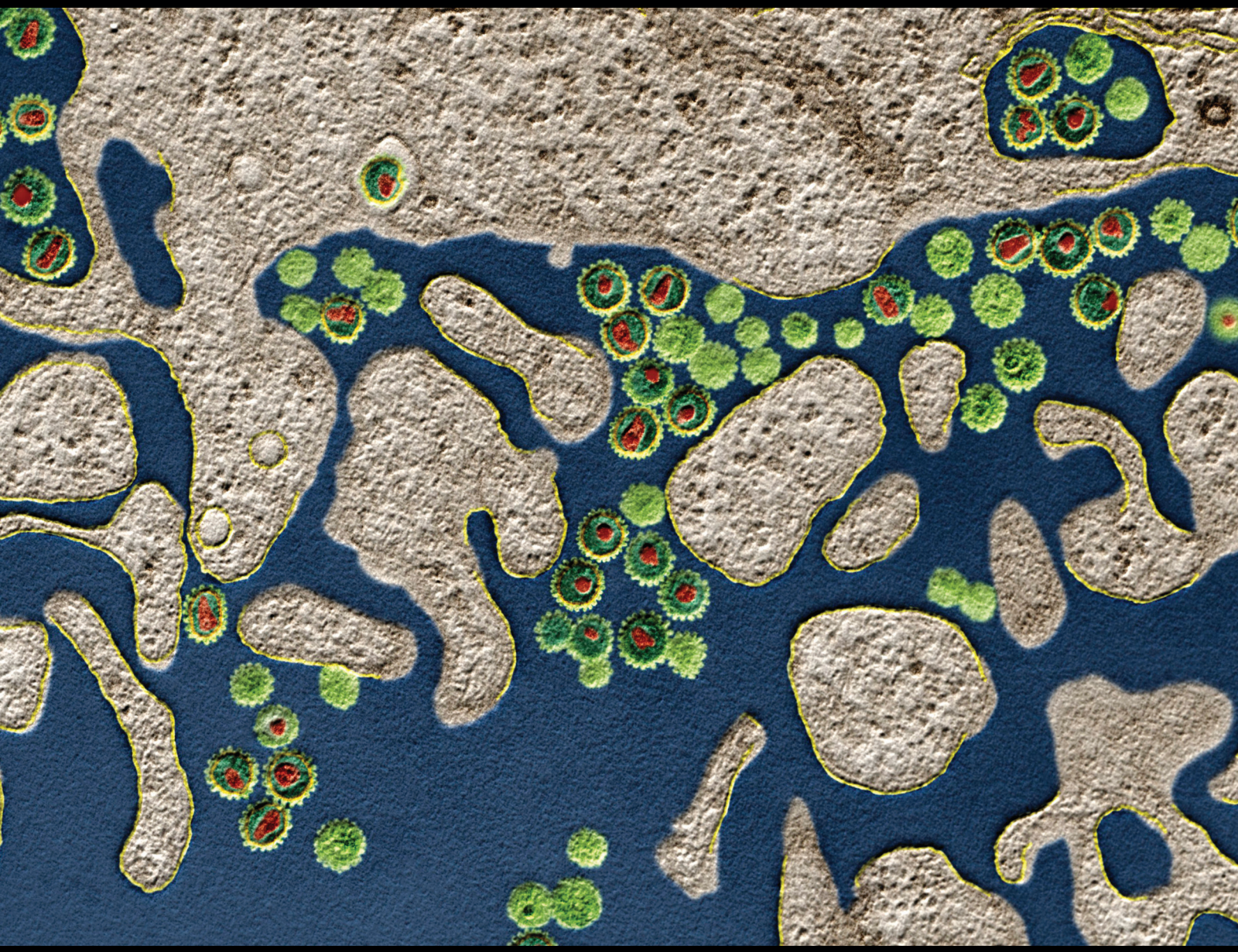
Journal of Immunology Research

Immunological Effects of Environmental Factors: Focus on the Fibrous and Particulated Materials

Guest Editors: Takemi Otsuki, Andrij Holian, and Mario Di Gioacchino



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Editorial Board

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 697438, 1 page
http://dx.doi.org/10.1155/2014/697438

Editorial

Immunological Effects of Environmental Factors: Focus on the Fibrous and Particulated Materials

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Received 2 November 2014; Accepted 2 November 2014; Published 21 December 2014

On behalf of guest editors for this special issue, we are very pleased to publish this special interest issue. The immunological effects of various fibrous and particulate materials such as asbestos and silica as well as nanoparticles and nanotubes have received increased attention in recent years. These materials are now well known for their biological effects that include lung fibrosis and carcinogenic potential, which is the basis for the scientific interest for environmental health. Furthermore, the immunological effects of these substances are also very important issues since, from the point of initial administration of these materials, the innate immune system will recognize these materials as foreign danger signals and they may affect the systemic immune system. In this special issue, various recent investigations regarding the above-mentioned viewpoints will provide readers with recent advances in the area of environmental immunology. We hope that this special issue will contribute to the better understanding and considerations for biological effects of fibrous and particulate materials, particularly on the human immune systems.

Takemi Otsuki
Andrij Holian
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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 937987, 8 pages
http://dx.doi.org/10.1155/2014/937987

Review Article

Cytokine Network Involvement in Subjects Exposed to Benzene

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Received 10 May 2014; Revised 30 July 2014; Accepted 4 August 2014; Published 18 August 2014

Academic Editor: Andrij Holian

Benzene represents an ubiquitous pollutant both in the workplace and in the general environment. Health risk and stress posed by benzene have long been a concern because of the carcinogenic effects of the compound which was classified as a Group 1 carcinogen to humans and animals. There is a close correlation between leukemia, especially acute myeloid leukemia, and benzene exposure. In addition, exposure to benzene can cause harmful effects on immunological, neurological, and reproductive systems. Benzene can directly damage hematopoietic progenitor cells, which in turn could lead to apoptosis or may decrease responsiveness to cytokines and cellular adhesion molecules. Alternatively, benzene toxicity to stromal cells or mature blood cells could disrupt the regulation of hematopoiesis, including hematopoietic commitment, maturation, or mobilization, through the network of cytokines, chemokines, and adhesion molecules. Today there is mounting evidence that benzene may alter the gene expression, production, or processing of several cytokines *in vitro* and *in vivo*. The purpose of this review was to systematically analyze the published cases of cytokine effects on human benzene exposure, particularly hematotoxicity, and atopy, and on lungs.

1. Introduction

Benzene is a clear, colorless, flammable liquid with a gasoline-like odor that can volatilize to vapors in air. It is an organic compound found most often in air as a result of emissions from burning coal and oil, gasoline vapors, motor vehicle exhaust, cigarette smoke, wood-burning fires, and other sources. Environmental contamination of benzene mainly comes from industrial uses through improper discharge and vehicle traffic. Means of transportation emissions represent the major source of benzene in urban ambient air, while its concentrations in indoor air are significantly increased in homes where people smoke.

The two primary sources of industrial exposure to benzene are activities associated with both its synthesis and its use to produce other chemicals. A number of other occupations may be exposed to benzene through the use of petroleum products or solvents (reviewed in [1]). In occupational settings the primary contact routes are generally inhalation and dermal absorption, and exposure assessment can be relatively straightforward. On the other hand, assessment of benzene exposure for general population is harder to quantify because of the differences in lifestyles, weather conditions, and living environments [1].

Human exposure to benzene can occur not only through inhalation and dermal absorption, but also through ingestion of food and drinking water [2]. Elevated concentrations of benzene in the groundwater could cause potential risk to human health and change the diversity and structure of ecological systems [3]. In addition, natural gas has been reported

as a source of benzene contamination in groundwater [4]. In both occupational and general populations another source of benzene exposure is the active and passive tobacco smoking [1].

Benzene is converted in the liver into toxic metabolites such as benzene oxide, phenol, hydroquinone (HQ), catechol, 1,2,4-benzenetriol, and a ring-opened product, *trans,trans*-muconic acid [5]. The selective toxicity of benzene for hematopoietic tissue is apparently connected with the capacity of these hepatic metabolites to specifically accumulate in the bone marrow [6].

Health risk and stress posed by benzene have long been a concern because of the carcinogenic effects of the compound which was classified as a Group 1 carcinogen to humans and animals [7]. For instance, epidemiological and laboratory studies have shown a close correlation between leukemia, especially acute myeloid leukemia (AML), and benzene exposure [8, 9]. Moreover, there is limited evidence that benzene may also cause acute and chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and multiple myeloma and aplastic anaemia [8, 10].

Recently, relatively low-level exposure to benzene was associated with an increased risk of myelodysplastic syndrome (MDS) [11].

In addition, exposure to benzene can cause harmful effects on immunological, neurological, and reproductive systems [12]. Benzene can reduce both B-cell and T-cell proliferation, decrease the host resistance to infection in animals exposed to it, and produce chromosomal aberrations in human peripheral lymphocytes [10].

Cytokines are signaling molecules that mediate and regulate immunity, inflammation, hematopoiesis, and many other cellular processes, forming a cytokine network. Cytokines can be produced by immune and nonimmune cells. T helper cell (Th) subsets are regulators of the adaptive immune response against infection. Th1-type cells produce cytokines which activate macrophages and promote cell-mediated immunity, protective against intracellular infections. Th2-type cells produce a variety of anti-inflammatory cytokines that promote humoral immune responses against extracellular pathogens. Th17 cells are a subset of $CD4^+$ T cells that play a critical role in clearing pathogens during host defence reactions and in inducing tissue inflammation in autoimmune disease. Regulatory T cells (T_{reg}) are thought to be the master regulators of the immune response in humans. Defects in the transcription factor forkhead box protein 3 (FoxP3), which defines the T_{reg} lineage, result in multiple autoimmune diseases and atopy [13].

Benzene can directly damage hematopoietic progenitor cells, which in turn could lead to apoptosis or may decrease responsiveness to cytokines and cellular adhesion molecules. Alternatively, benzene toxicity to stromal cells or mature blood cells could disrupt the regulation of hematopoiesis, including hematopoietic commitment, maturation, or mobilization, through the network of cytokines, chemokines, and adhesion molecules [14–18]. Accordingly, hematotoxic effects could be enhanced among individuals exposed to benzene who have genetic variants that alter key pathways that regulate hematopoiesis (reviewed in [19]).

Today there is mounting evidence that benzene may alter the gene expression, production, or processing of several cytokines *in vitro* and *in vivo*.

The purpose of this review was to systematically analyze the published studies about cytokine effects on human benzene exposure.

1.1. Hematotoxicity. Benzene appears to cause hematotoxicity through multiple mechanisms that may involve alterations in the expression of numerous genes and proteins, DNA methylation patterns, and miRNA profiles, even at low doses [20].

Toxicogenomic studies, including genome-wide analyses of susceptibility genes (genomics), gene expression (transcriptomics), protein expression (proteomics), and epigenetic modifications (epigenomics), of human populations exposed to benzene are crucial for understanding gene environment interactions and providing the ability to develop biomarkers of exposure, early effect, and susceptibility [20].

Effects of benzene and its metabolites on the immune system can be investigated through the expression of cytokines and chemokines (Table 1). Stimulation of some inflammatory cytokines production by human peripheral blood mononuclear cells (PBMC) and the inhibition of others were observed in response to benzene treatment in the work by Gillis et al. [21]. The authors showed a dose-dependent increase in the production of TNF-α by activated PBMC for all benzene metabolites. Also, IL-6 concentration was increased only by treatment with catechol, benzenetriol, and BQ, while IFN-γ production was increased by HQ treatment. In contrast, secretion of IL-1β and GM-SCF was suppressed by HQ and catechol. IL-2 production was decreased by BQ treatment [21].

Renz and Kalf in 1991 demonstrated that HQ prevents the proteolytic conversion of 31 kDa pre-IL-lα to the mature cytokine by the processing protease calpain in purified murine stromal macrophages [22]. The first study that examined the role of HQ in the release of IL-1α and IL-1β by mononuclear phagocytes in humans was in 1995 [23]. The results of the study showed a dose-dependent reduction of IL-1 secretion by HQ that also determined the fall of total protein content. This suggests that reduction of IL-1 production caused by HQ results from a global impairment of monocytes' essential functions such as transcription or translation. Therefore, the inhibition of cytokines production by mononuclear phagocytes involved in the regulation of hematopoiesis can contribute to myelotoxicity [23]. In the same year another study reported the effects of HQ on IL-1 [24]. The authors showed that 1,4-benzoquinone, the oxidation product of HQ in the cell, causes a concentration-dependent inhibition of highly purified human platelet calpain [24]. Moreover, they demonstrated that HQ also inhibits the processing of pre-interleukin-lβ by interleukin-lβ convertase. The addition of HQ to Bl human cells, which undergo autocrine stimulation by interleukin-lβ, resulted in both cessation of autocrine cell growth and interleukin-lβ secretion into the culture medium [24].

Table 1: Summary of considered studies on hematotoxicity induced by benzene and/or its metabolites.

Cytokine involved	Substance(s)	Effect(s)	Reference
TNF-α	Benzene	Similar peripheral levels in exposed subjects and controls	Rothman et al., 1996 [28]
	HQ	Dose-dependent inhibition of TNF-α-induced activation of NF-kB	Kerzic et al., 2003 [26]
		Synergistic action of hydroquinone and TNF-α in producing apoptosis in human bone marrow cells.	Kerzic et al., 2003 [26]
	Benzene	−238 (G → A) polymorphism is associated with the development of persistent bone marrow dysplasia developing in patients previously exposed to benzene	Lv et al., 2007 [27]
	HQ, benzenetriol, BQ, and catechol	Dose-dependent increase in TNF-α production by activated PBMC	Gillis et al., 2007 [21]
	BTX	Dose-dependent reduction in TNF production by PBMC	Haro-García et al., 2012 [29]
IL-1	HQ	Prevention of the proteolytic conversion of pre-IL-lα to the mature cytokine by the processing protease calpain	Renz and Kalf, 1991 [22]
	HQ	Dose-dependent reduction of IL-1α and IL-1β by mononuclear phagocytes	Carbonnelle et al., 1995 [23]
	HQ	Inhibition of pre-interleukin-lβ production by interleukin-lβ convertase	Niculescu et al., 1995 [24]
	HQ, catechol	Suppression of IL-1β production by activated PBMC	Gillis et al., 2007 [21]
	Benzene	−889 (C > T) polymorphism is associated with the decrease of granulocyte count	Lan et al., 2005 [19]
IL-2	BQ	Decrease in IL-2 production by activated PBMC	Gillis et al., 2007 [21]
IL-3	Benzene	Nondetectable peripheral levels	Rothman et al., 1996 [28]
IL-4	Benzene	−1098 (T > G) polymorphism is associated with decreased granulocyte and total lymphocytes counts	Lan et al., 2005 [19]
IL-6	Benzene	Similar peripheral levels in exposed subjects and controls	Rothman et al., 1996 [28]
	Benzenetriol, BQ, and catechol	Increased IL-6 production by activated PBMC	Gillis et al., 2007 [21]
IL-10	Benzene	No statistically significant difference in peripheral levels of exposed subjects and controls	Spatari et al., 2013 [30]
	BMX	No statistically significant dose-dependent reduction in IL-10 production by PBMC	Haro-García et al., 2012 [29]
	HQ, catechol	Strong inhibition of IL-10 production by activated PBMC	Gillis et al., 2007 [21]
	Benzene	−819 (T > C) polymorphism is associated with the decrease of granulocyte count	Lan et al., 2005 [19]
IL-12	BMX	No statistically significant dose-dependent reduction in IL-10 production by PBMC	Haro-García et al., 2012 [29]
	Benzene	−8685 (G > A) polymorphism is associated with decreased granulocytes, total lymphocyte count, and $CD4^+$ and $CD8^+$ T-cell subsets.	Lan et al., 2005 [19]
IFN-γ	HQ	Increased IFN-γ production by activated PBMC	Gillis et al., 2007 [21]
VCAM1	Benzene	−1591 (T > C) polymorphism is associated with decreased B cells, natural killer cells, $CD4^+$ T cells, and monocytes and colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte progenitor cells	Lan et al., 2005 [19]
PF4	Benzene	Downregulation of the expression	Vermeulen et al., 2005 [31]
CTAP-III	Benzene	Downregulation of the expression	Vermeulen et al., 2005 [31]
CXCL12	BQ	Upregulation of gene expression	Zolghadr et al., 2012 [34]
	HQ	Downregulation of gene expression	

Table 1: Continued.

Cytokine involved	Substance(s)	Effect(s)	Reference
IL-8	HQ, catechol	Increased IL-8 production by PBMC Decreased IL-8 production by activated PBMC	Gillis et al., 2007 [21]
	Benzenetriol, BQ	Increased IL-8 production by PBMC	
Eotaxin, MIP1-α, and RANTES	HQ, benzenetriol, BQ, and catechol	Increased chemokines production by PBMC	Gillis et al., 2007 [21]
MCP-1	HQ, benzenetriol, and BQ	Increased MCP-1 production by PBMC	Gillis et al., 2007 [21]
	HQ, catechol	Decreased IL-8 production by activated PBMC	Gillis et al., 2007 [21]

HQ: hydroquinone; BQ: benzoquinone; BTX: benzene-toluene-xylene mixture; PBMC: peripheral blood mononuclear cells; PF4: platelet factor 4; CTAP-III: connective tissue activating peptide; CXCL: CXC chemokine ligand.

In the study of Gillis et al., the authors also noted strong inhibition of the production of the anti-inflammatory cytokine IL-10 by higher concentrations of HQ and catechol. Enhanced production of proinflammatory cytokines coupled with the suppression of anti-inflammatory cytokines could lead to tissue damage and could predispose an individual to the development of autoimmunity [21]. Interleukin-3 (IL-3) and granulocyte/macrophage-colony-stimulating factor (GM-CSF) are responsible for maintaining survival and stimulating growth of early dormant hematopoietic progenitor cells (HPC). These cytokines exhibit extensive overlap, with GM-CSF supporting growth and differentiation of myeloid HPC [25]. It has been demonstrated that pretreatment of $CD34^+$ cells, human bone marrow cells containing HPC, with HQ results in enhanced clonogenic response with GM-CSF but not IL-3 [25]. These findings suggest that an early step in chemical leukemogenesis may involve transient alterations in the regulation of cytokine response to GM-CSF. It seems that HQ activates a mechanism involving one or more secondary signals that are not sufficient to induce HPC into cycle but will synergize with GM-CSF to do so. In a rapidly dividing tissue, such as bone marrow in which control of stem and progenitor cell proliferation commands a high priority, changes in proliferation or survival may predispose susceptible target cells to replication-dependent damage and subsequent neoplastic transformation [25].

Another possible mechanism leading to suppression of hematopoiesis involves the inhibition of nuclear factor kappa B (NF-κB). It is known that tumor necrosis factor alpha (TNF-α) activates NF-κB in multiple cell types and inhibits proliferation and colony-forming activity of bone marrow cells. Activated NF-κB is required for many cells to escape apoptosis, including hematopoietic progenitor cells (HPC). HQ alters cytokine response, induces cell death in HPC, and inhibits NF-κB activation in T and B cells [26]. A study [26] demonstrated that in a hematopoietic cell line and primary HPC HQ exposure inhibits TNF-α-induced activation of NF-κB in a dose-dependent manner. The authors further investigated the ability of HQ to potentiate TNF-α-induced apoptosis in these cells and found that HQ sensitized the cells to the proapoptotic effect of TNF-α. These results suggest that NF-κB plays a key role in HPC survival and that HQ-induced inhibition of NF-κB leaves these cells susceptible to cytokine-induced apoptosis [26].

The same authors investigated the association of four single nucleotide polymorphisms (SNPs) in the promoter region of TNF-α on the development of a transient hematotoxicity induced by benzene (benzene poisoning, BP), a persistent bone marrow dysplasia with unique dysplastic and inflammatory features developing in individuals previously exposed to benzene (BID) and *de novo* myelodysplastic syndrome (MDS). Only the -238 (G $\rightarrow$ A) polymorphism was significantly associated with the development of BID and was specific for BID and not *de novo* MDS or BP [27]. These findings suggest the possibility that cell-specific alterations in TNF-α expression linked to this polymorphism may facilitate the escape of damaged hematopoietic progenitor cells from $CD8^+$ T-cell targeting and promote clonal selection in the evolution of neoplastic hematopoietic disease. It is also possible that -238A may be linked in an extended haplotype with other genes that play a role in influencing TNF-α expression in hematopoietic progenitor cells [27].

Another study on SNPs of 20 candidate genes of cytokines, chemokines, and cellular adhesion molecules involved 250 workers exposed to benzene and 140 unexposed controls [19]. The authors found that SNPs in five genes (four cytokines and one adhesion molecule) were associated with a statistically significant decrease in total white blood cell counts among exposed workers, and one SNP was associated with an increase in white blood cell counts. In particular, IL-1A and IL-10 variants were significantly associated with only granulocytes; the IL-4 promoter SNP was associated with a decline in both granulocytes and total lymphocytes, and the IL-12A SNPs were associated with declines in three white blood cell subtypes. The adhesion molecule VCAM1 variant was particularly noteworthy as it was associated with a decrease in B cells, natural killer cells, $CD4^+$ T cells, and monocytes. Further, VCAM1 and CSF3 were associated, respectively, with decreased and increased colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte progenitor cells in a subgroup of 29 benzene-exposed workers. Therefore, selected variants seemed to influence only granulocytes, whereas others altered cell types of both myeloid and lymphoid lineage, suggesting effects that could extend to earlier progenitor and possibly stem cells [19]. A Chinese study from 1996 [28] on peripheral cytokine levels, as IL-3, IL-6, erythropoietin, G-CSF, and TNF-α, in a group of 11 benzene-exposed healthy workers

and 11 unexposed controls showed that levels of IL-6, erythropoietin, and TNF-α were similar in exposed subjects and controls. Levels of G-CSF and IL-3 were nondetectable in almost all subjects. Neither benzene urinary metabolites nor cumulative benzene exposure was associated with any cytokine level [28]. Later, a Mexican study [29] evaluated the relationship between occupational exposure to benzene-toluene-xylene mixture (BTX) and IL-10, TNF, and IL-12 production by peripheral blood mononuclear cells. The study involved 54 workers divided into two exposure groups: high and low BTX accumulated potential dose. Workers highly exposed to BTX showed a significant fall in TNF production; although without statistical significance, a reduction for IL-10 and IL-12 was also observed [29]. A recent Italian study [30] evaluated IL-10 serum levels in 51 oil refinery employees exposed to benzene and in 16 nonexposed office workers who resided in the same area. The authors found no statistically significant difference between serum concentrations of IL-10 in both groups. Moreover, a statistically significant relationship in exposed subjects between age, working years, and serum concentration of IL-10 and the latter with the employment age was found [30].

Among chemokines, three proteins were consistently downregulated in workers exposed to benzene compared to control individuals. Two of these proteins were identified as platelet factor 4 (PF4), also downregulated at gene expression level, and connective tissue activating peptide CTAP-III, respectively, both platelet-derived CXC chemokines. Thus, reduced protein levels of PF4 or CTAP-III are potential biomarkers of benzene's early biologic effects and may play a role in the immunosuppressive effects of benzene [31].

CXCL12 chemokinesis is crucial for the homing process of haematopoietic stem cell (HSCs) [32]. Bone marrow CXC chemokine ligand- (CXCL12-) abundant reticular cells originate from mesenchymal stem cells (MSCs) as well. Short term ablation of these cells severely impairs the adipogenic and osteogenic differentiation capacity of marrow cells and gives rise to a marked reduction in cycling lymphoid and erythroid progenitors [33]. In a recent study the effects of a range of benzene concentrations along with those of its reactive metabolites, p-benzoquinone (BQ) and HQ, on the viability of MSCs were analyzed [34]. In particular, some genes such as CXCL12, which are expressed by MSCs and play roles in adipo-osteogenic differentiation of these cells and the regulation of haematopoiesis, were investigated. With regard to CXCL12, treatment with BQ caused slight upregulation and treatment with HQ led to downregulation. Enhanced CXCL12 expressions probably lead to an increase in the attraction and adhesion of HSCs to haematopoietic niche while downregulation of CXCL12 could have a converse effect. Moreover, the observed alterations in the expression of CXCL12 can also lead to disturbance of HCS niche which in turn can result in haematopoietic failure and contribute to leukemia development [34].

In another study significant concentration-dependent increases in the expression of most extracellular chemokines, such as IL-8, Eotaxin, MIP1-α, and RANTES, by PBMC were observed in response to four benzene metabolite (catechol, HQ, 1,2,4-benzenetriol, and 1,4-benzoquinone) treatments [21]. An increase in MCP-1 chemokine secretion was observed for cells treated with HQ, benzenetriol, and benzoquinone, but not catechol [21]. In addition, these treatments resulted in the induction of the inflammatory cytokine IL-6 [21]. This study is in agreement with other studies reporting increased IL-8 production by various types of cells in the presence of benzene metabolites [35, 36]. The study of Gillis et al. showed that PBMC stimulation with the PMA ionomycin mixture induced production of cytokines at 10-to-several-thousand-fold higher levels, resulting in a dramatic increase in cytokine levels in the medium. The effects of benzene metabolites on the secretion of soluble cytokines by activated PBMC varied. For example, dramatic concentration-dependent decreases in the production of the chemokines IL-8 and MCP-1 were observed for HQ and catechol, but not for benzenetriol and BQ. Concentrations of other chemokines either slightly increased or remained unchanged [21].

It is known that NAD(P)H : quinone oxidoreductase 1 (NQO1) deficiency due to a polymorphism is a risk factor for benzene-induced myeloid toxicity. A study demonstrated that inhibition or knockdown of NQO1 in human bone marrow endothelium leads to decreased TNF-α-induced adhesion molecule expression and adhesion of progenitors to endothelial cells via an NF-κB mechanism [37].

1.2. Atopy. Besides its direct toxicity, benzene exerts multiple effects after being converted to reactive metabolites such as HQ and benzoquinone. For instance, benzene and its metabolites can directly or indirectly influence the responses of mast cells and basophils (reviewed in [38]), which are primary effector cells involved in the development of respiratory allergies such as rhinitis and bronchial asthma. These compounds may act directly or together with other cells, such as T cells, macrophages, and monocytes, which are functionally connected. In particular, HQ and benzoquinone inhibit the release of preformed mediators, leukotriene synthesis, and cytokine production in human basophils stimulated by IgE- and non-IgE-mediated agonists. Furthermore, these metabolites reduce IgE-mediated degranulation of mast cells and the development of allergic lung inflammation in rats (reviewed in [38]). It is also known that benzene metabolites alter biochemical and functional activities of other immunocompetent cells and may impair immune responses in the lung. These inhibitory effects of benzene metabolites are primarily mediated by interference with early transduction signals. Together, the currently available studies indicate that benzene metabolites interfere by multiple mechanisms with the role of basophils and mast cells in innate immunity and in lung chronic inflammation (reviewed in [38]).

The studies on atopy induced by benzene and/or its metabolites are shown in Table 2. In a study performed on a group of both asymptomatic atopic and nonatopic women and a group of women residing in urban and suburban areas with almost uniform levels of toxic compounds produced by vehicular traffic and low air pollution produced by factories, urinary *trans,trans*-muconic acid, a metabolite of benzene, did not show differences but was significantly correlated with

Table 2: Summary of considered studies on atopy induced by benzene and/or its metabolites.

Population	Exposure substance(s)	Effect(s)	Reference
Nonatopic and atopic women	Vehicular traffic and low air pollution	No difference in urinary *trans,trans*-muconic acid	Boscolo et al., 2000 [39]
Nonatopic and atopic women	Vehicular traffic and low air pollution	Stimulation of NK activity	Boscolo et al., 2000 [39]
3-year-old children	Chlorobenzene	Contribution to the risk of allergic sensitization to the food allergens milk and egg white	Lehmann et al., 2001 [40]
3-year-old children	Benzene, ethylbenzene, and chlorobenzene	Increased IL-4 producing $CD3^+$ T cells	Lehmann et al., 2001 [40]
Healthy subjects	HQ, benzenetriol, BQ, and catechol	Increased secretion of IL-4	Gillis et al., 2007 [21]
Healthy subjects	HQ, catechol	Increased secretion of IL-5	Gillis et al., 2007 [21]

HQ: hydroquinone; BQ: benzoquinone.

NK $CD16^+CD56^+$ lymphocytes in both groups [39]. The correlation of NK cells with urinary *trans,trans*-muconic acid in women with low levels of environmental exposure suggests that low levels of exposure to benzene may stimulate NK activity [39].

Another study carried out in a group of 3-year-old children showed that exposure to aromatic compounds, such as chlorobenzene, may contribute to the risk of allergic sensitization to the food allergens milk and egg white [40]. Moreover, exposure to benzene, ethylbenzene, and chlorobenzene was associated with higher percentages of IL-4 producing $CD3^+$ T cells. Both an increase in IL-4 producing type 2 T cells and a reduction of IFN-gamma producing type 1 T cells may contribute to a type 2 skewed memory in response to allergens [40].

Gillis et al. reported that the treatment with four different benzene metabolites, such as catechol, HQ, 1,2,4-benzenetriol, and 1,4-BQ, increased secretion of Th2-type cytokines IL-4 and IL-5. All four benzene metabolites augmented production of IL-4, while increased expression of IL-5 was observed only for catechol and HQ treatments [21].

1.3. Effects on Lungs. HQ is present in large quantities in cigarette tar as a result of the combustion of tobacco leaf pigments. It has been demonstrated that HQ in concentrations comparable to those found in cigarette tar is a potent inhibitor of IL-2-dependent T-cell proliferation [41]. The authors demonstrated that exposure of primary human T lymphoblasts (HTL) to HQ *in vitro* blocked IL-2-dependent proliferation by 90% with no loss in viability. Moreover, HQ inhibited the IL-2-dependent progression of HTL through S phase of the cell cycle without blocking binding of IL-2 to the cells. Therefore, these results may help to explain the potent immunosuppressive effects of cigarette smoke on lung lymphocytes [41].

2. Discussion and Conclusions

Nowadays, the carcinogenic effects of benzene are well established in particular for AML and MDS. Low-risk MDS is characterized by increased apoptosis in the bone marrow with autoimmune characteristics whereas the advanced or high-risk stages involve immune evasion and secondary DNA damage, giving cells growth potential to progress into AML. Nevertheless, the causes of MDS remain poorly defined and it is not clear how the disease progresses from an early stage to advanced MDS and AML. Although there are clear indications for the role of the immune system, the exact mechanism by which the immune response contributes to the progression is not yet clear [42]. Some of the mechanisms of hematotoxicity involve the network of cytokines, chemokines, and adhesion molecules. This review shows that the actions of benzene and its metabolites on immune system can involve different kinds of cytokines both *in vivo* and *in vitro*.

Therefore, the increase in production of proinflammatory cytokines and the reduction of anti-inflammatory cytokines induce the development of chronic inflammation and autoimmunity that can be responsible for the onset of MDS and/or its progression to AML.

Moreover, benzene and its compounds not only produce toxic and/or cancerogenic effects but also can influence immune response in atopy.

In the future it could be useful to identify one or more biomarkers of exposure, early effect, susceptibility, and disease development in patients exposed to benzene, which will be useful as a novel target for therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 141378, 7 pages
http://dx.doi.org/10.1155/2014/141378

Research Article

Airborne Biogenic Particles in the Snow of the Cities of the Russian Far East as Potential Allergic Compounds

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Received 23 May 2014; Accepted 10 July 2014; Published 22 July 2014

Academic Editor: Takemi Otsuki

This paper presents an analysis of airborne biogenic particles (1 mkm–1 mm) found in the snow in several cities of the Russian Far East during 2010–2013. The most common was vegetational terraneous detritus (fragments of tree and grass leaves) followed by animal hair, small insects and their fragments, microorganisms of aeroplankton, and equivocal biological garbage. Specific components were found in samples from locations close to bodies of water such as fragments of algae and mollusc shells and, marine invertebrates (needles of sea urchins and shell debris of arthropods). In most locations across the Far East (Vladivostok, Khabarovsk, Blagoveshchensk, and Ussuriysk), the content of biogenic particles collected in the winter did not exceed 10% of the total particulate matter, with the exception of Birobidzhan and the nature reserve Bastak, where it made up to 20%. Most of all biogenic compounds should be allergic: hair, fragments of tree and grass leaves, insects, and microorganisms.

1. Introduction

Suspended particulate matter in air as abiotic factor has not been studied until recently. Its formation is a complex and multifaceted process that generally depends on soil erosion, volcanic activity, fire, and global air-mass circulation. In addition to quantifying air pollution (commonly measured by weight), which is occasionally the only criterion, particle size and composition in suspended material are critical for air monitoring.

For a long time, since the planetesimal occurrence that eventually became the Earth, air suspensions included predominantly materials other than organic (mineral and rock particles) and low-molecular organic compounds drawn by meteorites and asteroids. The recently discovered planetesimal (e.g., Lutecia 21) is now covered with nonorganic dust (layers up to 600 m) [1, 2], which arrived at the start of the solar system evolution.

Over the course of time, or more precisely, since the appearance of life on Earth and accumulation of large biomaterial deposits, air suspensions became enriched with residues of complex polymer organic matter, which was also constantly changing, being a dynamic system.

It should be noted that the particle composition in the snow samples is different from the samples collected in spring and summer which had an abundance of plant pollen in them [3]. Pollen in particular, as many researchers suggest, is believed to cause allergic diseases, whose frequency typically peaks from the middle of May to the middle of August. That is why the majority of research work to date deals with highly allergenic compounds in air suspensions (or aeroallergens) like fungi spores and plant pollen, produced during warm seasons [3–12].

