented. Values represent the mean of six rats per group.

Inflammatory Response in the Silica-Exposed Rats

The number of PMNs and AMs in the BALF of the silica-exposed rats was significantly ($p < 0.05$) higher than that of the controls (Figs. 5A and 5B, respectively) at most of the postexposure time intervals analyzed, suggesting the induction of pulmonary inflammation in response to silica exposure in the rats. The increase in the number of PMNs observed in the silica-exposed rats at the various postexposure time intervals followed the same U-shaped trend as those of the BALF parameters of pulmonary damage, histological changes, and differential blood gene expression profile. The BALF levels of inflammatory chemokines, MIP-2 and MCP-1, were significantly ($p < 0.05$) elevated in the silica-exposed rats compared with the controls at most of the postexposure time intervals analyzed (Figs. 5C and 5D, respectively), further supporting the activation of an inflammatory response in the silica-exposed rats as suggested by the blood gene expression data.

Detection of Silica Exposure/Pulmonary Toxicity Using Blood Gene Expression Signature

Exposure of rats to silica at the lower concentration of 2 mg/m^3, 6 h/day for 5 days, resulted in less severe but statistically significant pulmonary toxicity compared with those exposed to silica at 15 mg/m^3, 6 h/day for 5 days. This was evidenced by the observation of a significant ($p < 0.05$) elevation in the LDH activity and total protein and albumin content in the BALF of these rats (Figs. 6A–C). Lung histology of these rats was comparable to that of the controls (data not presented). Rats exposed to the lowest concentration of silica (1 mg/m^3, 6 h/day, 5 days) did not show any pulmonary toxicity as evidenced from results of BALF parameters of toxicity (Figs. 6A–C) and histological analysis (data not presented).

The blood gene expression signature consisting of the seven marker genes for pulmonary toxicity is presented in Table 2. Most of the marker genes (five out of seven) were involved in inflammatory response. All the genes belonging to the gene expression signature were differentially expressed in all eight rats that were exposed to silica at the highest dose of 15 mg/m^3, 6 h/day, 5 days (0-week postexposure group) (Table 3). Seven out of eight rats (87.5%) that were exposed to the lower, less toxic concentration of 2 mg/m^3 silica, 6 h/day, 5 days, were correctly identified as silica-exposed rats based on the expression pattern of the marker genes in their blood (Table 3). Six out of eight rats (75%) exposed to the nontoxic dose of 1 mg/m^3 silica, 6 h/day, 5 days, were correctly identified as belonging to silica-exposed groups by the blood gene expression signature (Table 3). The gene expression signature also identified the rats exposed to the higher, more toxic silica concentration (15 mg/m^3, 6 h/day, 5 days) at the various postexposure time intervals with significant accuracy (87.5% for the 1-week, 75% for the 2- and 4-week, and 100% for the 8- and 16-week postexposure time intervals) (data not presented).

DISCUSSION

Recent advances in toxicogenomics have demonstrated the superior sensitivity of gene expression changes taking place in target organs of laboratory animals as reliable and mechanistically informative indicators of target organ toxicity compared with the traditional biochemical and histological toxicity markers (Heinloth *et al.*, 2004). However, obtaining specimens of lung samples for routine monitoring of workers to determine their exposure to silica potentially capable of resulting in silicosis and other disease conditions is not feasible. This could be overcome by using surrogate tissues obtained by safe and minimally invasive procedures. For this to work, the gene expression changes taking place in the surrogate tissues must represent target organ toxicity accurately. Blood appears to be the most suitable surrogate tissue to detect gene expression changes relevant to target organ toxicity induced by toxic agents (Rockett, 2006). Therefore, studies were undertaken using a rat model to determine whether peripheral blood gene expression profiling can be employed as a minimally invasive surrogate approach to detect pulmonary damage induced by exposure to crystalline silica.

The present study was designed and conducted based on the results of several previous studies reported by investigators at NIOSH (Castranova *et al.*, 2002; Porter *et al.*, 2001, 2002a, 2002b). As expected and in agreement with the previous reports (Castranova *et al.*, 2002; Porter *et al.*, 2001, 2002a, 2002b), inhalation exposure of rats to silica resulted in mild to moderate pulmonary damage that was detected at all post-exposure time intervals with the exception of the 2-week interval. LDH activity—a sensitive indicator of general cytotoxicity—and protein and albumen contents—reliable indicators of alveolar epithelial integrity—were significantly elevated in the BALF of the silica-exposed rats compared with the time-matched controls. Similarly, lung histological changes, including type II pneumocyte hyperplasia, noticed

TABLE 1
Differentially Expressed Genes in the Blood of Silica-Exposed Rats Compared with the Time-Matched Controls

