

図2 ナノ安全科学研究の概要

担保したものに仕上げていく Nano-Safety Design (ナノ最適デザイン) 研究を両輪とした、独自のナノ安全性評価研究を推進している(図2)。本稿では、われわれの研究成果のなかから、化粧品分野において化粧品基材としてすでに実用化されている、ナノシリカを中心に、ナノシリカの経皮吸収性に関する検討とともに、曝露局所である皮膚細胞を対象としたハザード情報、および体内移行後のハザード情報について紹介する。

3 ナノシリカの経皮吸収性

本検討では、実験用グレードである、一次粒径が70nmのナノシリカ(nSP70)、および、300nm、1,000nmの従来型シリカ(nSP300、mSP1000)を用いた。まず、透過型電子顕微鏡(TEM)により、各シリカの形状を観察したところ、粒

子径にかかわらず、表面の滑らかな球状の素材であることが示された。また、動的光散乱法を用いて平均二次粒径を測定したところ、いずれの粒子も単一のピークを示し、その値はTEM解析の結果とほぼ同等の値を示したことから、これらの粒子の分散性が高いことが示された。次に、nSP70、nSP300、mSP1000の経皮吸収性を検討するため、各粒子をマウス耳介に28日間連続で経皮塗布し、投与局所や各種臓器への移行性をTEMにより観察した。その結果、nSP300やmSP1000は、表皮にすら到達しない一方で、nSP70のみが皮膚バリアを突破し、神経

や血管が豊富に存在する真皮層にまで侵入することが明らかとなった。さらに、nSP70は、投与局所近傍の所属リンパ節や脳組織へ移行することも見出した^{7,8)}。

これらの結果や、上述の報告などを踏まえると、ナノサイズにまで微小化された微粒子の中には、経皮的に吸収される粒子も存在することが示唆される。したがって今後、体内蓄積性・排泄などを考慮し、より長期の検討などを実施する必要があると考えられる。さらに、nSP70のみが皮膚バリアを突破するという可能性は、ナノマテリアルを化粧品に適用することで、皮膚表面のみならず、表皮や真皮にまで化粧品の美白効果や老化防止効果を発揮できるという、ナノマテリアルの有用性を示すものと考えられる。すなわち、将来的に、ナノマテリアルの分散性・凝集状態などの物性や溶媒などを適切に制御し、各標的部位に効率的にナノマテリアルを送達することで、有用性と安全性を具備した新規経皮適用製品が開発可能であると期待される。

4 ナノシリカ曝露によるハザード同定

先述のように、われわれはナノ安全科学研究の一環から、ナノマテリアルの曝露実態情報に基づく、安全性評価を実施している。その検討のなかで、これまでに、多くのナノシリカに関しては安全であると考えられる一方で、特定の表面性状を持つものに関しては、従来型シリカでは認められないハザードを発現してしまう可能性を見出してきた。ここでは、これら知見について、われわれの検討結果を踏まえて紹介したい。

前項において、nSP70のみが皮膚バリアを突破するという可能性を見出したことから、経皮吸収後のナノシリカの動態を精査するため、ヒト皮膚角化細胞を用い、nSP70、nSP300、mSP1000の細胞内動態を調べた。その結果、nSP300、mSP1000がエンドソーム内に局在するのに対し、nSP70の一部は細胞質内もしくは核内にまで局在することが明らかとなった⁷⁾。さらに、大量にnSP70が細胞質内に侵入すると、活性酸素種 (ROS) が産生され、それに起因すると考えられる DNA 障害を生じる可能性が見出された⁹⁾。次に、実際に使用されているナノシリカがさまざまな表面修飾を施されている場合があることを考慮し、nSP70に加え、その表面をアミノ基やカルボキシル基で修飾したナノシリカ (nSP70-N、nSP70-C) を用い、先ほどと同様に細胞内動態を解析した。その結果、nSP70-N、nSP70-C が細胞外や細胞質内に留まっているのに対し、nSP70のみが細胞の核内にまで侵入している様子が観察された。また、nSP70作用群で認められた細胞傷害性や ROS の産生、DNA 障害は nSP70-N、nSP70-C 作用群では認められないことが

明らかとなった¹⁰⁾。したがって、現在、nSP70の核内・細胞内移行メカニズムを追究するとともに、細胞内での局在を定量的に解析することで、ハザード発現の機序解明に向けた検討を進めている。今後は、ナノシリカの表面性状と生体影響誘発との連関をより詳細に解析していくことで、ナノシリカの安全性を高度に担保するための基礎情報の集積につながるものと考えられる。

次に、上述のナノシリカの経皮吸収性評価における曝露局所の影響を詳細に解析したところ、皮膚バリアを突破したnSP70塗布群のみが、経皮塗布後の皮膚局所において、アポトーシスを誘発する可能性に注意する必要があることを明らかとした⁷⁾。一方で、本検討は過剰量を塗布した検討であることから、今後、ハザード発現の閾値を追究するなど、リスク解析に資する情報を集積していくことが必要不可欠であると考えられる。さらに、nSP70の経皮塗布の影響を解析するにあたっては、曝露局所の影響のみならず、全身を対象とした生体影響を評価する必要がある。しかし本検討時においては、体内吸収性を解析するために過剰量を投与したにもかかわらず、生化学検査などの一般毒性的解析においてはほとんど影響が認められなかった。

また、われわれは、ナノシリカが全身循環した際の影響について、ハザード同定を目的に、各種一般毒性試験、特殊毒性試験を実施している。その結果、nSP70-N や nSP70-C など、表面修飾を施したナノシリカでは、各検討において、目立ったハザードは認められなかった。一方で、静脈内投与という強制的な投与経路、かつ過剰量における検討ではあるものの、nSP70が、①消費性凝固障害を惹起し得ること¹¹⁾、②胎盤障害を誘発し、胎仔発育障害や流産を惹起し得ること¹²⁾、③経皮的に吸収させることで、アトピー性皮膚

炎の悪化などのアレルギー症状を誘発し得ること¹³⁾を認めている。なお、これらハザードは従来型シリカである nSP300、mSP1000では認められず、一部のナノシリカが従来型のシリカとは異なる生体影響を誘発する可能性を示している。

これら知見は、ナノシリカが、その物性によってはハザードの誘発に注意を払う必要がある一方で、逆に、表面性状を適切に制御することで、高度に安全性を担保できることを示唆するものである。今後は、各ナノシリカについて、体内吸収量の定量的解析や、ハザード発現の閾値などをより詳細に解析することで、リスク解析に資する情報を引き続き収集するとともに、ハザード発現メカニズムを詳細に解析することで、ナノ最適デザインに資する具体的情報を収集することが必要であると考えられる。

