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研究課題名：ナノマテリアル曝露による生体毒性の慢性移行及び遅発性に
関わる評価手法の開発研究

分担研究課題名：ナノマテリアルの慢性影響および生殖発生毒性評価系に関する研究

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研究要旨

　本研究では、ナノマテリアル曝露による生体毒性の慢性移行及び遅発性に関わる研究の一環として、多層カーボンナノチューブ（MWCNT）の腹腔内投与および気管内投与による中皮腫誘発性につてい繊維長が及ぼす影響および、ナノマテリアルの催奇形性について解析を行っている。26 年度は、気管反復投与により摂取された多層カーボンナノチューブ（MWCNT）の呼吸器系及び体腔膜中皮組織における作用発現に対する PBN の影響について病理組織学的な検討を行った。その結果、PBN の併用暴露は中皮腫や増殖性病変の発現頻度や程度に明らかな影響を示さず、MWCNT によるラットの中皮及び肺における増殖性病変の誘発機構には、酸化ストレスとは異なる要因が関与する可能性が示唆された。酸化ストレスとは異なる要因が関与する可能性が示唆された。また、サイズの異なる多層カーボンナノチューブのマウス腹腔内投与による死胚の誘発性を比較した結果、胎仔への影響は、長い繊維長の製品に偏っており、その強さは、母体の炎症性変化や体温低下などと関連していると考えられた。

A. 研究目的

　近年、新素材である産業用ナノマテリアルは、新たな用途や特性をもたらすと期待されている物理化学特性により、ヒト健康にも未知の影響を及ぼす可能性が指摘されているが、そのための適切な健康リスク評価を行えるほどの

知見は未だ十分に集積したとは言えない。国際的な取り組みとしてOECDが2006年から産業用ナノマテリアルの作業グループを設置し、加盟国によるスポンサーシッププログラムの基で、有害性情報の収集活動が行なわれている。現時点では、このプログラムで得られた情報を整理すると共に、更なる知見の必要性やガイドラインの見直し等の検討が行われているところである。一方、我々のこれまでの研究では、急性暴露による影響よりも、体内残留性に基づいた慢性影響がナノマテリアルの最も懸念すべき健康影響であるとの認識に則り、特にアスベスト様形状を持つ多層型カーボンナノチューブ（MWCNT）については中皮腫を誘発するポテンシャルを持つことを明らかにしてきた。さらに、我々はMWCNTの曝露が催奇形性ポテンシャルを持つこともこれまでの研究で明らかにしてきた。26年度はMWCNTによる気管内投与による慢性影響の病理学的解析、及びサイズの異なる多層カーボンナノチューブによる死胚の誘発性を検討することを目的とした。

B. 研究方法

多層カーボンナノチューブによるラット中皮及び肺増殖性病変誘発に対するphenyl N-tert-butyl nitrone (PBN)の影響

26年度は、気管反復投与により摂取された多層カーボンナノチューブ（MWCNT）の呼吸器系及び体腔膜中皮組織における作用発現に対するPBNの影響について病理組織学的な検討を行った。動物はHan:WIST系雄ラット10週齢を用いた。MWCNTは、MWNT-7を用い、経気管投与用に分散性を高めるため、ショ糖及びTween80を加えて、自動乳鉢を用い混和し、最終的に脱イオン水で、MWCNT 250 mg/L の懸濁液（ショ糖及びTween80濃度：5%）を調製し、実験に用いた。懸濁液中の MWCNT 繊維サイズ分布は、平均長 2 μm、平均径 75 nm で、5 μm以上の長さの繊維の占める割合が、きわめて少なかった（図1）。実験は 0mg/kg（対照群：5%ショ糖・Tween80 水溶液）及びMWCNT 0.25mg/kgを腹腔内単回投与、または 0、0.01、0.05、0.25mg/kg を1回/4週間・12 回経気管噴霧投与した。投与液量は 1mL/kg 体重. とした。PBN は 0及び 0.25 mg/kg の腹腔内及び経気管投与追加群に、0.065%の濃度で、実験期間を通して飲水投与した。動物はいずれも実験開始後 104 週間を目処に飼育した。途中解剖例及び実験終了時生存例について、常法により病理組織学的検査を行った。