Gene		Postexposure time intervals (weeks)					
		0	1	2	4	8	16
Reactive oxygen species							
CYBA	Cytochrome b-245, alpha polypeptide	Up	Up*	Up*	Dn	Up	Up
NCF1	Neutrophil cytosolic factor 1	Up*	Up*	Up	Dn	Up	Up
NCF2	Neutrophil cytosolic factor 2	Up	Up*	Dn	Up	Up*	Up*
SOD2	Superoxide dismutase 2, mitochondrial	Up*	Dn	Dn	Dn	Up	Up
CAT	Catalase	Dn	Dn*	Dn*	Up	Dn	Dn
XDH	Xanthine dehydrogenase	Up*	Dn	Dn	Dn	Up*	Up
NOS2	Nitric oxide synthase, inducible (NOS type II) nitric oxide synthase 2, inducible	Up*	Dn	Up	Up	Up*	Up*
Antioxidant							
NFE2L2	Nuclear factor, erythroid-derived 2, like 2	Up*	Up	Up	Dn	Up*	Up*
FOS	FBJ osteosarcoma oncogene	Up	Up	Up	Dn	Up*	Up*
JUNB	Jun B proto-oncogene	Up	Up	Dn	Up	Up*	Up*
Danger signals or alarmins							
S100A8	S100 calcium-binding protein A8	Up*	Up*	Up*	Up	Up*	Up*
S100A9	S100 calcium-binding protein A9	Up	Up	Up	Dn	Up*	Up*
Inflammation							
NLRP3	NLR family, pyrin domain containing 3	Up*	Up	Up	Up	Up*	Up*
TLR2	Toll-like receptor 2	Up*	Up*	Up	Up	Up*	Up*
CLEC4E	C-type lectin domain family 4, member e	Up*	Up*	Up*	Dn	Up*	Up*
FPR1	Formyl peptide receptor 1	Up*	Up*	Up	Up	Up*	Up*
TLR13	Similar to toll-like receptor 13	Up*	Up	Up	Dn	Up*	Up*
TREM1	Triggering receptor expressed on myeloid cells 1	Up*	Up*	Up*	Dn	Up	Up*
CD44	Cd44 molecule	Up*	Up*	Up*	Dn	Up*	Up*
P2RX4	Purinergic receptor P2X, ligand-gated ion channel 4	Up*	Up	Up	Up	Up*	Up*
MYD88	Myeloid differentiation primary response gene 88	Up*	Up	Up	Up	Up*	Up*
CASP1	Caspase 1	Up*	Up	Up	Dn	Up	Up
IL1β	Interleukin 1 beta	Up*	Up	Up*	Up	Up*	Up*
PTAFR	Platelet-activating factor receptor	Up*	Up*	Up	Up	Up*	Up*
CXCR2	Interleukin 8 receptor, beta	Up*	Up	Up	Up	Up*	Up*
LGALS3	Lectin, galactoside-binding, soluble, 3	Up*	Up*	Up*	Up	Up*	Up*
ILR17A	Interleukin 17 receptor A	Up*	Up*	Up*	Dn	Up*	Up*
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein	Up*	Up*	Up*	Up	Up*	Up*
Fibrosis/tissue remodeling							
ALOX5	Arachidonate 5-lipoxygenase	Dn	Up	Up	Up	Up	Up*
CCR2	Chemokine (C-C motif) receptor 2	Up	Dn	Dn	Up	Dn	Up*
CCL17	Chemokine (C-C motif) ligand 17	Up	Up	Up	Dn	Up	Up*
FAS	Fas (TNF receptor superfamily, member 6)	Up	Up	Up	Dn	Up	Up*
FOS	FBJ osteosarcoma oncogene	Up	Up	Up	Dn	Up*	Up*
JUNB	Jun B proto-oncogene	Up	Up	Dn	Up	Up*	Up*
MMP8	Matrix metallopeptidase 8	Up*	Up*	Up	Up*	Up*	Up*
MMP9	Matrix metallopeptidase 9	Up	Up*	Up*	Up	Up*	Up*

Note. Up, Increased; Dn, decreased gene expression in the silica-exposed rats compared with the time-matched controls.
*Statistically significant (FDR $p \leq 0.05$) compared with the time-matched control rats.

in the rats further indicated silica-induced pulmonary damage. It is worth mentioning that, in agreement with the results of several previous studies (Porter *et al*., 2001, 2002b), all the pulmonary damage parameters (BALF LDH, albumen and protein, and lung histology) exhibited a biphasic or U-shaped response in the silica-exposed rats during the postexposure periods. It has been fairly well established that the pulmonary response to silica exposure progresses well even after cessation of the exposure (Porter *et al*., 2004). Typically, inhalation exposure to silica results in an initial acute inflammatory