There are also reports about other aeroallergens—hair of cats and dogs and dust mites [13–21].

We have already discussed the issue of differences between natural and industrial suspensions across the Far East region and the ecological and hygienic value of this [22].

This paper considers a material research of biogenous particles in air suspensions found in snow samples from the urban areas in the Russian Far East to provide information about potential allergic features.

2. Materials and Methods

The methodology used to study airborne biogenous particles is commonly centered on analyzing plant pollen [23, 24], whereas other components need different techniques to be applied.

We have studied the airborne suspensions that were found in snow samples collected during 2010–2013 in the biggest cities in the Russian Far East: Vladivostok (at 13 points), Khabarovsk (12 points), Birobidzhan (5 points), Blagoveshchensk (25 points), Ussuriysk (10 points), and National Reserve Bastak (5 points).

To avoid secondary pollution, we collected the samples (atmospheric precipitation of ice crystals) only in snowfalls. Only the upper layer (5–10 cm) of new snow was used for this purpose. The snow was then placed in three-litre sterile containers. The containers were cleaned of dust with distilled water before use. The liquid received from melted snow was dried out in sterile and dust-free conditions. The light microscope Nikon SMZ1000 and the electronic microscope Hitachi S-3400N, equipped with an energy-dispersive spectrometer (EDAX, Thermo Scientific), were used to analyse airborne suspensions taken from those containers. Fifty particles were selected for the field of view for every sample. Biogenous particles were then singled out based on the morphology and the results of energy-dispersive analysis. Platinum was used to sputter coating and examine the samples under the electronic microscope.

3. Results

The biogenous particles found in the analyzed samples were classified as follows: vegetational terraneous detritus (fragments of tree and grass leaves), animal hair, small insects (lice, fleas) and their fragments, aeroplankton, and equivocal biological garbage.

Vegetational terraneous detritus was the most frequent, even in winter, when its amount was up to 70%. Particles of vegetational terraneous detritus mostly present themselves as undefinable materials of leaves, stems (Figures 1(a), 1(c), and 1(d)), wood (Figure 1(b)), diatoms, and phytolithic shells.

Phytoliths (biominerals of plants) and shell debris of diatoms were the rarest particle types in the samples. The snow samples occasionally included plant pollen, whose content was several times higher in the spring and summer periods.

3.1. Animal Hair. Hair of animals has a pronounced human health effect (in the form of allergies) as a factor in the presence of domestic animals (pets) at home [25]. Figures 2(a)–2(d) show that this type of airborne allergen can also be found outdoors.

Hairs of animal species were identifyed using [26, 27].

The allergic response to pets in humans, that is, to hair of pets, is known to be one of the most challenging issues in the modern allergy science [14, 15, 17–20]. Allergens of cats are the best studied types [21].

Gusareva and coauthors, for example, [17] note that most cases of bronchial allergy in patients (57.3%) have sensitization to the primary allergen of cats (Fel d 1).

Diette and coauthors [25], in their study, provide a sensitization correlation diagram for the Fel d 1. It should be noted that the circle of allergens relative to hair of animals has become wider more recently.

In addition to allergens in cats—Fel d 1, Fel d 2 (albumin), Fel d 4 (lipocalin), Fel d 5w [14, 16, 21]—, those in dogs have already been described, such as Can f 1 [13].

3.2. Small Insects. Small insects in airborne suspensions are a rare event (Figure 3), but if we consider their higher allergenic potential, this type of particle deserves a closer look.

Except for allergens of dust mites (Der p 1, Der f 1), there are also descriptions of allergic reactions to allergens of other insect species, for example, of cockroaches (Bla g 2) [13, 28, 29].

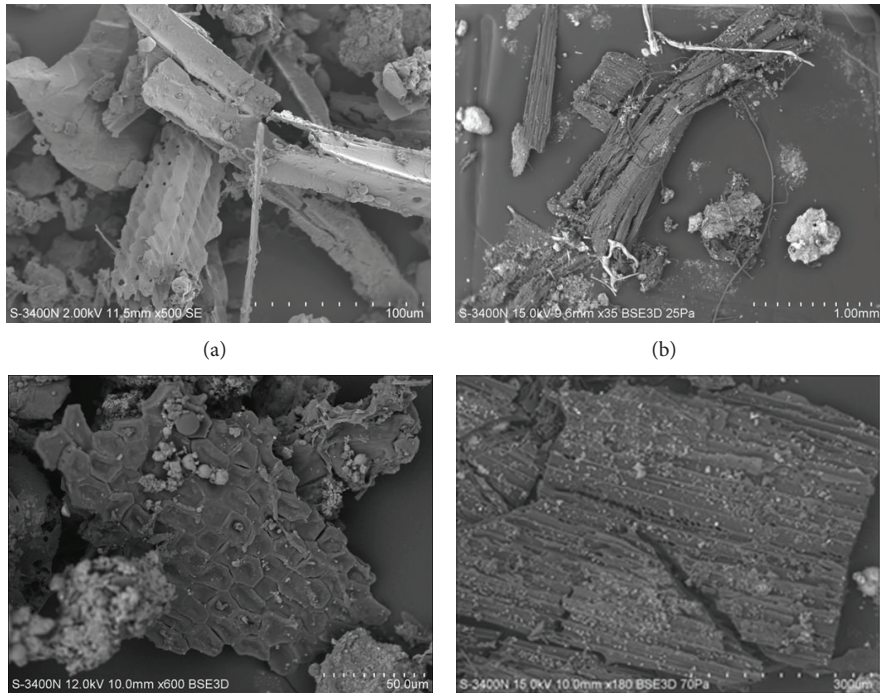
According to data from Gusareva and coauthors [17], almost one-third of bronchial allergy cases has sensitization to allergens of dust mites, while Huss and coauthors [28] report on the extremely high level of mite contaminations in dust from households in a few cities of the United States and Canada. For example, in San Diego, this level is 78.5% and in Toronto 59.3%. According to further investigation by this author, the presence of cockroach allergen in households of Boston was registered in 21.5% of the study cases, Saint Louis 16.3%, and Baltimore 13.4%.

Taking into account this fact, we can suggest that the air in these cities will be abundant with such allergens too.

3.3. Aeroplankton. Particles of air suspensions often become a living environment for a wide range of organisms—bacteria and fungi—some of which are sources of toxins, dangerous to human health [6, 30]. In general, all airborne living organisms (bacteria, fungi, moss, algae, spores, pollen, phytoplankton, tiny seeds, and arthropods) can be considered as aeroplankton [31]. Spores of many bacteria easily reach high atmospheric strata and can spread over large areas [32]. Aeroplankton in combination with dust is believed to seriously influence the weather; it often becomes a centre for atmospheric ice desublimation in particular [33]. Klyuzko and coauthors [34] demonstrated that, under laboratory conditions, bacteria contribute to the freezing of water by acting as typical condensation nuclei. Aeroplankton are found even at a height of 9000 meters [35].

We found some hyphae of unknown fungi in the snow sample taken in the Pervomayskiy park area in Blagoveshchensk (Figure 4(a)) and in Child Sanatorium "Detskiy" in Khabarovsk (Figure 4(b)).

The presence of water, micronutrients, oxygen, carbon oxide, and nitrogen inside clouds, as well as the presence of intensive radiation energy, creates favourable conditions for photosynthesis and metabolism and cell growth [36]. This is why the atmosphere and, more precisely, clouds represent a unique environmental system [37, 38], which influences the composition and properties of airborne solid particles.



(a) (b)

(c) (d)

Figure 1: Electronic micrography: (a) fragments of leaves from a snow sample taken in the area of the ring motorway in Birobidzhan. Magnification ×500. (b) Wood particles from a snow sample taken at the exhibition area in Blagoveshchensk. Magnification ×35. (c) Fragments of leaves from a snow sample taken in the center of Bastak nature reserve. Magnification ×600. (d) Wood particles from a snow sample taken at the intersection of Partizanskaya and Lenin streets in Blagoveshchensk. Magnification ×180.

Due to this fact, aeroplankton can be regarded not only as a carrier of various allergens but also as a potential ecosystem for pathogenic germs.

3.4. Sea Detritus. Any components of sea flora and fauna can be present in the air of seaside cities and settlements, especially if located close to the fish and seafood processing industry (Figures 5(a) and 5(b)).

Allergic reactions are most likely (and also are most studied) to the proteins of shrimps— tropomyosin (Pen a 1) [39], of fishes—, the cod allergen Cad c 1 [40, 41], and the perch allergen [42].

3.5. Equivocal Biological Garbage. According to the elementary analysis, this component of airborne suspensions contains a large amount of carbon (max. 90%), but it is not homeomorphous like charcoal and soot (Figure 6).

In equally possible chances, such indefinable organic garbage may contain digested residues of any organic detritus, including that of industrial origin (e.g., food industry or sawmill wastes). Possible source of garbage is the 2 big rivers: Amur and Zeya.

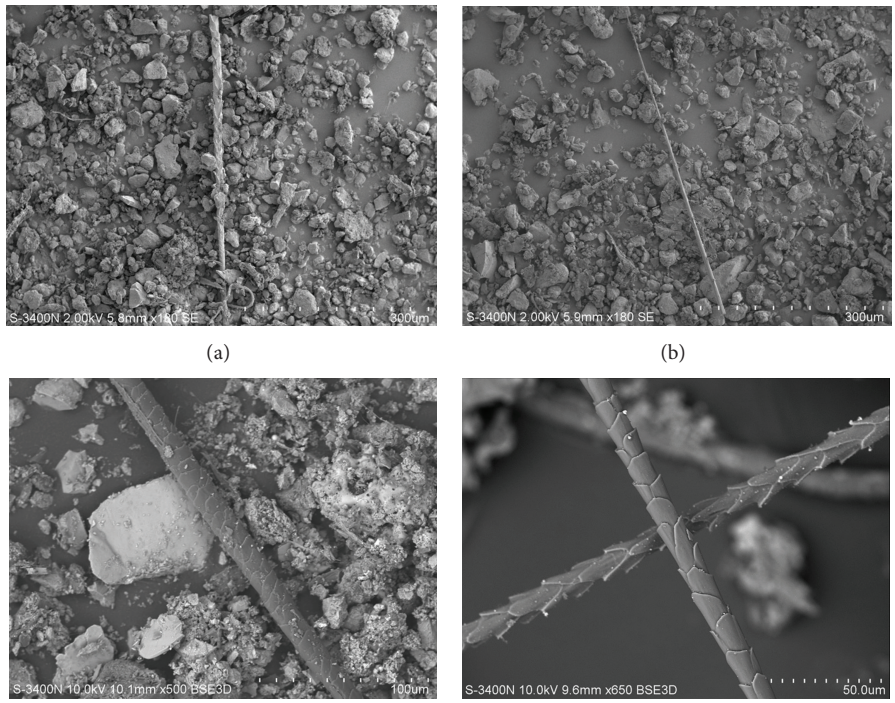
4. Discussion

The results above provide enough data for judgments and allow us to specify the components by the order of their occurrence in airborne suspensions from the snow samples taken in various locations across the Far East region of Russia: vegetational terraneous detritus (fragments of tree and grass leaves), animal hair, small insects and their fragments, microorganisms of aeroplankton (bacteria and fungi), and equivocal biological garbage [43].

Considering the literature data, the airborne biogenous particles found in our samples have the following order by allergenic potential: animal hair, fragments of insects, vegetational detritus, and pollen. The role of industrial garbage that contains biological components in allergies is completely unknown as there are no means to identify its precise composition.

Basic biogenous particles in air suspensions can be singled out and specified separately across every location of the Far East region according to this study. Particles of sea and land detritus, for example, prevail in Vladivostok, a major city and harbor on the Japan Sea. Airborne biogenous particles in air suspensions from Khabarovsk and Blagoveshchensk (both of which are located on the banks of the Amur River and the Zeya River) also in most cases belong to land detritus.

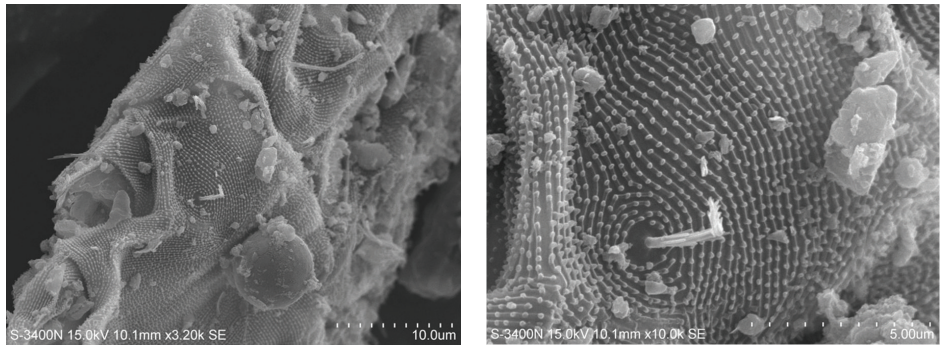
A similar trend, that is, predominance of land detritus particles, is found in Birobidzhan and the State Natural Reserve of Bastak. However, it should be noted that in all tested locations naturally occurring minerals and rocks with insignificant organic ingredients (5–10%) dominated in the whole picture. The samples taken in Birobidzhan and the Bastak reserve are noted for the highest levels of vegetational



(a) (b)

(c) (d)

Figure 2: Scanning electron microscopy in secondary electrons: (a) and (b)—hair of unknown animal (sheep *Ovis aries*?) from a snow sample collected in Zmeinka District, Vladivostok—; the sample was taken during a dust storm in Mongolia 04.09.2012. Magnification ×180. (c) Hair of a dog (*Canis familiaris*) from a snow sample taken in a one district of Blagoveshchensk. Magnification ×500. (d) Hair of a cat (*Felis domesticus*) from a snow sample taken around the Main Railway Station, Blagoveshchensk. Magnification ×650.



(a) (b)

Figure 3: (a) Fragments of unknown insect from a snow sample taken in the area of the Ring Motorway in Birobidzhan. (a) Magnification ×3200. (b) Magnification ×10000.

detritus among all tested populated locations in the Far East, with a total amount of 20–30% in the particles. This is likely due to weather conditions, geographic location, and vast woodlands.

Equivocal biological garbage is prevailing in the air suspensions taken form Ussuriysk (a land locked town, average by size, with much pollution from industry and transport).

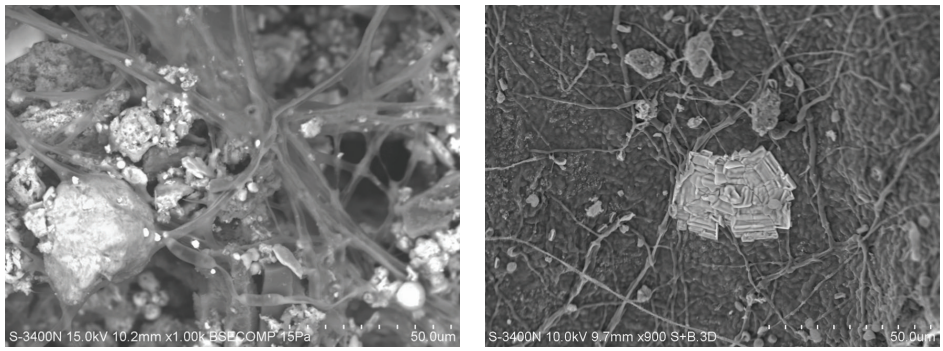
The analysis found particles of a biological origin and their possible allergic effect (considering quantity of finds) is provided in Table 1.

The share of biological components differs between the cities (5 to 25%), but we consider that settlements, where in air there are only a lot of parts of plants, threat of allergies will not be high.

The degree of "infection" of suspensions particles by aeroplankton was approximately equal in all cities (1 of 20–25 particles), but in Blagoveshchensk and Khabarovsk—is higher (1 of 10–15 particles). As shown in [44] patients with bronchial asthma in Amur region (Blagoveshchensk), mycotic allergy is accompanied by the domestic (air) sensitization. It is also worth noting that the surface of biogenous

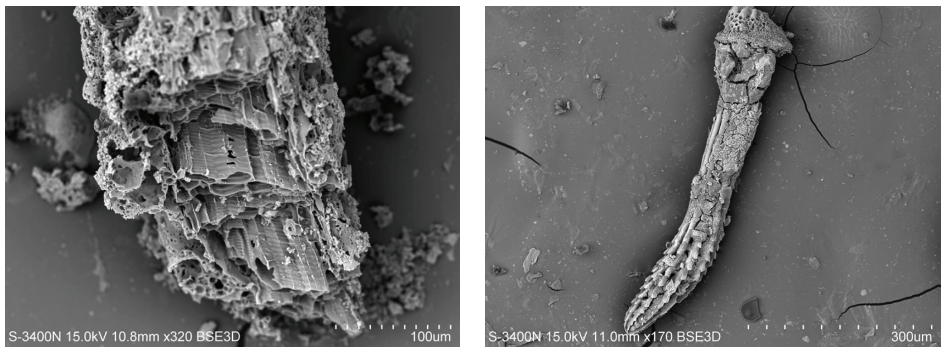
Table 1: Influence of different factors on structure of biological components of suspensions.

Cities	Water object	Forest area	Mail biological components	Share, %	Level of allergic threat
Vladivostok	Seaside	Small	Plant detritus, sea detritus	5	Average
Khabarovsk	One big river	Average	Plant detritus, aeroplankton	5	Average
Birobidzhan	—	Big	Plant detritus	20	Small
Blagoveshchensk	Two big rivers	Average	Equivocal biological Garbage, plant detritus, and aeroplankton	10	High
Ussuriysk	Three small rivers	Small	Equivocal biological garbage	10	High
Bastak	—	Big	Plant detritus	25	Small



(a) (b)

Figure 4: Hyphae of fungi from a snow sample taken: (a) at the Pervomayskiy park area in Blagoveshchensk, (b) at the Child Sanatorium "Detskiy" in Khabarovsk. Scanning electron microscopy. (a) Magnification ×1000. (b) Magnification ×900.



(a) (b)

Figure 5: (a) Fragments of sea organics (hypothetically a part of a sponge) and (b) needle of sea urchin Scaphechinus mirabilis from a snow sample taken in the Sadgorod District of Vladivostok. Scanning electron microscopy in secondary electrons. (a) Magnification ×320. (b) Magnification ×170.

particles often serves as an adsorbent for nanoscale particles of both natural and artificial origins.

We consider that existence of large water objects allows effectively clearing the atmosphere of polluting particles, including biogenous origin. But, on the other hand, water objects can be a source of allergens.

According to our results, "winter" air suspensions contain a considerable amount of allergenic organic matter that is consistent with the data of other studies [45]. In the course of the analysis, [46] revealed a progressive increase in the incidence of complaints of child population of Khabarovsk for urgent and emergency allergy care.

As a whole, we see that in the cities of Vladivostok and Khabarovsk growth of allergic diseases [44, 46] is noted. Unfortunately data on allergies of other studied cities in literature are absent; therefore, it is not possible today to draw a conclusion on correlation between types of biological particles and allergies.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

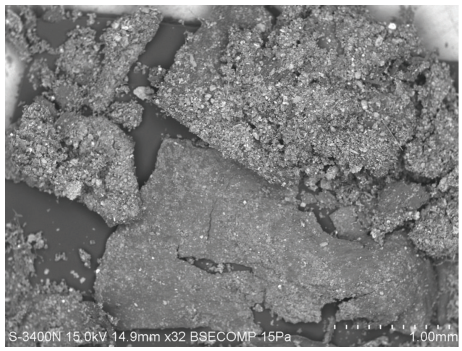


Figure 6: Aggregates from naturally occurring minerals, vegetational detritus, and equivocal biological garbage in a snow sample taken at the Pervomayskiy park area in Blagoveshchensk. Scanning electron microscopy in secondary electrons. Magnification ×32.

Acknowledgments

This study was carried out with support from the Scientific Fund of the Far Eastern Federal University and the Presidential Grant for Young Researchers MK-1547.2013.5.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 962871, 8 pages
http://dx.doi.org/10.1155/2014/962871

Review Article

Significance of Persistent Inflammation in Respiratory Disorders Induced by Nanoparticles

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Received 22 February 2014; Revised 17 June 2014; Accepted 20 June 2014; Published 7 July 2014

Academic Editor: Mario Di Gioacchino

Pulmonary inflammation, especially persistent inflammation, has been found to play a key role in respiratory disorders induced by nanoparticles in animal models. In inhalation studies and instillation studies of nanomaterials, persistent inflammation is composed of neutrophils and alveolar macrophages, and its pathogenesis is related to chemokines such as the cytokine-induced neutrophil chemoattractant (CINC) family and macrophage inflammatory protein-1α and oxidant stress-related genes such as heme oxygenase-1 (HO-1). DNA damages occur chemically or physically by nanomaterials. Chemical and physical damage are associated with point mutation by free radicals and double strand brake, respectively. The failure of DNA repair and accumulation of mutations might occur when inflammation is prolonged, and finally normal cells could become malignant. These free radicals can not only damage cells but also induce signaling molecules containing immunoreaction. Nanoparticles and asbestos also induce the production of free radicals. In allergic responses, nanoparticles act as Th2 adjuvants to activate Th2 immune responses such as activation of eosinophil and induction of IgE. Taken together, the presence of persistent inflammation may contribute to the pathogenesis of a variety of diseases induced by nanomaterials.

1. Persistent Inflammation and Harmful Effects

Reports on the toxicology of nanomaterials have been increasing recently, but the effect of nanomaterials on the human body is inconclusive. It is thought that, in general, inhaled dusts such as particles and fibrous materials in the lung repeatedly induce inflammation and finally lead to pulmonary fibrosis and respiratory cancer [1, 2]. It is considered that the presence of persistent inflammation leads to advanced stages such as fibrosis and tumors. Persistent inflammation, reported in animal exposure models using asbestos and silica, is important in the pathology of the formation of irreversible chronic lesions [1, 2]. In an examination of inhalation exposure of rat to chrysotile for 20 days, continuous inflammation and fibrosis containing mainly neutrophils were observed [3]. Intratracheal instillation of crystalline silica induced a persistent neutrophil inflammation in rat lung. This inflammation progressed time-dependently during 6 months after exposure [4]. Pulmonary persistent inflammation is also thought to be related to lung disorders induced by manufactured nanomaterials. Among nanoparticles, nickel oxide nanoparticles, a material with high toxicity, induced persistent inflammation in the lung [5, 6]. Nishi et al. [6] reported that nanoparticles of nickel oxides induced persistent neutrophil inflammation in the rat lung from 3 days to 3 months after intratracheal instillation. There are many reports that carbon nanotubes induced persistent inflammation in rats and mice after intratracheal instillation or inhalation.

In order to examine what kinds of cytokines are related to lung disorders induced by nanoparticles, Morimoto et al. measured the concentrations of 27 cytokines including inflammation, fibrosis, and allergy-related ones, in the lung and bronchoalveolar lavage fluid (BALF) following intratracheal instillation of well-dispersed nickel oxide nanoparticles [5]. The expression of macrophage inflammatory protein-1α (MIP-1α), heme oxygenase-1 (HO-1), cytokine-induced

neutrophil chemoattractant-1(CINC-1), and CINC-2 showed a continued increase in the lung tissue and BALF, while interleukin-1α (IL-1α), IL-1β in the lung tissue, and monocyte chemotactic protein-1 (MCP-1) in BALF showed transient increases. In another experiment, Fujita et al. [7] analyzed the comprehensive gene expression by microarrays and found that CINC-1, 2, MIP-1, HO-1, and matrix metalloproteinase-12 (Mmp-12) expressions increased with exposure to nickel oxide nanoparticles, while nearly no increase of other neutrophil chemokines was observed. This persistent expression of the CINC family suggests that chemokines are important in neutrophil inflammation in lung exposed to nanoparticles. Diesel particles [8], inhaled materials with inflammatory potentials, have been reported to persistently increase CINC-1 or CINC-2 expression in the lung following intratracheal instillation. On the other hand, TiO_2 (micron-size) and fullerene, which are less inflammogenic to the lung, revealed a mild and transient increase in CINC-1 and CINC-2$\alpha\beta$ expression only at an acute phase after intratracheal instillation [9]. Nickel oxide nanoparticles induced only a transient expression of CINC-3 in an intratracheal instillation study, although the nickel oxide nanoparticles induced persistent pulmonary inflammation in the rat lung [6, 10]. In that intratracheal instillation study, the maximum dose of nickel oxide nanoparticles was 0.2 mg/rat. We performed an intratracheal instillation study with a high dose, and 1 mg of nickel oxide nanoparticle induced a persistent increase in CINC-3 concentration and more severe neutrophil inflammation in rat lung. Taken together, we suspect that CINC-3 may play a role in enhancing pulmonary inflammation. There is a report [11] that a difference in biological activities of the CXC chemokine receptor 2 was observed between CINC-1, CINC-2, and CINC-3. CINC-3 induced the enhancement of cytoplasmic calcium concentration more potently than did CINC-1 and CINC-2 in the short-term incubation (<10 min) of bovine alveolar macrophage with quartz dust particles, a material with inflammatory potential.

If the high dose of nanomaterials was increased too much, even particles with low toxicity induced persistent inflammation, fibrosis, and tumor in the lung following not only intratracheal instillation but also inhalation. A 2-year inhalation exposure study of TiO_2 micron-sized particles, which are considered to be negative control, at concentrations of 0, 10, 50, and 250 mg/m^3 was conducted on rats, and bronchioloalveolar adenomas and cystic keratinizing squamous cell carcinomas occurred at 250 mg/m^3 exposure, while no compound-related lung tumors were found in rats exposed to either 10 or 50 mg/m^3 [12]. These data suggested that an overload of titanium dioxide, in spite of its toxicity, may induce lung tumors at high dose exposure. The overload of materials is due to a dysfunction of alveolar macrophage, and this phenomenon is accompanied by a delay of clearance of materials from the lung and the pulmonary response. As for nanoparticles, Bermudez et al. [13] performed a subchronic inhalation (13 weeks) of ultrafine TiO_2 particles at aerosol concentrations of 0.5, 2.0, and 10 mg/m^3. Exposure to 10 mg/m^3 induced the retardation of particle clearance and neutrophil infiltration in rat lung; on the other hand, exposure to 2 mg/m^3 caused neither reaction. The inflammation at 10 mg/m^3 was accompanied by delay of clearance, and the inflammation induced by the titanium dioxide nanoparticles may have been due to an overload of titanium dioxide. We speculate that the difference in deposition rate of nano- and micron-sized particles of TiO_2 (the amount of deposition of nanoparticles in the lung is more than that of micron-sized particles) may affect only nanoparticle to induce the pulmonary response in the lung in spite of same concentration (10 mg/m^3). Therefore, measurements of the harmful effect of nanoparticles may be performed using data from concentrations which are below the dose of overload, such as equal to or less than 2 mg/m^3 titanium dioxide nanoparticles.

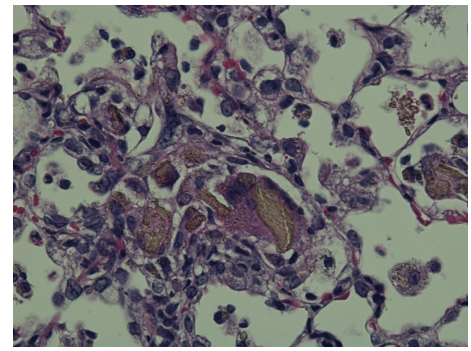

Figure 1: Hematoxylin and eosin staining of lung sections exposed to 3 mg TiO_2 nanoparticles at 3 days after instillation.

In cases of intratracheal instillation of particles, an excess dose induces the artificial effect of the bolus. We conducted an intratracheal instillation of 3 mg/rat of titanium dioxide nanoparticles [14]. Figure 1 shows a large granulomatous lesion including local accumulation of TiO_2 nanoparticles in the bronchoalveolar area 3 days after exposure. These lesions in the lung are not seen in usual inhalation studies. From this point of view, exposure of animals to excess doses of nanoparticles should be avoided. Although the most suitable dose of nanoparticles for the evaluation of harmful effects is not known, 0.2 mg/rat (0.67 mg/kg) of nickel oxide nanoparticles with high toxicity induced persistent neutrophil inflammation in rats [6, 15], and 1 mg (3.3–5 mg/kg) of fullerene and titanium dioxide nanoparticles with low toxicity induced transient inflammation [9, 14, 16]. If the relative harmful effect between nanoparticles is measured under the same weight base, pulmonary responses at doses from 0.2 mg/rat to 1 mg/rat (0.67–5 mg/kg) may be useful, at least partially.

It is important to estimate how long the persistent inflammation in an animal model is related to the toxicity of the nanoparticles. From previous studies, we think that 3 or 6 months of persistent inflammation from the end of exposure is related to a high or medium toxicity of nanoparticles [17].

We performed intratracheal instillations of different mineral fibers to rats and examined lung inflammation from 3 days up to 6 months [17]. Harmful respirable particles like

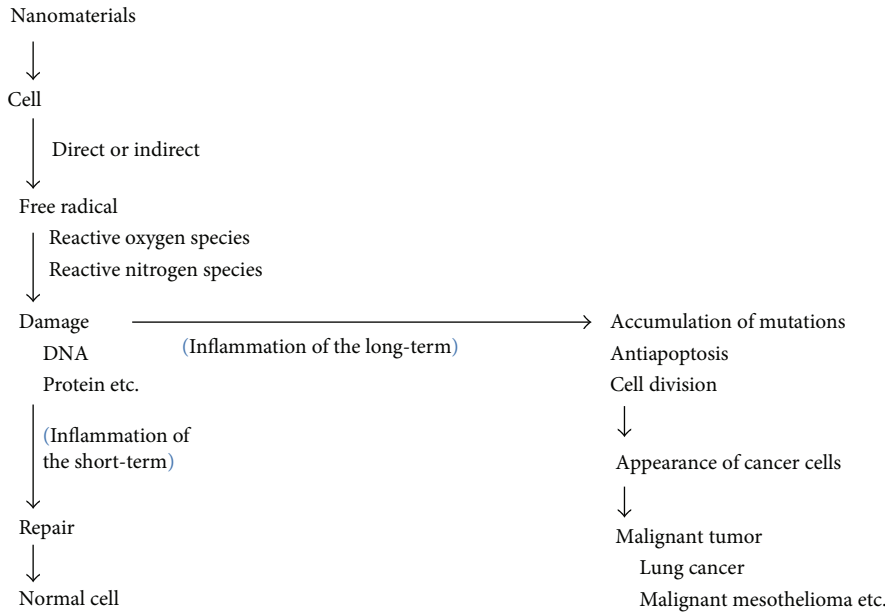

Figure 2: Tentative relationship between inflammation by nanomaterials and malignant tumor.

crystalline silica or crocidolite asbestos, which are kinds of asbestos, caused persistent inflammation from the initial instillation until 6 months later. However, when less harmful micron-sized titanium dioxide was inhaled, transient inflammation was only observed early in the instillation. As for crystalline silica, an exacerbation of inflammation was found in the lung after 1 or 2 months after exposure. Sellamuthu et al. [18] reported that when rats were exposed to inhalation of crystalline silica (15 mg/m^3, 6 h/day, 5 days), pulmonary damage was determined after the latent periods (0–16 weeks). The number of neutrophils and the concentration of MCP-1 in BALF were maximum after 16 weeks. Langley et al. [19] conducted a 6-week inhalation of silica with 27 weeks after exposure, and the number of neutrophils and lymphocytes in BALF increased 10 weeks after exposure, although not at 4 days, and LDH and protein concentration in BALF significantly increased at 10 and 17 weeks, but not at 4 days. Kobayashi et al. [16] showed that different evaluations of pulmonary toxicity by intratracheal instillation of titanium dioxide nanoparticles can be derived on the basis of observations up to 1 week after instillation and those after 1 month after instillation. Based on the results of intratracheal instillation studies and inhalation studies, both short-and long-term effects (from 3 days up to 6 months) should be evaluated when assessing the toxicity, including persistent inflammation, of nanoparticles. Therefore we speculate that exposure of the high toxic nanomaterial may induce persistent inflammation in the lung through the persistent production of chemokines, such as CINC, MIP, and MCP, and that sustained production of proteinases and ROS cause the lung injury during these chronic inflammations. Fujita et al. [7] reported that exposure to nickel oxide nanoparticle following intratracheal instillation induced persistent proteinases such as MMP-12 in rat lung.

2. Relationship between Inflammation and Malignant Tumor (Figure 2)

Malignant tumor is a polygenic abnormality disease caused by the accumulation of mutations in the genome of a normal cell, such as by single nucleotide substitution, deletion or insertion of a nucleotide, or translocation. Hanahan and Weinberg reported that malignant tumors should acquire six biological properties: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [20]. In 2011, four other biological properties (deregulation of cellular energy, avoidance of immune destruction, genome instability, and mutation and tumor-promoting inflammation) were added [21]. These properties are obtained when gene mutations accumulate. For the accumulation of genetic mutations, it is necessary to argue from two viewpoints: DNA damage and DNA repair. When DNA damage is caused by asbestos and nanomaterials containing nanoparticles and nanofibers as well as mutagens, and the damage cannot be repaired, genetic mutations accumulate and malignant tumors can occur. Interestingly, Xu et al. reported that multiwalled carbon nanotubes are similar to asbestos and have higher risk of causing asbestos-like pleural lesions [22]. For that reason, not only nanomaterials but also asbestos is reviewed.