サイズの異なる多層カーボンナノチューブによる胎仔毒性の差異の検討

26年度は、6種のサイズの異なる多層カーボンナノチューブの単一用量の、マウスへの腹腔内投与による死胚の誘発性を比較することで、胎仔への影響を検討した。用いた多層カーボンナノチューブは、W社製の WS および WL の2品、M社製の M、N社製の N、S社製の SD-1、T社製の T の6種類で、表1に各製品の概要を示す。各多層カーボンナノチューブは、2％カルボキシメチルセルロースナトリウム/りん酸緩衝生理食塩水に懸濁し、高圧蒸気滅菌した。妊娠9日の ICR マウス（Crlj:CD-1）に、4mg/kg 体重を10mL/kg体重で、腹腔内投与し、投与直前と投与2時間後の直腸体温をシチズン電子体

温計で測定した。また、投与 2 時間後に、尾静脈を注射針で穿刺して数 μL の血液を採取し、一部で血液塗抹標本を作製し、残りの血液を－20℃で保存した。妊娠 18 日に母体をイソフルラン（日本薬局方）で軽麻酔し、大腿動脈から抗凝固剤（EDTA2K）入り試験管に採血し、帝王切開にて、胎仔・死胚数を計数した。得られた血液から、自動血液計数装置（SYSMEX KX-21NV）で白血球数を計測し、血液塗抹標本を作製した。血液塗抹標本は、Diff-Quik（SYSMEX）で染色し、光学顕微鏡下で白血球サブタイプ百分率を計数した。投与後 2 時間の保存血で、インターロイキン-6（IL-6）および単球走化性因子（MCP-1）を、Thermo Scientific 社製の ELISA キットで測定した。

C. 研究結果

多層カーボンナノチューブによるラット中皮及び肺増殖性病変誘発に対する phenyl N-tert-butyl nitrone（PBN）の影響

経気管投与用に用いた試料による腹腔内投与実験では、中皮腫の発現が 0.25mg/kg で 4/20（P<0.05、vs 対照群）、PBN 併用群で 3/20 に認められ、MWCNT の中皮腫誘発性に対する PBN の作用は明らかでなかった（表1）。

経気管投与実験では、投与期間及び投与後飼育期間を通して、途中死亡・瀕死例の発現、臨床症状または体重増加経過に MWCNT 投与に関連した異常は認められなかった。組織学的に、MWCNT の各投与群及び PBN 併用群で心嚢膜、胸膜（臓側及び横隔膜胸腔面）、腹膜に単発的に中皮腫や中皮細胞肥大・過形成が認められ（表1）、経気管投与された MWCNT の中皮組織に対する影響の可能性が考えられた。肺実質では、MWCNT 繊維は、主に繊維を貪食したマクロファージに貪食された状態で、肺胞腔内や肺胞壁、また胸膜間質、肺内リンパ組織、血管及びや細気管支周囲間質内にも認められ、沈着量は 0.25mg 及び 0.25mg/kg・PBN 併用群で顕著に多く認められた。0.25mg 及び 0.25mg/kg・PBN 併用群では沈着量に関連して軽度な肺胞の炎症（好中球の浸潤）や線維の増生が見られた。さらに投与に関連して細気管支-肺胞領域及び肺胞領域で過形成が有意に認められた（表2）が、壁側胸膜や肺実質における腫瘍の発現は認められなかった。 4) MWCNT 投与群では、主に腹膜中皮腫と、肺の細気管支末梢・肺胞の増殖性病変を認めたが、PBN 併用は、これらの病変の発現に明らかな影響を示さなかった。

サイズの異なる多層カーボンナノチューブによる胎仔毒性の差異の検討

多層ナノチューブ投与後 2 時間の直腸体温が、WL、N、M、および SD-1 投与群で有意に低下し、特にNおよびM群で低下が著しかった。この時、NおよびM群の母体は極度に活動性が鈍っていた。またWL および SD-1 群の母体では、いくらか活動性が鈍っていた。しかし、WS および T 群の母体は、対照群と変わらない活動性を示していた。投与後 2 時間の白血球サブタイプ百分率では、WL 群のリンパ球％が下がり、好中球％が上がっていた。