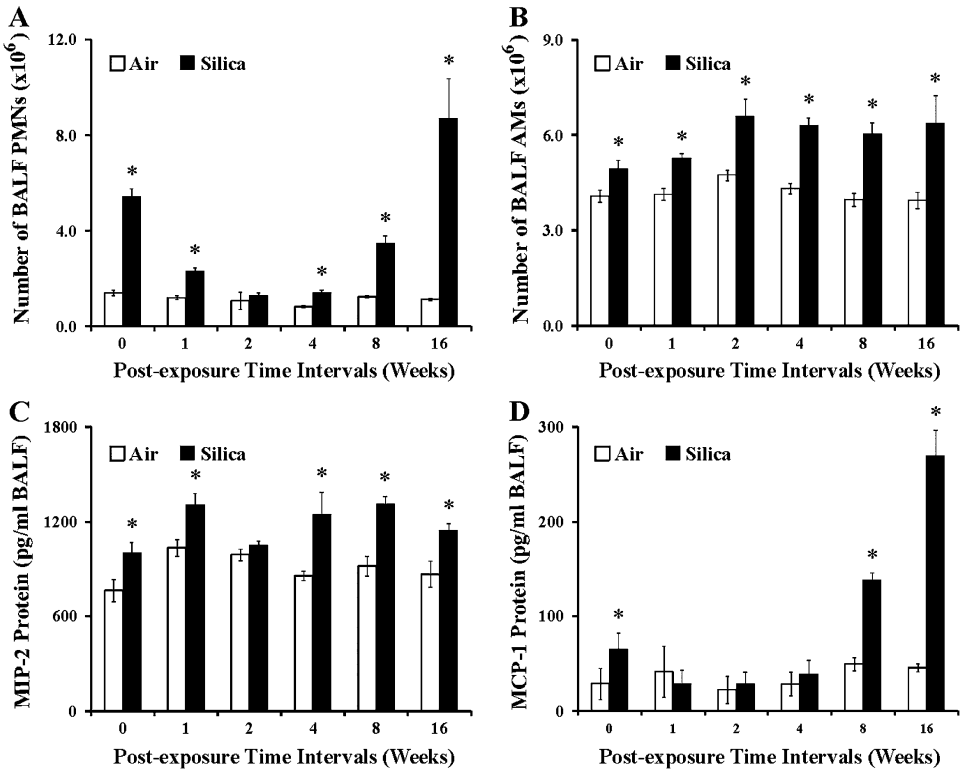

FIG. 5. BALF parameters of pulmonary inflammation in the control and silica-exposed rats. Rats were exposed to silica (15 mg/m^3, 6 h/day, 5 days) or air (control). BALF parameters of pulmonary inflammation, viz, number of PMNs (A), number of AMs (B), MIP-2 protein (C), and MCP-1 protein (D), were determined at the indicated postexposure time intervals. Values represent mean ± SE of eight rats per group. *Statistically significant ($p < 0.05$) compared with the corresponding control rats.

response, which is followed by further progression of the response culminating in the development of pulmonary fibrosis and silicosis. Such a response to silica exposure is attributed to the initial engulfment of silica particles by AMs and their later release along with apoptotic bodies, autoantigens, and inflammatory molecules following excessive apoptotic death of the AMs (Fubini and Hubbard, 2003; Hamilton *et al*., 2008). In addition, the relatively long persistence of silica in the body, continued recruitment of neutrophils and macrophages into lung alveoli, and the continued generation of biochemical mediators such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) and inflammatory cytokines that are involved in silica-induced pulmonary toxicity may account for the biphasic response (Absher *et al*., 1989; Castranova *et al*., 2002; Porter *et al*., 2001, 2002a, 2002b).

Traditionally, blood has been employed as the preferred surrogate tissue to detect toxicity induced by chemicals in target organs that are difficult to access. Thus, alterations in the blood level of certain biomolecules, e.g., specific enzymes, have been employed as reliable surrogate markers of target organ toxicity (Zira *et al*., 2009). Significant structural and/or functional impairment of the target organs has often been a prerequisite for observing alterations in the blood level of the majority of the traditionally employed toxicity biomarkers. In contrast, alterations in the global gene expression profile taking place in response to target organ toxicity have been detected in the blood even prior to the observation of histological and biochemical alterations indicative of target organ toxicity (Bushel *et al*., 2007; Umbright *et al*., 2010). This, along with the fact that small quantities of blood may be obtained by a minimally invasive procedure from almost every human being, has prompted the potential use of blood gene expression profiling as an attractive surrogate approach to detect target organ toxicity subclinically. In the present study, the number of significantly differentially expressed genes in the blood correlated with the various pulmonary damage parameters, viz, lung histology, LDH activity, and albumin and protein content in BALF. The observation that the number of significantly differentially expressed blood genes at various postexposure time intervals in the silica-exposed rats exhibited a biphasic or U-shaped response similar to the various parameters of pulmonary toxicity suggests the potential use of blood gene expression profiling as an alternative approach to detect silica-induced pulmonary damage.

In addition to demonstrating the potential of crystalline silica to cause pulmonary toxicity, numerous studies conducted over the past several years have provided valuable information regarding the mechanisms potentially underlying silica-induced pulmonary toxicity (Castranova *et al*., 2002; Porter *et al*., 2001, 2002a, 2002b). It is very well known that crystalline silica

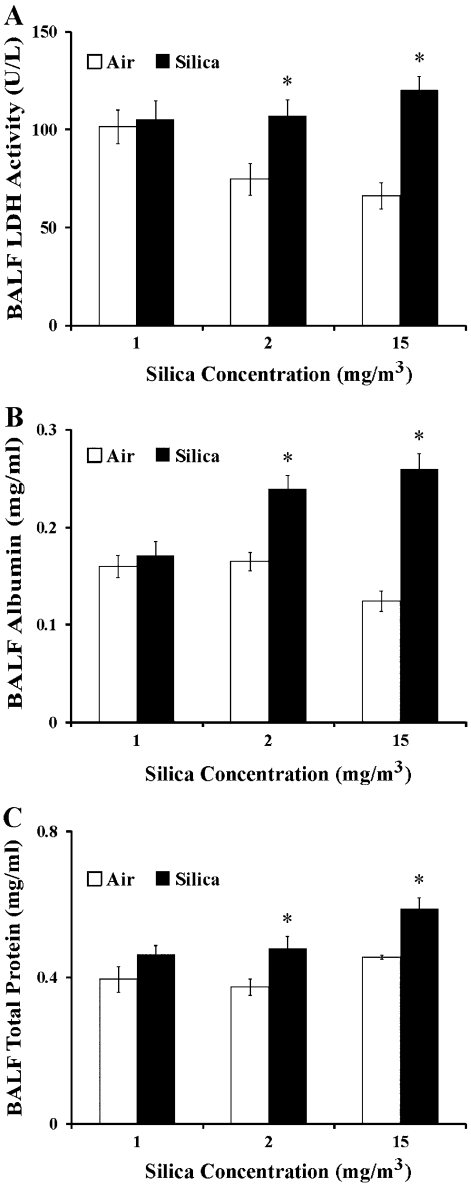

FIG. 6. BALF parameters of pulmonary damage in the control and silica-exposed rats. Rats were exposed to silica (1, 2, or 15 mg/m^3, 6 h/day, 5 days) or air (control). At 16 h following the last day of exposure to silica or air, all the rats were sacrificed and BALF parameters of pulmonary damage, viz, LDH activity (A), total protein (B), and albumin (C) were determined. Values represent mean ± SE of eight rats per group. *Statistically significant ($p < 0.05$) compared with the corresponding control rats.