There are two kinds of mutations in tumors: passenger mutations and oncogenic driver mutations [23]. Passenger mutation occurs only by chance, and oncogenic driver mutation occurs in important genes involved in the phenotype of cancer. Oncogenic driver mutations contain EGF receptor, K-ras, HER2, AKT1, and so forth [24–26]. In addition, there is a cancer that is completely dependent on the oncogenic signal

associated with cell proliferation, and survival of cancer by only one mutated gene is possible [27]. This state is called oncogene addiction, and a representative example is L858R mutation in the EGFR gene.

It is known that exposure to asbestos and nanomaterials induces cell dysfunctions at various levels, such as cell death by oxidative stress, DNA damage, and protein damage. DNA damage is associated with malignant tumors and is closely related to inflammation. Nanomaterials in inhalation or intratracheal instillation can cause acute and chronic inflammation to the respiratory tract and pulmonary alveolar space [28, 29]. In particular, persistent inflammation causes fibrosis of the lung and pleura and progresses to lung cancer or malignant mesothelioma [30]. Persistent inflammation causes lung damage, in which the production of free radicals due to inflammation is the most important cause. There are two types of free radicals, reactive oxygen species and reactive nitrogen species [31]. In the reactive oxygen species, there are superoxide ion (O_2^-), hydroxyl radicals ($^\bullet OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). $^\bullet OH$ is the most reactive of these reactive oxygen species. On the other hand, in the reactive nitrogen species, there are nitric oxide (NO), nitrosonium ion (NO^+), nitrite ion (NO_2^-), and peroxynitrite ($ONOO^-$). $ONOO^-$ is the most reactive of these reactive nitrogen species. Free radicals are produced spontaneously in the energy metabolism of the cell. Asbestos and nanomaterials are taken into the body, and free radicals are produced on their surface by inflammatory cells or epithelial cells phagocytizing them. TiO_2 [32], asbestos, and silica [33] have been reported as examples of radicals produced on the surface of asbestos and nanomaterials, while phagocytic cells such as neutrophils and macrophages play a role essential to the host defense to produce superoxides by the active oxygen production enzyme system, such as NADPH oxidase [34]. 8-hydroxydeoxyguanosine (8-OHdG) and 8-nitroguanine (8-NG) are known as DNA damages caused by reactive oxygen and nitrogen species, respectively [35, 36]. It has been reported that nanomaterials cause these damages [37–39]. It was reported that reactive oxygen species generated by titanium expressed Fas, Bcl-2-associated X protein (Bax), IL-1 beta, and induced apoptosis [40, 41]; those generated by silica induced DNA damage and autophagy [42, 43], those generated by polyvinylpyrrolidone (PVP)-coated silver nanoparticles and silver ions induce apoptosis and necrosis in THP-1 monocytes [44], and those generated by carbon nanotube activated p38 MAPK and NF-κB signaling and induced fibrogenic and angiogenic responses [45–47].

Mutations of tumor protein p53 (TP53), Kirsten rat sarcoma viral oncogene homolog (K-ras), epidermal growth factor receptor (EGFR), and neurofibromatosis 2 (NF2) caused by asbestos and nanomaterials are frequently reported. K-ras mutation plays an important role in signal transduction of EGFR. Both K-ras and EGFR mutations are oncogenic driver mutations. Andujar et al. [48] reported that TP53, EGFR, and K-ras mutations were found in non-small-cell lung cancer without association with asbestos exposure and that NF2 was only altered in MPM. Nelson et al. [49] reported that asbestos exposure increases the mutation at K-ras codon 12 and that this process occurs independently of the induction of interstitial fibrosis. Husgafvel-Pursiainen et al. reported that asbestos exposure alone was not significantly associated with an increased occurrence of K-ras mutations. However, a strong and significant association was found between adenocarcinoma and K-ras mutation in a group of smokers with asbestos exposure [50, 51]. Recently, Shvedova et al. reported that single-wall carbon nanotube and carbon nanofibers, but not asbestos exposures, increased the incidence of K-ras oncogene mutations in the lung [52]. However, there are few reports associated with nanoparticles and oncogene mutation. To investigate the occurrence of oncogene mutations with long exposure of nanoparticles in lung is required.

Free radicals can not only damage cells but also induce signaling molecules containing immunoreaction [53], remodeling of the extracellular matrix [54, 55], regulation of cell proliferation [56], and malignant transformation [57].

DNA damage caused by free radicals is an indirect damage caused by asbestos and nanomaterials. On the other hand, low soluble nanomaterials that are phagocytosed may contact DNA directly. Mu et al. reported that multiwalled carbon nanotubes (MWCNTs) could perform nuclear translocation [58]. This result suggests that the fibers could induce DNA damage directly. But it is not clear whether the penetration of the nuclear membrane by MWCNTs is active or passive. It may be possible that nanomaterials contact DNA directly even in the cytoplasm, because the nuclear membrane disappears in the chromosomal distribution in cell division. DNA cleavage is one of the predicted DNA damages caused by contact with DNA. Translocation may occur if DNA cleavage is not repaired correctly. The occurrence of malignant tumors by translocation has been reported in various leukemias [59]. It was recently reported that genomic translocation in lung cancer was observed and that the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) fusion gene plays an important role in lung cancer [60]. However, it is not known how the EML4-ALK fusion gene occurs.

It is conceivable that gene mutations accumulate when repair is not in time for a lot of DNA damages by nanomaterials or when nanomaterials are not cleared because of their physical and chemical properties. Various pathogenic substances cause acidic conditions around the inflammation [61]. Various proteins forming a complex work in the DNA repair system, including nucleotide excision repair, base excision repair, mismatch repair, homologous recombination, and nonhomologous end-joining [62]. It is thought that an acidic condition inhibits the complex formation of proteins working on DNA damage recognition and DNA repair. Yuan et al. [63] reported that DNA repair was diminished and mutagenesis was elevated in mammalian cells by exposure of hypoxia and low pH. Therefore, gene mutations might accumulate easily by asbestos and nanomaterials where inflammation is sustained (Figure 2). Further research is needed to determine whether persistence of inflammation is a cause of cancer.

3. Allergic Response

It has been revealed that the presence of inflammation plays a key role in the formation of allergic disease. It is thought that persistent inflammation, especially allergic inflammation, is related to the onset and progression of allergic disease. In bronchial asthma, persistent allergic inflammation induces airway remodeling, including deposition of collagen, and progresses to intractable asthma suggesting that sustained allergic inflammation contributes to the pathogenesis of the disease [64–67].

Particle materials, unlike allergens, are reported to have properties to enhance the immune response against antigens, the so-called adjuvant effect, and act as Th2 adjuvants to activate Th2 immune responses such as activation of eosinophil and induction of IgE [68]. There are reports that crystalline silica, nickel oxide nanoparticles, and carbon nanotubes induced IgG1 and IgE [69, 70]. Because nanoparticles and fibrous materials also induce Th2 immune responses, the surface area and length of fibrous materials may affect not only nonspecific responses but also immune-specific responses. The molecular and immunological mechanisms of action of particles in immune responses are poorly understood. Almost all particles preferentially induce Th2 immune responses; therefore it has been hypothesized that the specific signals evoked by particles in immune cells are involved in triggering in Th2 immune responses.

In 2008, several reports focused on the discovery that particles activate the NLRP3 inflammasome [71, 72]. The inflammasome is one of the pattern recognition receptors and is expressed in intracellular. The NLRP3 inflammasome is activated by particles such as aluminum salts, crystalline silica, and asbestos. Activated NLRP3 inflammasome promotes the production of IL-1β and IL-18, and these cytokines are considered to be involved in the induction of immune responses.

Some particles have cytotoxic activities and induce cell death. Dead cell-derived factors, what we call damage-associated molecular patterns (DAMPs), are known to stimulate immune cells. Uric acid is a purine catabolite that is released from dying cells. It is reported that uric acid or monosodium urate crystal (MSU) stimulates immune cells [73]. In addition, uric acid is released at the site of administration of aluminum particle [73].

Not only uric acid, it is also reported that the DNA released from damaged cells mediates the adjuvant activity of particles. Released host DNA is considered to be recognized by intracellular DNA sensors, but the detailed mechanisms by which the host DNA triggers the immune responses are unclear. Several reports have shown that stimulator of interferon genes (STING), interferon regulatory factor 3 (IRF3), and TANK-binding kinase 1 (TBK1), which are molecules associated with the signal pathway activated by host DNA, is required for the adjuvant activity of aluminum particle [74, 75].

Another unique mechanism is reported that lipid mediator, prostaglandin E_2 (PGE_2), is released by macrophages in response to particles and activates immune responses. PGE_2 is the well-characterized proinflammatory lipid mediator synthesized from arachidonic acid. It is reported that PGE_2 released by particles stimulates B cells to induce IgE [70].

Many factors are likely to be involved in the adjuvant activity of particles, and a consistent mechanism has not been found. We expect to elucidate what kinds of factors are involved in persistent allergic inflammation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work is partially supported by "Development of Innovative Methodology for Safety Assessment of Industrial Nanomaterials" by Ministry of Economy, Trade and Industry (METI) of Japan.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 295092, 8 pages
http://dx.doi.org/10.1155/2014/295092

Research Article

Palladium Nanoparticles Induce Disturbances in Cell Cycle Entry and Progression of Peripheral Blood Mononuclear Cells: Paramount Role of Ions

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Received 5 March 2014; Revised 6 June 2014; Accepted 8 June 2014; Published 3 July 2014

Academic Editor: Andrij Holian

There is concern about the possible toxicity of palladium nanoparticles (Pd-NP), as they are released in the environment through many applications. We previously studied the toxicity of Pd-NP at high concentrations; here we address the possible toxicity of Pd-NP at low, subtoxic doses. In particular, we have exposed normal human PBMC entering into the first in vitro mitotic division to Pd-NP and to Pd(IV) ions to evaluate ROS generation and cell cycle progression. We have measured a statistically significant increase of intracellular ROS in Pd(IV) exposed cells, but not in Pd-NP exposed cells. TEM revealed accumulation of lipid droplets and autophagic and mitophagic vacuoles, which appeared more conspicuous in cells exposed to Pd(IV) ions than to Pd-NP. Pd-NP were visible in the cytoplasm of Pd-NP exposed cells. Pd-NP addition was associated with a significant increase of cells within the G0/G1-phase and a significant reduction in GS- and G2/M-phases. Cells exposed to Pd(IV) ions showed a significant amplification of these cell cycle alterations. These results suggest that ions, per se or released by NPs, are the true inducers of Pd toxicity. It will be essential to verify whether the observed disturbance represents a temporary response or might result in permanent alterations.

1. Introduction

Palladium (Pd) is a component of the catalytic converters that promotes reduction and oxidation of pollutants in exhaust gases to less harmful ones [1]. Catalytic converters emit Pd mainly as particulate matter in the breathable size range ($d < 2.5\,\mu$m) [2, 3], containing also Pd-NP ($d < 100$ nm) [4]. Indeed, after the introduction of catalytic converters based on the Pt group elements, the level of Pd (also in the form of nanoparticles) rapidly increased in the areas bordering roads [5–10]. At the same time, in Europe, allergic sensitization to Pd increased [11–14]. A causative role for Pd(II) ions in the development of allergic symptoms has been deduced from studies on occupationally exposed people [14–16] and people

wearing Pd-containing dental alloys [17] and is sustained by laboratory data [18]. Pd being chemically related to nickel, one of the most important sensitizing metals, phenomena of cross-reactivity might contribute to the increased frequency of immune reactions to Pd [19–21]. Few data are available to explain the role of Pd-NP for the allergic sensitization. Our recent data confirm the sensitizing power of Pd(II) and Pd(IV) ions [22, 23]. Moreover, we observed that Pd-NP can induce secretion of INF-γ, a Th1 cytokine involved in type IV immune reactions [24] suggesting a role for polluting Pd-NP in the raised frequency of allergic contact dermatitis to Pd in people living in urban settings [14].

On the other hand, no data are available for the possible involvement of Pd in cancer development, which is one of the major concerns about metal-based NPs. In the ionic form, Pd(II) does not induce significant genotoxicity [25], but no extrapolations are possible to Pd-NP. In fact, NPs have peculiar physical properties that may not be simply predicted by data from the corresponding ions. However, ions can be released from NPs and contribute to NP toxicity. Cobalt represents an example of these events. Recently, our group found that highly cytotoxic Co^{2+} ions are released from Co-NPs, but only nanoparticles are rapidly accumulated within the nucleus and interact with DNA [26], induce transformation [27], and affect molecular pathways implicated in carcinogenesis and inflammation [28]. All these findings were obtained at rather high exposure concentrations. In the present work, we have studied the effects of low, subtoxic Pd-NP doses. As a target, we used cells of the immune system, taking into account that palladium can act as hapten able to induce an immune reaction. In particular, to study the possible interference of Pd-NP in the cell cycle progression, we have exposed to Pd-NP normal human PBMCs entering the first in vitro mitotic division.

2. Materials and Methods

2.1. Palladium Model Nanoparticles and Palladium Ions. Zerovalent Pd-NP (2–8 nm diameter) were produced and characterized as described previously [29]. The Pd-NP stock solution did not contain Pd ions, even after long time from preparation, as assessed by low resolution inductively coupled plasma mass spectrometry. To exclude the presence of agglomerates, the Pd-NP stock solution was treated by ultrasonic bath for 10 min at 100 Hz (Elmasonic S, Elma, Singen, Germany) and then immediately diluted in complete culture medium at working concentrations and given to the cells straight away. Potassium hexachloropalladate (K_2PdCl_6, Sigma Aldrich, Milan, Italy) (Pd(IV) ion) was used at subtoxic concentration, for comparison.

2.2. Cells. Human peripheral blood mononuclear cells (PBMCs) from 3 healthy donors were isolated by density gradient centrifugation using a commercial separation medium consisting of a mixture of Ficoll 400 and sodium diatrizoate (Lymphoprep, StemCell Technologies, Voden Medical Instruments, Milan, Italy), according to standard manufacturer procedure. Cells were seeded at 500,000 cells mL^{-1} in RPMI-1640 medium supplemented with 10% fetal calf serum (complete culture medium) containing 5 $\mu g\,mL^{-1}$ phytohemagglutinin-L (PHA-L) (Sigma Aldrich, Milan, Italy), an initiator of mitosis of normal human leukocytes. To evaluate the effect of the mitogen on cell viability and number, the PHA-stimulated cells were examined every 24 h for 5 days by trypan blue exclusion using a Neubauer counting chamber as described [30]. After 48 hours of culture in the presence of PHA, Pd-NP (or Pd(IV) ions or an equal volume of the vehicle, as controls) were added to the cultures for further 48 hours and finally harvested appropriately for analysis. Experiments were done in triplicate.

2.3. Cytotoxicity Evaluation. Cytotoxicity was assessed by MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test according to the standard procedure. Briefly, PBMCs (40,000 per well in 200 μL culture medium, four replicates) were seeded in a 96-well culture plate, incubated at 37°C, 5% CO_2, and exposed to increasing concentrations of Pd-NP or Pd(IV) ion (0.1, 1, 5, 10, 20, 40, 80 $\mu g\,mL^{-1}$) or to an equal volume of vehicle (RPMI, 10% FCS) to assess 100% viability.

At the end of the exposure time, a washing step was performed to remove the incubation medium containing unreacted MTT and Pd-NP or Pd(IV) ion. Then, 20 μL of the MTT solution (5 $mg\,mL^{-1}$) was added to each well and incubated for an additional 3 hours. The colored formazan crystals produced from MTT reduction were dissolved in 200 μL of dimethyl sulfoxide (DMSO). The optical density (O.D.) values of the solutions were measured at 540 nm as excitation wavelength using a spectrophotometer plate reader (Applied Biosystems, Life Technologies, Monza, Italy). Also, negative controls (i.e., complete culture medium alone or complete culture medium containing Pd-NP) have been tested and no interference with the colorimetric assay was observed, at all concentrations used. Cells without any treatment were assumed as positive control (100% viability).

The highest concentration that, after 48 h incubation, caused signal reduction (cell death) by less than 10% compared to the control was used for the experiments.

2.4. Detection of Intracellular Reactive Oxygen Species. The production of intracellular reactive oxygen species (ROS) was indirectly measured using dichlorodihydrofluorescein diacetate (DCFH-DA), a nonfluorescent and cell-permeant substance converted by endogenous esterases to dichlorofluorescein (DCFH), and nonfluorescent nonpermeant compound that cellular ROS oxidize proportionally to fluorescent dichlorofluorescein (DCF). Briefly, 200,000 PBMCs per well were seeded, in quadruplicate samples. After 48 hours, cells were washed with phosphate-buffered saline (PBS) and incubated with 10 μM DCFH-DA, for 30 min, at 37°C. Finally, the fluorescence emitted by the oxidized form of DCFH-DA was measured using a CytoFluor fluorescence multiwell plate reader (Applied Biosystems, Life Technologies, Monza, Italy) with the excitation fluorescence wavelength at 485 nm and the emission one at 530 nm.

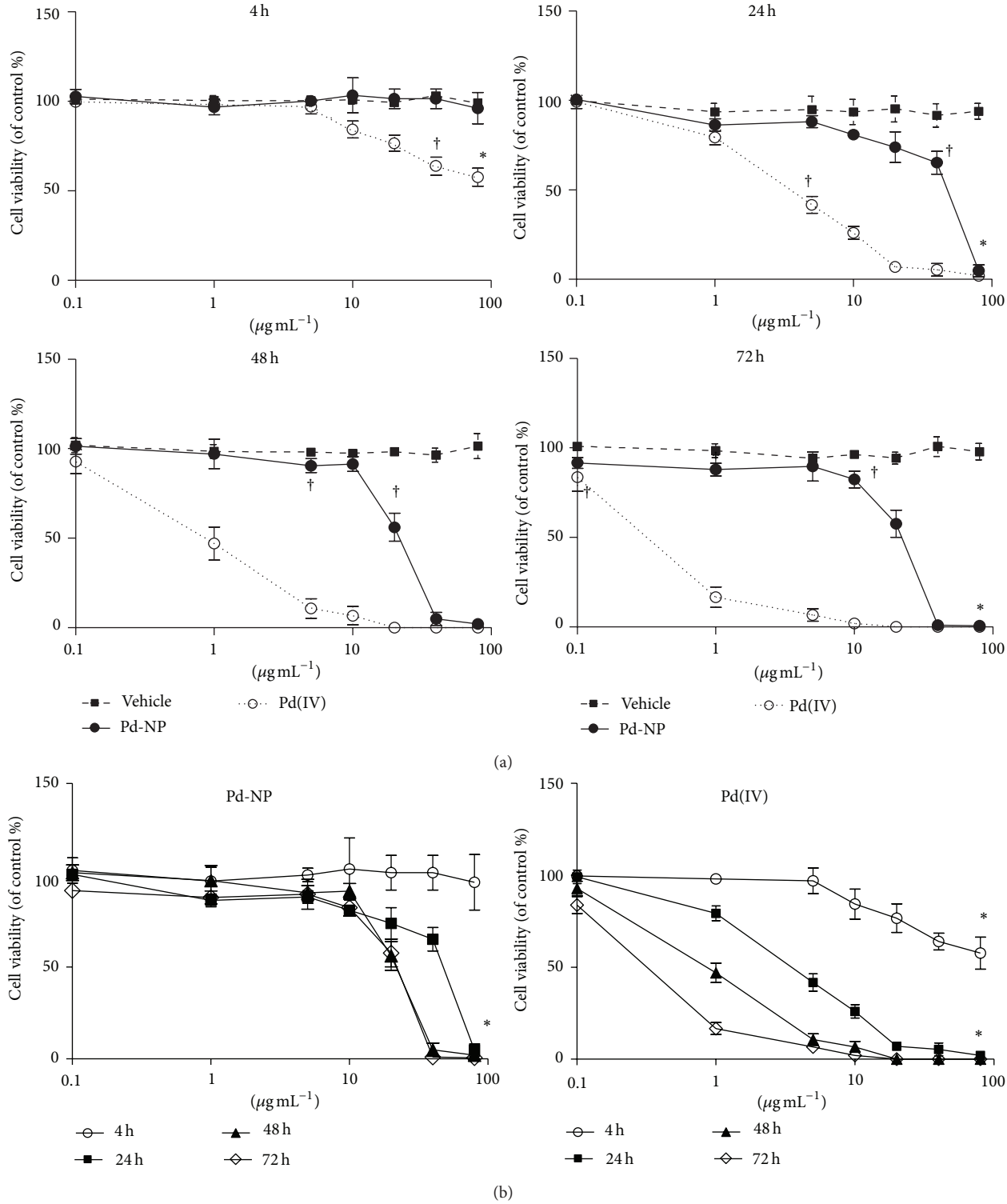

Figure 1: Evaluation of in vitro cytotoxicity by MTT assay. Different concentrations of Pd-NP (circles) (0.1, 1, 5, 10, 20, 40, and 80 μg mL^{-1}) or Pd(IV) ions (open circles) were added to PHA-stimulated PBMCs from healthy donors ($n = 3$) on the 3rd day of culture, in quadruplicate wells. As reference, parallel control wells were set by adding the vehicle alone (culture medium, 10% FCS) (squares). The extent of cell growth was measured after 4, 24, 48, and 72 hours of incubation. The vehicle did not significantly affect cell viability. (a) Data comparing the different compounds (vehicle, Pd-NP, and Pd(IV)) are plotted in logarithmic scale and shown as mean ± S.D., as a function of concentration. (b) Data comparing the incubation time for Pd(IV) and Pd-NP are plotted in logarithmic scale and shown as mean ± S.D., as a function of concentration. The differences in cell viability related to Pd(IV) versus vehicle reached statistical significance ($P < 0.05$) starting from the following concentration/time exposure combinations: 40 μg mL^{-1} after 4 hours, 5 μg mL^{-1} after 24 h, 1 μg mL^{-1} after 48 h, and 0.1 μg mL^{-1} after 72 h. Pd-NP-induced cytotoxicity was statistically significant ($P < 0.05$) versus vehicle for the following conditions: 80 μg mL^{-1} after 24 h, 20 μg mL^{-1} after 48 h, and 10 μg mL^{-1} after 72 h. $^{*}P < 0.001$ three-way ANOVA with Tukey correction, $^{\dagger}P < 0.05$ t-tests Pd-NP and Pd(IV) vs not exposed.

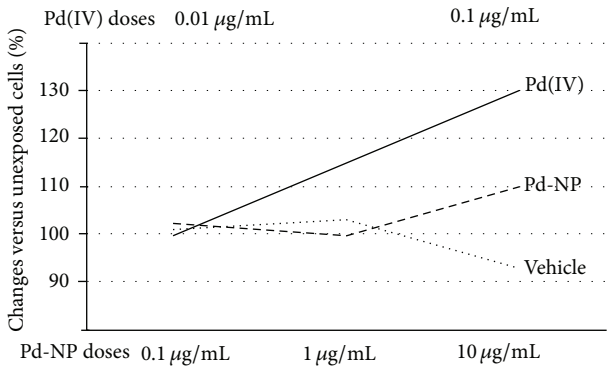

Figure 2: ROS production. Production of ROS by cells exposed to vehicle was similar to that of unexposed cells. Nontoxic concentration of Pd-NP induced a moderate, but not significant, change in ROS production in respect to unexposed and vehicle exposed cells, whereas cells stimulated by Pd(IV) ions showed a significant increase in ROS production (by 30% in respect to unexposed cells. $P < 0.05$).

2.5. Transmission Electron Microscopy. PBMCs were fixed overnight at 4°C in 2% paraformaldehyde and 2% glutaraldehyde in PBS, postfixed in 1% osmium tetroxide in veronal acetate buffer (pH 7.4) for 2 h at room temperature, stained with 2% uranyl acetate, dehydrated in acetone, and embedded in Epon 812. Thin sections were examined under a Philips CM10 TEM after poststaining with uranyl acetate and lead hydroxide.

2.6. Cell Cycle Assay. Cells were fixed in 70% cold ethanol, stained with 50 μg/mL propidium iodide in PBS buffer containing 200 μg/mL RNase (DNase-free, Sigma Aldrich, Milan, Italy), and analyzed using a FACSCalibur flow cytometer equipped with the CellQuest software (Becton Dickinson). Debris were excluded from the analysis after gating them out by setting the forward scatter versus side scatter plot on the viable cells area. Cell doublets and aggregates were excluded by gating FL2-area versus FL2-width. The low flow rate mode (400–500 events sec^{-1}) was used to record 20,000 nondebris events for each sample. PI fluorescence data were collected using the linear amplification mode. DNA content was assessed by placing the G1 peak at channel 400. Finally, data were analysed using ModFit LT software (Verity Software House, Toshan, ME, USA).

2.7. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5.0 software (La Jolla, CA, USA). MTT and ROS data were analyzed by Student's t-test; cell cycle data were analyzed by three- and four-way analysis of variance (ANOVA) with Tukey multiple comparisons correction, when appropriate. The comparison between the different groups in different concentrations was performed with the t-test. In all cases the threshold for significance was set at $P < 0.05$.

3. Results

PBMCs, which are recognized as quiescent cells (G0 state), were induced to enter the cell cycle by in vitro stimulation with PHA, a well-known mitosis inducer. After a latency period of 48 hours, the number of cells started to increase and doubled within further 72 hours. Pd-NP were added 48 hours after PHA, at the beginning of the growth phase. After 4 hours of exposure to Pd-NP, no evident changes in cell viability were observed at any tested dose. Prolongation of the exposure time to 24 hours showed a concentration-dependent decrease of viability up to 40 $\mu g\,mL^{-1}$ and death at 80 $\mu g\,mL^{-1}$. At 48 hours, also 40 $\mu g\,mL^{-1}$ treated cells were incapable of metabolic conversion of MTT, and the same result was obtained after 72 hours of exposure. The viability data for Pd-NP are shown in Figure 1. Pd(IV) ions were more toxic, causing almost complete loss of cell viability earlier, at 48 hours and at lower dose (5 $\mu g\,mL^{-1}$). Therefore, the following experiments were performed using the nontoxic doses of 10 $\mu g\,mL^{-1}$ Pd-NP and 0.1 $\mu g\,mL^{-1}$ Pd(IV) ions, for 48 hours.

A moderate, but not statistically significant increase of intracellular ROS was measured both in controls and in Pd-NP exposed cells, whereas ROS significantly ($P < 0.05$) increased after Pd(IV) exposure by more than 30% of the control (Figure 2). Electron microscopy revealed that, as compared to controls, PBMCs exposed to Pd-NP and to Pd ions shared marked subcellular alterations (Figure 3). Most notably, these included the presence of numerous autophagosomal vacuoles containing damaged mitochondria and/or undigested cytoplasmic material, as well as the accumulation of multilamellar bodies and lipid droplets. Such alterations were more marked in PBMCs exposed to Pd ions, being already well evident at our nontoxic concentration. With both treatments, mitochondria showed evidence of damage, as indicated by condensation and loss or swelling of cristae. Furthermore, in both cases, there was evidence of increased plasma membrane ruffling and nuclei were more indented and/or convoluted compared to controls. In PBMCs exposed to Pd-NP, electron-dense agglomerates of nanosized particulate material within or adjacent to cytoplasmic vesicles were consistent with the internalization of Pd-NP.

The examination of cell cycle (Figure 4) confirmed the quiescent/resting state of all unstimulated PBMCs either freshly isolated (w/o PHA, 0 hours) or cultured for the entire experimental period (w/o PHA, 96 hours), as they were found in the G0 state. The addition of the polyclonal activator PHA stimulated cell division, as shown by their distribution throughout all phases of the cell cycle (G0/G1: 43.1 ± 1.9%; S: 48.8 ± 2.3%; G2/M: 8.1 ± 1.2%). The exposure of PHA-activated PBMCs to 10 $\mu g\,mL^{-1}$ Pd-NP for the time allowing the completion of at least one cell division (48 h) was associated with a significant reduction of cells synthesizing DNA (S-phase) (35.4 ± 1.5% versus 48.8 ± 2.3%, $P < 0.05$), a significant increase of cells within the G0/G1-phase (59.8 ± 1.8% versus 43.1 ± 1.9%, $P < 0.05$), and a significant reduction of cells in G2/M-phase (4.8 ± 0.1% versus 8.1 ± 1.2%, $P < 0.05$), compared with unexposed control cells. A different cell cycle outline was observed for Pd(IV) ion exposed cells, with

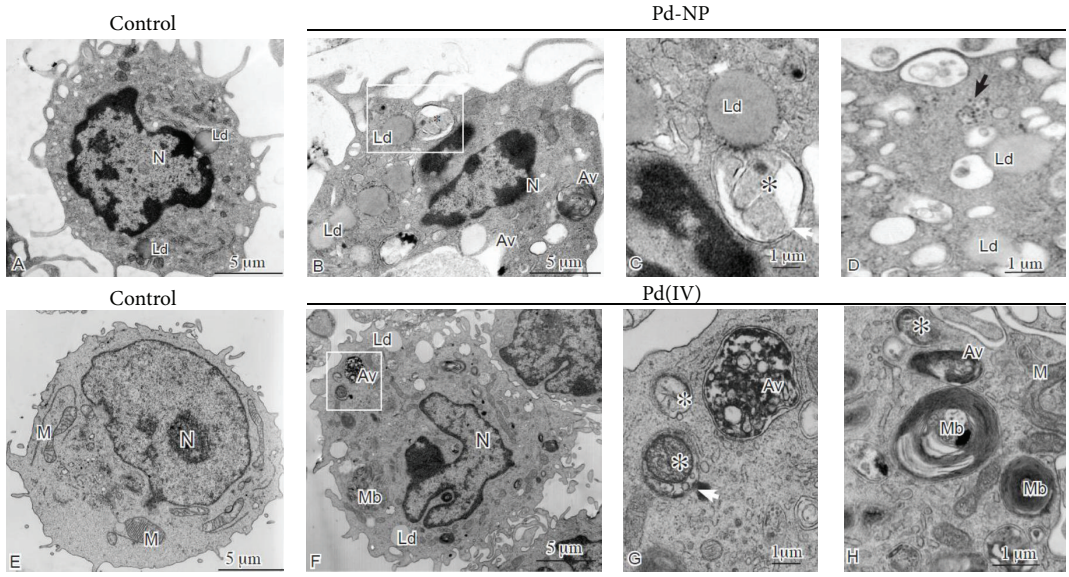

Figure 3: Ultrastructural alterations observed in peripheral blood mononuclear cells exposed to Pd-NP and ions. The upper panels show alterations observed in cells exposed to 10 μg mL^{-1} Pd-NP (B-D) relative to unexposed control (A); the lower panels (F-H) exemplify, relative to control (E), the alterations seen in PBMCs exposed to 0.1 μg mL^{-1} Pd (IV) ions. PBMCs exposed to cobalt NPs and ions show numerous autophagic vacuoles (Av) at different stages of maturation, often enclosing damaged mitochondria (indicated by asterisks in C and G and white arrow in G). Accumulation of lipid droplets (Ld) and multilamellar bodies (Mb), membrane-bound structures composed of concentric membrane whorls, is also readily evident. Agglomerates of electron-dense nanosized particles, consistent with internalized Pd-NP, are present within or near cytoplasmic vacuoles in PBMCs (indicated by black arrows in D). Compared to the untreated controls, the PBMCs exposed to Pd-NP and ions show increased membrane ruffling and more indented nuclei (B and F).

significantly higher G0/G1 accumulation compared to Pd-NP exposed cells (74.9 ± 2.2% versus 59.8 ± 1.8%, $P < 0.05$), lower percent of cells in S-phase (25.1 ± 1.2% versus 35.4 ± 1.5%, $P < 0.05$), and no cells engaged in the cell division (G2/M). The vehicles alone did not cause significant changes of the cell cycle distribution. Also, PBMCs were evaluated for cell cycle distribution after exposure to Pd-NP and Pd(IV), in absence of PHA stimulation; in no case were these compounds found to induce cell cycle initiation and all cells remained in the G0 phase (data not shown).

Sub-G0/G1 cells, indicative of cell death, were largely absent from the analyzed populations, for all tested samples. All cell cycle data are summarized in (Table 1).

4. Discussion

In the present work, we have found that Pd-NP exposure is associated with an increased percentage of mitogen-activated PBMCs with a diploid DNA content, indicative of maintenance of G0 state or prolongation/arrest in G1-phase. Studying the influence of potential toxicants on the G1-phase of the cell cycle is of central importance; in fact, most, if not all, human cancer types show a deregulated control of G1 progression, a period in which cells decide whether to start proliferation or stay quiescent [31, 32]. Important processes, such as increase in cell size and centrosome duplication, are initiated in G1 and are thus controlled. Control is conducted by checkpoint complexes that stall the cell cycle until the appropriate repair is completed. If such repair is not achieved, the cell may pause or undergo senescence or apoptosis. Alteration of repair (controlled by caretakers) and checkpoint complexes (controlled by gatekeepers genes) is central in oncogenesis [33].

In our experiments, PBMCs, which are mostly quiescent cells in the G0 state and prompted to enter the cell cycle by in vitro stimulation with PHA, were exposed to low noncytotoxic dose-time combinations of Pd-NP and Pd ions. Under these experimental conditions, Pd-NP gain entry to the cytosol, where they appear in clusters lacking a membranous envelope, suggesting the possibility to exert a catalysing redox activity [34] on cellular elements.

At the ultrastructural level the alterations observed in the PBMCs exposed to Pd-NP largely corresponded to those observed in the PBMCs exposed to Pd ions. Most notably, in both cases the cells showed evidence of mitochondrial damage and of mitophagy, that is, autophagic elimination of damaged mitochondria. Mitophagy is a key protective mechanism against mitochondrial damage and the consequent ROS-induced cellular alterations [35]. The accumulation of abundant multilamellar bodies is also in agreement with the macroautophagic response, as these structures originate via autophagy and reflect the accumulation of membrane lamellar material selectively resistant to lysosomal degradation within autophagolysosomes. Lipid droplets, specialized organelles for the deposition and storage of neutral lipids, are associated with common pathologies linked to lipid accumulation and mitochondrial damage. Their accumulation in

Table 1: Cell cycle distribution data of quiescent and PHA-activated PBMCs exposed to Pd-NP or Pd(IV) ion.