5 今後の課題

昨今、ナノマテリアルの実用化は、あらゆる分野において拡大の一途を辿っており（ナノマテリアルの世界市場が、2015年には250兆円にまで成長するとの見通しもある）、われわれの豊かな生活の成立にもはや必須のものとなっている。したがって、ナノマテリアルの安全・安心な使用に向けては、ナノマテリアルの安全性を保証するガイドラインの整備・策定が待望されている。ナノマテリアル特有の規制・ガイドラインの策定にあたり、ナノマテリアルの分散／凝集状態や純度といった品質情報と体内動態および安全性の関連について関心が高まっている。この点を考慮して、今後はナノマテリアルのこれら物性・品質情報と体内動態および安全性の関連を明確化することで、ナノマテリアル特有の物性・品質

に応じた規制・ガイドラインの策定に貢献できるであろう。知財技術立国を目指すわが国としては、ナノマテリアルの開発・実用化をやみくもに規制するのではなく、ナノテクノロジーの恩恵を社会が最大限に享受できるよう、ナノ産業の育成や発展を強力に支援しつつ、ナノマテリアルの安全性を高度に保障し、ヒトの健康環境を確保していかなければならない。

日本化粧品工業連合会によると、化粧品原料に適用されている多くのナノマテリアルは、製造工程では、いずれもナノサイズに分散している一方で、原料の保管段階あるいは製品への適用段階においては、粒子が凝集状態で存在していると考えられている。このことはナノマテリアルの安全性評価研究を進めるうえで考慮すべき点であり、ナノマテリアルの安全性確保に向けては、実サンプルを用いた安全性評価が必須であることはいうまでもない。この点われわれは、既実用化・実用化予定のナノマテリアルの安全性評価、さらには安全なナノマテリアルの開発支援をより一層加速させることを計画している。

また、今回は詳細には述べなかったが、動物愛護の観点から、ナノマテリアルの安全性評価においても、今後、動物実験に替わる代替法の確立、利用が重要となると考えられる。この点においてわれわれは、iPS細胞に加え、ES細胞を用いた *in vitro* におけるナノマテリアルの安全性予測（EST法）に向けた基礎情報の収集や基盤技術開発も試みている。今後、より詳細な検討が必要ではあるものの、これらEST法を用いることで、ナノマテリアルの安全性を精度よく予測・評価することが可能となり、安全なナノマテリアルを効率よくスクリーニングし、ナノマテリアルの開発支援の一助となることを期待している。



おわりに

本稿には、われわれが推進するナノ安全科学研究のなかから、実験用グレードのナノシリカを用いた検討を中心として、従来までのサブミクロンサイズの素材とは異なる、ナノマテリアルの体内動態や生体影響に関する情報の一部を紹介した。今後、さらに小さな「サブナノマテリアル(1~10nm)」の台頭、まさに、アルブミン~抗体のサイズ領域の素材が加速度的に開発、実用化されることを一層鑑み、ナノ安全科学研究をさらに推し進め、ナノマテリアルの物性と生体影響との連関を追究することで、安全なナノマテリアルの創製に資する有用な情報を得られるものと考えている。そうして得られた知見が広く公表され、産業応用されることが、有用かつ安全なナノマテリアル製品の開発、ひいてはナノマテリアル産業界のさらなる発展につながると期待している。

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Intestinal absorption and biological effects of orally administered amorphous silica particles

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Abstract

Although amorphous silica nanoparticles are widely used in the production of food products (e.g., as anticaking agents), there is little information available about their absorption and biological effects after oral exposure. Here, we examined the *in vitro* intestinal absorption and *in vivo* biological effects in mice of orally administered amorphous silica particles with diameters of 70, 300, and 1,000 nm (nSP70, mSP300, and mSP1000, respectively) and of nSP70 that had been surface-modified with carboxyl or amine groups (nSP70-C and nSP70-N, respectively). Analysis of intestinal absorption by means of the everted gut sac method combined with an inductively coupled plasma optical emission spectrometer showed that the intestinal absorption of nSP70-C was significantly greater than that of nSP70. The absorption of nSP70-N tended to be greater than that of nSP70; however, the results were not statistically significant. Our results indicate that silica nanoparticles can be absorbed through the intestine and that particle diameter and surface properties are major determinants of the degree of absorption. We also examined the biological effects of the silica particles after 28-day oral exposure in mice. Hematological, histopathological, and biochemical analyses showed no significant differences between control mice and mice treated with the silica particles, suggesting that the silica nanoparticles evaluated in this study are safe for use in food production.

Keywords: Biological effects; Everted gut sac method; Intestinal absorption; Silica nanoparticles

Background

Nanomaterials are currently used in a variety of applications, including the production of food products. For example, amorphous silica nanoparticles are widely used as stabilizers, anticaking agents, and carriers for fragrances and flavors [1-3]. With the growing commercialization of nanomaterial-derived food additives, the opportunities for human oral exposure to nanomaterials are substantially increasing; however, there have been few studies conducted, examining the safety of nanomaterials in food products. Therefore, assessments of the absorption and biological effects of nanomaterials after oral exposure are urgently needed.

The information currently available about the biological effects and absorption of nanomaterials after oral exposure is limited. For example, 56-nm silver nanoparticles have been found to be distributed in rat liver, kidney, brain, lung, and blood after 90 days of oral exposure at doses of 30, 125, or 500 mg/kg, and a no-observed-adverse-effect level (NOAEL) of 30 mg/kg and a lowest-observed-adverse-effect level (LOAEL) of 125 mg/kg have been suggested for these nanoparticles [4]. However, there is little information about the intestinal absorption and biological effects of silica nanoparticles after oral exposure. Furthermore, an efficient method to determine the absorption of silica particles in the human body is yet to be established. Therefore, to evaluate the safety of silica nanoparticles after oral exposure, practical *in vitro* methods that allow prediction of the *in vivo* intestinal absorption and biological effects of silica nanoparticles after long-term oral exposure are urgently needed.

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The everted gut sac method is a useful tool for modeling the in vitro absorption and intestinal metabolism of drugs. This method, which was first described by Wilson and Wiseman in 1954 and then later improved by Barthe et al., has been extensively used to investigate the pharmacokinetics of drug absorption, drug metabolism, and pro-drug conversion in gastrointestinal segments [5-7]. This method has also recently been used to obtain information about the absorption of drugs contained in nanocarriers such as liposomes [8]. The results of these studies suggest that this method could be used to accurately measure the intestinal absorption of silica nanoparticles.

In the present study, we used the everted gut sac method to evaluate the intestinal absorption of amorphous silica particles of various diameters. In addition, we investigated the in vivo biological effects of 28-day oral administration of the amorphous silica particles in mice.

Methods

Silica particles

We evaluated the following particles purchased from Micromod Partikeltechnologie, Rostock/Warnemünde, Germany: amorphous silica nanoparticles (nSP) with a diameter of 70 nm (nSP70), microsilica particles (mSP) with diameters of 300 or 1,000 nm (mSP300 and mSP1000, respectively), and nSP70 that had been surface-modified with carboxyl or amine groups (nSP70-C and nSP70-N, respectively). All the silica particles were sonicated for 5 min and vortexed for 1 min prior to use.