妊娠 18 日の母体の主要臓器（肝、腎、脾、胸腺および卵巣）中、N および M 群で脾臓重量が有意に増加していた。M 群の妊娠 18 日の白血球数が有意に上昇して、サブタイプ実数（白血球数×サブタイプ百分率）では、N および M 群の好中球数、M および WL 群の好酸球数、M 群の単球数が有意に上昇していた。各群とも、卵巣の黄体数および総着床数は変化がなかったが、N および M 群で、早期死胚数が有意に増加し、結果として生存胎仔数が有意に減少していた。同様の傾向は、WL および SD-1 群で見られたが、統計学的に有意ではなかった。後期死胚については、各群で変化は見られなかった。また、生存胎仔の平均重量も変化は見られなかった。（表 4）。投与後 2 時間の母体血中の IL-6 および MCP-1 濃度は、N および M 群で有意に上昇していた。次いで WL および SD-1 群、また WS および T 群でもわずかに上昇する傾向が見られたが、有意差はなかった。（図 2）

D. 考察

ラット中皮腫発現には、繊維を貪食した大食細胞による炎症反応と酸化ストレスの関与が提唱されている。一方、PBN は活性酸素捕捉などにより、酸化ストレスによる毒性・発がん性を抑制する。今回は経気管反復投与により摂取された多層カーボンナノチューブ（MWCNT）の呼吸器系及び体腔膜中皮組織における作用発現に対する PBN の影響について病理組織学的な検討を行った。PBN の併用は、中皮腫や増殖性病変の発現頻度や程度に明らかな影響を示さなかった。したがって、今回投与に用いた MWCNT 繊維サイズは短いものであったものの、MWCNT によるラットの中皮及び肺における増殖性病変の誘発機構において、酸化ストレスとそれによるシグナル異常とは異なる要因が関与する可能性が示唆した。

一方、24 年度に報告したように、M 社製と N 社製の多層カーボンナノチューブは、マウス腹腔内投与（M 社製および N 社製）あるいは気管内投与（M 社製）で、用量相関性に奇形を誘発する。しかしながら、M 社製多層カーボンナノチューブと N 社製多層カーボンナノチューブは、サイズが似通っており、サイズの違いによる催奇形性の差異を類推出来なかった。そこで、26 年度は、6種のサイズの異なる多層カーボンナノチューブの単一用量の、マウスへの腹腔内投与による死胚の誘発性を比較することで、胎仔への影響を検討した。その結果、多層カーボンナノチューブの胎仔への影響は、ある程度の長さ（2μm を越えて、20μm 程度までの長さ）の製品に偏って見られ、その強さは、母体の炎症性変化（白血球数、特に好中球数の増加、脾臓の腫大、IL-6 および MCP-1 の上昇）や投与後 2 時間のショック様状態（体温低下や活動性低下）と関連していると考えられた。

E. 結論

気管反復投与により摂取された多層カーボンナノチューブ（MWCNT）の呼吸器系及び体腔膜中皮組織における作用発現に対する PBN の影響について病理組織学的な検討を行った結果、PBN の併用暴露は中皮腫や増殖性病変の発現頻度や程度に明らかな影響を示さなかった。MWCNT によるラットの中皮及び肺におけ

る増殖性病変の誘発機構には、酸化スト
レスとは異なる要因が関与する可能性が
示唆された。また、サイズの異なる多層
カーボンナノチューブのマウス腹腔内投
与による死胚の誘発性を比較した結果、
胎仔への影響は、長い繊維長の製品に
偏っており、その強さは、母体の炎症性
変化や体温低下などと関連していると考
えられた。

F. 健康危機情報
該当無し

G. 研究発表
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H. 知的財産権の出願・登録状況
（予定を含む）
1. 特許取得
    （該当なし）
2. 実用新案登録
    （該当なし）
3. その他
    （該当なし）