Phase	PHA	PHA + Pd(IV) ion	PHA + Pd-NP	PHA + vehicle	w/o PHA 0 h	w/o PHA 48 + 48 h
G0/G1	43.1 ± 1.9	74.9 ± 2.2*	59.8 ± 1.8*	44.4 ± 2.1	98.9 ± 0.2	99.0 ± 0.1
S	48.8 ± 2.3	25.1 ± 1.2*	35.4 ± 1.5*	47.3 ± 1.7	0	0
G2/M	8.1 ± 1.2	0	4.8 ± 0.1	8.3 ± 1.4	0.9 ± 0.2	1.0 ± 0.1

Data from three experiments are shown as mean ± S.D. *$P < 0.05$ Pd-NP and Pd(IV) vs not exposed.

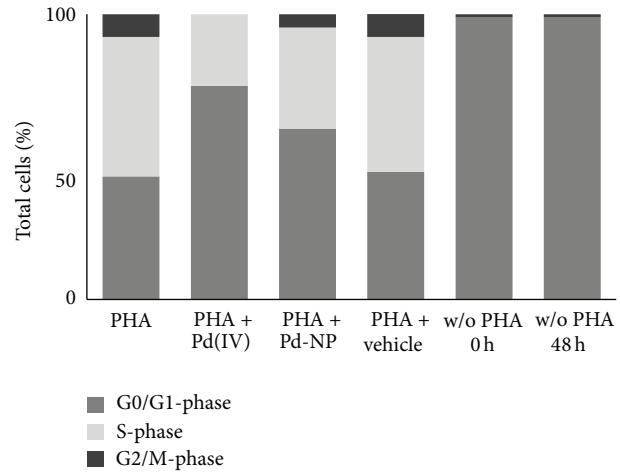

Figure 4: Cell cycle analysis of human PBMCs exposed to Pd-NP. PBMCs ($n = 3$) were cultivated in the presence or absence of PHA for 48 hours and after being exposed or not to Pd-NP (10 μg mL^{-1}) or Pd(IV) ion (50 ng mL^{-1}) for 48 hours. For comparison, an equal volume of vehicle was used in parallel cultures. The proportion of cells in the different phases was quantitated using ModFit software and represented with partitioned bars as the average of three experiments. G0/G1-phase: dark grey; S-phase: light grey; G2/M-phase: black.

PBMCs exposed to Pd is fully consistent with the loss of mitochondrial fatty acid beta oxidation due to mitochondrial damage, that, in turn, results in lipid accumulation and loss of ATP production.

In conclusion, the morphological evidence strongly suggests that Pd ions, per se or dissolved from NPs, accumulate in the mitochondria, as we recently demonstrated in the case of cobalt [26]. Here, acting as catalyst, ionic Pd may interfere with the oxidative phosphorylation (OXPHOS). The mitochondria, whose respiratory activity is damaged by the influx of Pd ions, will be poor ATP producers and will release excessive amounts of ROS. The ROS-induced cellular stress is predicted to arrest the cell cycle, acting via oxidative stress-sensitive cell cycle regulator genes such as P53 and CDKN2a, which we have recently found to be implicated in the response to Co particles in Balb/3T3 cells [28]. In turn, ROS and ATP depletion activate cell death pathways, which induce the release of proapoptotic proteins from mitochondria. Macroautophagy may mitigate these effects by the selective sequestration and subsequent degradation of the dysfunctional mitochondria and other ROS-damaged subcellular structures before the activation of prodeath pathways [30, 35, 36]. Thus, macroautophagy, and, in particular, mitophagy, most likely represents a key prosurvival pathway in cells exposed to palladium.

Autophagy has been demonstrated to be marker of heavy metals toxicity for human hematopoietic progenitor cells [30], representing a protecting and recycling catabolic pathway removing damaged organelles and molecules [37]. The cellular outcome of inducing autophagy in response to stress is complex and depends on the cellular context, type, and magnitude of stress. In mild stress conditions, autophagy may enhance cell survival by allowing the cell to engage DNA repair mechanisms and checkpoint activation [38]. Nontoxic dose of Pd ions induced a more marked production of ROS relative to Pd-NP, contrary to almost all other metal-based NPs [39]. However, local ROS generation at sites of Pd-NP accumulation, not measurable by the overall method used, might be possible at the nontoxic doses applied in our experiments. In fact, local concentration is important for the cellular function of ROS [40]. On the other hand, changes in ROS generation may modulate autophagic processes, as ROS can modify activating molecules to stimulate or inhibit autophagy and engages in cross-talk with autophagy in both cell signaling and protein damage [41].

Mechanisms of cell damage by Pd-NP can be also explained through cell cycle disturbance by the withdraw/inactivation of nutrients and/or PHA or by modulation/inhibition of downstream signaling due to their catalytic activity, as described for polystyrene-coated Pd nanoparticles [34]. Therefore, Pd-NP might produce an effect similar to nutrient depletion in inducing autophagy in the G1 phase [42]. It is possible that Pd-NP interference with mitogen/growth factors would prolong the G1-phase checkpoint called restriction point that prevents cells from entering the cycle until they have accumulated a certain threshold of mitogen-induced events [43].

5. Conclusions

The described findings suggest a potentially harmful role for low dose Pd ions and Pd-NP in hematopoietic cells leaving quiescence. However, it will be essential to verify whether the observed disturbance of the transition from quiescence to cell division represents a temporary response or might lead to permanent alterations of the cell functions. To this aim, long-term exposure to low dose of Pd (ions and NPs) and single cell-based technologies are envisaged.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 304180, 10 pages
http://dx.doi.org/10.1155/2014/304180

Research Article

Role of Lysosomes in Silica-Induced Inflammasome Activation and Inflammation in Absence of MARCO

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Received 30 April 2014; Accepted 3 June 2014; Published 26 June 2014

Academic Editor: Takemi Otsuki

MARCO is the predominant scavenger receptor for recognition and binding of silica particles by alveolar macrophages (AM). Previously, it was shown that mice null for MARCO have a greater inflammatory response to silica, but the mechanism was not described. The aim of this study was to determine the relationship between MARCO and NLRP3 inflammasome activity. Silica increased NLRP3 inflammasome activation and release of the proinflammatory cytokine, IL-1β, to a greater extent in MARCO$^{-/-}$ AM compared to wild type (WT) AM. Furthermore, in MARCO$^{-/-}$ AM there was greater cathepsin B release from phagolysosomes, Caspase-1 activation, and acid sphingomyelinase activity compared to WT AM, supporting the critical role played by lysosomal membrane permeabilization (LMP) in triggering silica-induced inflammation. The difference in sensitivity to LMP appears to be in cholesterol recycling since increasing cholesterol in AM by treatment with U18666A decreased silica-induced NLRP3 inflammasome activation, and cells lacking MARCO were less able to sequester cholesterol following silica treatment. Taken together, these results demonstrate that MARCO contributes to normal cholesterol uptake in macrophages; therefore, in the absence of MARCO, macrophages are more susceptible to a greater inflammatory response by particulates known to cause NLRP3 inflammasome activation and the effect is due to increased LMP.

1. Introduction

Silica is a basic component of soil, sand, and most types of rocks. Silica forms a tetrahedral structure and there are two common forms of silica—crystalline silica and amorphous silica. Crystalline silica has a regular tetrahedral structure, while amorphous silica has an irregular structural arrangement. Environmental or occupational exposure to crystalline silica particles over an extended period of time results in pulmonary inflammation, which plays a vital role in pathological development of silicosis. Silicosis is a lung disease characterized by inflammation and fibrosis and remains a prevalent health problem throughout the world. Currently, treatment choices for silicosis are limited and at present no cure exists for silicosis [1, 2]. Consequently, it is important to further define mechanisms of silica-induced inflammation on which new therapeutic approaches could be developed.

Inhaled silica particles are encountered by alveolar macrophages (AM) in the lungs. The AM are the primary innate immune phagocytic cells at the air tissue interface responsible for clearance of particles through the mucociliary escalator and/or lymphatic systems [2]. Previous studies have demonstrated that AM recognize and bind silica particles through class A scavenger receptors (SR) expressed on their surface [3–6]. The class A SR family includes SR-A and MARCO, which function primarily as phagocytic receptors binding to a variety of microbial components and can also modulate inflammatory signaling by Toll-like receptors [7, 8]. SR-AI, SR-AII, and MARCO are associated with silica binding, and MARCO is the predominant receptor for binding and uptake of unopsonized particles such as silica [5, 9–13].

Previous *in vivo* results showed an increased inflammatory response in MARCO$^{-/-}$ mice compared with WT mice following 24 hrs of silica exposure [14]. There was an increase in total protein levels and total number of lavage cells and a significant increase in infiltration of immune cells such as AM, DC, and neutrophils in MARCO$^{-/-}$ mice compared with WT mice, all indicating an increase in inflammation in

MARCO$^{-/-}$ mice. However, the mechanism to explain the increased inflammatory response in the absence of MARCO was not clear.

Recent advances in understanding the NLRP3 inflammasome have explained the role of this pathway in inflammation and fibrosis. Particles phagocytized by AM are confined to intracellular vesicles called phagosomes, which undergo a series of interactions with endosomes and lysosomes [15–18]. Certain particles, such as silica, permeabilize the lysosome leading to release of lysosomal enzymes, including cathepsin B, to the cytoplasm [19, 20]. This triggers the assembly of the NLRP3 inflammasome—a multiprotein complex [21]. The inflammasome assembly consists of NALP3 protein, the adaptor protein ASC (apoptosis—associated speck-like protein containing a Caspase recruitment domain (CARD)), and pro-Caspase-1 [22, 23]. Adaptor protein ASC connects a NALP3 protein to pro-Caspase-1 leading to cleavage of the CARD domain of pro-Caspase-1 resulting in activation of Caspase-1. At this point another signal is required, which is triggered by endotoxins like LPS to activate the NF-κB pathway. NF-κB, a transcription factor, is responsible for formation of pro IL-1β and pro IL-18 [24]. Active Caspase-1 catalyzes cleavage of the inactive precursor molecules pro IL-1β and IL-18 to their active forms, IL-1β and IL-18 [24, 25]. IL-1β and IL-18 have been associated with inflammation and it is evident from the literature that inflammation and fibrosis development are closely linked to the maturation and release of these inflammasome cytokines [26–30].

We propose that the MARCO receptor can modulate the sphingomyelin pathway, leading to the lysosomal accumulation of sphingomyelin, phosphatidylcholine, and cholesterol and a decrease in ceramide. The sphingomyelin pathway is a ubiquitous signaling system, which activates multiple signal transduction pathways associated with both physiological and pathological processes that include cell growth, cell death, autophagy, angiogenesis, cancer, and inflammatory responses [31]. This pathway is initiated by the hydrolysis of membrane phospholipid sphingomyelin to ceramide. Increased ceramide production has been indicated in inflammation in response to a large variety of stressors [32]. It is anticipated that accumulation of cholesterol in the lysosomal membrane can contribute to lysosomal membrane stabilization and prevention of inflammasome activation and downstream inflammation [33–35]. Furthermore, we propose that in the absence of MARCO receptor there is decreased cholesterol accumulation in the lysosomal membrane making them susceptible to membrane permeabilization upon exposure to silica. In this study we examined the effect of MARCO on inflammasome activation upon exposure to silica. For this study, AM isolated from C57BL/6 wild-type (WT) and MARCO null mice were used to determine the role of MARCO in silica-induced inflammasome activation and inflammation.

2. Results and Discussion

2.1. Evaluation of the Effects of Silica on IL-1β Production in C57BL/6 WT and MARCO$^{-/-}$ AM. Since the molecular mechanisms involved in increased inflammation in MARCO$^{-/-}$ compared to WT mice are not completely understood, an *in vitro* study was conducted to measure release of key cytokines and activity of key enzymes in AM of WT and MARCO$^{-/-}$ mice. AM from MARCO$^{-/-}$ and WT mice were stimulated with LPS (20 ng/mL) and then treated with 0, 25, 50, 100, or 200 μg/mL of silica for 24 hrs. Silica caused a dose-dependent increase in IL-1β release from MARCO$^{-/-}$ and WT AM. IL-1β release was significantly higher in MARCO$^{-/-}$ AM than WT AM at dosage values 100 μg/mL and 200 μg/mL (Figure 1(a)). Maximum IL-1β was released at 100 μg/mL and decreased at 200 μg/mL in both MARCO$^{-/-}$ and WT AM, most likely due to cytotoxicity. The increased production of IL-1β by MARCO$^{-/-}$ AM relative to WT AM is consistent with the observation of increased inflammation *in vivo* in MARCO$^{-/-}$ mice [14].

In order to determine whether the increase in IL-1β release from MARCO$^{-/-}$ AM was due to the absence of MARCO receptor and not an unknown compensatory mechanism, MARCO Ab was used to block MARCO function in WT AM. IL-1β release from WT AM treated with LPS, silica, and MARCO Ab was also significantly higher than WT AM treated with LPS and silica only (Figure 1(b)). Furthermore, the resulting levels of IL-1β release by blocking MARCO receptor function using MARCO Ab with WT AM were similar to levels of IL-1β release from MARCO$^{-/-}$ AM treated with LPS and silica. This result implies that the difference in IL-1β release between the WT AM and MARCO$^{-/-}$ AM can be attributed to the absence of MARCO function.

2.2. Effect of Silica on Cathepsin B Activation. Lysosomal membrane permeabilization (LMP) leads to cathepsin B release from the lysosomal lumen to the cytoplasm [19, 20]. Release of cathepsin B to the cytosol triggers NLRP3 inflammasome assembly and subsequent downstream inflammation. In order to determine whether the effect of MARCO was at LMP, the contribution of silica on cathepsin B release to the cytosol was determined (Figure 2). Primary AM from MARCO$^{-/-}$ and WT mice were stimulated with or without LPS and then treated with silica (100 μg/mL) for 2 hrs prior to cathepsin B assay. Cathepsin B activation was higher in AM treated with silica alone or silica plus LPS treated AM from MARCO$^{-/-}$ mice compared to WT AM. The results indicate that silica exposure increases cathepsin B activity and corresponds to increased lysosomal membrane permeabilization, which was increased in the absence of MARCO.

2.3. Effect of MARCO on IL-1β. The activation of NLRP3 inflammasome is known to be essential for maturation of IL-1β [29] and cathepsin B has been proposed to work upstream of NLRP3 inflammasome assembly [36]. Therefore, the contribution of cathepsin B to silica-induced IL-1β release from AM was examined. As expected, AM pretreated with LPS and a cathepsin B inhibitor (10 μM CA-074-Me added 30 min prior to silica exposure) showed a significant reduction in IL-1β release compared to the absence of the cathepsin B

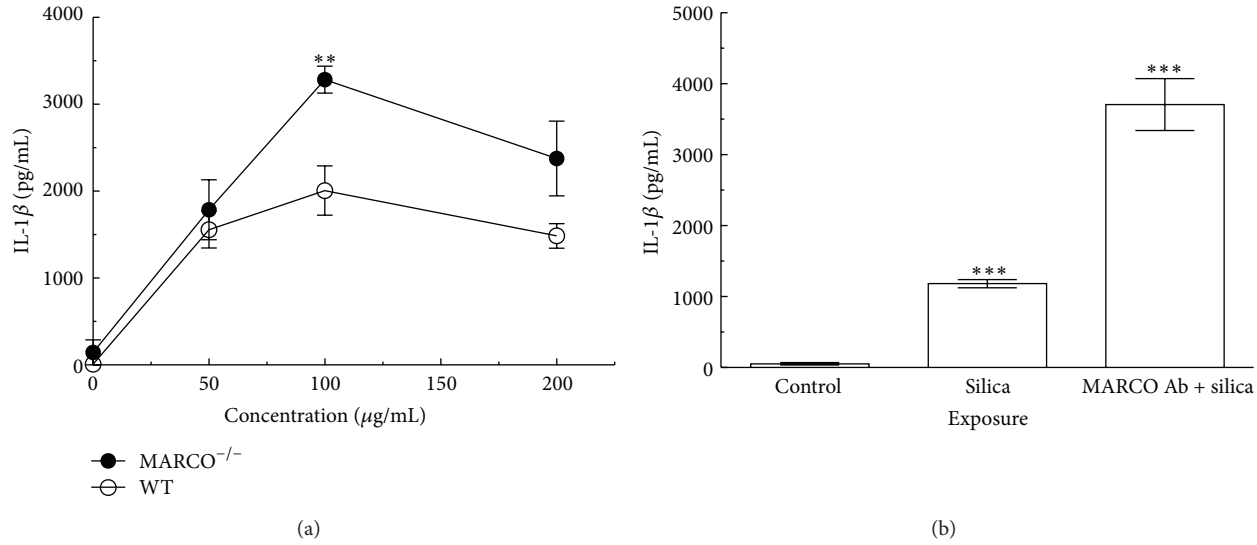

Figure 1: *Evaluation of the effects of doses of silica and IL-1β production in AM from WT and MARCO$^{-/-}$ mice.* (a) Dose response to silica-stimulated IL-1β release from AM cocultured with endotoxin (LPS at 20 ng/mL) 24 hrs was measured by ELISA. Mean ± SEM IL-1β from cultured macrophages where filled circle ● indicates MARCO$^{-/-}$ AM and open circle ○ indicates C57BL/6 WT AM. Double asterisk ** indicates $P < 0.01$ compared to WT control at the corresponding concentration, $n = 4$ per experimental group. (b) IL-1β release from WT AM with MARCO Ab. AM were pretreated with or without MARCO Ab (5 μg/mL) then exposed to silica (100 μg/mL) in the presence or absence of LPS (20 ng/mL) 24 hrs. Mean ± SEM IL-1β release from cultured macrophages was analyzed by ELISA. Triple asterisk *** indicates $P < 0.001$ compared to control at the corresponding treatment, $n = 4$ per experimental group. Unstimulated cells were used as control.

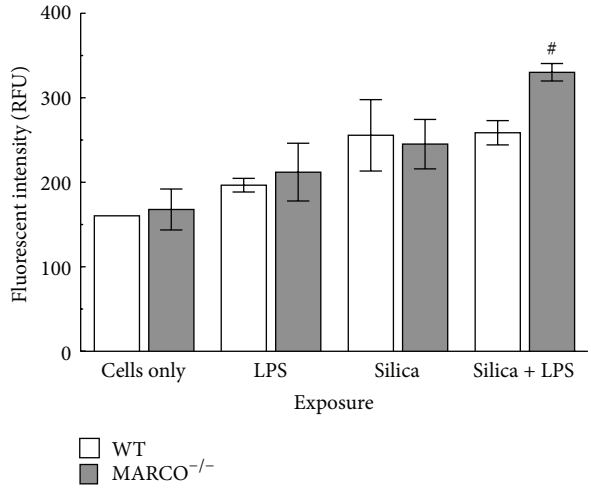

Figure 2: *Cathepsin B activation in MARCO$^{-/-}$ and WT AM exposed to silica.* MARCO$^{-/-}$ AM and WT AM were stimulated with or without LPS (20 ng/mL) and then exposed to silica (100 μg/mL) for 2 hrs prior to cathepsin B activity assay. Mean ± SEM cathepsin B activity where white bar indicates WT AM and grey bar indicates MARCO$^{-/-}$ AM. Hashtag # indicates $P < 0.05$ compared to MARCO$^{-/-}$ unstimulated (cells only) group.

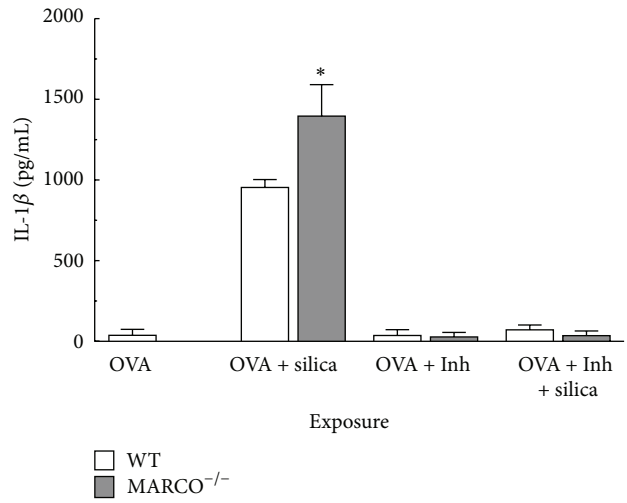

Figure 3: *Effect of MARCO on NLRP3 activation using IL-1β as an indicator* and *OVA as signal 1.* IL-1β release from WT and MARCO$^{-/-}$ AM in response to silica (100 μg/mL) cocultured with OVA (10 mg/mL) and with or without cathepsin B inhibitor (10 μM CA-074-Me). Mean ± SEM IL-1β from cultured macrophages where white bars indicate WT AM and grey bars indicate MARCO$^{-/-}$ AM. Asterisk * indicates $P < 0.05$ compared to WT, $n = 3$ per experimental group.

inhibitor (Figure 3). The MARCO receptor is known to bind LPS [3], and in the absence of MARCO, more LPS could be expected to bind to CD14 and signal more pro-IL-1β production [37, 38]. In order to rule out the possibility that the differences in IL-1β release in the absence of MARCO were due to increased availability of LPS for signaling through CD14, ovalbumin (OVA) was used as an alternate stimulant to increase pro-IL-1β levels in AM [39]. As shown in Figure 3, IL-1β release with OVA as a stimulant was comparable to

LPS. Furthermore, CA-074-Me was effective in blocking IL-1β release. Therefore, the results with OVA and the cathepsin B inhibitor indicate that the difference in IL-1β maturation in MARCO$^{-/-}$ and WT was not due to differences in availability of LPS acting on CD14 and that regardless of the source of signal 1 (LPS or OVA) inhibiting cathepsin B blocked IL-1β maturation and release.

2.4. Effect of Silica on Caspase-1. Measuring IL-1β release is a proxy measure of NLRP3 inflammasome activation since it requires both signal 1 (NF-κB mediated pro-IL-1β generation) and signal 2 (NLRP3 inflammasome activation). Therefore, a more direct assay for NLRP3 inflammasome activation is to measure Caspase-1 activity. Primary AM from MARCO$^{-/-}$ and WT were stimulated with or without LPS and then treated with silica (100 μg/mL) for 4 hrs prior to Caspase-1 assay. The pattern of results for Caspase-1 activity was similar to IL-1β release in that it was higher in MARCO$^{-/-}$ AM compared to WT AM (Figure 4). Therefore, the results suggest that the effect of MARCO on the NLRP3 inflammasome or signal 2 pathway does not involve signal 1, consistent with the above findings using OVA as a signal 1 initiator. Furthermore, the findings are consistent with the cathepsin B results, which also suggested the effect of MARCO on modulating the NLRP3 inflammasome pathway. Taken together, the results suggest that the target of the contribution of MARCO in regulating IL-1β release with silica may well be at lysosomal membrane permeability since in the absence of MARCO there is less silica being taken up although there is more cathepsin B release and correspondingly greater Caspase-1 activity.

2.5. Induction of Pro-IL-1β Expression in AM. Pro-IL-1β mRNA expression in MARCO$^{-/-}$ and WT AM was measured in order to evaluate the contribution of MARCO to signal 1 activation. Primary AM from MARCO$^{-/-}$ and WT mice were treated with LPS, silica, or left untreated. Pro-IL-1β expression in AM was measured by RTPCR and the results are shown in Figure 5. Pro-IL-1β expression was higher in MARCO$^{-/-}$ AM compared to WT following LPS stimulation. This finding is consistent with the increased binding of LPS to CD14 in the absence of MARCO, although as the results presented in Figure 4 illustrated that primary contribution of MARCO was to regulate signal 2. Silica treatment produced negligible expression of pro-IL-1β in MARCO$^{-/-}$ and WT AM tested. These results confirm that LPS is a stimulant for pro-IL-1β expression, while silica exposure has minimal to no effect on pro-IL-1β expression. Consequently, activation of signal 2 has little impact on signal 1.

2.6. Effect of Inflammasome Activation on IL-6 Release. In order to evaluate whether the effect of MARCO was specific (restricted to inflammasome cytokines) or more global on macrophage cytokine production, the effect of silica on MARCO$^{-/-}$ and WT AM IL-6 production, which does not require NLRP3 inflammasome activation, was also measured after 24 hrs (Figure 6). In contrast to the IL-1β production

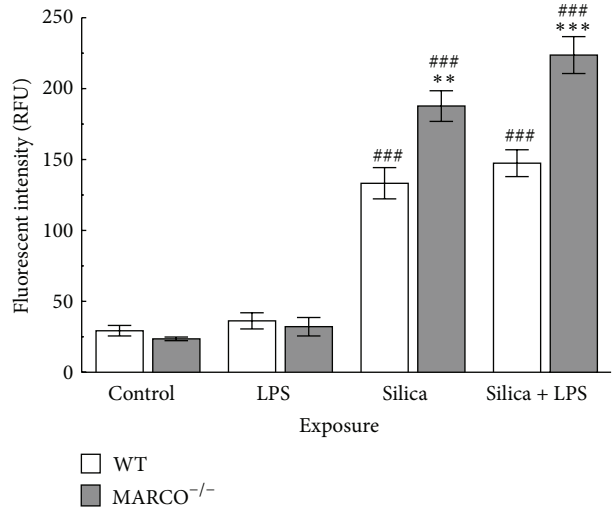

Figure 4: *Caspase-1 activation in MARCO$^{-/-}$ and WT AM exposed to silica.* Caspase-1 activation in WT and MARCO$^{-/-}$ AM was measured 4 hrs after treatment with silica (100 μg/mL) with or without LPS (20 ng/mL) as described in Methods. Mean ± SEM white bars indicate WT AM and grey bars indicate MARCO$^{-/-}$ AM. Double asterisk ** indicates $P < 0.01$ and triple asterisk *** indicates $P < 0.001$ compared to WT at corresponding treatment. $^{\#\#}$ indicates $P < 0.01$ compared to control in the same mice strain. $n = 3$ per experimental group.

results, IL-6 levels in the LPS-treated group in MARCO$^{-/-}$ AM were significantly lower than WT AM. There was also significantly less IL-6 in the LPS- and silica-treated groups, compared to LPS stimulated group. These results are consistent with the known ability of LPS to stimulate IL-6 production and IL-6 production does not depend on inflammasome activation. However, IL-6 production is not upregulated by MARCO.

2.7. Effect of Silica on Acid Sphingomyelinase Activity. Acid sphingomyelinase is a lipid-metabolizing enzyme localized in lysosomes [32]. Acid sphingomyelinase hydrolyses membrane phospholipid sphingomyelin to ceramide [31]. In addition acid sphingomyelinase is an important regulator of the sphingolipid metabolism pathway [40]. Acid sphingomyelinase levels were measured following silica treatment of AM because of its reported association with inflammation and acute lung injury [41]. Primary AM from MARCO$^{-/-}$ and WT mice were treated with or without silica (100 μg/mL) for 1 hr. After 1 hr cell lysates were prepared for sphingomyelinase activity and the results are shown in Figure 7. There was a significant increase in acid sphingomyelinase activity in MARCO$^{-/-}$ AM treated with silica compared to WT AM incubated with silica. These results indicate that acid sphingomyelinase activity was significantly higher in MARCO$^{-/-}$ AM than WT supporting the notion that silica exposure affects the sphingolipid pathway and consequently may decrease sphingomyelin and increase ceramide levels in the absence of MARCO [32].

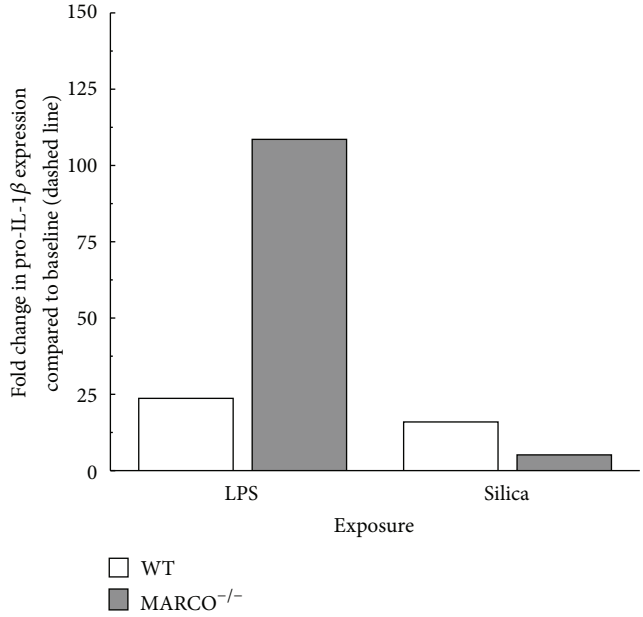

Figure 5: *Pro-IL-1β mRNA expression in WT and MARCO$^{-/-}$ AM analyzed using RT-PCR.* White bars indicate WT AM and grey bars indicate MARCO$^{-/-}$ AM. Fold change in pro-IL-1β expression was compared to unstimulated control. The data represent one experiment with 2 replications.

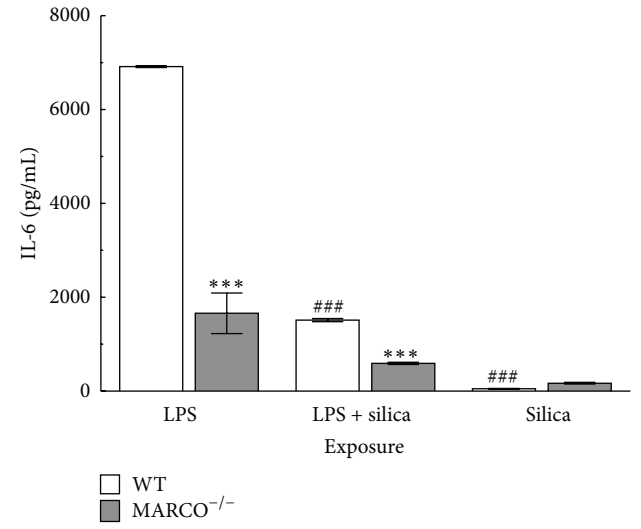

Figure 6: *Effect of inflammasome activation on IL-6 release.* IL-6 release from WT and MARCO$^{-/-}$ AM in response to silica (100 μg/mL), LPS (20 ng/mL), or silica and LPS. Mean ± SEM IL-6 from cultured AM where white bars indicate WT AM and grey bars indicate MARCO$^{-/-}$ AM. Triple asterisk *** indicates $P < 0.001$ compared to WT at corresponding treatment, $^{###}$ indicates $P < 0.001$ compared to control in the corresponding mouse strain, $n = 3$ per experimental group.

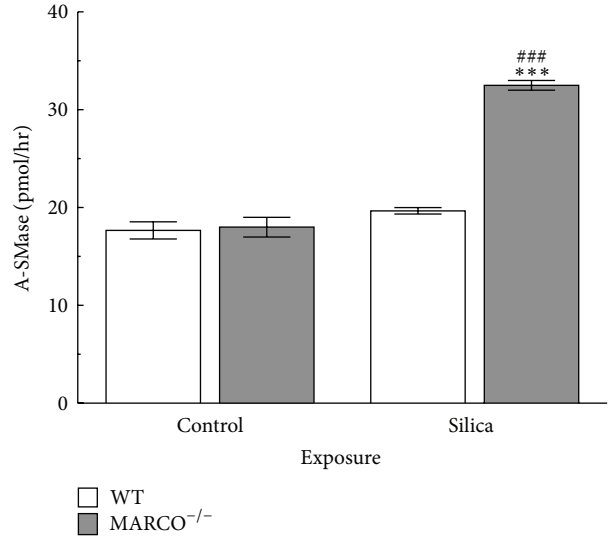

Figure 7: *Effect of silica on acid sphingomyelinase activity.* Acid sphingomyelinase activity in WT and MARCO$^{-/-}$ AM, 1 hr after treatment with silica (100 μg/mL). White bars indicate WT AM and grey bars indicate MARCO$^{-/-}$ AM. Triple asterisk *** indicates $P < 0.001$ compared to WT at corresponding treatment. $^{###}$ indicates $P < 0.001$ compared to control at the corresponding mouse strain, $n = 3$ per experimental group.

2.8. Effect of Silica on Intracellular Cholesterol. The results above demonstrated that silica exposure impacted lipid metabolism, which is associated with lysosomes. Since the critical impact of silica signaling appears to be on lysosomal membrane permeability and cholesterol is known to affect lysosomal integrity [33] the potential role of cholesterol content in AM was examined as a potential mechanistic explanation for LMP. Therefore, cholesterol levels in WT and MARCO$^{-/-}$ AM were assessed by loading cells with 1 μg/mL TopFuor cholesterol for 24 h and then treated with or without silica for 4 hrs. As expected, there is very large background fluorescence due to the naturally high cholesterol content in AM (Figure 8). There was a significant increase in intracellular cholesterol (represented as an increase in TopFuor fluorescence) in WT AM in response to silica exposure compared to control. This outcome is consistent with the proposal that silica is inducing cholesterol uptake and could contribute to protection against LMP. However, intracellular cholesterol in MARCO$^{-/-}$ AM in response to silica exposure did not change significantly compared to the baseline control and was less than that taken up by WT AM (although it was not significantly different from WT AM treated with silica). This result is consistent with the proposal that AM lacking MARCO were not able to take up as much cholesterol making them more susceptible to LMP. This would also explain why the difference in IL-1β release was only evident at the higher doses of silica.