Animals

BALB/c mice (female, 6 weeks) were purchased from Japan SLC (Shizuoka, Japan) and Wistar rats (male, 8 weeks) were purchased from Shimizu Laboratory Supplies Co (Kyoto, Japan). The animals were housed separately in a ventilated animal room that was maintained at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under a 12-h light/12-h dark cycle. All the animal experiments in this study were performed in accordance with the National Institute of Biomedical Innovation and the Osaka University Guidelines for the Welfare of Animals.

Everted gut sac analysis

Wistar rats (male, 8 weeks) were fasted for 12 h (ad libitum access to water) prior to the experiment. The rats were anesthetized with pentobarbital, subjected to abdominal section, and exsanguinated by transection of the descending aorta. The whole small intestine was isolated and gently flushed with Tyrode's buffer (NaCl, 137 mM; KCl, 5.4 mM; NaH_2PO_4 , 0.16 mM; MgCl_2 , 0.5 mM; CaCl_2 , 1.8 mM; HEPES, 5 mM; pH 7.4). A 3- to 4-cm segment of the small intestine was removed and everted over a silicone tube. The bottom portion was tied with thread and the segment was filled with 0.6 to 0.8 mL of Tyrode's buffer. The filled segment was then placed in 2.5 mL of

Tyrode's buffer only (control group) or a solution of silica particles (12.5 mg/mL) in Tyrode's buffer or/and incubated at 37°C for 45 min. After incubation, the solution on the serosal side of the segment was collected. An inductively coupled plasma optical emission spectrometer (ICP-OES; 735-ES, Agilent Technologies, Tokyo, Japan) was used to determine the silicon content in the solution.

Oral exposure of mice to silica particles

BALB/c mice (female, 6 weeks) were orally exposed to nSP70, mSP300, mSP1000, nSP70-C, nSP70-N, or water (control group) at a daily dose of 2.5 mg/mouse administered by means of oral gavage for 28 days. The mice were weighed on days 7, 14, 21, and 28 of the study period.

Blood biomarker assay

Twenty-four hours after the final oral administration of the silica particles, a blood sample was collected from the heart of each mouse by means of a plastic syringe containing 5 IU/mL of heparin sodium. Plasma was harvested by centrifuging the collected blood at $1,750 \times g$ for 15 min. The plasma levels of alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were determined with a biochemical auto-analyzer (Fuji Dri-Chem 7000, Fujifilm, Tokyo, Japan).

Histopathological examination

Twenty-four hours after the final oral administration of the silica particles, the liver, kidney, brain, lung, spleen, heart, stomach, small intestine, or large intestine of the mice were excised and fixed immediately in 4% paraformaldehyde. The tissues were embedded in paraffin blocks and sectioned, and the sections were mounted on glass slides.

Hematological analysis

Twenty-four hours after the final oral administration of the silica particles, a blood sample was collected from the heart of each mouse by means of a plastic syringe containing 0.1 mM EDTA, and the numbers of total white blood cells, lymphocytes, monocytes, red blood cells, granulocytes, and platelets in whole blood were determined with an auto-analyzer (VetScan HMII Hematology System, Abaxis, Sunnyvale, CA, USA).

Statistical analysis

Differences between the treated groups and the control groups were compared with Tukey's test after analysis of variance.

Results

Physicochemical properties of silica particles

Previously, we confirmed by means of dynamic laser scatter analysis that the mean secondary particle diameters of

nSP70, mSP300, mSP1000, nSP70-C, and nSP70-N are 65, 322, 1,140, 70, and 72 nm, respectively [9]. Transmission electron microscopy revealed that the silica particles are well-dispersed smooth-surfaced spheres, indicating that they remain stable and well dispersed in solution and do not aggregate [9].

Intestinal absorption of silica particles

We used the everted gut sac method to evaluate the intestinal absorption of silica particles. Tissue viability is a limiting factor in everted gut sac analysis; however, the viability and metabolic activity of intestinal tissue has been reported as being retained for approximately 2 h under physiological conditions [10]. Everted gut sac analysis revealed that the absorption of nSP70-C and nSP70-N from the mucosal side to the serosal side of the sacs was significantly greater than the absorption of the other silica particles after incubation for 45 min. And the absorption of nSP70-C was significantly greater than that of nSP70 (Figure 1). This suggested that silica nanoparticles are absorbed through the intestine and that the surface property of the particles is one of a determinant of the degree of absorption.

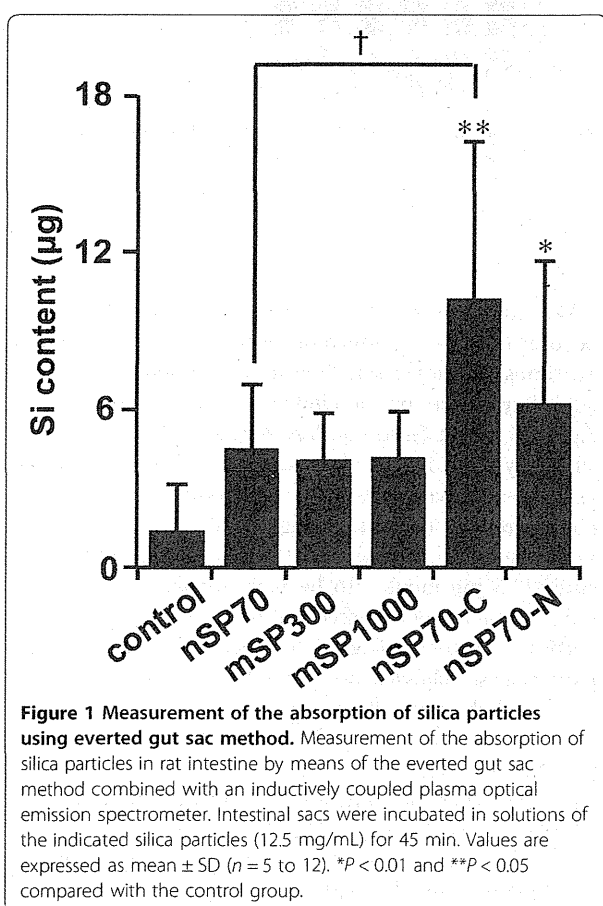


Figure 1 Measurement of the absorption of silica particles using everted gut sac method. Measurement of the absorption of silica particles in rat intestine by means of the everted gut sac method combined with an inductively coupled plasma optical emission spectrometer. Intestinal sacs were incubated in solutions of the indicated silica particles (12.5 mg/mL) for 45 min. Values are expressed as mean \pm SD ($n = 5$ to 12). * $P < 0.01$ and ** $P < 0.05$ compared with the control group.