particles, following inhalation exposure, interact with AMs and alveolar epithelium to result in the generation of ROS and RNS (Porter *et al.*, 2002a, 2002b), induction of inflammation (Castranova *et al.*, 2002), and pulmonary damage including fibrosis (Castranova *et al.*, 2002; Porter *et al.*, 2001) culminating in silicosis. As presented and discussed above, significant pulmonary damage was noticed in the rats exposed to crystalline silica in our study. Functional analysis of the blood genes that were differentially expressed in response to silica exposure indicated many cellular events taking place in the blood that are relevant to the silica-induced pulmonary damage. This, too, supports the use of blood gene expression profiling as an appropriate surrogate approach to detect and study the pulmonary effects of silica exposure.

Inhaled crystalline silica particles, either directly or due to their interaction with the AMs, result in the generation of ROS and RNS that play a significant role in silica-induced pulmonary damage (Porter *et al.*, 2002a, 2002b). Functional analysis of the differentially expressed genes in the blood of the silica-exposed rats supported the existing evidence for the involvement of ROS and RNS in the 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 silica-exposed rats. The nicotinamide adenine dinucleotide phosphate, reduced (NADPH) oxidase (NOX) family of NADPH oxidases (*NCF1, NCF2, CYBA,* and *CYBB*), *NOS2, XDH,* and *SOD2* were significantly overexpressed, whereas the expression of *catalase* was significantly downregulated in the blood of the silica-exposed rats. The NOX family of NADPH oxidases is involved in the generation of toxic superoxide anion (Bedard and Krause, 2007; Nunoi *et al.*, 1988). Similarly, through its involvement in purine metabolism, XDH plays a significant role in the generation of ROS (Bai *et al.*, 2005; Dobson *et al.*, 1988). Significant *SOD* overexpression along with downregulated catalase expression, as noticed in the blood of the 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 (Hamilton *et al.*, 2008). 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 silica-exposed rats (Chan *et al.*, 2001; Vulin and Stanley, 2004).

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 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 (Hamilton *et al.*, 2008). This is often associated with the release of intracellular molecules, referred to commonly as endogenous danger signals or alarmins (Cantin *et al.*, 1992; Eklund *et al.*, 1991), that play a significant role in the inflammatory response. The transcripts for two such alarmins, *S100A8* and *S100A9* (Foell *et al.*, 2007), were significantly overexpressed in the blood of the silica-exposed rats. The alarmins are recognized by innate immune receptors such as the pattern recognition receptors (PRRs) that

TABLE 2
Blood Genes Comprising the Prediction Signature for Crystalline Silica Exposure and Pulmonary Toxicity

Gene		Postexposure time interval (weeks)					
		0	1	2	4	8	16
LDHC	Lactate dehydrogenase C	Up*	Up*	Up*	Up*	Up*	Up*
CLEC4D	C-type lectin domain family 4, member D	Up*	Up	Dn	Up*	Up*	Up*
CLEC4E	C-type lectin domain family 4, member E	Up*	Up*	Up*	Dn	Up*	Up*
BST1	Bone marrow stromal cell antigen 1	Up*	Up*	Up*	Up	Up*	Up*
MMP8	Matrix metallopeptidase 8	Up*	Up*	Up	Up*	Up*	Up*
CD97	CD97 molecule	Up*	Up*	Up	Up	Up*	Up*
PIRA2	Leukocyte immunoglobulin-like receptor, subfamily B (with transmembrane and immunoreceptor tyrosine-based inhibitory motif domains), member 3-like; paired Ig-like receptor A2	Up*	Up*	Up*	Up	Up*	Up*

Note. Up, Increased and Dn, decreased gene expression in the silica-exposed rats compared with the time-matched controls.
*Statistically significant (FDR $p \leq 0.05$) compared with the time-matched control rats.

are involved in the induction of inflammation (Chen and Nunez, 2010). Transcripts for the *PRRs*, viz, *NLRP3, CLEC4E, TLR2,* and *FPR1*, were significantly overexpressed in the blood of the silica-exposed rats suggesting their potential activation and involvement in the 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* (Babelova *et al*., 2009; Sharif and Knapp, 2008; Yamasaki *et al*., 2009) were also significantly overexpressed in the blood of the silica-exposed rats suggesting their potential involvement in silica-induced inflammatory response. Transcript for *MyD88*, an adaptor molecule for *TLR* (Xiang and Fan, 2010), and *CASP1*, an adaptor molecule for the *NLRP3* inflammasome complex (Cassel *et al*., 2008), were also significantly overexpressed in the blood of the silica-exposed rats. A definite role for the *NLRP3* inflammasome complex in silicosis and asbestosis has been demonstrated previously (Cassel *et al*., 2008). 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 silica-induced pulmonary inflammation and damage, was significantly overexpressed in the blood of the silica-exposed rats (Table 1).