2.9. Effect of Lysosomal Cholesterol on NLRP3 Inflammasome. The above results suggested that cholesterol in the lysosomal membrane can influence lysosomal membrane stabilization,

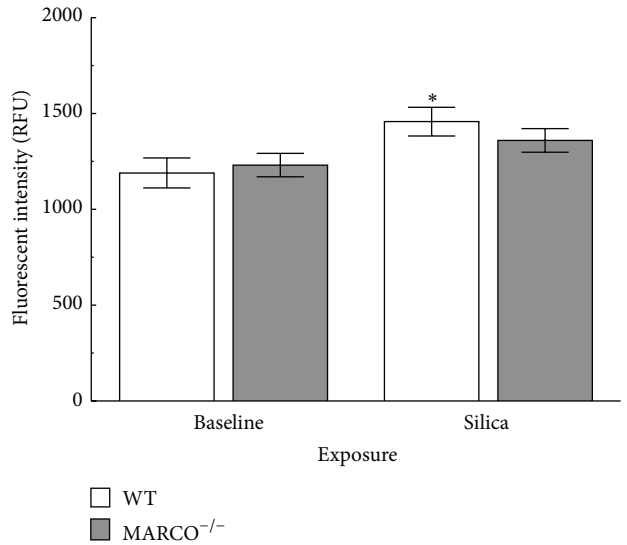

Figure 8: *Increased cholesterol uptake in WT AM in response to silica*. Isolated AM were loaded with TopFluor cholesterol (1 μg/mL) for 24 hrs prior to silica (50 μg/mL) exposure in RPMI media with 5% delipidated FBS. Silica exposure was 4 hrs in regular RPMI culture media. The wells were read in a microplate reader at 488 nm ex/525 nm em. Asterisk * indicates statistical significance at $P < 0.05$ compared to baseline WT response, $n = 7$.

inflammasome activation, and downstream inflammation. In order to confirm the role of cholesterol on silica-induced LMP leasing to IL-1β release, the cholesterol trafficking modifier U18666A was used. U18666A blocks the intracellular trafficking of cholesterol and the exit of free cholesterol from the late endosomal compartment [42]. It also inhibits oxidosqualene cyclase and desmosterol reductase eliminating cholesterol biosynthesis [42]. Primary AM from WT mice were treated with U18666A for 24 hrs and then exposed to silica followed by LPS for another 24 hrs. Cell viability was also measured at 24 hrs after particle exposure by the MTS assay. Silica caused a significant decrease in cell viability; however, there was significant increase in cell viability in silica-U18666A exposed group compared to baseline silica group demonstrating protection against cell death (Figure 9(a)). There was the expected significant IL-1β release following silica exposure (Figure 9(b)) compared to control. However, IL-1β release was significantly less in the silica-U18666A exposed group compared to silica treatment alone. Taken together, these results indicate that lysosomal cholesterol accumulation can decrease LMP, NLRP3 inflammasome activation, and IL-1β release.

3. Experimental Section

3.1. Mice. Breeding pairs of C57Bl/6 were originally purchased from the Jackson Laboratory (Bar Harbor, ME), while MARCO null breeders were kindly provided by Dr. Lester Kobzik (Harvard School of Public Health, Boston, MA). MARCO null mice were on C57BL/6 background. Animals were housed in microisolators on a 12-hr light/dark cycle. The mice were maintained on an ovalbumin-free diet and given deionized water ad libitum. Age matched (6–8 week) male and female mice were used in all studies. All mice were maintained in the University of Montana specific pathogen free laboratory animal facility. The University of Montana Institutional Animal Care and Use Committee approved all animal procedures.

3.2. Particles. Crystalline silica (Min-U-Sil-5, average particle size 1.5–2 μm in diameter) was obtained from Pennsylvania sand glass corporation (Pittsburgh, PA) and was acid-washed in 1 M HCl at 100°C for 1 hr. The silica was washed in sterile water three times and dried in an oven at 200°C to remove all water. A stock suspension of 5 mg/mL in phosphate-buffered saline (PBS) was made. Silica suspensions were sonicated for 1 min at half max power in a Masonix cup-horn sonicator (XL2020, Farmingdale, NY) attached to a ThermoForma circulating water-bath at 550 watts and 20 Hz (8000 Joules).

3.3. Alveolar Macrophage Isolation and Culture. Mice were euthanized by a lethal injection of Euthasol (Virbac Corp, Fort Worth, TX). The lungs were removed with the heart. The lungs were lavaged five times with 1 mL of cold PBS. Pooled cells were centrifuged at 1500 ×g for 5 min. The lavage fluid was aspirated and discarded. The cells were resuspended in 1 mL of RPMI 1640 culture media supplement with 10% fetal bovine serum, 100 IU penicillin, and 100 μg/mL streptomycin (Media tech, Inc., Herdon, VA). Total nucleated lavage cell fractions were counted by lysing the red blood cells with Zap-globin reagent II lytic reagent (Beckman Coulter, Fullerton, CA) and by using Z2 Coulter particle count and size analyzer (Beckman Coulter, Fullerton, CA). The concentration of cells was then adjusted to 10^6 cells/mL before doing any further steps. Later the cells were treated with or without silica or endotoxin or a combination of the two. The cells were cultured in a cell culture plate overnight at 37°C in a water-jacketed CO_2 (5%) incubator (ThermoForma, Mariette, OH). For determining specificity of MARCO in IL-1β release experiment, AM from WT mice were treated with MARCO Ab (polyclonal goat IgG, rmMARCO; aa 70–518 assession number: Q60754, Catalog number: AF2956 obtained from R&D Systems, Minneapolis, MN) 30 min prior to exposure to silica or endotoxin for 24 hr. IL-1β was measured as described below.

3.4. Cytokine Assays. Culture supernatants were assayed for cytokines with commercially available kits according to the manufacturer's protocol. IL-1β measurements were determined by using Duo-set kits (BD and R&D systems). IL-6 measurements were determined using Duo-set kits (R&D systems). Colorimetric analysis was done using a Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Data are expressed as mean ± SEM picograms/mL of retrieved culture supernatant.

3.5. RNA Isolation. RNA Isolation was performed using Trizol according to the manufacturer's protocol (Trizol

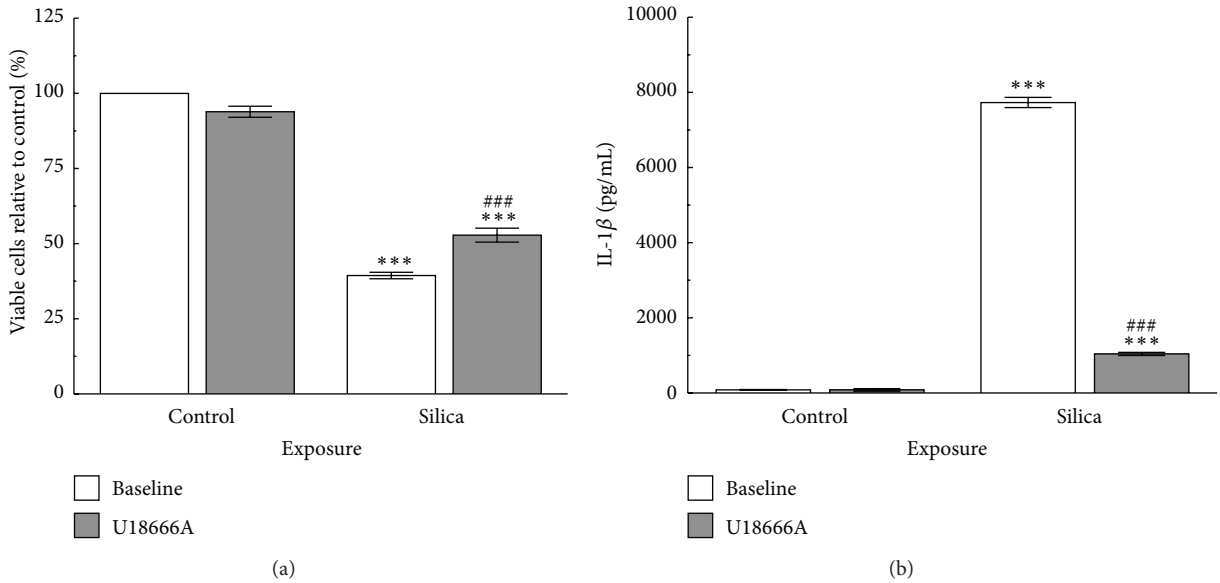

Figure 9: *Cholesterol trafficking modifier U18666A decreases WT AM response to silica (50 μg/mL).* (a) Cell viability at 24 hrs after particle exposure by MTS assay. (b) IL-1β release 24 hrs following silica exposure cocultured with LPS (20 ng/mL) to stimulate NF-κB activation. Asterisks *** indicate statistical significance at $P < 0.001$ compared to corresponding control condition. Hashtags $^{\#\#\#}$ indicate significance at $P < 0.001$ compared to baseline silica-exposed response, $n = 3$.

Reagent, Invitrogen, Carlsbad, CA). Further purification was performed using the E.Z.N.A. Total RNA Kit I according to the RNA cleanup protocol from Omega Bio-Tek, Inc. (Norcross, GA). The final elution step was performed with 40 μL of RNase free water. RNA quantity was determined using a NanoDrop ND 1000 Spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE).

cDNA production was performed according to the qScript cDNA protocol from Quanta BioSciences (qScript cDNA SuperMix, Gaithersburg, MD).

3.6. Real Time PCR. Pro IL-1β message expression was measured using a Stratagene Mx3005p instrument with MxPro software (Agilent Technologies, Santa Clara, CA). Primers (Integrated DNA Technologies, Coraville, IA, USA) were as follows: β-actin forward sequence (5′-ACACTGTGCCCATCTACGAG-3′), reverse sequence (5′-TCAACGTCACACTTCATGATG-3′), and IL-1β primers were derived from IL-1β sequence (accession number: NM_008361.3), forward primer sequence (GGTACATCAGCACCTCACAA), reverse primer sequence (TTAGAAACAGTCCAGCCCATAC). Reactions were performed using FastStart Universal SYBR Green Master (ROX) mix (Roche Diagnostics Corp., Indianapolis, IN) according to manufacturer's protocol. Expression levels were normalized to Actb expression, and fold changes were calculated using the ΔΔCt method.

3.7. Acid Sphingomyelinase Assay. Cell lysates were prepared from alveolar macrophages using a Dounce homogenizer. Cell lysates were assayed for acid sphingomyelinase with commercially available kits (K-3200 Echelon biosciences Inc., Salt Lake City, UT 84108) according to the manufacturer's protocol. Colorimetric analysis was done using a Gemini XS plate reader (Molecular Devices, Sunnyvale CA) at 360 nm excitation and 460 nm emission. Data are expressed as pmol/hour of active acid sphingomyelinase.

3.8. Cathepsin B Activation Assay. Isolated AM were used to determine cathepsin B activation. Cathepsin B activation was determined in the AM after 2 hr of incubation with silica, silica and LPS, LPS and unstimulated cells. Cathepsin B activity was determined using Magic Red cathepsin B Detection Kit (ImmunoChemistry Technologies, Bloomington, MN 55431). The Gemini XS plate reader was used to analyze cathepsin B activation at 590 ex and 630 em (Molecular Devices, Sunnyvale CA).

3.9. Caspase-1 Activation Assay. Caspase-1 activation was determined in isolated AM after 4 hr of incubation with silica, silica and LPS, LPS and nontreated cells. Caspase-1 activity was determined using FAM-FLICA *in vitro* Caspase-1 kit (ImmunoChemistry Technologies, LLC, Bloomington, MN 55431). The Gemini XS plate reader was used to analyze Caspase-1 activation at 492 ex and 520 em (Molecular Devices, Sunnyvale, CA).

3.10. TopFluor Cholesterol Loading Experiments. Isolated AM from C57BL/6 WT and MARCO$^{-/-}$ mice (process described above) were cultured at 100×10^3 cells per well in 96-well tissue culture plates for 24 hrs prior to silica exposure. The cells were cultured with 1 μg/mL TopFuor cholesterol (Avanti Polar Lipids, Alabaster, AL) in RPMI media with 5% delipidated FBS. After the 24 hr loading period, the cells were switched to normal media described above and exposed to

silica at 50 μg/mL for 4 hr. The plates were read in a Gemini fluorometric plate reader (Molecular Devices, Sunnyvale CA) at 488 ex and 525 em.

3.11. U18666A Cholesterol Modification Experiments. Isolated AM from C57BL/6 WT mice (process described above) were cultured at 100×10^3 cells per well in 96-well tissue culture plates for 24 hr $\pm$ 1 μM U18666A (Sigma, St Louis, MO) prior to silica exposure. U18666A is an amphipathic steroid 3-β-[2-(diethylamine)ethoxy] androstenone. It blocks the intracellular trafficking of cholesterol and the exit of free cholesterol from the late endosomal compartment. The mechanism of action for the accumulation of cholesterol in late endosomes and lysosomes can be attributed to the amphipathic property of the compound [42]. U18666A also inhibits oxidosqualene cyclase and desmosterol reductase eliminating cholesterol biosynthesis [42]. After the 24 hr loading period, the cells were exposed to silica at 50 μg/mL, in addition to a 20 ng/mL LPS coculture, for another 24 hr. The LPS was used to stimulate NF-κB, leading to pro-IL-1β production. The culture media was retrieved for IL-1β assay (R&D Systems, Minneapolis MN) and the cells were tested for viability using a MTS assay according to the manufacturer's instructions (Promega, Madison, WI).

3.12. Statistical Analysis. Statistical analyses involved comparison of means using a one- or two-way *ANOVA* followed by Dunnett's test or Bonferroni's test to compensate for increased type I error. All probabilities were two-tailed unless otherwise stated. Statistical power was greater than 0.8. Statistical significance was defined as a probability of type I error occurring at less than 5% ($P < 0.05$). The minimum number of experimental replications was 3-4 depending on the experiment. Graphics and analyses were performed on PRISM 6.0.

4. Conclusions

This study focused on understanding the role of MARCO in inflammation on silica exposure. In the present study we demonstrated that in the absence of MARCO receptor or when MARCO Ab was used to block MARCO receptor function, silica increased NLRP3 inflammasome activation and proinflammatory cytokine release in AM from MARCO$^{-/-}$ mice compared to AM from WT mice. Exposure to silica caused more LMP and greater cathepsin B release in MARCO$^{-/-}$ AM compared to WT AM. These results suggested that the absence of MARCO enhanced AM susceptibility to silica at the level of LMP. The mechanism of the increased LMP in the absence of MARCO was attributed to changes in cholesterol trafficking. In WT AM silica treatment resulted in increased cholesterol uptake that was greatly reduced in the absence of MARCO. Furthermore, blocking cholesterol trafficking with U186666A greatly decreased silica-induced cytotoxicity and inflammasome activation. Taken together, these results demonstrate the important role that cholesterol plays in phagolysosomal stability and suggest a potential therapeutic site to decrease particle-induced inflammation.

Conflict of Interests

The authors have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Rupa Biswas designed and conducted the experiments in the C57Bl/6 and MARCO null mice. Raymond F. Hamilton Jr. conducted lysosomal cholesterol content experiments and dose-response study in the C57Bl/6 and MARCO null mice and helped with the statistical analysis. Rupa Biswas wrote the paper with Raymond F. Hamilton Jr. and Andrij Holian. Andrij Holian contributed to the overall project design.

Acknowledgments

The work was supported by a research Grant from NIEHS (R01ES015294) and Center Grants from NCRR and NIGMS, P20 RR017670 and P30 GM103338, respectively. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors also acknowledge the technical support of the CEHS Inhalation and Pulmonary Physiology Core. They would like to thank Britten Postma, Corbin Schwanke, and Kevin Trout for their technical help and expertise.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 670140, 10 pages
http://dx.doi.org/10.1155/2014/670140

Research Article

Functional Properties of $CD8^+$ Lymphocytes in Patients with Pleural Plaque and Malignant Mesothelioma

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Received 7 February 2014; Revised 24 May 2014; Accepted 26 May 2014; Published 18 June 2014

Academic Editor: Andrij Holian

It is known that asbestos exposure can cause malignant mesothelioma (MM) and that $CD8^+$ T cells play a critical role in antitumor immunity. We examined the properties of peripheral blood $CD8^+$ lymphocytes from asbestos-exposed patients with pleural plaque (PL) and MM. The percentage of $CD3^+CD8^+$ cells in PBMCs did not differ among the three groups, although the total numbers of PBMCs of the PL and MM groups were lower than those of the healthy volunteers (HV). The percentage of IFN-γ^+ and $CD107a^+$ cells in PMA/ionomycin-stimulated $CD8^+$ lymphocytes did not differ among the three groups. Percentages of $perforin^+$ cells and $CD45RA^-$ cells in fresh $CD8^+$ lymphocytes of PL and MM groups were higher than those of HV. Percentages of granzyme B^+ and $perforin^+$ cells in PMA/ionomycin-stimulated $CD8^+$ lymphocytes were higher in PL group compared with HV. The MM group showed a decrease of perforin level in $CD8^+$ lymphocytes after stimulation compared with patients with PL. These results indicate that MM patients have characteristics of impairment in stimulation-induced cytotoxicity of peripheral blood $CD8^+$ lymphocytes and that PL and MM patients have a common character of functional alteration in those lymphocytes, namely, an increase in memory cells, possibly related to exposure to asbestos.

1. Introduction

Asbestos fibers exhibit tumorigenicity. It is commonly believed that the cause of lung cancer and malignant mesothelioma induced by inhaled asbestos might be due to its tumorigenic activity [1, 2]. Previous reports have shown that asbestos induces oxidization of nucleotide bases and increases mutation frequencies [3, 4]. However, it takes a long period of about forty years to develop malignant mesothelioma after exposure to asbestos [5–7]. These findings suggest that asbestos might gradually impair antitumor immunity [8–14].

In antitumor immunity, cytotoxic T lymphocytes (CTL) and natural killer (NK) cells play a role as effectors, which kill tumor cells [15]. CTL are known to be differentiated from naïve $CD8^+$ T cells. When these naïve cells recognize antigen, they are immediately activated, expand, and differentiate into antigen-specific CTL [16]. Following conjugation of CTL with an appropriate target cell, lytic granules are transported to the point of contact with the target cell, and the granule contents, perforin, and granzyme B are released into the immune synapse between CTL and the target. Once CTL release granule contents, they act on target cells and induce apoptosis to eliminate these cells [17]. The granule core is surrounded

by a lipid bilayer containing lysosome-associated membrane glycoprotein, CD107a, which is known to be transiently expressed on the T cell surface upon degranulation [18].

We reported recently that asbestos exposure suppressed the differentiation of human CTL during mixed lymphocyte reactions (MLR), which was accompanied by decreases in IFN-γ and tumor necrosis factor-α [19]. Several additional findings include low cytotoxicity and altered expression of NK cell-activating receptors on NK cells and a decrease in $CD4^+$ $CXCR3^+$ T cells in patients with mesothelioma [8, 9]. These findings raise the possibility that $CD8^+$ lymphocytes might show a functional decline in asbestos-exposed patients with pleural plaque (PL) or malignant mesothelioma (MM). Pleural plaques are an objective sign of previous asbestos inhalation, are known to be whitish, sharply circumscribed, fibrous, hyaline, sometimes calcified, form patches involving parietal pleura, and there is a report that pleural plaques are harmless in themselves [20].

Therefore, in the present study, we examined the functional properties of $CD8^+$ lymphocytes of patients with PL or MM by flow cytometry (FCM) and compared results with those of healthy volunteers (HV). Freshly prepared peripheral blood monocyte cells (PBMCs) were assayed for the percentage and number of $CD3^+CD8^+$ cells and percentages of granzyme B^+, $perforin^+$, and $CD45RA^-$ cells in $CD8^+$ lymphocytes. PBMCs were also stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 4 h and assayed for the percentages of IFN-γ^+, $CD107a^+$, granzyme B^+, and $perforin^+$ cells in $CD8^+$ lymphocytes.

2. Materials and Methods

2.1. Isolation of PBMCs. Blood from HV and patients with PL or MM was collected in citrate phosphate dextrose, and PBMCs were isolated from blood using a Ficoll-Hypaque density gradient. Specimens were taken from 16 HV (mean ± S.D. age, 57.2 ± 6.4 years; 16 men), 27 patients with PL (mean ± S.D. age, 66.7 ± 5.1 years; 27 men), and 18 patients with MM (mean age, 67.3 ± 9.0 years; 18 men). Isolated PBMCs were assayed for the percentage and number of $CD3^+CD8^+$ cells and percentages of $CD45RA^-$, granzyme B^+, and $perforin^+$ cells in $CD8^+$ lymphocytes. A portion of PBMCs were cultured and stimulated as described below and assayed for the percentages of IFN-γ^+, $CD107a^+$, granzyme B^+, and $perforin^+$ cells in $CD8^+$ lymphocytes. All donors provided their informed consent and the Institutional Ethics Committees of Kawasaki Medical School, Okayama Rosai Hospital, and Hyogo College of Medicine approved the project.

2.2. Cell Culture. For staining of intracellular IFN-γ, granzyme B, perforin, and cell-surface CD107a, isolated 2.0×10^5 PBMCs were stimulated with 50 ng/mL PMA and 250 ng/mL ionomycin (both Sigma-Aldrich, St. Louis, MO) in RPMI-1640 medium supplemented with 10% FBS (Molecular and Biological laboratories, Co., Ltd.), 100 μg/mL streptomycin, and 100 U/mL penicillin (both Meiji, Tokyo, Japan) in 96-well flat-bottomed plates. The plates were incubated at 37°C for 4 h in a humidified atmosphere of 5% CO_2. Golgi Stop Protein Transporter inhibitor (containing monensin) (BD Biosciences, San Jose, CA) was also added to the medium for the staining of IFN-γ.

2.3. Assay for Expression Levels of Cell-Surface and Intracellular Molecules. Isolated PBMCs were stained with the following antibodies (Abs): CD8-phycoerythrin cychrome 5 (PC5) (Beckman Coulter, Inc., Brea, CA) and CD3-FITC (BD Biosciences), CD45RA-phycoerythrin (PE) (BioLegend, San Diego, CA), granzyme B-RPE (AbD Serotec, Oxford, UK), IgG1 negative control: RPE (AbD Serotec), perforin-RPE (Ancell Corporation, Bayport, MN), or IgG2b-RPE (Ancell Corporation) Abs. The stimulated PBMCs were harvested and stained with CD8-PC5 and IFN-γ-PE Abs (BD Biosciences) using a Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences). The stimulated cells were also stained with CD8-PC5 and perforin-RPE or granzyme B-RPE Abs. Before staining with granzyme B-RPE or perforin-RPE Ab, the cells stained with CD8-PC5 Ab were fixed with 3.7% formaldehyde and then permeabilized with 0.1% Triton X-100. The percentage of cells positive for each parameter was analyzed using a FACSCalibur (BD Biosciences).

2.4. Detection of Degranulation. The degranulation of $CD8^+$T lymphocytes stimulated by PMA and ionomycin was detected by the increase in expression of cell-surface CD107a using the FACSCalibur. Isolated PBMCs were stimulated in the presence of PMA and ionomycin for 4 h, as described above. At the end of the stimulation period, cells were washed and stained with CD107a-PE (BD Biosciences) and CD8-PC5 Abs. Cells positive for CD107a expression were defined by comparison with unstimulated control cells.

2.5. Statistical Analysis. The significance of differences was determined using an analysis of variance (ANOVA) with a *post hoc* Student-Newman-Keuls test. The statistical analysis was performed using StatView 5.0 (SAS Institute Inc., Cary, NC) software.

3. Results

3.1. Percentage of $CD3^+CD8^+$ Cells in PBMCs and the Number of $CD3^+CD8^+$ Cells in Blood. We isolated fresh PBMCs from the peripheral blood of HV, PL, and MM groups and examined the percentage of $CD3^+CD8^+$ cells in PBMCs and the number of $CD3^+CD8^+$ cells in blood. The percentage of $CD3^+CD8^+$ cells in PBMCs did not differ among HV, PL, and MM groups (mean ± S.D., 11.3 ± 3.5, 10.7 ± 5.1, and 9.9 ± 6.7, resp.) (Figure 1(a)). However, statistical analysis showed a significant difference between groups ($P < 0.01$) for the number of $CD3^+CD8^+$ cells. The number of $CD3^+CD8^+$ cells per 1 mL of blood in PL and MM groups was significantly lower than that of HV (mean ± S.D., 7.2 ± 3.6, 5.6 ± 4.9, and 11.5 ± 4.9, resp.) (Figure 1(b)). These results reflect the decrease in total number of PBMCs, which was significantly lower for the PL and MM groups than the HV group

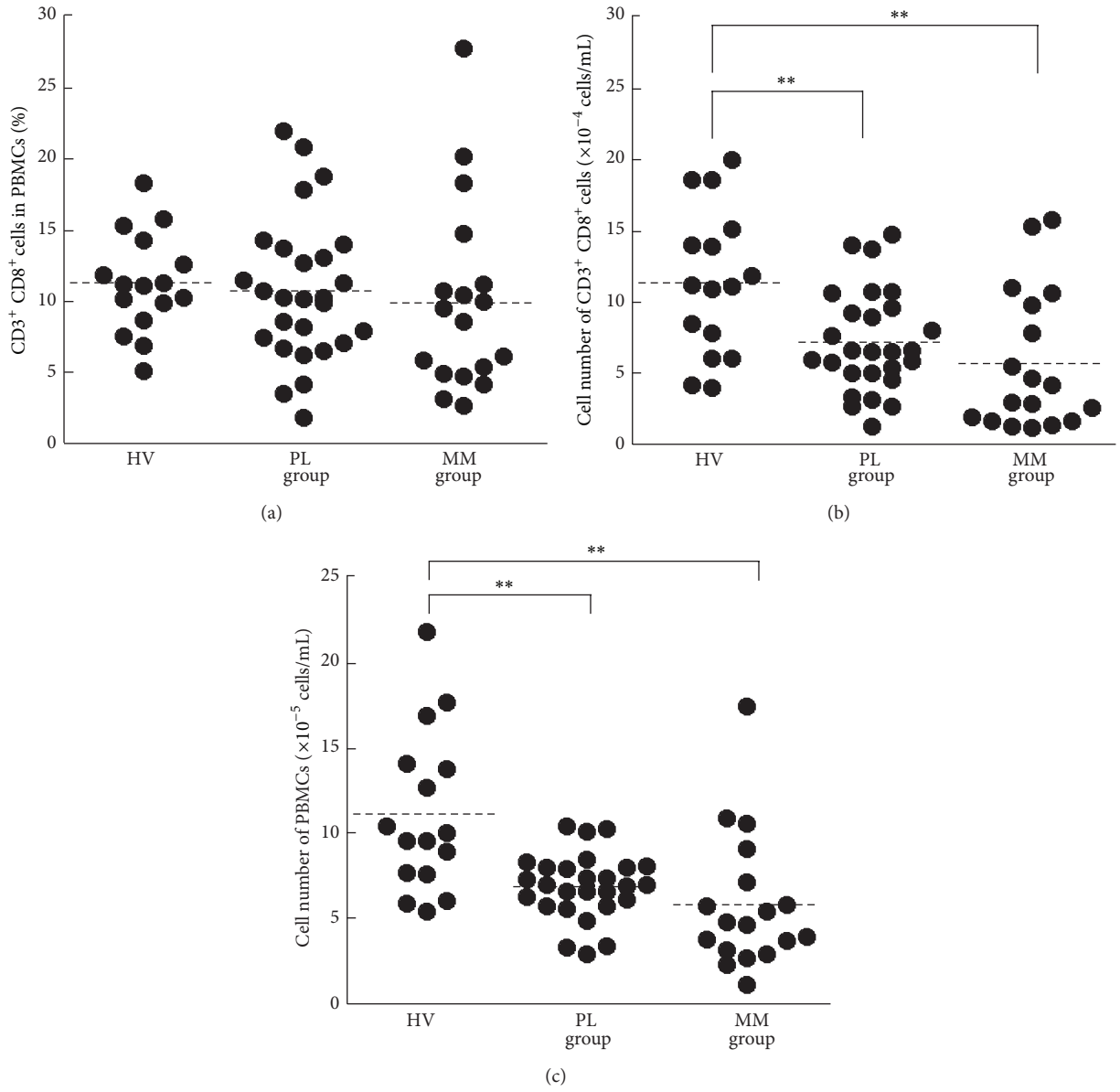

Figure 1: The percentage and number of $CD3^+CD8^+$ cells for HV, PL, and MM groups. Freshly isolated PBMCs were assayed for the percentage of $CD3^+CD8^+$ cells in PBMCs (a) and the number of $CD3^+CD8^+$ cells per 1 mL of blood (b) and the number of PBMCs per 1 mL of blood (c). Horizontal dotted bars indicate the mean percentage (a) and mean number ((b), (c)). Data represent values from 16 HV individuals, 27 individuals of the PL group, and 18 individuals of the MM group. Significant differences are indicated by asterisks ($^{**}P < 0.01$).

(Figure 1(c)). Thus, the proportion of $CD3^+CD8^+$ cells in PL and MM patients did not differ, although the total number of PBMCs decreased.

3.2. CD45RA-Negative Cells in $CD8^+$ Lymphocytes. CD45RA-negative cells in $CD8^+$ T cells are known as memory cells in peripheral blood [21, 22]. We considered the possibility that the percentages of CD45RA-negative cells in $CD8^+$ lymphocytes of asbestos-exposed patients with PL and MM might be lower than those of HV because our previous study using MLR showed that asbestos exposure suppressed the differentiation of human CTL [19]. To examine this possibility, we assayed the percentages of CD45RA-negative cells in $CD8^+$ T cells among the three groups (Figure 2(a)). There was a significant difference in percentages of CD45RA-negative cells in fresh CD8 lymphocytes among the groups. Contrary to our expectation, the percentages of CD45RA-negative cells in fresh $CD8^+$ lymphocytes of the PL and MM groups were significantly higher than those of HV (mean ± S.D., 63.4 ± 19.5, 61.7 ± 10.0, and 46.5 ± 8.0, resp.) (Figure 2(b)).

3.3. Production of IFN-γ by $CD8^+$ Lymphocytes. $CD8^+$ T cells have been shown to be crucial for the immune response against tumors through production of effector molecules including granzyme B, perforin, and IFN-γ [23, 24]. To

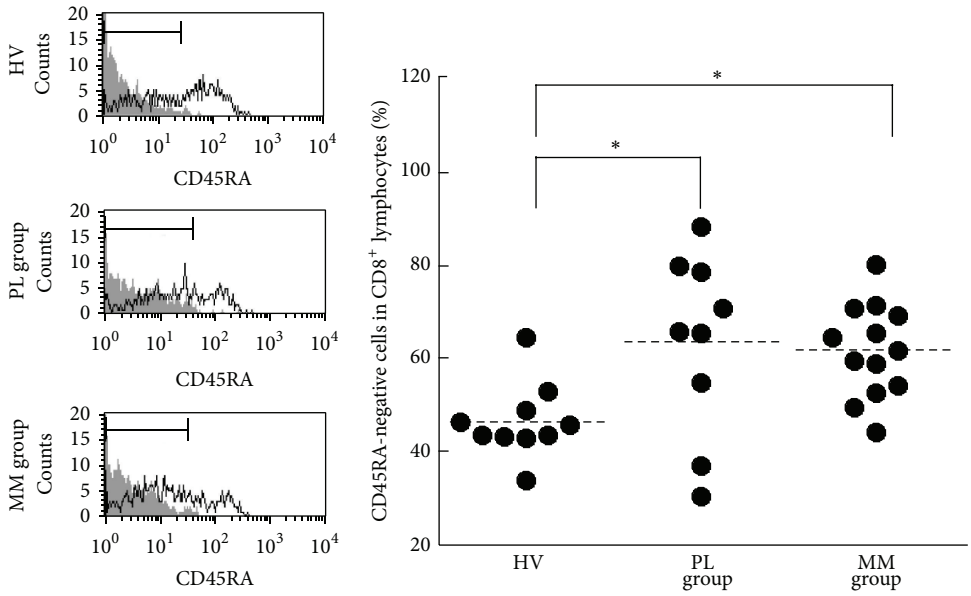

Figure 2: The percentages of CD45RA-negative cells in $CD8^+$lymphocytes of HV, PL, and MM groups. Freshly isolated PBMCs were assayed for the percentage of CD45RA-negative cells in $CD8^+$ lymphocytes. (a) Representative histograms of cell-surface CD45RA (solid line) and nonstaining control (gray) in $CD8^+$ lymphocytes. For the nonstaining control, PBMCs from arbitrary HV samples were used. (b) Representative graph showing the percentage of CD45RA-negative cells in $CD8^+$ lymphocytes. Horizontal dotted bars indicate the mean percentage. Data represent values from 10 HV individuals, 9 individuals of the PL group, and 13 individuals of the MM group. Significant differences are indicated by asterisks ($^*P < 0.05$).

examine the ability of these cells to produce IFN-γ in patients with PL and MM, we assayed the percentages of cells positive for intracellular IFN-γ in stimulated $CD8^+$ lymphocytes of individuals in the PL, MM, and HV groups. After PBMCs were stimulated with PMA/ionomycin as a substitute for antigenic stimulation for 4 h, they were analyzed for the percentage of IFN-γ-positive cells in $CD8^+$ lymphocytes and the results were compared between the HV, PL, and MM groups (Figure 3(a)). There was no statistically significant difference among the groups, although the percentage of IFN-γ^+ cells in the MM group showed a tendency to be lower than that of the HV and PL groups (mean ± S.D., 34.3 ± 18.0, 44.5 ± 15.5, and 45.8 ± 18.7, resp.) (Figure 3(b)).