Biological effects of silica particles

Oral exposure of BALB/c mice to nSP70, mSP300, mSP1000, nSP70-C, and nSP70-N at 2.5 mg/mouse for 28 days produced no significant differences in body weight in the treated mice compared with in the control mice (Figure 2A). We observed no significant changes in the plasma levels of ALT (marker of liver function) or BUN (sensitive indicator of kidney damage) after oral administration of the silica particles in treated mice compared with control mice (Figure 2B,C). Although ALT values of silica nanoparticle-treated group are slightly higher than control group, the change of the ALT value was within healthy range (the range was about below 43 U/L) among all the groups. Therefore, we speculate that the level of ALT among all the groups does not effect on liver's function. Histopathological examination revealed no abnormalities in the liver, kidney, and large intestine (Figure 3) or any other tissue (the brain, lung, spleen, heart, stomach and small intestine; data not shown) in the treated mice. Furthermore, the counts of total monocytes, granulocytes, or platelets in the treated mice were also not significantly different from those in the control mice (Figure 4). Although the white blood cells, lymphocytes, and the red blood cell counts in the treated mice were significantly different from those in the control group, they were within the normal, healthy range or slightly increase (the white blood cells: 6 to 15 $\times 10^9$ /L, lymphocytes: 3.7 to 7.4 $\times 10^{12}$ /L, red blood cells: 7 to 12 $\times 10^{12}$ /L) in all groups (Figure 3A,B,F). Taken together, our results indicate that oral administration of nSP70, mSP300, mSP1000, nSP70-C, or nSP70-N for 28 days does not produce any abnormal biological effects, suggesting that the silica nanoparticles tested are safe for use in food production.

Discussion

In the present study, we attempted to quantify the absorption of silica particles by using ICP-OES to measure the silicon content in the liver and blood after 28-day oral exposure. However, we did not detect the silicon content in the biological tissue with this analytical protocol (data not shown; detection limit of the protocol, 0.1 μ g). ICP-OES protocols are regarded to be suitable for measuring silica; however, our attempt to measure ultratrace levels of the silicon content derived from silica particles may have been hindered by interference from nitrogen present in the air. Other groups have also been unable to determine the level of the silicon content derived from with ICP-OES after oral exposure because of interference [11]. We therefore tried to measure the absorption of silica particles through the intestine by means of a combination of the everted gut sac method and ICP-OES. The advantages of this model are that there is a relatively large surface area available for absorption and that a mucus layer is present. The absorption of nSP70-C and nSP70-N were

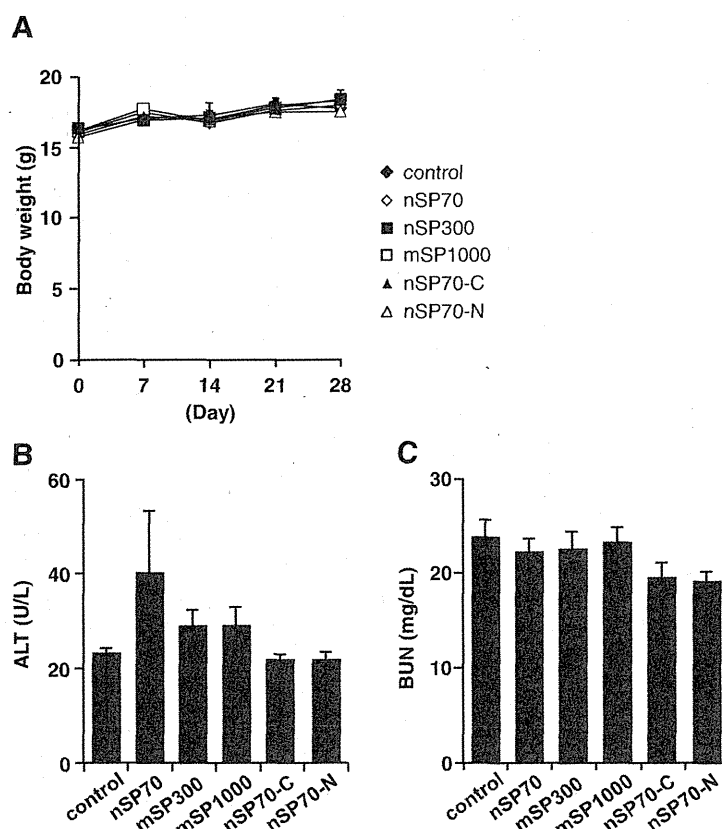


Figure 2 Body weight, histopathological, and biochemical analyses after 28-day oral administration of the indicated silica particles. (A) Body weight during 28-day oral administration of silica particles; plasma concentrations of (B) alanine aminotransferase (ALT) and (C) blood urea nitrogen (BUN). Values are expressed as mean \pm SE ($n = 4$ to 11).

significantly higher than that of the other silica particles, and the absorption of nSP70-C was also significantly higher than that of nSP70 (Figure 1). This indicates that the differences in intestinal absorption of nSP70-C and nSP70-N may be a result of the particles being absorbed via different proteins. Indeed, some groups have suggested that interactions between proteins and nanomaterials play important roles in the biological effects and biodistribution of nanomaterials [12]. In the future, to understand the mechanism of intestinal absorption, we will need to examine the relationships between intestinal absorption and the binding proteins of nSP70, nSP70-C, and nSP70-N. We found that the everted gut sac method was effective for assessing the intestinal absorption of nanomaterials. Although the relationship between models of *in vivo* absorption and the everted gut sac model of *in vitro* absorption must be further elucidated, we expect our results to contribute to the development of methods for determining the absorption of silica particles after oral exposure.

We next exposed BALB/c mice to silica particles at an oral dose of 2.5 mg/mouse, which is approximately 10 times the upper safe limit for consumption of silica by adult humans set by the United Kingdom Food Standards Agency's Expert Group on Vitamins and Minerals (700 mg silica/day) [13]. Since silica nanoparticles are already used as food additives in some food products, for example, coffee creamer (silica nanoparticle content, 1.0 g/kg) and instant soup [1], we consider the dose used in the present study (2.5 mg/mouse) to be high enough for the evaluation of the biological effects of oral administration of the particles. Histopathological, biochemical, and hematological analyses showed no differences between the treated mice and the control mice (Figures 2, 3, and 4). However, another research group has reported that silica nanoparticles (diameter, 30 nm) have toxic effects on the liver when administered orally at a total dose of 140 g/kg over 10 weeks [14], which is a dose approximately 20 times that used in the present study. Therefore, to determine the NOAEL for orally administered silica