TABLE 3
Prediction of Crystalline Silica Exposure/Pulmonary Toxicity in Rats Using Blood Gene Expression Signature

Prediction accuracy		
15 mg/m^3 (training set)	2 mg/m^3 (test set 1)	1 mg/m^3 (test set 2)
8/8 = 100%	7/8 = 87.5%	6/8 = 75%

Induction of inflammation as a major biological signal in response to silica exposure, as indicated by the blood gene expression data, was further confirmed by results of pulmonary inflammation analysis in the rats. An increase in the number of AMs, as observed in the BALF of the silica-exposed rats in the present study, may be considered an adaptive inflammatory response to remove silica particles from the lungs. The significant increase in the pro-inflammatory cytokines, MIP-2 and MCP-1, in the BALF (Figs. 5C–D, respectively) as well as the significant increase in the number of infiltrating PMNs (Figs. 2 and 5A) in the lungs of the silica-exposed rats further supported the findings of the blood gene expression analysis. Thus, as expected and in agreement with the results of previous studies (Castranova *et al*., 2002; Porter *et al*., 2002a, 2002b) and as suggested by the blood gene expression data of the present study, 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 silica exposure, is characterized by the development of fibrosis resulting in progressively restrictive pulmonary function. Type II cell hyperplasia noticed in the lungs of the silica-exposed rats at the 16-week postexposure time interval may be considered an early indication of fibrosis (Degryse *et al*., 2010). Functional analysis of the differentially expressed genes in the blood of the 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 silica-exposed rats at the 8- and 16-week postexposure time intervals. Many tissue remodeling and fibrosis-related genes, e.g., *CCl17* (Belperio *et al*., 2004), *CCR2* (Moore *et al*., 2001; Okuma *et al*., 2004), *Fas*

(Chapman, 1999; Matute-Bello *et al*., 2007), *MMP8* (Garcia-Prieto *et al*., 2010), *MMP9* (Scabilloni *et al*., 2005), and *MyD88* (Gasse *et al*., 2007), were significantly overexpressed in the blood of the silica-exposed rats, especially at the 16-week postexposure time interval.

The findings of this study demonstrate the potential application of blood gene expression profiling to develop minimally invasive and, therefore, practical tests to detect subclinical silicosis that may not be detected by the currently available techniques. The blood gene expression signature consisting of the seven marker genes for pulmonary toxicity (Table 2) identified the rats that were exposed to slightly toxic (2 mg/m^3) and nontoxic (1 mg/m^3) concentrations of silica with significant accuracy (88 and 75%, respectively). This may be attributed to the superior sensitivity of blood gene expression markers, compared with lung histology and BALF biochemical parameters of toxicity, to detect silica-induced pulmonary damage. This argument is supported by previous reports (Bushel *et al*., 2007; Umbright *et al*., 2010) suggesting the superior sensitivity of blood gene expression changes as indicators of target organ toxicity compared with the already established biochemical and histological endpoints of toxicity. However, it remains to be determined whether some or all the gene expression changes that are noticed in the absence of significant biochemical and histological changes indicative of target organ toxicity may also represent physiological adaptive changes to protect the organs against the toxicity or repair processes involved in frank target organ toxicity.

The superior sensitivity of blood gene expression profiles to detect silica-induced subclinical pulmonary damage may have implications in early detection and prevention of occupational silicosis. Occupational exposure to silica typically takes place at very low concentrations and over a prolonged period of time. Chest x-ray and pulmonary function test, the currently employed techniques to detect silicosis, do not detect the disease subclinically. Because silicosis is an irreversible and potentially life-threatening pulmonary condition, the ability to detect subclinical silicosis by employing peripheral blood gene expression markers may provide the opportunity to apply strategies to intervene or prevent silicosis.

The findings of our study, for the first time, have demonstrated that peripheral blood gene expression profiling can be employed to detect pulmonary response to crystalline silica exposure. A major factor that would determine the potential application of blood gene expression profiling to detect the pulmonary effects of silica exposure in human is the specificity of the gene expression signature, i.e., whether the peripheral blood gene expression signature can specifically detect the pulmonary response induced by silica exposure. In this regard, 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 (Brown *et al*., 2002; 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 pulmonary toxicity in spite of the predominance of inflammatory response genes in both the signatures. Because most of the genes constituting the 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 silica-responsive blood gene expression signature was found differentially expressed in the blood of cigarette smokers (Charlesworth *et al*., 2010). Similarly, none of the blood signature genes identified for the silica-induced pulmonary toxicity was 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 silica (Cobben *et al*., 1997; Kuempel *et al*., 2003). The available evidence, therefore, suggests that the blood gene expression signature developed in our study may be specific to the pulmonary effects of silica exposure. Currently, we are conducting additional animal and human studies to determine the potential application of peripheral blood gene expression profiling as a practical approach to monitor human exposure to silica and the possible detection of preclinical silicosis. The outcome of these investigations is expected to have a major implication in the prevention/intervention of silicosis.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

ACKNOWLEDGMENTS

The authors thank Drs Anthony Laney, Michael Luster, and Ainsley Weston for critical review of the manuscript. The microarray data have been deposited in the Gene Expression

Omnibus Database, http://www.ncbi.nlm.nih.gov/geo (accession number GSE27023).

REFERENCES

Absher, M. P., Trombley, L., Hemenway, D. R., Mickey, R. M., and Leslei, K. O. (1989). Biphasic cellular and tissue response of rat lungs after eight-day aerosol exposure to the silicon dioxide cristobalite. *Am. J. Pathol.* **134,** 1243–1251.

Antonini, J. M., and Roberts, J. R. (2007). Chromium in stainless steel welding fume suppresses lung defense responses against bacterial infection in rats. *J. Immunotoxicol.* **4,** 117–127.

Babelova, A., Moreth, K., Tsalastra-Greul, W., Zeng-Brouwers, J., Eickelberg, O., Young, M. F., Bruckner, P., Pfeilschifter, J., Schaefer, R. M., Grone, H. J. *et al.* (2009). Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. *J. Biol. Chem.* **284,** 24035–24048.