3.4. Storage and Retention/Enhancement of Granzyme B by $CD8^+$ Lymphocytes. To examine the storage level of intracellular granzyme B and its retention/enhancement in $CD8^+$ lymphocytes of patients with PL and MM, we assayed the percentage of cells positive for intracellular granzyme B in fresh or stimulated $CD8^+$ lymphocytes (Figure 4(a)). The percentages of granzyme B^+ cells in fresh CD8 lymphocytes did not differ among HV, PL, and MM groups (mean ± S.D., 24.6 ± 14.5, 29.6 ± 20.8, and 38.0 ± 22.8, resp.) (Figure 4(b)). In contrast to results for fresh $CD8^+$ lymphocytes, there was a significant difference in the percentages of granzyme B^+ cells in PMA/ionomycin-stimulated CD8 lymphocytes among the groups. The PL group showed a higher percentage of granzyme B^+ cells in stimulated $CD8^+$ cells compared to HV (mean ± S.D., 37.0 ± 26.3, and 15.2 ± 10.4, resp.), although the percentage did not differ from that of the MM group (mean ± S.D., 29.2 ± 25.3) (Figure 4(c)). To examine the retaining/enhancing performance of granzyme B molecules in $CD8^+$ lymphocytes after stimulation, the percentage of granzyme B^+ cells in fresh $CD8^+$ lymphocytes was subtracted from the percentage of granzyme B^+ cells in stimulated cells of each individual (Figure 4(d)). Figure 4(d) reveals a significant difference in the subtracted percentages of granzyme B^+ cells among the groups and shows that the subtracted percentage of granzyme B^+ cells of the PL group was significantly higher than that of the HV or MM group. These results indicate that $CD8^+$ lymphocytes in PL patients have an increased ability to retain and enhance intracellular granzyme B following stimulation, compared with HV and MM patients.

3.5. Storage and Retention/Enhancement of Perforin by $CD8^+$ Lymphocytes. In a manner similar to the analysis of granzyme B level, the percentages of cells positive for intracellular perforin in fresh or stimulated $CD8^+$ lymphocytes were assayed, and each percentage and subtracted percentage were compared among the PL, MM, and HV groups (Figure 5(a)). The percentages of perforin$^+$ cells in fresh CD8 lymphocytes of the PL and MM groups were significantly higher than those of the HV group (mean ± S.D., 38.8 ± 26.3, 44.2 ± 24.9, and 17.8 ± 14.5, resp.) (Figure 5(b)). In addition, the PL group, but not the MM group, showed a higher percentage of perforin$^+$ cells in stimulated $CD8^+$ lymphocytes compared to the HV group (mean ± S.D., 27.4 ± 20.9, 12.6 ± 16.2, and 9.1 ± 7.0, resp.)

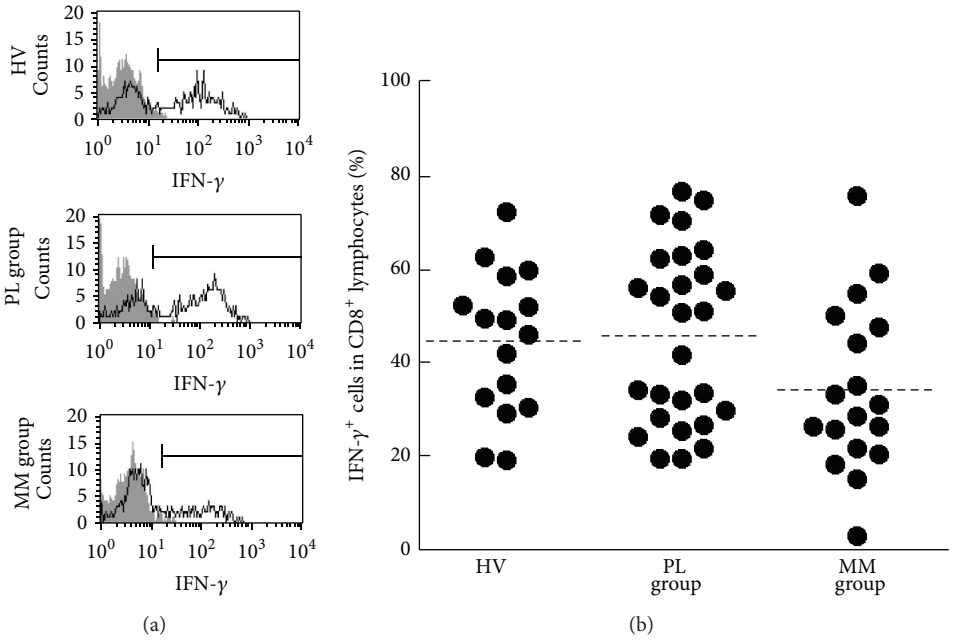

Figure 3: The percentages of IFN-γ-positive cells in CD8$^+$lymphocytes of HV, PL, and MM groups. (a) Representative histograms of intracellular IFN-γ (solid line) and nonstaining control (gray) in CD8$^+$ lymphocytes. (b) Representative graph showing the percentage of IFN-γ^+ cells in CD8$^+$ lymphocytes. Horizontal dotted bars indicate the mean percentage. Data represent values from 16 HV individuals, 27 individuals of the PL group, and 18 individuals of the MM group.

(Figure 5(c)). Moreover, there was a significant difference in the subtracted percentage, calculated by subtraction of the amount in fresh cells from that in stimulated cells, among the groups. The MM group showed significantly lower subtracted percentages of perforin$^+$ cells compared with the PL and HV groups (Figure 5(d)). These results indicate that CD8$^+$ lymphocytes in MM patients have a decreased ability to retain and enhance perforin following stimulation, compared with PL patients and HV.

3.6. Degranulation of CD8$^+$ Lymphocytes. The results shown above suggested the possibility that CD8$^+$ lymphocytes in MM have an insufficient ability to retain a suitable perforin level. However, we cannot exclude the possibility that the decrease in perforin in stimulated cells of MM patients might be related to augmented degranulation, by which perforin and granzymes are released outside to injure target cells. Therefore, to examine degranulation we cultured PBMCs in the presence of PMA/ionomycin stimulation for 4 h and then collected and stained cells with fluorescence-labeled anti-CD8 and anti-CD107a Abs before performing an analysis using FCM (Figure 6(a)), as described in Section 2. The percentages of CD107a$^+$ cells in stimulated CD8 lymphocytes did not differ statistically in multiple comparisons among HV, PL, and MM groups (mean ± S.D., 7.4 ± 2.8, 7.8 ± 3.1, and 11.6 ± 5.4, resp.), although the percentages of CD107a$^+$ cells in the MM group tended to be higher than those in PL and HV groups (Figure 6(b)). Additionally, although the MM group included some individuals who showed an enhanced decrease in perforin level after stimulation, they did not show the higher percentages of CD107a$^+$ cells shown by others in the MM group or individuals of the PL and HV groups (data not shown). These findings exclude the possibility that augmented degranulation in MM patients might lead to the decrease in perforin level after stimulation.

4. Discussion

We previously confirmed the suppressive effect of asbestos on CD8$^+$ T cells in an *in vitro* study. We therefore examined the functional properties of CD8$^+$ lymphocytes in asbestos-exposed patients with PL and MM in the present study, focusing on cellularity, production of IFN-γ, and intracellular levels of effector molecules affecting cytotoxicity. In particular, CD8$^+$ lymphocytes were also analyzed for the ability to retain/enhance the effector molecules perforin and granzyme after stimulation, as well as the storage level of those molecules in fresh cells. PL patients showed higher percentages of granzyme B$^+$ and perforin$^+$ cells in stimulated CD8$^+$ lymphocytes, compared with HV. In contrast, the MM group showed a decrease in the percentage of perforin$^+$ cells following stimulation, which differed significantly from that of the PL and HV groups. On the other hand, the PL and MM groups showed higher percentages of CD45RA$^-$ and perforin$^+$ cells in fresh CD8$^+$ lymphocytes. These findings indicate that the characteristics of CD8$^+$ lymphocytes in PL and MM patients differ, although they have several points in common. The commonality between PL and MM patients means that the increases in CD45RA$^-$ and perforin$^+$ cells may be related to asbestos exposure, whereas the difference in post-stimulation maintenance of perforin between these patients underlines the immunological states of PL and MM,

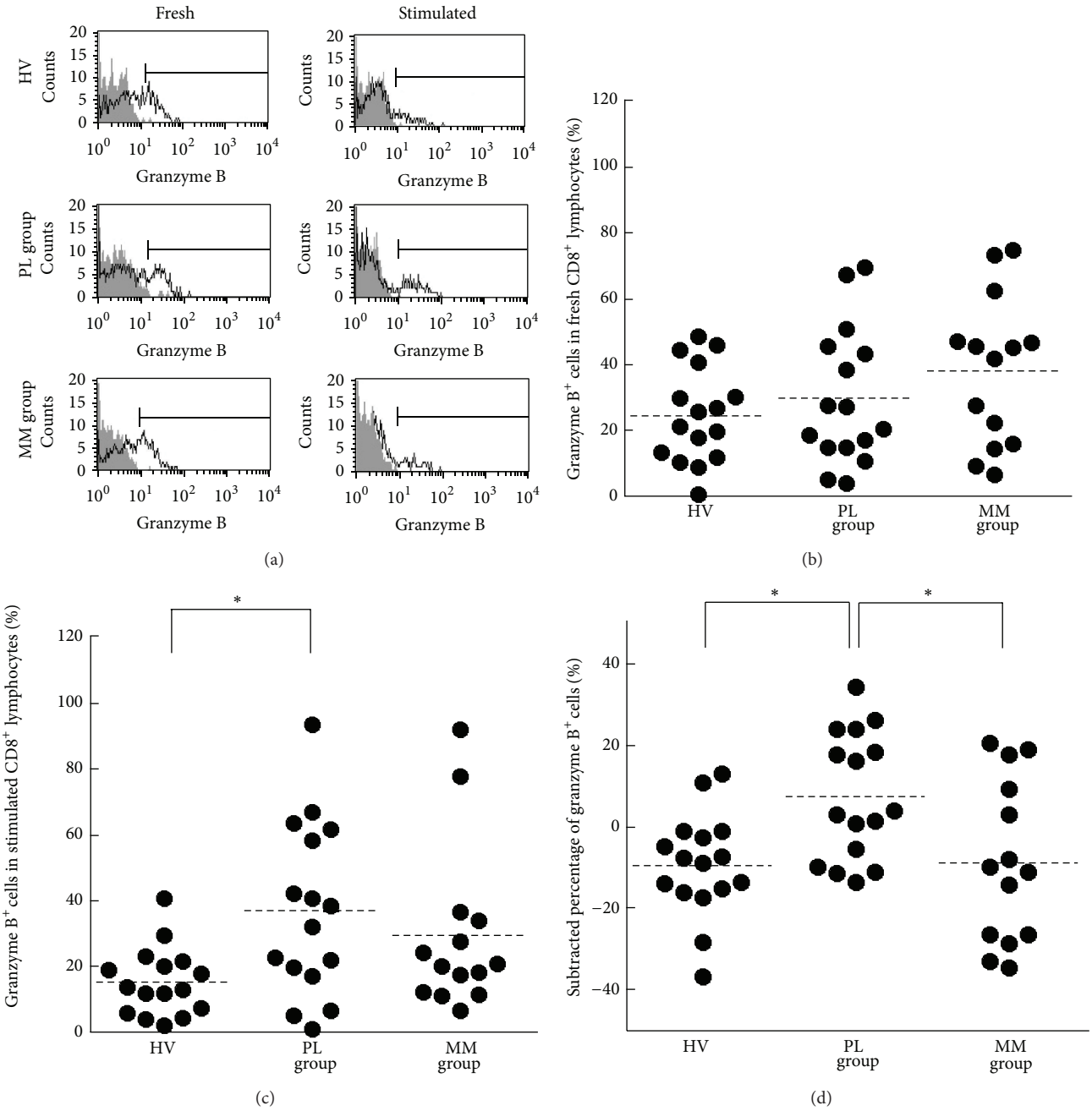

Figure 4: The percentages of granzyme B-positive cells in $CD8^+$lymphocytes of HV, PL, and MM groups. (a) Representative histograms of intracellular granzyme B (solid line) and isotype control (gray) in fresh or stimulated $CD8^+$ lymphocytes. For staining with an isotype control Ab, PBMCs from arbitrary HV samples were used. (b) Representative graph showing the percentage of granzyme B^+ cells in fresh $CD8^+$ lymphocytes. (c) Representative graph showing the percentage of granzyme B^+ cells in PMA/ionomycin-stimulated $CD8^+$ lymphocytes. (d) Representative graph showing the subtracted percentages of granzyme B^+ cells. ((b)–(d)) Horizontal dotted bars indicate the mean percentage. Data represent values from 16 HV individuals, 16 individuals of the PL group, and 14 individuals of the MM group. Significant differences are indicated by asterisks ($^*P < 0.05$).

related to the pathologies of asbestos exposed patients not or suffering from malignant mesothelioma, respectively.

The MM group showed a decrease in perforin level of $CD8^+$ lymphocytes after PMA/ionomycin stimulation whereas this was not shown in PL and HV (Figure 5(d)), although the percentage of perforin$^+$ cells in stimulated $CD8^+$ did not differ from that of HV (Figure 5(c)). These results imply impairment in stimulation-induced persistent cytotoxicity of $CD8^+$ lymphocytes in patients with MM compared to PL patients. Asbestos exposure might result in impaired function of $CD8^+$ lymphocytes in patients with MM because our previous study showed the suppressive effect of asbestos

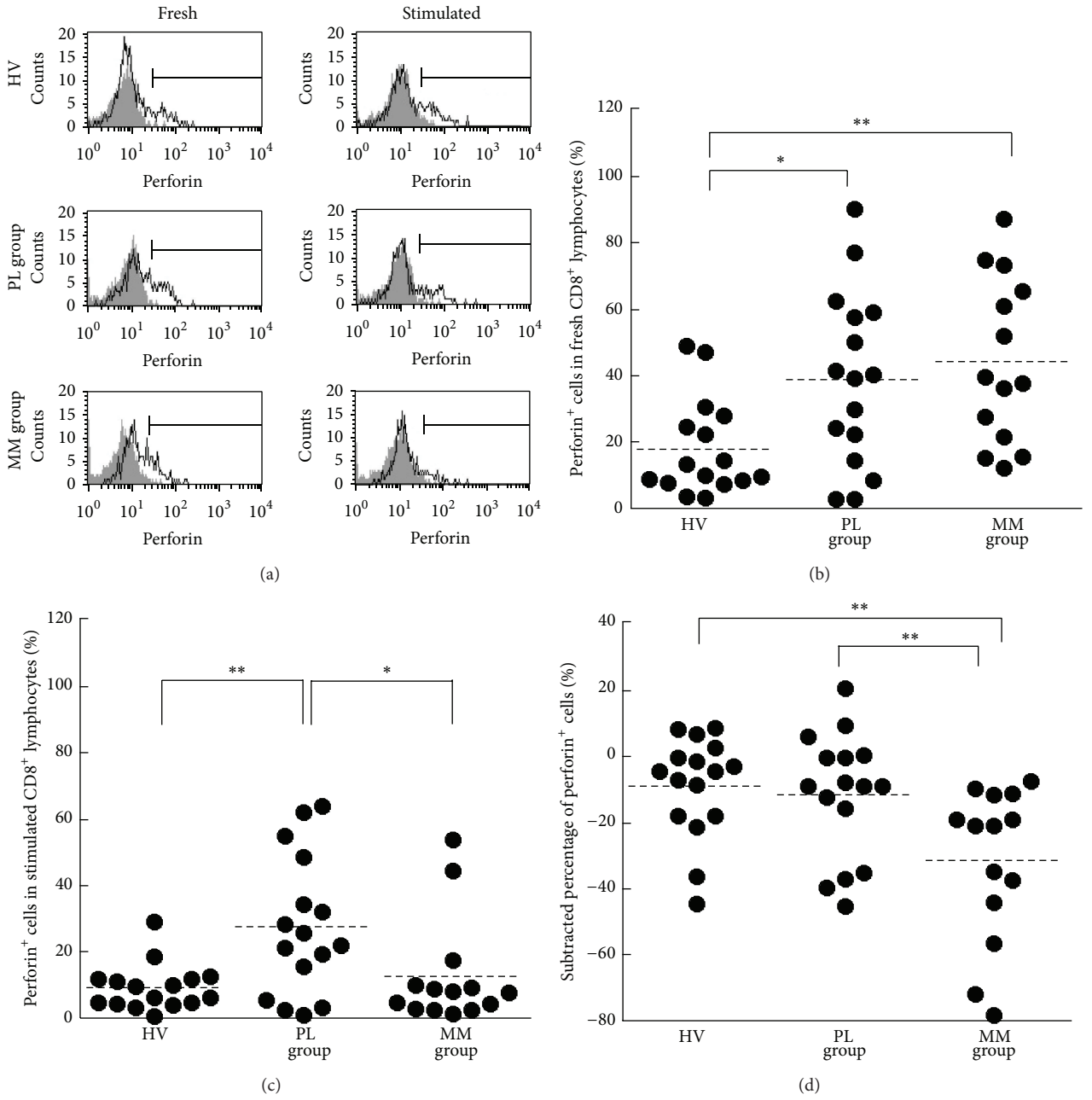

Figure 5: The percentages of perforin-positive cells in $CD8^+$lymphocytes of HV, PL, and MM groups. (a) Representative histograms of intracellular perforin (solid line) and isotype control (gray) in fresh or stimulated $CD8^+$ lymphocytes. For staining with an isotype control Ab, PBMCs from arbitrary HV samples were used. (b) Representative graph showing the percentage of perforin$^+$ cells in fresh $CD8^+$ lymphocytes. (c) Representative graph showing the percentage of perforin$^+$ cells in PMA/ionomycin-stimulated $CD8^+$ lymphocytes. (d) Representative graph showing the subtracted percentages of perforin$^+$ cells. ((b)–(d)) Horizontal dotted bars indicate the mean percentage. Data represent values from 16 HV individuals, 16 individuals of the PL group, and 14 individuals of the MM group. Significant differences are indicated by asterisks ($^*P < 0.05$, $^{**}P < 0.01$).

exposure on *in vitro* induction of CTL. This phenomenon cannot explain why the $CD8^+$ lymphocytes of patients with PL, namely, asbestos-exposed individuals without tumor, did not show functional impairment. However, as discussed below, the difference between the MM and PL groups might be explained by understanding that inhalation of asbestos might not always result in the suppressed function of $CD8^+$ lymphocytes, unlike addition of asbestos into a culture of PBMCs, and that an adequate immune response might have been induced to protect tumor disease in individuals of the PL group analyzed in this study. Alternatively, the impaired function of $CD8^+$ lymphocytes in MM patients might be related to immune suppression by tumor cells [25]. In fact, it has been shown that PBMCs isolated from blood samples

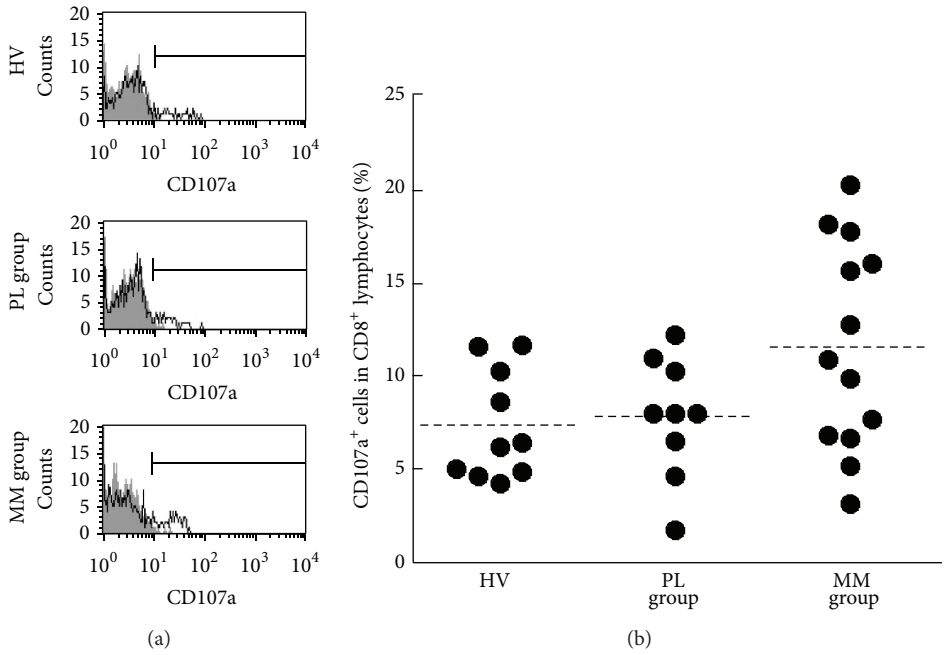

Figure 6: The percentages of CD107a-positive cells in CD8$^+$lymphocytes of HV, PL, and MM groups. (a) Representative histograms of cell-surface CD107a in CD8$^+$ lymphocytes cultured with PMA/ionomycin (solid line) and without stimulation (gray) for the HV, PL, and MM groups. (b) Representative graph showing the percentage of CD107a$^+$ cells in CD8$^+$ lymphocytes. Horizontal dotted bars indicate the mean percentage. Data represent values from 10 HV individuals, 9 individuals of the PL group, and 13 individuals of the MM group.

of patients with carcinoma exhibited a significant decrease in T cell receptor-mediated cytotoxicity compared with HV [26]. Therefore, although we cannot draw a conclusion with absolute certainty, it is a notable finding that the CD8$^+$ lymphocytes of MM patients, but not PL patients, showed an enhanced decrease in perforin level after stimulation, as first demonstrated by our present study.

CD45RA-negative cells in CD8$^+$ T cells are defined as memory cells and can be divided into two types of cells, central memory cells and effector memory cells in peripheral blood [21, 22]. Both PL and MM groups showed higher percentages of CD45RA-negative cells in CD8$^+$ lymphocytes compared with HV, and both groups also showed a higher percentage of perforin$^+$ cells in these lymphocytes. Perforin expression is also known to be restricted to effector memory cells, but not central memory cells [21, 22]. Therefore, the findings of our study should be interpreted to indicate that PL and MM patients have an increased proportion of effector memory cells in peripheral blood CD8$^+$ lymphocytes. It is suggested that this character of CD8$^+$ lymphocytes might be related to asbestos exposure because both PL and MM patients should have been exposed to asbestos. However, the increase in effector memory CD8$^+$ lymphocytes in peripheral blood may not result from the direct effect of asbestos exposure on the immune system because the results obtained by our *in vitro* study showed the suppressive effect of asbestos addition on cytotoxicity of CD8$^+$ lymphocytes. It is possible that the increase in effector memory CD8$^+$ lymphocytes might represent an immune response against abnormal cells and molecules caused by asbestos exposure. As mentioned above, it is noteworthy that CD8$^+$ lymphocytes in MM patients showed impairment in stimulation-induced persistent cytotoxicity. Insufficient action of tumor immunity might be related to acquisition of MM following exposure to asbestos. In contrast, tumor immunity might function successfully in PL patients, who have not currently acquired MM.

Before beginning the present study, we predicted that PL patients might show the suppressed function of CD8$^+$ lymphocytes because we reported previously that asbestos exposure suppressed the induction of human CTL during MLR [19]. Other researchers have observed that lymph nodes contained an amount of asbestos that was comparable to that of lung parenchyma and pleural plaques [27]. The lymph node is known as a place where naïve CD8$^+$ T cells differentiate into CTL [28]. These findings support our idea that induction of CTL might suffer from exposure to asbestos *in vivo*. Contrary to our expectation, CD8$^+$ T cells in PL patients showed increased percentages of granzyme B$^+$ cells and maintained a high percentage of perforin$^+$ cells following stimulation, which differed from HV and MM groups. As mentioned above, these results supported the possibility that tumor immunity might function well in PL patients. However, further discussion on this point is needed. In the study using MLR, CD8$^+$ lymphocytes were exposed to asbestos during stimulation with allogenic targets and then examined for the effect of asbestos on cytotoxicity of these lymphocytes. On the other hand, CD8$^+$ lymphocytes prepared from peripheral blood in the present study may include some lymphocytes that had been exposed to asbestos

fiber in lymph nodes or lungs, and then they were stimulated *in vitro* for the analyses. This difference in timing concerning the effects of asbestos exposure on lymphocytes might have caused the variation in results between the MLR experiments and analyses of patient specimens.

5. Conclusion

Our present investigation is the first to show that MM patients have characteristics of impairment in stimulation-induced cytotoxicity of peripheral blood $CD8^+$ lymphocytes and that both PL and MM patients have a common character of functional alteration in those lymphocytes, namely, an increase in memory cells, possibly related to exposure to asbestos. Our findings regarding the immunological properties of $CD8^+$ lymphocytes related to asbestos exposure or mesothelioma might assist in the early detection of MM. The present method of diagnosis for MM is based mainly on image analysis by X-ray and CT-scan, which require training to obtain accurate results. Information about these lymphocytes can be easily and safely taken from peripheral blood. The combination of imaging and immunological analyses might carve out a new future for the diagnosis of MM.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

Parts of this study were supported by Special Coordination Funds for Promoting Science and Technology (H18-1-3-3-1), JPSS KAKENHI Grants (20890270, 20390178, 19659153, and 23790679), The Takeda Science Foundation (Tokutei Kenkyu Josei I, 2008), Kawasaki Medical School Project Grants (23B-66, 23P-3, 22-A29, and 21-107), Okayama-Ken (Tokubetsu Dengen Syozai Ken Kagaku Gijyutsu Sinkou Jigyou Kenkyu Itaku, 2010-2012), Research Project Grant for Young Investigator (2010) in the Japanese Society of Hygiene, and Strategic Research Foundation Grant-Aided Project for Private Universities from Ministry of Education, Culture, Sport, Science, and Technology, Japan. The authors thank Ms. Tamayo Hatayama, Minako Kato, Naomi Miyahara, and Shoko Yamamoto for their technical help.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 138751, 9 pages
http://dx.doi.org/10.1155/2014/138751

Research Article

Proinflammatory Effects of Diesel Exhaust Nanoparticles on Scleroderma Skin Cells

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Received 20 February 2014; Accepted 9 May 2014; Published 1 June 2014

Academic Editor: Takemi Otsuki

Autoimmune diseases are complex disorders of unknown etiology thought to result from interactions between genetic and environmental factors. We aimed to verify whether environmental pollution from diesel engine exhaust nanoparticulate (DEP) of actually operating vehicles could play a role in the development of a rare immune-mediated disease, systemic sclerosis (SSc), in which the pathogenetic role of environment has been highlighted. The effects of carbon-based nanoparticulate collected at the exhaust of newer (Euro 5) and older (Euro 4) diesel engines on SSc skin keratinocytes and fibroblasts were evaluated *in vitro* by assessing the mRNA expression of inflammatory cytokines (IL-1α, IL-6, IL-8, and TNF-α) and fibroblast chemical mediators (metalloproteases 2, 3, 7, 9, and 12; collagen types I and III; VEGF). DEP was shown to stimulate cytokine gene expression at a higher extent in SSc keratinocytes versus normal cells. Moreover, the mRNA gene expression of all MMPs, collagen types, and VEGF genes was significantly higher in untreated SSc fibroblasts versus controls. Euro 5 particle exposure increased the mRNA expression of MMP-2, -7, and -9 in SSc fibroblasts in a dose dependent manner and only at the highest concentration in normal cells. We suggest that environmental DEP could trigger the development of SSc acting on genetically hyperreactive cell systems.

1. Introduction

Autoimmune diseases are complex disorders of unknown etiology characterized by immune responses to self-antigens and are thought to result from interactions between genetic and environmental factors. Many independent lines of investigation suggest that the environment, acting on genetically susceptible individuals, plays a causative role in the development of such diseases [1–3]. The study of epigenetic mechanisms in the pathogenesis of autoimmune diseases is receiving unprecedented attention. Epigenetic mechanisms control gene expression and are influenced by external stimuli, linking environment and gene function. A variety of environmental agents, such as viral infection, hormones, certain drugs, and pollutants, have been found to influence the development of autoimmune diseases. On the other hand, there is considerable evidence of epigenetic changes, particularly DNA methylation alterations, in diseases like systemic lupus erythematosus, rheumatoid arthritis, or multiple sclerosis [4, 5]. Systemic sclerosis (SSc) is a complex immune-mediated disease of unknown etiology associated with early inflammation, immune dysfunction, and vascular injury, followed by progressive fibrosis of the skin and internal organs. In this disease the cell infiltration correlates with skin thickening, suggesting a relation between inflammation and fibrosis. Fibrosis, the distinguishing pathological hallmark of SSc, is characterized by an excessive connective accumulation and matrix remodeling [6, 7]. SSc is generally divided into

two categories based on the extent of skin fibrosis: diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc) [8, 9]. A possible causative role of environmental factors in the pathogenesis of the disease has been suggested and the importance of several occupational factors in the development of SSc such as crystalline silica dust, white spirit, aromatic solvents, chlorinated solvents, trichlorethylene, ketones, and welding fumes has been highlighted [3]. The association between SSc and occupational exposure may be variable according to gender and the risk of SSc appears to be markedly associated with high cumulative exposure [10–12]. Particulate emissions from diesel exhaust engines (DEP) have been associated with a variety of pathological conditions, including fibrosis. Adverse effects of DEP on human health are currently a serious concern and have been shown to include a higher risk for cancer and pulmonary and cardiovascular diseases [13–16]. Diesel exhaust particles are a category of particulate matter (PM) derived from diesel fossil fuels and combustible engines. PM is divided into three major size categories: ultrafine (<0.1 μm), fine (<2.5 μm), and coarse (<10 μm and >2.5 μm). Particles of nanosized dimension, as small aggregates of carbonaceous particles less than 100 nm, constitute the most part of DEP and represent the greatest concern to human health because they remain in the atmosphere for long periods, invade the indoor air environment, and can be breathed most deeply into the lungs where they are likely to be more toxic than coarse particles [13]. In the last years, as a consequence of the increasing awareness of the public of the potential cytotoxicity of soot particulates, as recently confirmed by the World Health Organization (WHO), the legislative body has imposed increasingly more strict emission levels. The modifications made to the diesel engine to fulfill the low emission standard have essentially changed the morphology and surface functionalization of the soot drastically increasing the chemical reactivity of the carbon surface, thus making it more cytotoxic [17, 18]. Like airway epithelial cells, the epidermal cells, as constitutive elements of the outermost barrier in direct contact with air pollutants, are among the cell populations most exposed to chemical pollutants, including DEP. These cells, when stimulated by foreign body materials and/or particles, can activate and release a cascade of proinflammatory and prooxidant mediators which in turn stimulate other cell types, especially fibroblasts, to produce a series of fibrogenic molecules responsible for the development of fibrous tissue [19, 20]. These fundamental functions result from the location of the skin, which is the largest body's organ at the interface between external and internal environment and is devoted to put in place efficient sensory and effector capabilities to differentially react to aggressive stimuli in external environment [21]. In a recent study we demonstrated that DEP are internalised by both monocyte-derived macrophages from peripheral blood and keratinocytes from healthy subject and possess oxidative, profibrotic, and proinflammatory potential [22, 23]. Our previous findings suggest that DEP could have a key role in inducing inflammatory responses and promoting fibrosis in normal human skin. Based on these results, we hypothesized that DEP, by chronically stimulating the release of proinflammatory and profibrotic mediators (cytokines, chemokines, reactive oxygen species, etc.) from skin cells (keratinocytes and fibroblasts), could represent a risk factor for the development of fibrosis in SSc subjects, genetically predisposed to have a dysregulation of the complex cellular inflammatory network [24]. We aimed to investigate whether airborne factors, represented by carbonaceous nanoparticles from both Euro 4 and Euro 5 light duty diesel engines, can stimulate the release of proinflammatory and profibrotic mediators by activated and genetically upregulated keratinocytes and fibroblasts derived from subjects affected with dcSSc and to compare the response *in vitro* to that obtained with cells from healthy subjects.

2. Materials and Methods

2.1. Particle Collection and Characterization

2.1.1. Experimental Setup. The experimental activities were conducted on a prototype single cylinder research engine which has a modern combustion system design derived from a Euro 5 compliant four-cylinder engine which represents the state of the art of light duty (LD) diesel engine technology.

The engine-out exhaust gases for pollutant and particle analysis were sampled at the same point, upstream of the typical emissions after-treatment systems (diesel oxidation catalyst (DOC) and diesel particulate filter (DPF)). From the same point the exhaust gases were drawn off and collected on a filter where the soot was collected. The gaseous emissions were recorded with an ABB, EMERSON, and Ecophysics device for UHCs, CO, CO_2, O_2, and NO_x , respectively. The counting and sizing of particles were performed by means of a differential mobility spectrometer (Cambustion DMS 500) in which measurement principle is based on a deflection of electrically charged particles combined with electrical counting. The measurement range is from 5 to 1000 nm [25].

2.1.2. Test Methodology. The test procedure and points were chosen in order to provide additional experimental information on soot characteristics. The operating points were performed using a Euro 4 and Euro 5 engine calibration (derived from the real four-cylinder engine of equal unit displacement) to ensure the value for practical application in the field of LD engines.