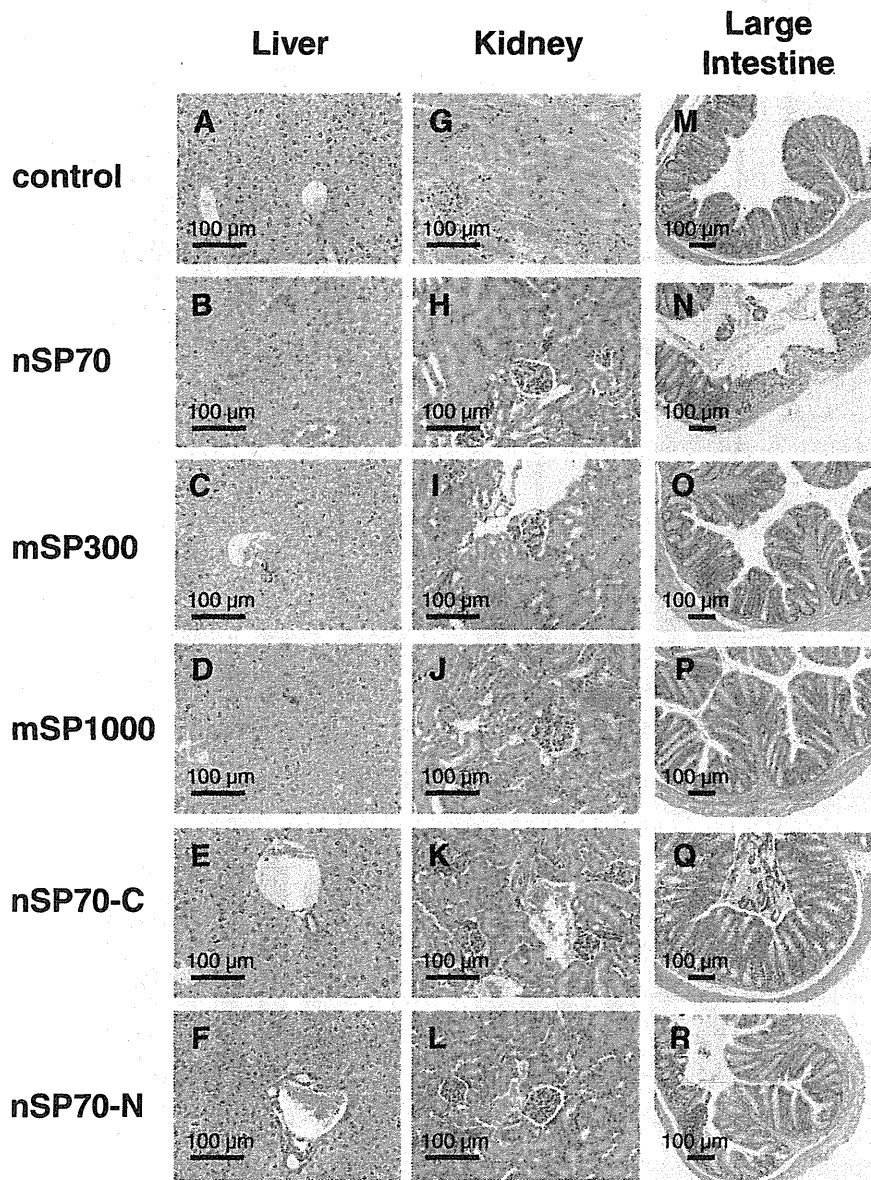


Figure 3 Histopathological analysis after 28-day oral administration of the indicated silica particles. (A to F) liver, (G to L) kidney, (M to R) large intestine.

nanoparticles, their effects on the liver must be precisely elucidated.

Our previous study showed that intravenous or intracutaneous administration of silica nanoparticles caused pregnancy complications [9] and modulation of the immune response [15]. Furthermore, it is possible that intranasally administered silica nanoparticles induce abnormal activation of the coagulation system and modulate the immune response [16,17]. Therefore, in which tissues silica nanoparticles localize after oral exposure,

as well as their chronic, reproductive, and immune toxicities, should be determined by using an oral exposure model.

Conclusions

By using the everted gut sac method coupled with ICP-OES, we examined the intestinal absorption of silica particles and found that surface properties were major determinants of the degree of intestinal absorption. None of the particles tested showed toxic biological

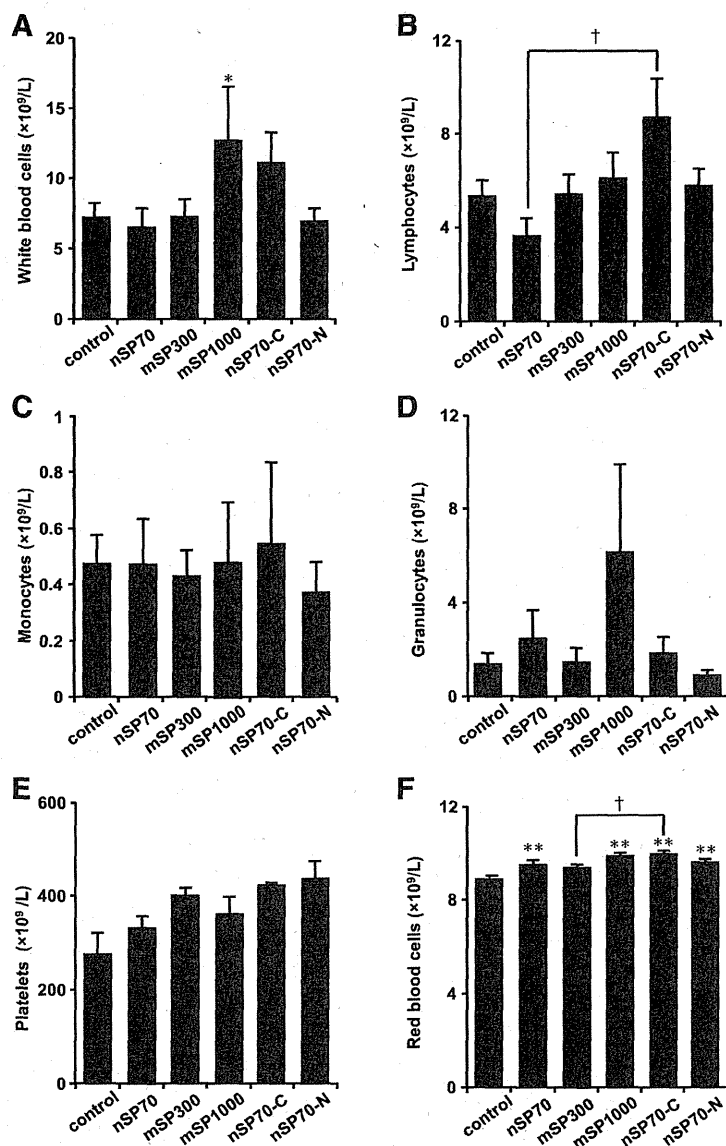


Figure 4 Hematological analysis after 28-day oral administration of the indicated silica particles. Hematological analysis of (A) white blood cells, (B) lymphocytes, (C) monocytes, (D) granulocytes, (E) platelets, and (F) red blood cells. Values are expressed as mean \pm SE ($n = 4$ to 11). * $P < 0.01$ and ** $P < 0.05$ compared with the control group; † $P < 0.05$ compared with the nSP70 group, as indicated by Tukey's test.

effects after 28-day oral administration. Our results suggest that the everted gut sac method coupled with ICP-OES could be one of the effective methods for assessing the absorption of silica particles. The results of this study will be useful for the development of methods for assessing the safety of silica nanoparticles and for the creation of safer nanoparticles.