Bai, X. C., Lu, D., Liu, A. L., Zhang, Z. M., Li, X. M., Zou, Z. P., Zeng, W. S., Cheng, B. L., and Luo, S. Q. (2005). Reactive oxygen species stimulates receptor activator of NF-kappaB ligand expression in osteoblast. *J. Biol. Chem.* **280,** 17497–17506.

Bedard, K., and Krause, K. H. (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol. Rev.* **87,** 245–313.

Belperio, J. A., Dy, M., Murray, L., Burdick, M. D., Xue, Y. Y., Strieter, R. M., and Keane, M. P. (2004). The role of the Th2 CC chemokine ligand CCL17 in pulmonary fibrosis. *J. Immunol.* **173,** 4692–4698.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser.* **57,** 289–300.

Bhalla, D. K., Hirata, F., Rishi, A. K., and Gairola, C. G. (2009). Cigarette smoke, inflammation, and lung injury: a mechanistic perspective. *J. Toxicol. Environ. Health B. Crit. Rev.* **12,** 45–64.

Brown, H. R., Ni, H., Benavides, G., Yoon, L., Hyder, K., Giridhar, J., Gardner, G., Tyler, R. D., and Morgan, K. T. (2002). Correlation of simultaneous differential gene expression in the blood and heart with known mechanisms of adriamycin-induced cardiomyopathy in the rat. *Toxicol. Pathol.* **30,** 452–469.

Bushel, P. R., Heinloth, A. N., Li, J., Huang, L., Chou, J. W., Boorman, G. A., Malarkey, D. E., Houle, C. D., Ward, S. M., Wilson, R. E. *et al.* (2007). Blood gene expression signatures predict exposure levels. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 18211–18216.

Calvano, S. E., Xiao, W., Richards, D. R., Felciano, R. M., Baker, H. V., Cho, R. J., Chen, R. O., Brownstein, B. H., Cobb, J. P., Tschoeke, S. K., *et al.* (2005). A network-based analysis of systemic inflammation in humans. *Nature* **437,** 1032–1037.

Canettieri, G., Coni, S., Della Guardia, M., Nocerino, V., Antonucci, L., Di Magno, L., Screaton, R., Screpanti, I., Giannini, G., and Gulino, A. (2009). The coactivator CRTC1 promotes cell proliferation and transformation via AP-1. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 1445–1450.

Cantin, A. M., Larivee, P., Martel, M., and Begin, R. (1992). Hyaluronan (hyaluronic acid) in lung lavage of asbestos-exposed humans and sheep. *Lung* **170,** 211–220.

Cassel, S. L., Eisenbarth, S. C., Iyer, S. S., Sadler, J. J., Colegio, O. R., Tephly, L. A., Carter, A. B., Rothman, P. B., Flavell, R. A., and Sutterwala, F. S. (2008). The Nalp3 inflammasome is essential for the development of silicosis. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 9035–9040.

Castranova, V., Porter, D., Millecchia, L., Ma, J. Y., Hubbs, A. F., and Teass, A. (2002). Effect of inhaled crystalline silica in a rat model: time course of pulmonary reactions. *Mol. Cell. Biochem.* **234–235,** 177–184.

Castranova, V., and Vallyathan, V. (2000). Silicosis and coal workers' pneumoconiosis. *Environ. Health Perspect.* **108**(Suppl. 4), 675–684.

Chan, K., Han, X. D., and Kan, Y. W. (2001). An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 4611–4616.

Chapman, H. A. (1999). A Fas pathway to pulmonary fibrosis. *J. Clin. Invest.* **104,** 1–2.

Charlesworth, J. C., Curran, J. E., Johnson, M. P., Goring, H. H., Dyer, T. D., Diego, V. P., Kent, J. W., Jr., Mahaney, M. C., Almasy, L., MacCluer, J. W. *et al.* (2010). Transcriptomic epidemiology of smoking: the effect of smoking on gene expression in lymphocytes. *BMC Med. Genomics.* **3,** 29.

Chen, G. Y., and Nunez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* **10,** 826–837.

Cobben, N. A., Drent, M., Schols, A. M., Lamers, R. J., Wouters, E. F., and Van Dieijen-Visser, M. P. (1997). Serum lactate dehydrogenase and its isoenzyme pattern in ex-coalminers. *Respir. Med.* **91,** 616–623.

Degryse, A. L., Tanjore, H., Xu, X. C., Polosukhin, V. V., Jones, B. R., McMahon, F. B., Gleaves, L. A., Blackwell, T. S., and Lawson, W. E. (2010). Repetitive intratracheal bleomycin models several features of idiopathic pulmonary fibrosis. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **299,** L442–L452.

Dobson, M. E., Stern, R. O., and Kane, A. B. (1988). Selective purine release from P388D1 macrophages injured by silica. *J. Cell. Physiol.* **135,** 244–252.

Drent, M., Cobben, N. A., Henderson, R. F., Wouters, E. F., and van Dieijen-Visser, M. (1996). Usefulness of lactate dehydrogenase and its isoenzymes as indicators of lung damage or inflammation. *Eur. Respir. J.* **9,** 1736–1742.

Duda, R. O. (2000). Linear discriminant functions. In *Pattern Classification*, 2nd ed. (D. G. Stock, Ed.), pp. 215–281. Wiley Interscienses, Hoboken, NJ.

Eklund, A., Tornling, G., Blaschke, E., and Curstedt, T. (1991). Extracellular matrix components in bronchoalveolar lavage fluid in quartz exposed rats. *Br. J. Ind. Med.* **48,** 776–782.