表1. MWCNT経気管投与群の組織所見−中皮細胞増殖性病変

実験群/用量	開始時動物数	観察例数	組織所見									中皮腫合計
			心嚢膜			胸膜			腹膜			
			肥大	過形成	中皮腫	肥大	過形成	中皮腫	肥大	過形成	中皮腫	
気管内投与群												
対照群	20	18	0	0	0	0	0	0	0	0	0	0
対照群+PBN	20	19	0	0	0	0	0	0	0	0	0	0
0.01mg/kg	20	16	0	0	0	0	0	1(6)[a]	0	0	0	1(6)
0.05mg/kg	20	17	0	0	0	0	0	0	0	0	1(6)	1(6)
0.25mg/kg	20	17	1(6)	0	1(6)	1(6)	1(6)	0	0	0	1(6)	2(12)
0.25mg/kg+PBN	20	19	1(5)	0	1(5)	0	1(5)	0	0	0	0	1(5)
腹腔内投与群												
対照群	20	16	0	0	0	0	0	0	0	0	0	0
0.25mg/kg	20	20	0	0	0	0	0	0	6	1	4	4(20)*
0.25mg/kg+PBN	20	20	0	0	0	0	0	0	5	1	3	3(15)

a) 発現数・発現率(%).　　* p<0.05; Chi square test and Fisher's test

表2. 肺気道/肺胞上皮細胞組織所見

実験群/用量	観察例数	細気管支	終末細気管支−肺胞移行部		肺胞領域		
		非線毛性細胞	非線毛性細胞	線毛性細胞（細気管支上皮化）	腫大/増生	過形成	腺腫
対照群	18	3 (16)	0	1 (6)	0	0	3
対照+PBN	19	3 (19)	0	1(5)	0	1(5)	0
0.01mg/kg	16	5 (31)	2 (14)	1 (6)	1(6)	0	0
0.05mg/kg	17	4 (24)	4 (24)	4 (24)	11 (65)	2 (12)	0
0.25mg/kg	17	4 (24)	3 (18)	7 (41)*	17 (100)*	10 (59)*	0
0.25mg/kg+PBN	18	1 (6)	2 (18)	8 (44)*	18 (100)*	10 (56)*	1(6)

a) 発現例数/発現率(%).
* p,0.05, vs 対照群/対照＋PBN群（2x2 Chi square test and Fisher exact probability）

表 3　胎児毒性の比較に用いた多層カーボンナノチューブの概要

	長さ(μm)	直径(nm)	純度（%）	鉄含有量（%）
WS	0.5-2	40-70	-	0.046
WL	0.5-10	85-200	-	6.504
N	1-4; 50.9% 5-20; 27.5% 平均 3.5	50-80; 94.8%	>98	0.046
M	1-4; 38.6% 5-20; 58.3% 20-; 3.1% 平均 3.3	50-80; 97.2%	95	0.35
SD-1	平均 6	平均 150	>9.95	-
T	数 10-数 100	20-100	85-94	3.822