The tests were performed at fixed engine (2000 rpm) speed and load (5-bar brake mean effective pressure (BMEP))

2.1.3. Soot Sampling and Pretreatment. Total particulate was collected from the exhaust pipe by isokinetic sampling. The sampling line comprised a Teflon filter (Millipore, pore diameter 0.45 μm) placed in temperature controlled system (360 K) to avoid gas condensation. The solid particulate collected on the filter was washed with dichloromethane (DCM) in order to remove condensable species and fuel residuals (soluble organic fraction (SOF)). The soluble organic fraction (SOF) was further quantified by drying the DCM extracts. It was found that SOF accounts for 8–10 wt.% of the total particulate in both cases. The carbonaceous solid after DCM extraction (soot) was dried, weighted, and characterized. Soot

spectral features and hydrodynamic diameter were evaluated in N-methyl pyrrolidinone (NMP) suspensions obtained by using an ultrasonic bath.

2.1.4. Soot Characterization. The chemical-physical characterization of the soot has been performed on soot washed with DCM in order to probe the soot surface without the interference of physisorbed species (unburned hydrocarbons and tar species). The thermal stability of the samples was evaluated by using thermogravimetric analysis (TGA) performed on a Perkin-Elmer Pyris 1 Thermogravimetric Analyzer in oxidative environment (air, 30 mL min^{-1}). Fourier transform infrared (FTIR) spectra were recorded on a Nicolet iS10 spectrometer using the attenuated total reflectance (ATR) method. The hydrodynamic diameter of the carbonaceous materials was measured by using a Malvern Zetasizer Nano ZS instrument on soot suspension in NMP (0.01 mg mL^{-1}) [26]. Transmission electron microscopy (TEM) and high resolution transmission electron microscopy (HRTEM) imaging were performed on a FEI Tecnai G2 F20 transmission electron microscope equipped with a field-emission gun. Electronic structure measurements were performed using EELS. Spectra were recorded with the Gatan Imaging Filter (GIF 100) with an energy resolution of 1 eV measured at the full width at half maximum (FWHM) of the zero loss. Several spectra are acquired from different areas (0.028 mm^2) of each sample. Each spectrum is background-subtracted and corrected for multiple scattering. An average of the spectra is calculated. Acquisition of EELS spectra is done using the diffraction mode in order to reduce the current density of the beam on the sample, without decreasing the signal-to-noise ratio in the acquired spectrum. UV-Vis spectra of soot, suspended in NMP, were acquired on an HP 8453 diode array spectrophotometer. With the soot molecular mass being unknown, the absorption coefficients have been expressed on a mass basis (m^2/g). The concentration of each suspension was 0.01 mg mL^{-1}. For the *in vitro* studies, the Euro 4 and Euro 5 soots were sterilized by heating at 180°C, at a temperature lower than that of the exhaust gas at the collection position (200°C), in order to avoid affecting the particle properties. Then the particles were washed three times in distilled water, suspended in PBS at a stock concentration of 1 mg/mL, and sonicated in a water bath at low intensity for 48 h before the use, in order to obtain a better dispersion of the particles that tend to agglomerate.

2.2. Cell Cultures. Primary cultures of keratinocytes and fibroblasts from dcSSc patients, classified according to LeRoy et al. [27, 28], and healthy controls were isolated and grown as previously described in [29]. The study was conducted according to the Declaration of Helsinki Principles. Participants gave their written informed consent.

2.3. RNA Extraction and Real Time RT-PCR. Primary cultures of keratinocytes and fibroblasts from healthy and dcSSc subjects were stimulated with Euro 4 and Euro 5 soot particles (30 and 60 μg/mL) for 6 h. These concentrations were selected as the lowest concentration able to affect the examined parameters, without inducing excessive cytotoxicity.

After treatment, cells were washed with PBS and harvested for real time RT-PCR analysis. Total RNA was then isolated by the RNAse kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The amount and the quality of mRNA were determined using a μQUANT spectrophotometer (BIOTEK, Instruments Inc., Winooski, VT, USA). According to the manufacturer's protocol 1 μg of template RNA was reverse-transcribed to complementary DNA using RevertAid M-MuLV reverse transcriptase (Fermentas Life Sciences, Hanover, MD, USA). Quantitative real time RT-PCR was performed in a total volume of 15 μL with SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) and a 200 nM concentration of each primer. Sequences of all primers used are indicated in Table 1. Amplification reactions were performed in triplicate for each sample, and analysis of relative gene expression data, normalized to endogenous control (GAPDH), was calculated applying the $2^{-\Delta\Delta CT}$ on the real time detection system (iQ5 Bio-Rad) supplied with iCycler IQ5 optical system software version 2.0 (Bio-Rad). Expression of each gene was assessed by at least three independent PCR analyses.

2.4. Statistical Analysis. All analyses were performed in at least three independent experiments performed in duplicate. Data were reported as mean ± SD followed by statistical significance (Student's t-test for unpaired experiments). P value of 0.05 was considered to be statistically significant.

3. Results

The particle size distribution function (PSDF) of diesel was in the range 10–500 nm (Figure 1). The aerodynamic diameter of soot emitted from Euro 5 configuration appeared slightly larger (90 ± 5 nm) than Euro 4 soot (80 ± 5 nm). This finding was confirmed by the estimation of hydrodynamic diameter measured by DLS (115 ± 5 and 95 ± 5 nm) in NMP suspensions. The soot appeared quite monodisperse, exhibiting a polydispersion index (PI) less than 0.3 in both cases (values of PI < 0.1 are typically obtained for highly homogeneous dispersion, whereas PI > 0.3 for highly heterogeneous dispersions).

TEM and HRTEM images of soot nanoparticles emitted in Euro 4 and Euro 5 calibration settings are depicted in Figure 2. Soot consists of irregularly shaped compact soot aggregates of almost spherical primary particles. The primary particles sizes are very low and keep quite constant (15–20 nm) for both Euro 4 and Euro 5 soots. No variation in dimension is clearly discernible. The interior structure of soot (Figures 2(b) and 2(d)) appears quite similar in both cases, indicating that the different calibration settings negligibly affect the formation of the nuclei cores at the early stage of the soot formation. The irregular soot surface suggests defects in the carbonaceous network arising from the presence of sp3-hybridized carbon in the aromatic moiety.

The impact of the two types of carbonaceous nanoparticles was evaluated on keratinocytes and fibroblasts form

Table 1: Primers used for the real time reverse transcriptase-PCR analysis.

Human	Oligonucleotide sequences (5′-3′)
GAPDH	
Sense:	TGCACCACCAACTGCTTAGC
Antisense:	GGCATGGACTGTGGTCATGAG
MMP-2	
Sense:	TCTCCTGACATTGACCTTGGC
Antisense:	CAAGGTGCTGGCTGAGTAGATC
MMP-3	
Sense:	GCTGCAAGGGGTGAGGACAC
Antisense:	GATGCCAGGAAAGGTTCTGAAGTG
MMP-7	
Sense:	TGAGCTACAGTGGGAACAGG
Antisense:	TCATCGAAGTGAGCATCTCC
MMP-9	
Sense:	TTGACAGCGACAAGAAGTGG
Antisense:	GCCATTCACGTCGTCCTTAT
MMP-12	
Sense:	GAATTGATCCGTTTAGAAGTTTAC
Antisense:	GGCTTGTAGAGCTGTTCAG
VEGF	
Sense:	GTTGACCTTCCTCCATCC
Antisense:	TTCTCTGCCTCCACAATG
Col I	
Sense:	CAGCCGCTTCACCTACAGC
Antisense:	AATCACTGTCTTGCCCCAGG
Col III	
Sense:	TCCAACTGCTCCTACTCGCC
Antisense:	GAGGGCCTGGATCTCCCTT
IL-1α	
Sense:	CGCCAATGACTCAGAGGAAGA
Antisense:	AGGGCGTCATTCAGGATGAA
IL-6	
Sense:	AGCCACTCACCTCTTCAGAACG
Antisense:	GGTTCAGGTTGTTTTCTGCCAG
IL-8	
Sense:	CTTGGCAGCCTTCCTGATTTC
Antisense:	TTCTGTGTTGGCGCAGTGTG
TNF-α	
Sense:	CAACCTCTTCTGGCTCAA
Antisense:	CGAAGTGGTGGTCTTGTT

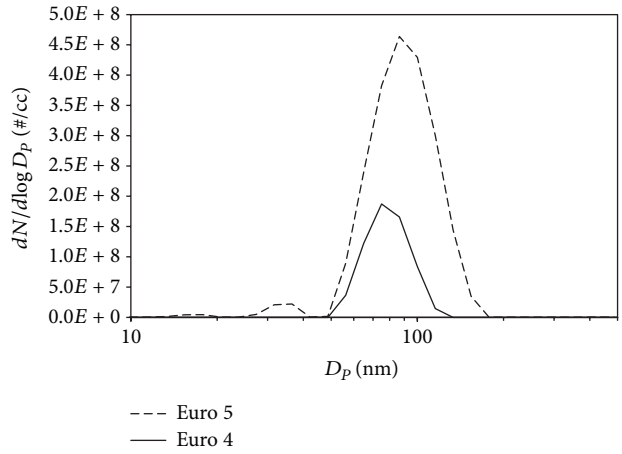

Figure 1: The particle size distribution function (PSDF) of diesel in the range 10–500 nm.

healthy and dcSSc subjects. We assessed the proinflammatory potential of Euro 4 and Euro 5 soot particles evaluating their effect on the induction of several proinflammatory cytokine (IL-1α, IL-6, IL-8, and TNF-α) mRNA expressions by keratinocytes from healthy and sclerodermic subjects. The IL-1α and IL-8 mRNA levels were significantly induced in soot particles treated cells from healthy controls only at the highest particle concentration (60 μg/mL) (Figures 3(a) and 3(d)). In dcSSc keratinocytes both soot particles significantly induced the increase of the mRNA level of IL-1α and IL-8 cytokines in a dose dependent manner. The IL-6 mRNA expression was significantly induced in Euro 4 treated cells from healthy controls only at the highest concentration, while in Euro 4 treated cells from dcSSc subjects it strongly increased in a dose dependent manner. On the contrary, the Euro 5 treatment significantly induced the IL-6 mRNA expression in a dose dependent manner in both normal and dcSSc cells (Figure 3(c)). Finally, TNF-α mRNA expression resulted strongly induced in both cell types treated with Euro 4 and Euro 5 soots (Figure 3(b)).

We further investigated the effects of these soot nanoparticulates on fibroblast activation, evaluating the mRNA expression of different metalloproteases (MMP-2, MMP-3, MMP-7, MMP-9, and MMP-12), collagen types (Col I and Col III), and vascular endothelial growth factor (VEGF). We found that the mRNA expression of all genes was higher in untreated cells from dcSSc subjects compared with healthy cells (Figure 4(a)). No significant changes in the mRNA expression of MMP-3 and MMP-12, Col I and Col III, and VEGF were observed following treatment with Euro 4 and Euro 5 both in normal and dcSSC cells (data not shown). On the contrary, the mRNA expression of MMP-2 and MMP-9 was induced at the highest dose of Euro 4 (60 μg/mL) in both cell types but was significantly higher in fibroblasts from SSC subjects than in normal fibroblasts (Figures 4(b), 4(c), and 4(d)). The Euro 4 treatment did not induce a significant expression of MMP-7 mRNA level in both cell types (Figure 4(c)). The Euro 5 exposure upregulated the mRNA expression of MMP-2, MMP-7, and MMP-9 in cells from dcSSc subjects in a dose dependent manner (Figures 4(b) and 4(d)). In cells from healthy subjects only the highest dose of Euro 5 induced the expression of MMP-9 mRNA levels.

4. Discussion and Conclusions

In the last few years advances in the field of autoimmunity have led to an understanding of the natural history of autoimmune diseases. Autoimmune diseases occur when

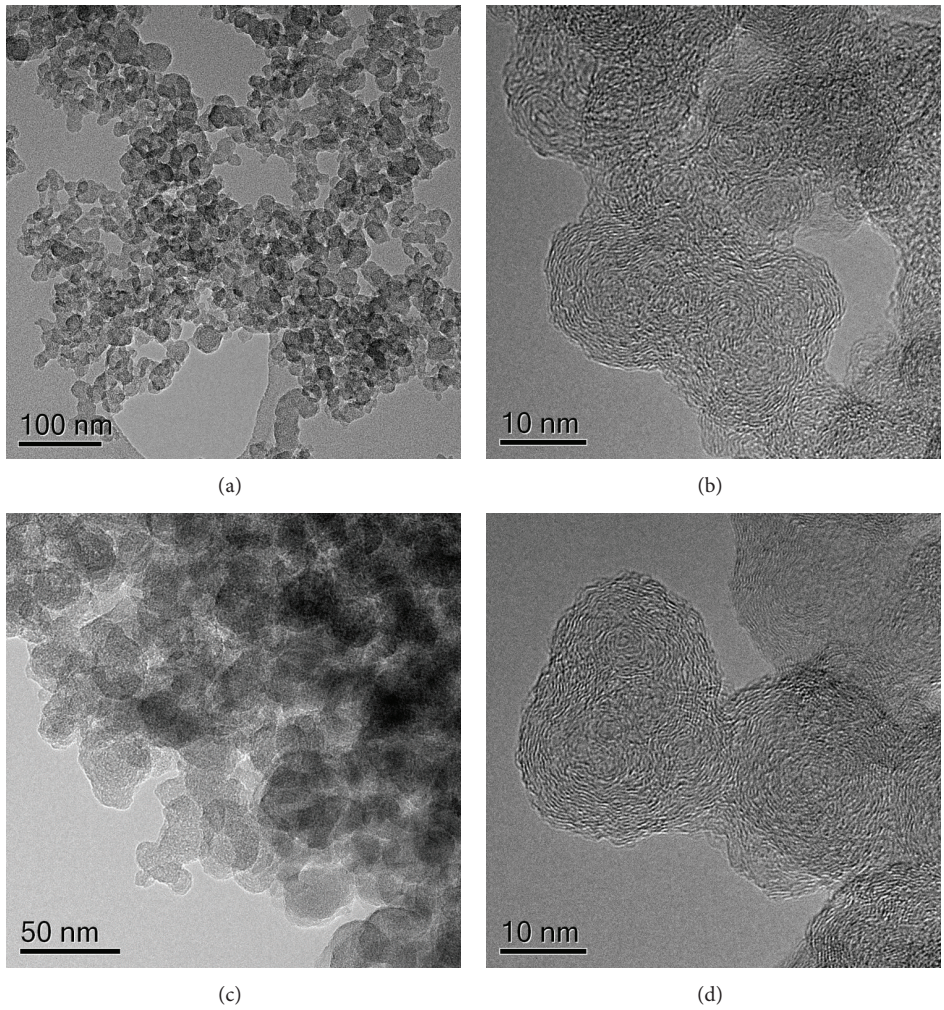

Figure 2: TEM and HRTEM images of the soot particles emitted in Euro 4 (a, b) and Euro 5 (c, d) calibration settings.

a genetically susceptible individual encounters an environmental trigger that could be responsible for inducing autoimmunity [30, 31]. Environmental exposures that could play a role in autoimmune disease pathogenesis include infectious agents, ultraviolet light, chemicals and compounds capable of modulating immune responses such as occupational/environmental pollutants or drugs, and behavioral factors, such as smoking and diet [32–36]. Environmental pollution due to nanoparticulate from diesel engine exhaust gases has been hypothesized to be responsible for several bronchopulmonary, cardiac, and vascular diseases. Moreover, numerous epidemiological studies have shown the higher incidence of these diseases in populations living in close proximity to highways, airports, and high density urban traffic zones [37–40]. The present study was performed by challenging *in vitro* skin dcSSc cells with carbonaceous nanoparticles collected from Euro 4 and 5 light duty diesel engines exhaust. Our main purpose was to verify the hypothesis that environmental pollution derived from automotive diesel engine exhaust of actually operating vehicles could have a role in the activation of inflammatory cell pathways in a model of immune-mediated disease. The highly specific characterization and description of particle shape and surface physical-chemical characteristics that have been performed help to better address the influence of one of the multiple constituents of vehicle exhaust emissions in order to make it possible to identify its own effects and to potentially modify its dangerous properties. DEP nanoparticulate consists of irregularly shaped compact aggregates of almost spherical primary particles. The primary particle sizes are very low and keep quite constant (15–20 nm) for both Euro 4 and Euro 5 soots. The very low dimensions of these particles enable them to be easily and spontaneously internalised by skin keratinocytes, as we previously demonstrated [23] and thus to induce a severe inflammatory response by these cells. We observed that DEP nanoparticles activated keratinocytes and fibroblasts of patients with dcSSc at a higher extent as compared to normal cells. In dcSSc keratinocytes both Euro 4 and 5 soot particles significantly induced the increase of IL-1α and IL-8 mRNA as well as IL-6 levels even at the lower particle concentration and in a dose dependent manner. A quite different behaviour was observed when cells were

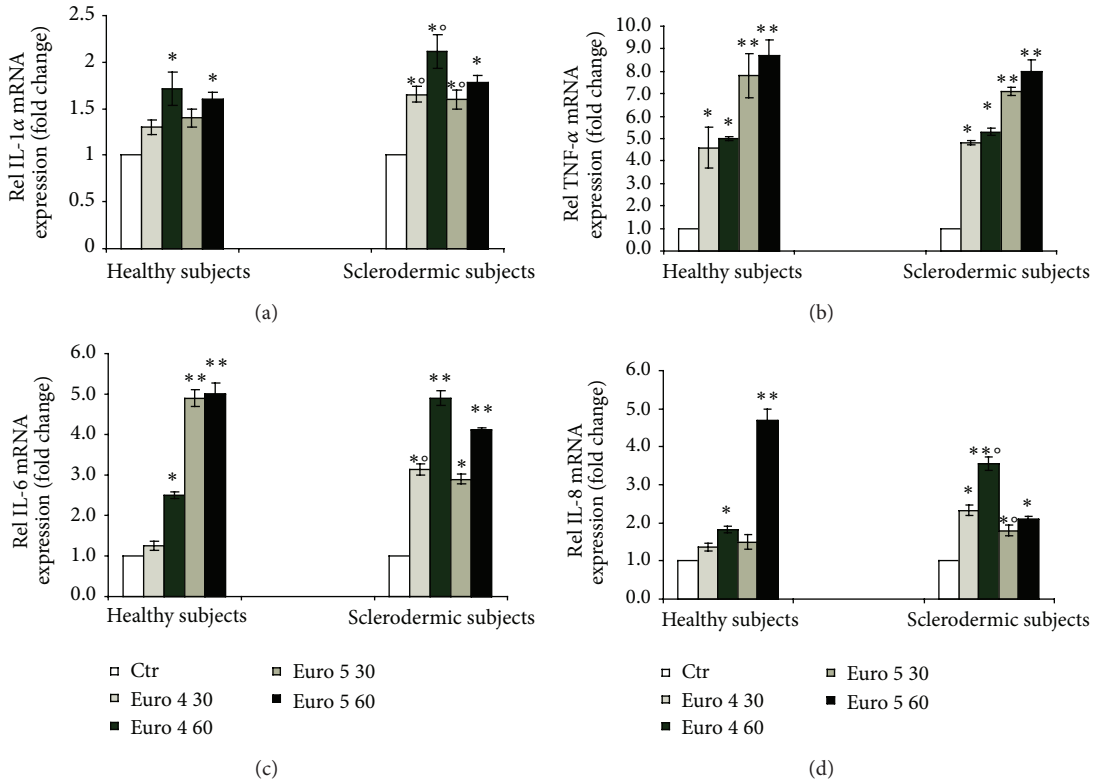

Figure 3: Real time PCR analysis of the expression of IL-1α (a), TNF-α (b), IL-6 (c), and IL-8 (d) in primary cultures of keratinocytes from sclerodermic and healthy subjects stimulated with Euro 4 and Euro 5 nanoparticles (30 and 60 μg/mL) for 6 h. ($^{*}P < 0.01$ and $^{**}P < 0.001$ versus control; $^{\circ}P < 0.01$ versus healthy subjects).

treated with Euro 5 particles. In this case the nanoparticle treatment significantly induced IL-6 as well as TNF-α mRNA expression in a dose dependent manner in both normal and SSc cells. This cell response could be attributed to the higher proinflammatory potential of Euro 5 with respect to Euro 4 nanoparticles. It can be speculated that a slightly higher graphitic degree of Euro 5 soot, as demonstrated by electron energy loss spectroscopy (EELS) and UV-visible absorption spectroscopy analysis (data not shown), responsible for a different stabilization of radical species on soot surface, could account for this effect. This hypothesis deserves a more focused investigation that is planned in the future.

The higher reactivity of dcSSc keratinocytes, revealed by the increased gene expression of all cytokine evaluated even at the lowest diesel particle concentration, has to be related to the genetically induced upregulation of dcSSc cell inflammatory pathways. Recently, IL-1α has been found to be greatly increased in SSc epidermis and it is believed to initiate keratinocyte-fibroblast interactions leading to fibroblast activation. The coculture of the keratinocytes from SSc patients with normal human fibroblasts was found to promote fibroblast activation. A model of chronic injury in SSc in which keratinocyte activation in turn promotes and sustains fibroblast activation and scarring through IL-1α has been proposed [41]. Several reports have reported that IL-6 levels are elevated in culture supernatants of dermal fibroblasts and serum from patients with SSc. Its prominent expression in the skin was observed in dermal fibroblasts, mononuclear cells, and endothelial cells in patients with early dcSSc; *in vitro* experiments supported a potent profibrotic effect of IL-6 transsignaling pathways [42–44]. Our findings, demonstrating that DEP induced the increase of IL-1α and Il-6 gene expression, both these cytokines possessing a profibrogenic capacity, suggest that this environmental stimulus could represent a key factor capable of initiating a proinflammatory/profibrogenic cascade in SSc subjects. Moreover, these results highlight the fundamental role played by keratinocytes in the development of this disease. In addition, because of the fundamental role played by fibroblasts in the pathogenesis and clinical manifestations of the disease, we evaluated the fibroblast response to DEP treatment. The observation that in untreated dcSSc fibroblasts all metalloproteinases, collagen types I and III, and VEGF genes were overexpressed is in agreement with the literature data and represents the hyperresponsiveness of these cells to unknown stimuli [45, 46]. In the same cells after DEP stimulation, significantly higher levels of MMP-2, MMP-7, and MMP-9 compared to controls were observed. Matrix metalloproteinases (MMPs) constitute a large group of endoproteases that are able not only to cleave all protein components of the extracellular matrix but also to activate or inactivate many other signaling molecules, such as receptors, adhesion molecules, and growth factors. Elevated MMP levels are associated with an increasing number of injuries

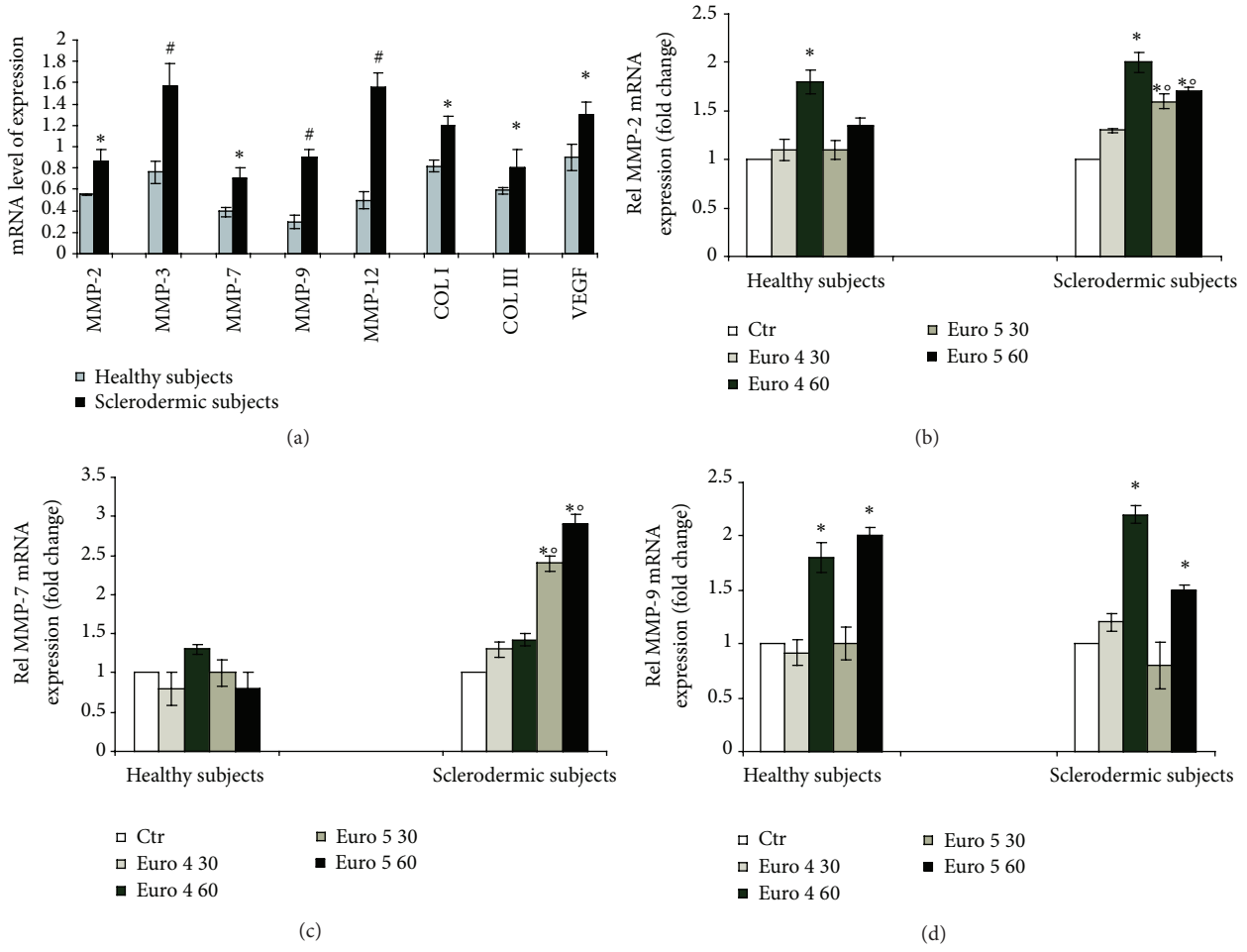

Figure 4: Real time PCR analysis of the expression of MMP-2, MMP-3, MMP-7, MMP-9, and MMP-12, Col I and Col III, and VEGF in primary culture of fibroblasts from sclerodermic and healthy subjects ($^{*}P < 0.01$; $^{\#}P < 0.001$) (a). Real time PCR analysis of the expression of MMP-2 (b), MMP-7 (c), and MMP-9 (d) in primary culture of fibroblasts from sclerodermic and healthy subjects stimulated with Euro 4 and Euro 5 nanoparticles (30 and 60 μg/mL) for 6 h. ($^{*}P < 0.01$ versus control; $^{\circ}P < 0.01$ versus healthy subjects).

and disorders, such as cancer, inflammation, and autoimmune diseases. Yet, MMP upregulation has also been implicated in many physiological functions such as embryonic development, wound healing, and angiogenesis and therefore, these proteinases are considered to be crucial mediators in many biological processes [47]. MMP-2, or gelatinase A, a constitutive enzyme, is found in almost all cell types and it degrades denatured collagen (gelatin) and collagen type IV (a component of the basement membrane) as well as other extracellular matrix proteins. MMP-9, or gelatinase B, has been implicated in the pathogenesis of cancer, autoimmune disease, and various pathologic conditions characterized by excessive fibrosis [48, 49]. Dermal fibroblasts from patients with SSc were observed to produce more MMP-9 than those from healthy controls when they were stimulated with IL-1β, TNF-α, or TGF-β. Moreover, the serum MMP-9 concentrations were found to be elevated in SSc patients and to correlate with skin scores. The increased MMP-9 concentrations were attributed to overproduction by dermal fibroblasts [45]. Moreover, serum MMP-9 concentrations were observed to be significantly higher in the diffuse type than the limited type of SSc [50]. SSc is mainly characterized by microvascular damage and excess organ fibrosis. The damage is caused by a massive deposition of collagen and other connective tissue components. In general, tissue fibrosis reflects an imbalance between collagen production and degradation. Excessive accumulation of extracellular matrix (ECM) components, especially types I and III collagen, is the most prominent pathological manifestation of the disease [51, 52]. Numerous studies have shown that an abnormally increased synthesis of these constituents in SSc skin *in vivo* and in cells cultured from the skin of SSc patients grown in tissue culture may be involved in the development of SSc [53, 54]. The observed hyperexpression by dcSSc fibroblasts, before DEP treatment, of metalloproteinase 2, 7, 9, and 12 genes, and their reaction with a further highly significant increase in metalloproteinases 2, 7, and 9 gene expression in response to DEP particles, can be considered as the expression of a tentative, put in place by these cells, to counteract the excessive collagen production in response to inflammatory

stimuli. Fibroblast behavior not only confirms that they are hyperreactive to external substances but also demonstrates that diesel nanoparticulate could have a key role in triggering a pathologic response in genetically up-responsive skin cells.

In conclusion, these preliminary results show that environmental factors due to traffic-derived pollution can play a key role in triggering an inflammatory-fibrogenic response by upregulated cells in genetically predisposed individuals that is significantly higher as compared to that of normal cells exposed to the same stimuli.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 856154, 9 pages
http://dx.doi.org/10.1155/2014/856154

Research Article

Asian Dust Particles Induce Macrophage Inflammatory Responses via Mitogen-Activated Protein Kinase Activation and Reactive Oxygen Species Production

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Received 17 February 2014; Accepted 18 May 2014; Published 29 May 2014

Academic Editor: Takemi Otsuki

Asian dust is a springtime meteorological phenomenon that originates in the deserts of China and Mongolia. The dust is carried by prevailing winds across East Asia where it causes serious health problems. Most of the information available on the impact of Asian dust on human health is based on epidemiological investigations, so from a biological standpoint little is known of its effects. To clarify the effects of Asian dust on human health, it is essential to assess inflammatory responses to the dust and to evaluate the involvement of these responses in the pathogenesis or aggravation of disease. Here, we investigated the induction of inflammatory responses by Asian dust particles in macrophages. Treatment with Asian dust particles induced greater production of inflammatory cytokines interleukin-6 and tumor necrosis factor-α (TNF-α) compared with treatment with soil dust. Furthermore, a soil dust sample containing only particles ≤10 μm in diameter provoked a greater inflammatory response than soil dust samples containing particles >10 μm. In addition, Asian dust particles-induced TNF-α production was dependent on endocytosis, the production of reactive oxygen species, and the activation of nuclear factor-κB and mitogen-activated protein kinases. Together, these results suggest that Asian dust particles induce inflammatory disease through the activation of macrophages.

1. Introduction

Asian dust (also yellow sand) is a dominant springtime aerosol in East Asia. The dust originates from the deserts of East Asia, such as the Takla Makan and Gobi, and is spread by prevailing winds not only locally to China and Mongolia but also throughout East Asia to countries such as Korea, Taiwan, and Japan. A similar phenomenon originating from the Sahara has also been observed in Europe and the United States [1, 2], raising concerns throughout the world about the impacts of aeolian dust [3, 4].

Asian dust not only is responsible for financial loss through crop damage, tree collapse, and flight cancellations due to low visibility, but also it poses a major threat to human health. Recent reports have shown that, in addition to harmful chemicals, Asian dust particles contain lipopolysaccharides and β-glucan, which are components of the bacterial cell membrane and fungal cell wall, respectively,

and that bacteria can adhere to the outer surface of the dust particles [5, 6]. Indeed, morbidity rates for cardiovascular and respiratory diseases in Central East Asian countries increase during Asian dust storms [7–9]; in Korea, Asian dust storms are correlated with a 2.2% increase in the rate of patients over the age of 65 presenting with respiratory symptoms [10]. The association between Asian dust levels and the pathogenesis of childhood asthma has been clearly demonstrated; Asian dust likely triggers the onset of this and other inflammatory conditions through the induction of excessive antigen-specific or nonspecific inflammatory responses [11].

Although most of the information regarding Asian dust-related health problems is based on epidemiological investigations, some experimental studies have demonstrated the effects of Asian dust on allergic respiratory diseases *in vivo* [12, 13]. For example, Asian dust particles are reported to enhance both ovalbumin-induced eosinophil recruitment in the alveoli and airway submucosa in mice [14] and nasal allergic reactions in guinea pigs [15]. However, detailed information on the mechanisms of these inflammatory responses remains limited. To further our knowledge on the mechanisms through which Asian dust affects human health, it is essential to evaluate the interplay among the physical characteristics and the biological responses it provokes.

It is generally accepted that, like bacteria and viruses, particulate matter such as Asian dust particles is eliminated from the human body by phagocytes such as macrophages [16, 17]. Macrophages ingesting exogenous materials produce interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and, through the activation of signal pathways such as the mitogen-activated protein kinase (MAPK) pathways, expedite the elimination of particulate matter by inducing inflammatory responses; however, excessive or chronic macrophage activation results in inflammatory diseases such as bronchitis or pneumonia [18]. It is therefore important to assess the biological responses of macrophages to Asian dust particles, in particular the inflammatory responses. In the present study, we collected samples of suspended Asian dust particles from Beijing, China, and soil dust from Loess Plateau, China, which is a source of Asian dust, and evaluated their effects on the macrophage inflammatory response.