Erdely, A., Liston, A., Salmen-Muniz, R., Hulderman, T., Young, S.-H., Ziedler-Erdely, P., Castranova, V., and Simeonova, P. (2011). Identification of systemic markers from a pulmonary carbon nanotube exposure. *J. Occup. Environ. Med.* **53,** S80–S86.

Foell, D., Wittkowski, H., Vogl, T., and Roth, J. (2007). S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *J. Leukoc. Biol.* **81,** 28–37.

Fubini, B., and Hubbard, A. (2003). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. *Free Radic. Biol. Med.* **34,** 1507–1516.

Garcia-Prieto, E., Gonzalez-Lopez, A., Cabrera, S., Astudillo, A., Gutierrez-Fernandez, A., Fanjul-Fernandez, M., Batalla-Solis, E., Puente, X. S., Fueyo, A., *et al.* (2010). Resistance to bleomycin-induced lung fibrosis in MMP-8 deficient mice is mediated by interleukin-10. *PLoS One* **5,** e13242.

Gasse, P., Mary, C., Guenon, I., Noulin, N., Charron, S., Schnyder-Candrian, S., Schnyder, B., Akira, S., Quesniaux, V. F., Lagente, V. *et al.* (2007). IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. *J. Clin. Invest.* **117,** 3786–3799.

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., *et al.* (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5,** R80.

Glatt, S. J., Everall, I. P., Kremen, W. S., Corbeil, J., Sasik, R., Khanlou, N., Han, M., Liew, C. C., and Tsuang, M. T. (2005). Comparative gene expression analysis of blood and brain provides concurrent validation of SELENBP1 up-regulation in schizophrenia. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 15533–15538.

Hall, M. (1999). *Correlation-Based Feature Selection for Machine Learning*. PhD Thesis. Department of Computer Science, Waikato University. Waikato, NZ.

Hamilton, R. F., Jr., Thakur, S. A., and Holian, A. (2008). Silica binding and toxicity in alveolar macrophages. *Free Radic. Biol. Med.* **44,** 1246–1258.

Heinloth, A. N., Irwin, R. D., Boorman, G. A., Nettesheim, P., Fannin, R. D., Sieber, S. O., Snell, M. L., Tucker, C. J., Li, L., Travlos, G. S., *et al.* (2004). Gene expression profiling of rat livers reveals indicators of potential adverse effects. *Toxicol. Sci.* **80,** 193–202.

Ihaka, R., and Gentleman, R. (1996). R: a language for data analysis and graphics. *J. Comput. Graph. Stat.* **5,** 299–314.

Kira, K., and Rendel, L. A. (1992). A practical approach to feature selection. In *Procedings of the International Conference on Machine Learning* (D. Sleeman and P. Edwards, Eds.), pp. 249–256. Morgan Kauffman, San Francisco, CA.

Kononeko, I. (1994). Estimating attributes: analysis and extensions of relief. In *Machine Learning: ECML-94* (L. De Raedt and F. Bergadano, Eds.), pp. 171–182. Springer Verlag, New York, NY.

Kuempel, E. D., Attfield, M. D., Vallyathan, V., Lapp, N. L., Hale, J. M., Smith, R. J., and Castranova, V. (2003). Pulmonary inflammation and crystalline silica in respirable coal mine dust: dose-response. *J. Biosci.* **28,** 61–69.

Lobenhofer, E. K., Auman, J. T., Blackshear, P. E., Boorman, G. A., Bushel, P. R., Cunningham, M. L., Fostel, J. M., Gerrish, K., Heinloth, A. N., Irwin, R. D., *et al.* (2008). Gene expression response in target organ and whole blood varies as a function of target organ injury phenotype. *Genome Biol.* **9,** R100.

Matute-Bello, G., Wurfel, M. M., Lee, J. S., Park, D. R., Frevert, C. W., Madtes, D. K., Shapiro, S. D., and Martin, T. R. (2007). Essential role of MMP-12 in Fas-induced lung fibrosis. *Am. J. Respir. Cell. Mol. Biol.* **37,** 210–221.

McKinney, W., Chen, B., and Frazer, D. (2009). Computer controlled multi-walled carbon nanotube inhalation exposure system. *Inhal. Toxicol.* **21,** 1053–1061.

Miyamoto, M., Yanai, M., Ookubo, S., Awasaki, N., Takami, K., and Imai, R. (2008). Detection of cell-free, liver-specific mRNAs in peripheral blood from rats with hepatotoxicity: a potential toxicological biomarker for safety evaluation. *Toxicol. Sci.* **106,** 538–545.

Moore, B. B., Paine, R., III., Christensen, P. J., Moore, T. A., Sitterding, S., Ngan, R., Wilke, C. A., Kuziel, W. A., and Toews, G. B. (2001). Protection from pulmonary fibrosis in the absence of CCR2 signaling. *J. Immunol.* **167,** 4368–4377.

National Institute for Occupational Safety and Health (NIOSH). (2002). *Hazard Review: Health Effects of Occupational Exposures to Respirable Crystalline Silica.* US Department of Health and Human Services (NIOSH). Publication No. 2002-129 Available at: http://www.cdc.gov/niosh/docs/2002-129/02-129a.html. Accessed January 21, 2011.

Nunoi, H., Rotrosen, D., Gallin, J. I., and Malech, H. L. (1988). Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. *Science* **242,** 1298–1301.

Okuma, T., Terasaki, Y., Kaikita, K., Kobayashi, H., Kuziel, W. A., Kawasuji, M., and Takeya, M. (2004). C-C chemokine receptor 2 (CCR2) deficiency improves bleomycin-induced pulmonary fibrosis by attenuation of both macrophage infiltration and production of macrophage-derived matrix metalloproteinases. *J. Pathol.* **204,** 594–604.