Abbreviations

ALT: alanine aminotransferase; BUN: blood urea nitrogen; EDTA: ethylenediaminetetraacetic acid; ICP-OES: inductively coupled plasma optical emission spectrometer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

T.Yoshida and YY designed the study. T.Yoshida, HT, KM, TM, and TH performed the experiments. T.Yoshida and YY collected and analyzed the data. T.Yoshida and YY wrote the manuscript. KN, YA, YM, HK, ST, HN, T. Yoshikawa, and KH provided technical support and conceptual advice. YT supervised the project. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Research Article

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

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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 $\leq 10 \mu\text{m}$ in diameter provoked a greater inflammatory response than soil dust samples containing particles $> 10 \mu\text{m}$. 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 $\leq 10 \mu\text{m}$ 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 $\mu\text{g}/\text{mL}$ of a suspension of Asian dust particles or soil dust or DMEM (negative control) or 1.5 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ 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

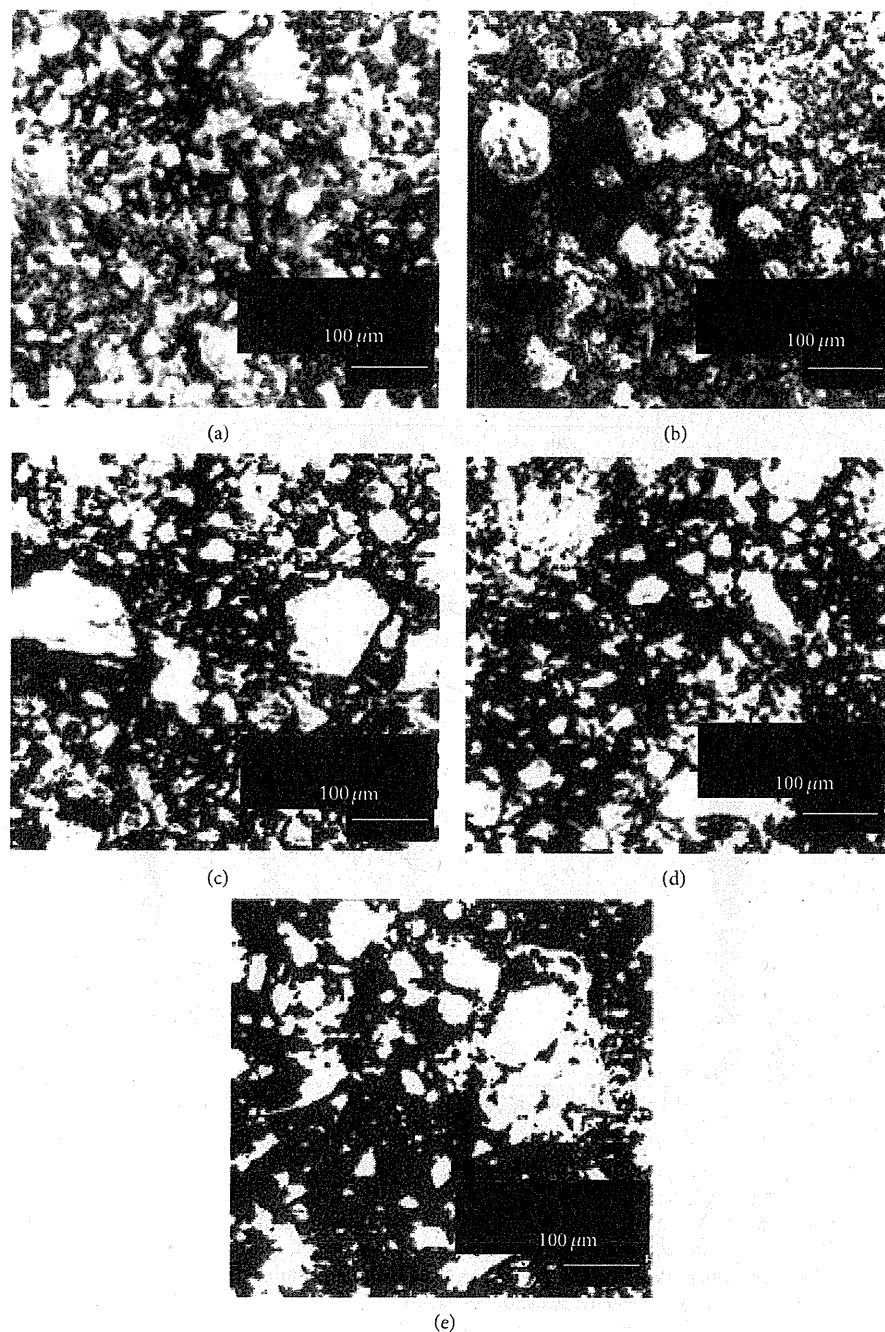


FIGURE 1: Scanning electron micrographs of two Asian dust particles (ADP1 (a) and ADP2 (b)) and three soil dust (SDP1 (c), SDP2 (d), and SDP3 (e)) samples. Prior to analysis, samples were adjusted to 0.25 mg/mL with deionized water and dried on a hotplate. Scale bars: 100 μm .

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 $\mu\text{g}/\text{mL}$ 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 \pm 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