2. Materials and Methods

2.1. Reagents. Samples of suspended Asian dust particles which fell on the top of the building of the Beijing inner city were collected each day for three successive days from March 24, 2010 (defined as ADP1), and once on March 20, 2010 (ADP2). Samples of soil dust were collected on June 30, 2009, from three locations at Loess Plateau, China, as follows: site 1: lat 35° 35′ 450″N, long 109° 10′ 074″E (SDP1); site 2: lat 35° 42′ 324″N, long 109° 25′ 390″E (SDP2); and site 3: lat 35° 42′ 279″N, long 109° 25′ 450″E (SDP3). These soil dust particles were the same samples previously used by Yamaguchi et al. in [6]. In addition, sample SDP2 was partitioned by using a soundwave vibrating screen (Tsutsui Rikagaku Kikai Co., Ltd., Tokyo), and particles ≤10 μm in diameter were collected (SDP4). Lipopolysaccharide (LPS), butylated hydroxyanisole (BHA), broad-spectrum ROS scavenger, and diphenyleneiodonium chloride (DPI), a specific inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, were purchased from Sigma-Aldrich (St. Louis, MO). U0126, an extracellular signal-regulated kinase (ERK) inhibitor, and SP600125, a c-Jun N-terminal kinase (JNK) inhibitor, were obtained from Merck (Darmstadt, Germany). SN50, a nuclear factor-κB (NF-κB) inhibitor, was purchased from Calbiochem (La Jolla, CA).

2.2. Cells. RAW264.7 cells (mouse monocyte/macrophage cell line) were obtained from the American Type Culture Collection (Manassas, VA) and cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum and antibiotics.

2.3. Scanning Electron Microscopy Analysis. The Asian dust particles and soil dust samples were adjusted to 0.25 mg/mL with deionized water. Aliquots (50 μL) of each sample were then dried on a hot plate (Cimarec, Barnstead Thermolyne, Dubuque, IA) and observed under a scanning electron microscope (Jeol, Ltd., Tokyo, Japan).

2.4. Cytotoxicity and Cytokine Production Assays. RAW264.7 cells (1.5×10^4 cells/well) were seeded in 96-well plates (Nunc, Rochester, NY), cultured at 37°C for 24 h, and then treated with 6.25, 25, or 100 μg/mL of a suspension of Asian dust particles or soil dust or DMEM (negative control) or 1.5 μg/mL of LPS at 37°C for 24 h. The cytotoxicity of Asian dust was assessed by methylene blue assay. The cells were fixed with glutaraldehyde and stained with 0.05% methylene blue for 15 min. After washing with water, the methylene blue in the wells was eluted using 200 μL of 0.33 N HCl for each of the wells and absorbance was measured at 655 nm (subwave length, 415 nm). Cell viability was calculated as the ratio of absorbance in the treated cultures compared to the control untreated cultures. Enzyme-linked immunosorbent assay (ELISA) kits were used in accordance with the manufacturer's instructions to determine the levels of IL-6 (BD Pharmingen, San Diego, CA) and TNF-α (eBioscience, San Diego, CA) in the culture supernatants.

2.5. Inhibition Assay. RAW264.7 cells (1.5×10^4 cells/well) were seeded in 96-well plates (Nunc), cultured at 37°C for 24 h, and then preincubated for 0.5 h with Cytochalasin D (5 or 10 μM), BHA (250 μM), DPI (1 or 2 μM), U0126 (30 μM), SP600125 (50 μM), or SN50 (50 μM). The cells were then treated with 100 μg/mL of a suspension of Asian dust particles, soil dust, or DMEM (negative control) for 6 h. TNF-α in the culture supernatants were assessed by means of an ELISA according to the manufacturer's instructions.

2.6. Evaluation of Reactive Oxygen Species (ROS) Production. RAW264.7 cells (1.5×10^4 cells/well) were seeded in 96-well plates (Nunc), cultured at 37°C for 24 h, washed three times with phosphate buffered saline, and then incubated in phenol

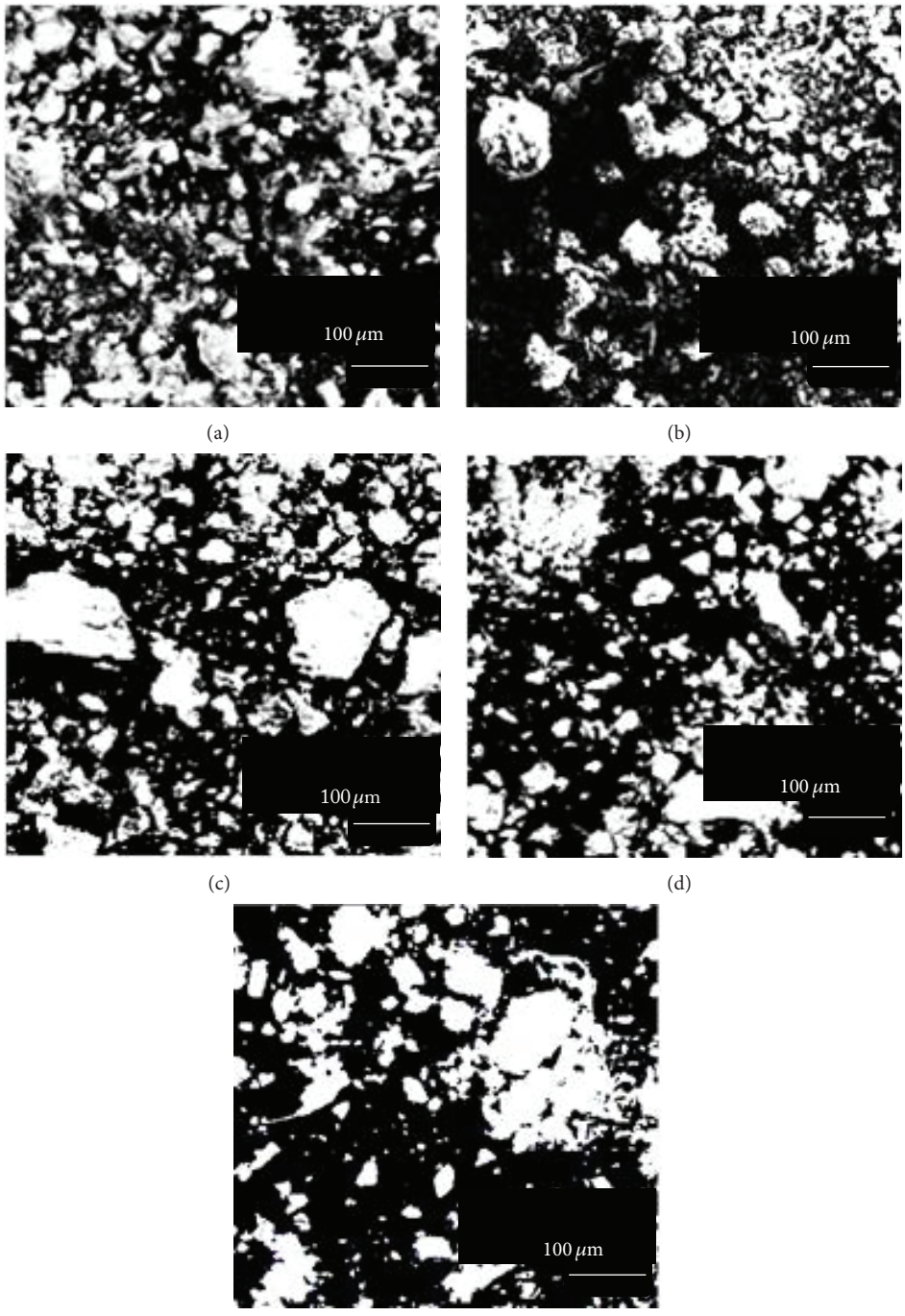

Figure 1: Scanning electron micrographs of two Asian dust particles (ADP1 (a) and ADP2 (b)) and three soil dust (SDP1 (c), SDP2 (d), and SDP3 (e)) samples. Prior to analysis, samples were adjusted to 0.25 mg/mL with deionized water and dried on a hotplate. Scale bars: 100 μm.

red-free DMEM containing 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (Cell Biolabs, Inc., San Diego, CA) for 30 min at 37°C. After incubation, the cells were treated with 100 μg/mL a suspension of Asian dust particles, soil dust, or DMEM (for negative control) for 6 h and fluorescence was measured at 485 nm (subwave length, 530 nm).

2.7. Statistical Analysis. All results are expressed as mean ± SD. Differences were compared by using Bonferroni method.

3. Results and Discussion

3.1. Characteristics of the Asian Dust Particles and Soil Dust Samples. First, the two Asian dust particles samples (ADP1 and ADP2) and three soil dust samples (SDP1, SDP2, and SDP3) were examined under a scanning electron microscope (Figure 1). We used soil dust samples as reference dust because satellite information indicated that the source regions of Asian dust are considered to be loess plateau, China

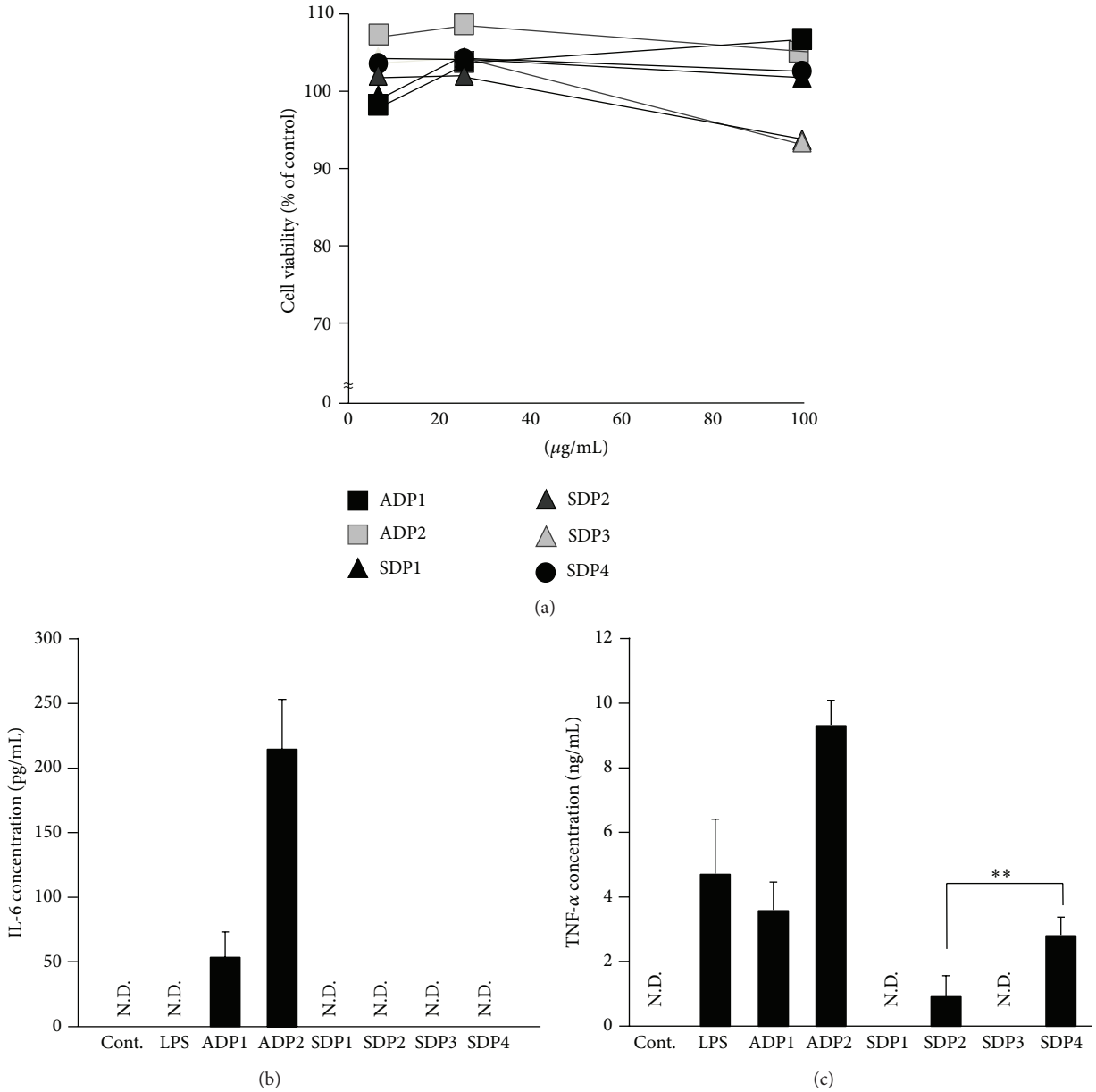

Figure 2: Assessment of cytotoxicity and inflammatory responses induced by Asian dust particles and soil dust in RAW264.7 cells. (a) RAW264.7 cells were treated for 24 h with 6.25, 25, or 100 μg/mL of Asian dust particles ADP1, ADP2, or with soil dust SDP1, SDP2, SDP3, or SDP4 (particle diameter, ≤10 μm). Cell viability was assessed by means of a methylene blue assay. (b, c) RAW264.7 cells were treated with 100 μg/mL of each dust sample or 1.5 μg/mL of lipopolysaccharide (LPS) for 24 h. Levels of interleukin-6 (IL-6) (b) and tumor necrosis factor-α (TNF-α) (c) in culture supernatants were assessed by means of enzyme-linked immunosorbent assay. DMEM was used as control (Cont.). Results are expressed as mean ± SD; $n = 6$; $^{**}P < 0.01$; N.D., not detected.

[6]. Yamaguchi et al. demonstrated that all samples contained particles with a coarse surface texture, that the particles varied in form, and that some particles were condensed by scanning electron microscopic analysis. They also showed that silicon and aluminum were major components of Asian dust particles and that significant quantities of iron, calcium, and magnesium were also present [6]. In addition, large numbers of particles with a diameter ≥100 μm were observed in the three soil dust samples (Figures 1(c), 1(d), and 1(e)) but were not observed in the two Asian dust particles samples (Figures 1(a) and 1(b)). Previously, Yamaguchi et al. showed that the mean particle size of Asian dust particles was <1 μm [6]. It represented that the mean size of the Asian dust particles in our samples would be ≤1 μm.

3.2. Asian Dust Particles Induce an Inflammatory Response in RAW264.7 Cells. Next, we evaluated the potential of Asian dust particles or soil dust to induce an inflammatory response in macrophages. Because our previous data show that particle-induced inflammatory responses are dependent on particle size [19–21], we also investigated whether the induction of an inflammatory response by soil dust was dependent on

particle size by partitioning the particles with a diameter ≤10 μm (SDP4) from SDP2.

To evaluate the cytotoxicity of the Asian dust particles and soil dust samples, RAW264.7 cells were treated with ADP1, ADP2 (Asian dust particles), SDP1, SDP2, SDP3 (soil dust), or SDP4 (soil dust; particle diameter ≤10 μm) and cell viability was assessed. No significant cytotoxicity was observed in any of the experimental groups (Figure 2(a)).

Next, to examine the macrophage inflammatory response to Asian dust particles or soil dust, RAW264.7 cells were exposed to 100 μg/mL of each sample for 24 h and cytokine production was assessed. The levels of IL-6 (Figure 2(b)) and TNF-α (Figure 2(c)) in the culture supernatants after treatment with ADP1 or ADP2 were markedly higher than those of the control group. Moreover, they were equivalent to or higher than those of lipopolysaccharide- (LPS-) treated group. However, the levels of IL-6 after treatment with SDP1, SDP2, or SDP3 were almost equal to those of the control group. In addition, the levels of TNF-α after treatment with SDP1 or SDP3 were almost equal to those of the control group but treatment with SDP2 could induce elevation of TNF-α production. Furthermore, the level of TNF-α in the culture supernatant after treatment with SDP4 was significantly higher than that after treatment with SDP2. These results suggest that Asian dust particles have greater potential to induce inflammation compared with soil dust and that particle size may affect the soil dust-induced macrophage inflammatory response. Materials such as lipopolysaccharides or β-glucan and chemicals such as ammonium or nitrates have been previously reported to adhere to Asian dust particles [5, 22]. Since the number of molecules that can adhere to particles per unit weight increases as particle size decreases, this may account for the increased production of IL-6 and TNF-α in macrophages exposed to smaller soil dust.

3.3. Asian Dust Particles-Induced ROS Production Mediates TNF-α Production in RAW264.7 Cells. Next, to assess the mechanisms of the Asian dust particles-induced macrophage inflammatory response, we evaluated the association between inflammatory response and endocytosis. RAW264.7 cells were pretreated for 30 min with Cytochalasin D, an inhibitor of phagocytosis, and then treated for 6 h with 100 μg/mL of ADP1 or ADP2; TNF-α production was assessed. Cytochalasin D significantly suppressed the production of TNF-α induced by ADP1 or ADP2 in a dose-dependent manner (Figure 3), suggesting that phagocytosis is a key aspect of the Asian dust particles-induced macrophage inflammatory response.

ROS activate various signal pathways, such as the NF-κB signaling pathway and the MAPK pathways, involved in the production of inflammatory cytokines [23, 24]. To evaluate the involvement of ROS in the macrophage inflammatory response to Asian dust particles, RAW264.7 cells were incubated with Asian dust particles (ADP1 or ADP2) or soil dust (SDP1, SDP2, SDP3, or SDP4) for 6 h and the fluorescence intensity of 2′,7′-dichlorodihydrofluorescein was measured as an index of ROS production. All dust samples induced ROS production (Figure 4(a)). ADP1 and ADP2 induced significantly higher ROS production compared with that

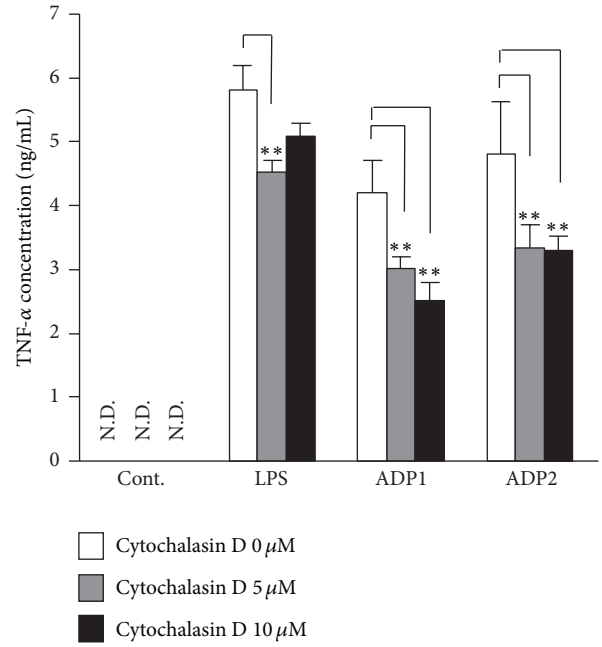

Figure 3: Cytochalasin D inhibited Asian dust particles-induced tumor necrosis factor-α (TNF-α) production in RAW264.7 cells. RAW264.7 cells were pretreated for 30 min with Cytochalasin D, an inhibitor of phagocytosis, and then treated for 6 h with 100 μg/mL of Asian dust particles (ADP1 or ADP2) or 1.5 μg/mL of LPS. Dimethyl sulfoxide (0.1%) vehicle was used as control (Cont.). The level of TNF-α in culture supernatants was assessed by means of an enzyme-linked immunosorbent assay. Results are expressed as mean ± SD; $n = 6$; $^{**}P < 0.01$; N.D., not detected.

induced by SDP1, SDP2, or SDP3. Furthermore, although not statistically significant, SDP4 did tend to induce greater ROS production than SDP1, SDP2, or SDP3, suggesting that particle size may be an important factor for soil dust-induced ROS production.

ROS are mainly produced by cell membrane, or endosome membrane, bound NADPH oxidase or by mitochondria [25, 26]; to investigate the involvement of ROS in Asian dust particles-induced TNF-α production, we measured TNF-α production induced by Asian dust particles in the presence of BHA, a broad-spectrum ROS scavenger, or DPI, a specific inhibitor of NADPH oxidase. BHA significantly suppressed the TNF-α production induced by ADP1 and ADP2 to almost the same level as that of the control group (Figure 4(b)). Furthermore, pretreatment with DPI also resulted in a dose-dependent decrease in the TNF-α production induced by ADP1 and ADP2 (Figure 4(c)). These results suggest that the Asian dust particles-induced production of inflammatory cytokines was mediated by ROS and that Asian dust particles-induced ROS production is dependent on NADPH oxidase.

3.4. Asian Dust Particles-Induced TNF-α Production Is Dependent on the MAPK and NF-κB Signal Pathways. The MAPKs are a family of proteins that includes the p38, ERK, and JNK, which activate transcription factors involved in the production of inflammatory cytokines in response to external

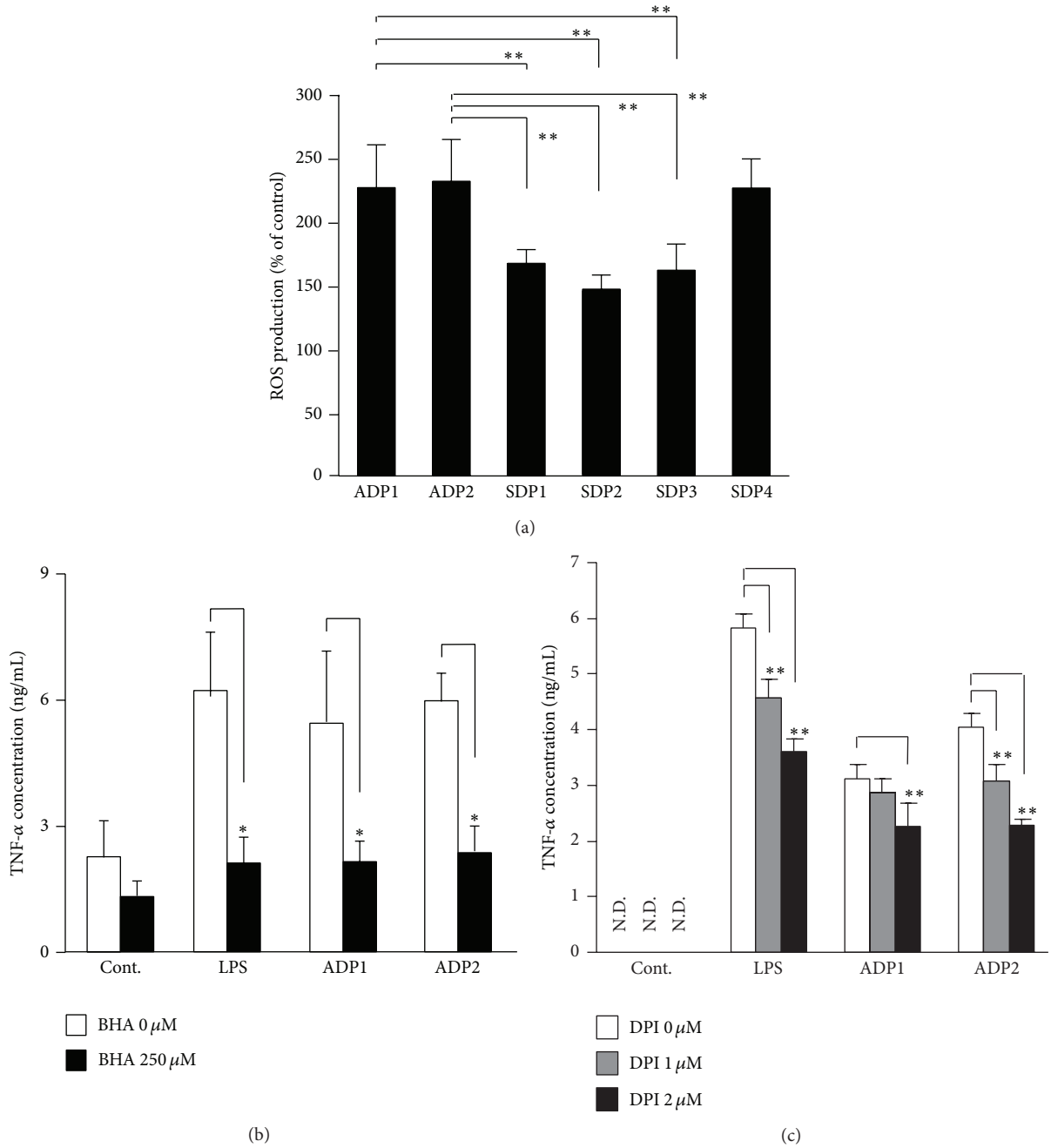

Figure 4: Asian dust particles-induced reactive oxygen species (ROS) production mediates tumor necrosis factor-α (TNF-α) production in RAW264.7 cells. (a) RAW264.7 cells were incubated in phenol red-free Dulbecco's modified Eagle's medium containing 20 μM 2′,7′-dichlorodihydrofluorescein diacetate for 30 min. The cells were then treated for 6 h with 100 μg/mL of one of the Asian dust particles (ADP1 or ADP2) or soil dust samples (SDP1, SDP2, SDP3, and SDP4) or culture medium. ROS production was measured as the fluorescence intensity of dichlorodihydrofluorescein. (b, c) RAW264.7 cells were preincubated for 30 min with (b) 250 μM of butylated hydroxyanisole (BHA), a broad-spectrum ROS scavenger, or (c) 1 or 2 μM of diphenyleneiodonium chloride (DPI), a specific inhibitor of NADPH oxidase. The cells were then treated for 6 h with 100 μg/mL of ADP1 or ADP2 or 1.5 μg/mL of LPS. Dimethyl sulfoxide (0.1%) vehicle was used as control (Cont.). The level of TNF-α in the culture supernatants was assessed by means of an enzyme-linked immunosorbent assay. Results are expressed as mean ± SD; $n = 6$; $^{**}P < 0.01$; N.D., not detected.

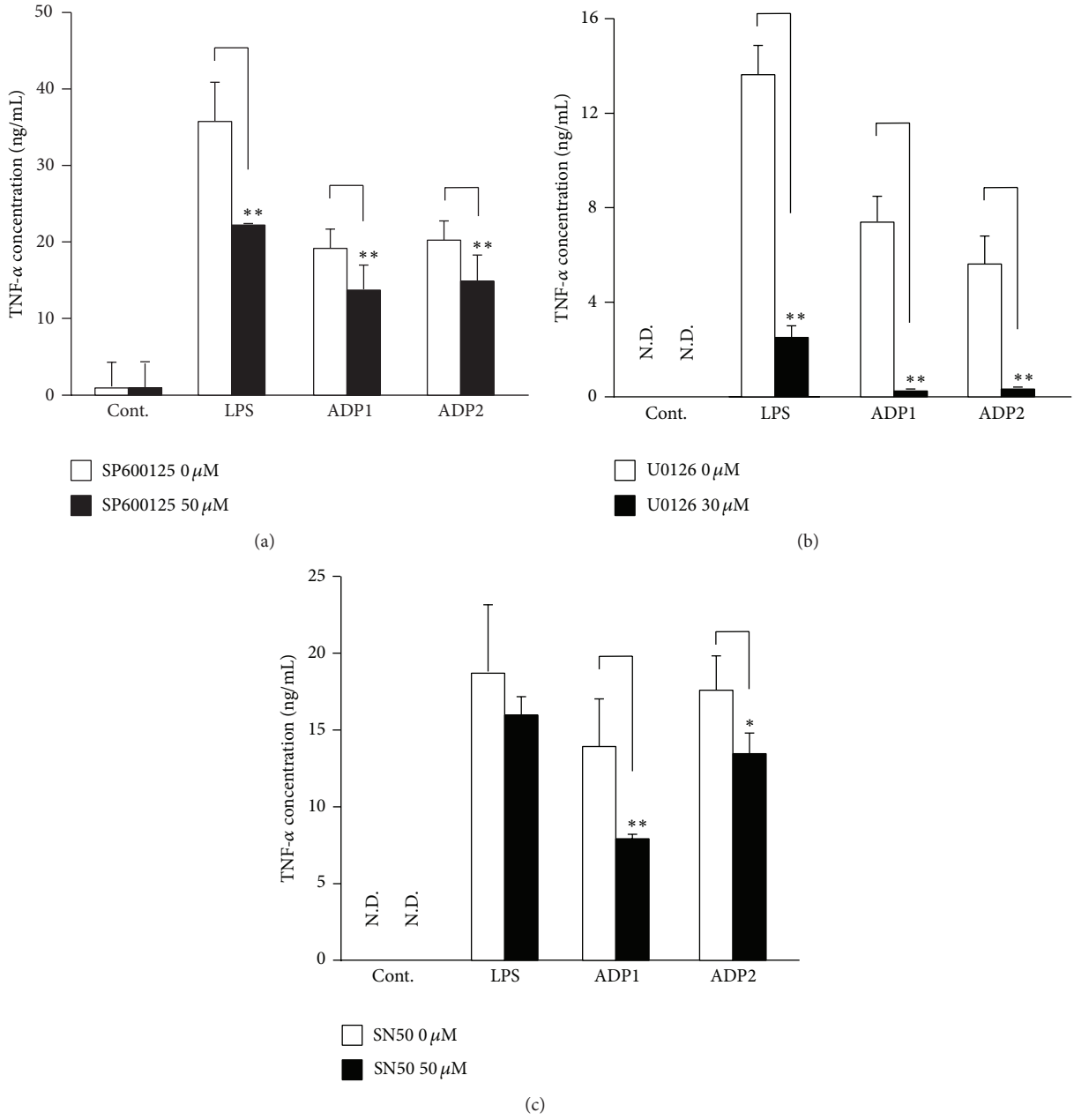

Figure 5: Asian dust particles-induced tumor necrosis factor-α (TNF-α) production is dependent on mitogen-activated protein kinase and nuclear factor-κB signaling pathways. RAW264.7 cells were pretreated for 30 min with (a) 50 μM of SP600125, an inhibitor of c-Jun N-terminal kinase; (b) 30 μM of U0126, an extracellular signal-regulated kinase (ERK) inhibitor; or (c) 50 μM of SN50, a nuclear factor-κB (NF-κB) inhibitor and then treated for 6 h with 100 μg/mL of ADP1 or ADP2 or 1.5 μg/mL of LPS. Dimethyl sulfoxide (0.1%) vehicle was used as control (Cont.). The level of TNF-α in the culture supernatants was assessed by means of an enzyme-linked immunosorbent assay. Results are expressed as mean ± SD; $n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$; N.D., not detected.

stresses or cytokine stimulation [27]. To investigate the association between the MAPKs and Asian dust particles-induced macrophage activation, RAW264.7 cells were incubated with ADP1 or ADP2 in the presence or absence of an inhibitor of the JNKs (SP600125) or ERKs (U0126), and the level of TNF-α in the cell culture supernatant was measured. Both SP600125 (Figure 5(a)) and U0126 (Figure 5(b)) significantly suppressed the TNF-α production induced by ADP1 or ADP2, suggesting that MAPK activation plays an important role in Asian dust particles-induced TNF-α production. Since the MAPKs stimulate transcription factors such as NF-κB, which in turn regulate the expression of genes encoding cytokines such as IL-1β, IL-8, and TNF-α, RAW264.7 cells were incubated with ADP1 or ADP2 in the presence or absence of an NF-κB inhibitor (SN50). SN50 significantly, but not completely, suppressed the TNF-α production induced

by ADP1 or ADP2 (Figure 5(c)), indicating that NF-κB may be partially involved in Asian dust particles-induced TNF-α production.

Asian dust particles contain chemical substances such as sulfates or nitrates derived from alkaline soil and microbiological materials [28] that may cause serious respiratory health problems in humans. Heat treatment of Asian dust particles has been reported to suppress allergic responses, which suggests that these adhered materials contribute to Asian dust particles-induced inflammation [29]. Our data suggests that as particle size decreases, the amount of chemicals or other materials that adheres to Asian dust particles increases, which may account for the different inflammatory responses to soil dust seen in the present study. Further data is needed on the attachment of these materials to Asian dust particles and on their effects on biological responses. Furthermore, it has been recently revealed that aerosols such as diesel exhaust or Asian dust particles contain not only microsized particles but also nanosized particles [30, 31]. Our recent research demonstrates that particles with diameter ≤100 nm show different biological responses and kinetics both *in vivo* and *in vitro* compared with microsized particles [32–34], suggesting that fine Asian dust particles have the potential to induce adverse biological effects. As shown in the present study, TNF-α production after treatment with SDP4 (soil sample; particle diameter, ≤10 μm) was significantly higher than that after treatment with the other soil samples (SDP1, SDP2, or SDP3). This implies that particle size contributed to the macrophage inflammatory response to the soil samples; therefore, it is necessary to evaluate the biological effects of not only fine particles in the environment but also of fine particles with diameters ≤100 nm.

Recent studies have shown that crystalline silica disturbs the host immune system by activating the nucleotide-binding oligomerization domain, leucine-rich repeat pyrin domain containing 3 (NLRP3) inflammasome. The NLRP3 inflammasome has been examined for its role in the initial inflammatory response to a diverse range of stimuli [35–37]; however, the mechanisms of the activation of host immunity by Asian dust particles remain poorly understood. Therefore, further analyses are necessary to clarify this mechanism.

It has been reported that particulate matter with a diameter ≤1 μm reaches the alveoli of the lung when aspirated [38]. However, there is little information on a global scale about the *in vivo* kinetics of these particles, including whether they infiltrate the body or not. The lack of data on particulate matter has raised concerns on their effect on human health. It is therefore important to clarify not only the *in vivo* kinetics and biological responses of tissues or cells to particulate matter with a diameter ≤1 μm, but also the specific mechanisms of the biological effects of Asian dust particles.

4. Conclusions

Our results indicate that Asian dust particles have greater potential to induce inflammation compared with soil dust and that the size of soil dust particles affects soil sample-induced inflammatory responses. Furthermore, Asian dust particles-induced activation of macrophages is dependent on ROS production and involves the activation of the MAPK signal pathway.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This research was supported by the Environment Research and Technology Development Fund of the Ministry of the Environment, Japan (B-0902).

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