Parks, C. G., Conrad, K., and Cooper, G. S. (1999). Occupational exposure to crystalline silica and autoimmune disease. *Environ. Health Perspect.* **107**(Suppl. 5), 793–802.

Peretz, A., Peck, E. C., Bammler, T. K., Beyer, R. P., Sullivan, J. H., Trenga, C. A., Srinouanprachnah, S., Farin, F. M., and Kaufman, J. D. (2007). Diesel exhaust inhalation and assessment of peripheral blood mononuclear cell gene transcription effects: an exploratory study of healthy human volunteers. *Inhal. Toxicol.* **19,** 1107–1119.

Porter, D. W., Hubbs, A. F., Mercer, R., Robinson, V. A., Ramsey, D., McLaurin, J., Khan, A., Battelli, L., Brumbaugh, K., Teass, A. *et al.* (2004). Progression of lung inflammation and damage in rats after cessation of silica inhalation. *Toxicol. Sci.* **79,** 370–380.

Porter, D. W., Millecchia, L., Robinson, V. A., Hubbs, A., Willard, P., Pack, D., Ramsey, D., McLaurin, J., Khan, A., Landsittel, D., *et al.* (2002a). Enhanced nitric oxide and reactive oxygen species production and damage after inhalation of silica.. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **283,** L485–L493.

Porter, D. W., Ramsey, D., Hubbs, A. F., Battelli, L., Ma, J., Barger, M., Landsittel, D., Robinson, V. A., McLaurin, J., Khan, A. *et al.* (2001). Time course of pulmonary response of rats to inhalation of crystalline silica: histological results and biochemical indices of damage, lipidosis, and fibrosis. *J. Environ. Pathol. Toxicol. Oncol.* **20**(Suppl. 1), 1–14.

Porter, D. W., Ye, J., Ma, J., Barger, M., Robinson, V. A., Ramsey, D., McLaurin, J., Khan, A., Landsittel, D., Teass, A. *et al.* (2002b). Time course of pulmonary response of rats to inhalation of crystalline silica: NF-kappa B activation, inflammation, cytokine production, and damage. *Inhal. Toxicol.* **14,** 349–367.

Rockett, J. C. (2006). Blood-derived transcriptomic profiles as a means to monitor levels of toxicant exposure and the effects of toxicants on inaccessible target tissues. In *Surrogate Tissue Analysis* (M. E. Burczynski and J. C. Rockett, Eds.), pp. 65–76. Taylor & Francis, Boca Raton, FL.

Sanderson, W. (1986). The U.S. population-at-risk to occupational respiratory diseases. In *Occupational Respiratory Diseases* (J. A. Merchant, Ed.). Department of Health and Human Services (NIOSH). Publication 86-102, Washington, DC.

Scabilloni, J. F., Wang, L., Antonini, J. M., Roberts, J. R., Castranova, V., and Mercer, R. R. (2005). Matrix metalloproteinase induction in fibrosis and fibrotic nodule formation due to silica inhalation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **288,** L709–L717.

Scherzer, C. R., Eklund, A. C., Morse, L. J., Liao, Z., Locascio, J. J., Fefer, D., Schwarzschild, M. A., Schlossmacher, M. G., Hauser, M. A. *et al.* (2007). Molecular markers of early Parkinson's disease based on gene expression in blood. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 955–960.

Sharif, O., and Knapp, S. (2008). From expression to signaling: roles of TREM-1 and TREM-2 in innate immunity and bacterial infection. *Immunobiology* **213,** 701–713.

Steenland, K., Mannetje, A., Boffetta, P., Stayner, L., Attfield, M., Chen, J., Dosemeci, M., DeKlerk, N., Hnizdo, E., Koskela, R., *et al.* (2001). Pooled exposure-response analyses and risk assessment for lung cancer in 10 cohorts of silica-exposed workers: an IARC multicentre study. *Cancer Causes Control* **12,** 773–784.

Umbright, C., Sellamuthu, R., Li, S., Kashon, M., Luster, M., and Joseph, P. (2010). Blood gene expression markers to detect and distinguish target organ toxicity. *Mol. Cell. Biochem.* **335,** 223–234.

Vulin, A. I., and Stanley, F. M. (2004). Oxidative stress activates the plasminogen activator inhibitor type 1 (PAI-1) promoter through an AP-1 response element and cooperates with insulin for additive effects on PAI-1 transcription. *J. Biol. Chem.* **279,** 25172–25178.

Wetmore, B. A., Brees, D. J., Singh, R., Watkins, P. B., Andersen, M. E., Loy, J., and Thomas, R. S. (2010). Quantitative analyses and transcriptomic profiling of circulating messenger RNAs as biomarkers of rat liver injury. *Hepatology* **51,** 2127–2139.

Xiang, M., and Fan, J. (2010). Pattern recognition receptor-dependent mechanisms of acute lung injury. *Mol. Med.* **16,** 69–82.

Yamasaki, K., Muto, J., Taylor, K. R., Cogen, A. L., Audish, D., Bertin, J., Grant, E. P., Coyle, A. J., Misaghi, A., Hoffman, H. M. *et al.* (2009). NLRP3/cryopyrin is necessary for interleukin-1beta (IL-1beta) release in response to hyaluronan, an endogenous trigger of inflammation in response to injury. *J. Biol. Chem.* **284,** 12762–12771.

Zira, A., Mikros, E., Giannioti, K., Galanopoulou, P., Papalois, A., Liapi, C., and Theocharis, S. (2009). Acute liver acetaminophen toxicity in rabbits and the use of antidotes: a metabonomic approach in serum. *J. Appl. Toxicol.* **29,** 395–402.