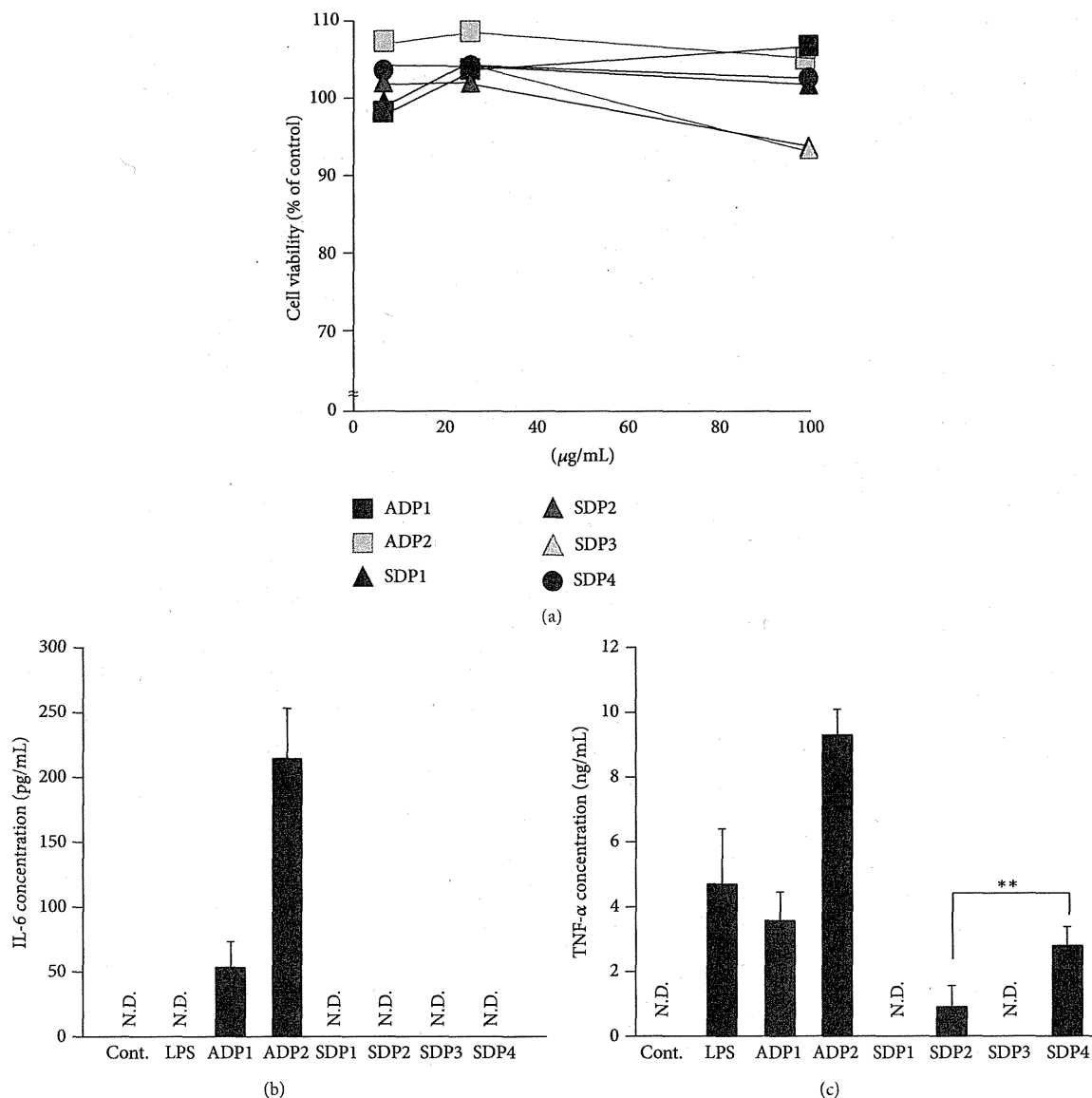


FIGURE 2: Assessment of cytotoxicity and inflammatory responses induced by Asian dust particles and soil dust in RAW264.7 cells. (a) RAW264.7 cells were treated for 24 h with 6.25, 25, or 100 $\mu\text{g/mL}$ of Asian dust particles ADP1, ADP2, or with soil dust SDP1, SDP2, SDP3, or SDP4 (particle diameter, $\leq 10 \mu\text{m}$). Cell viability was assessed by means of a methylene blue assay. (b, c) RAW264.7 cells were treated with 100 $\mu\text{g/mL}$ of each dust sample or 1.5 $\mu\text{g/mL}$ 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 \pm 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 $\geq 100 \mu\text{m}$ 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 $< 1 \mu\text{m}$ [6]. It represented that the mean size of the Asian dust particles in our samples would be $\leq 1 \mu\text{m}$.

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 $\leq 10 \mu\text{m}$ (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 $\leq 10 \mu\text{m}$) 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 $100 \mu\text{g/mL}$ 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 $100 \mu\text{g/mL}$ 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- κ B 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

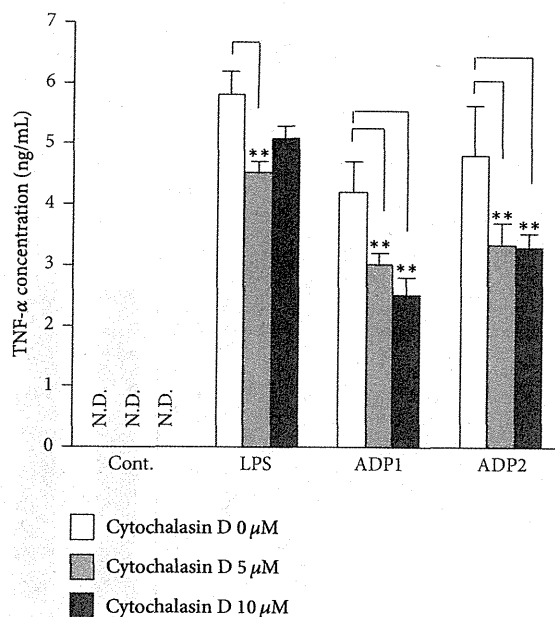


FIGURE 3: Cytochalasin D inhibited Asian dust particles-induced tumor necrosis factor- α (TNF- α) production in RAW264.7 cells. RAW264.7 cells were pretreated for 30 min with Cytochalasin D, an inhibitor of phagocytosis, and then treated for 6 h with $100 \mu\text{g/mL}$ of Asian dust particles (ADP1 or ADP2) or $1.5 \mu\text{g/mL}$ 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 \pm 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- κ B 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

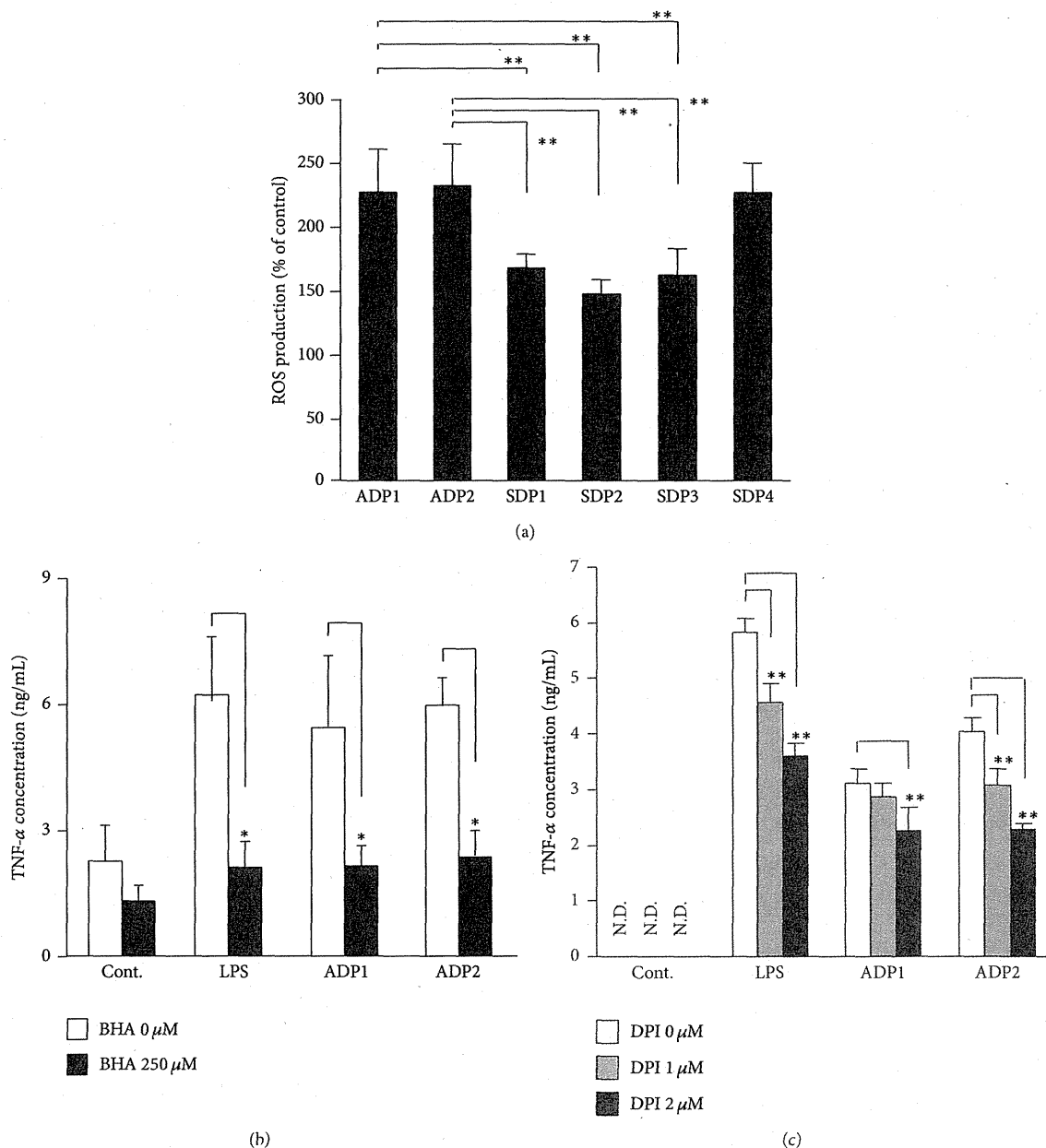


FIGURE 4: Asian dust particles-induced reactive oxygen species (ROS) production mediates tumor necrosis factor- α (TNF- α) production in RAW264.7 cells. (a) RAW264.7 cells were incubated in phenol red-free Dulbecco's modified Eagle's medium containing $20 \mu\text{M}$ 2',7'-dichlorodihydrofluorescein diacetate for 30 min. The cells were then treated for 6 h with $100 \mu\text{g}/\text{mL}$ 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) $250 \mu\text{M}$ of butylated hydroxyanisole (BHA), a broad-spectrum ROS scavenger, or (c) 1 or $2 \mu\text{M}$ of diphenyleiiodonium chloride (DPI), a specific inhibitor of NADPH oxidase. The cells were then treated for 6 h with $100 \mu\text{g}/\text{mL}$ of ADP1 or ADP2 or $1.5 \mu\text{g}/\text{mL}$ 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 \pm SD; $n = 6$; ** $P < 0.01$; N.D., not detected.

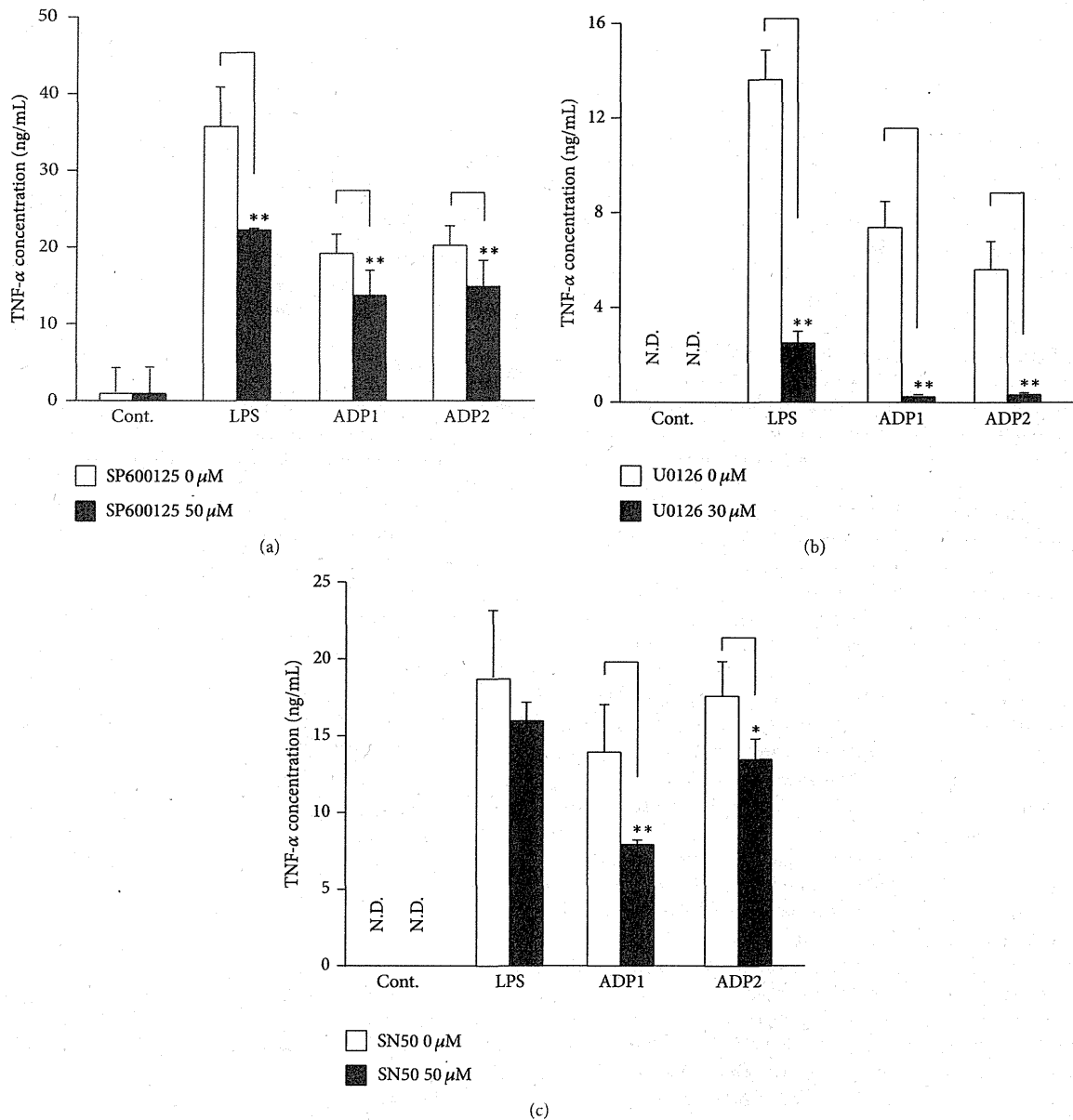


FIGURE 5: Asian dust particles-induced tumor necrosis factor- α (TNF- α) production is dependent on mitogen-activated protein kinase and nuclear factor- κ B signaling pathways. RAW264.7 cells were pretreated for 30 min with (a) 50 μ M of SP600125, an inhibitor of c-Jun N-terminal kinase; (b) 30 μ M of U0126, an extracellular signal-regulated kinase (ERK) inhibitor; or (c) 50 μ M of SN50, a nuclear factor- κ B (NF- κ B) inhibitor and then treated for 6 h with 100 μ g/mL of ADP1 or ADP2 or 1.5 μ g/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 \pm 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- κ B, which in turn regulate the expression of genes encoding cytokines such as IL-1 β , IL-8, and TNF- α , RAW264.7 cells were incubated with ADP1 or ADP2 in the presence or absence of an NF- κ B inhibitor (SN50). SN50 significantly, but not completely, suppressed the TNF- α production induced

by ADP1 or ADP2 (Figure 5(c)), indicating that NF- κ B 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 micro-biological 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 micro-sized 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 micro-sized 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, $\leq 10 \mu\text{m}$) 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 $\leq 1 \mu\text{m}$ 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 $\leq 1 \mu\text{m}$, 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.

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