表 4　多層カーボンナノチューブ腹腔内投与による母動物と胎児数への影響

	対照群	WS	WL	N	M	SD-1	T
直腸体温	37.4±0.4	36.8±0.7	34.4±1.2 ***	33.3±0.9 **	33.1±0.7 ***	34.8±0.5 ***	36.9±0.8
脾臓(mg)	182±36	154±33	246±36	474±110 ***	360±104 ***	243±34	169±36
総白血球	63±13	48±21	76±14	82±16	109±37 **	76±14	76±14
早期死胚	0.1±0.3	0.4±0.5	3.6±5.2	14.6±1.8 ***	8.8±6.3 ***	3.8±2.9	0.9±0.8
生存胎仔	12.9±2.5	13.9±2.2	10.0±5.1	0 ***	5.5±5.6 **	12.6±3.9	14.2±2.0
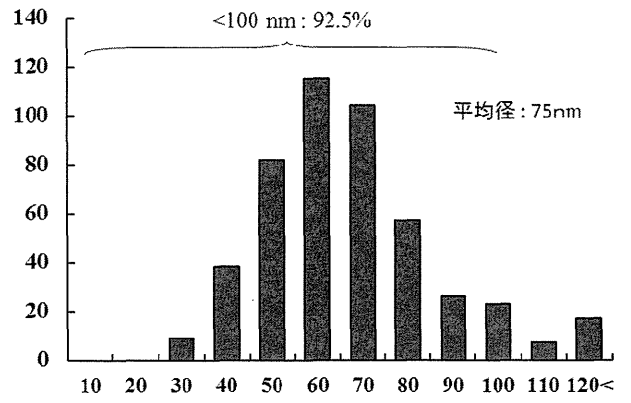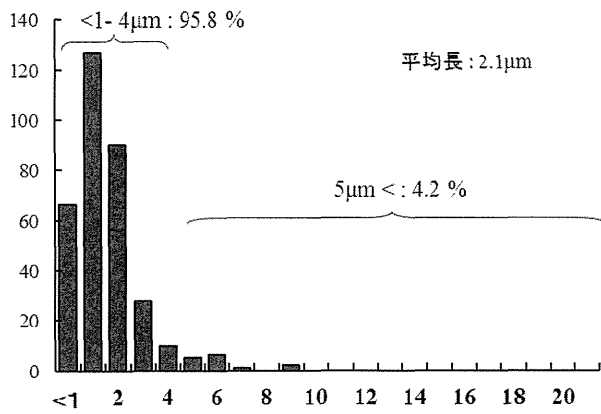
図1.経気管投与分散液のMWCNTサイズ分布
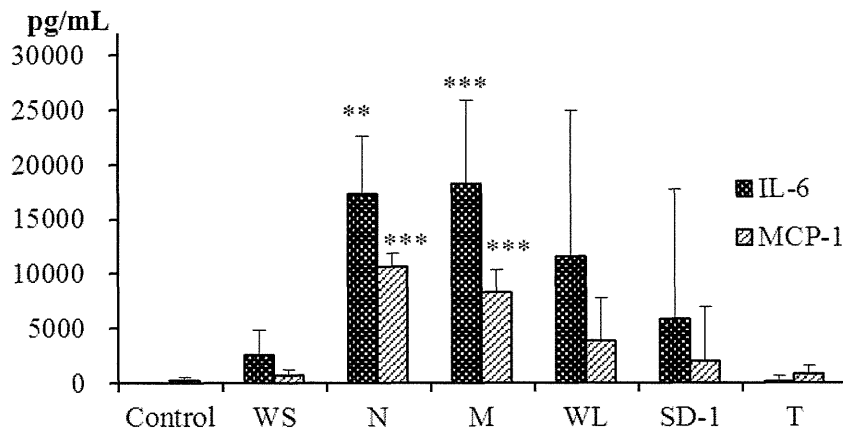
図2 投与後2時間の母体血中のIL-6およびMCP-1濃度

Ⅲ．研究成果の刊行に関する一覧表

書籍

著者名	論文タイトル	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

著者名	論文タイトル	発表誌名	巻号	ページ	出版年
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Xu J, Alexander DB, Futakuchi M, Numano T, Fukamachi K, Suzui M, Omori T, Kanno J, Hirose A, Tsuda H.	Size- and shape-dependent pleural translocation, deposition, fibrogenesis and mesothelial proliferation by multi-walled carbon nanotubes.	Cancer Sci.	105(7)	763-9	2014

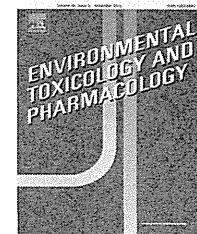|					
Grosse Y1, Loomis D1, Guyton KZ1, Lauby-Secretan B1, El Ghissassi F1, Bouvard V1, Benbrahim-Tallaa L1, Guha N1, Scoccianti C1, Mattock H1, Straif K1;International Agency for Research on Cancer Monograph Working Group.Kane AB, Debia M, Dion C, Møller P, Savolainen K, Canu IG, Jaurand MC, Comba P, Fubini B, Kobayashi N, Morimoto Y, Tsuda H, Yu IJ,Vermeulen R, Bugge MD, Bateson TF, Kuempel ED, Morgan DL, Pinkerton KE, Sargent LM, Stayner L.	Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes.	Lancet Oncol.	15	1427-1428	2014
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IV. 研究成果の刊行物・別冊

ELSEVIER

CrossMark

Titanium dioxide nanoparticles exacerbate pneumonia in respiratory syncytial virus (RSV)-infected mice

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ARTICLE INFO

ABSTRACT

To reveal the effects of TiO_2 nanoparticles, used in cosmetics and building materials, on the immune response, a respiratory syncytial virus (RSV) infection mouse model was used. BALB/c mice were exposed once intranasally to TiO_2 at 0.5 mg/kg and infected intranasally with RSV five days later. The levels of IFN-γ and chemokine CCL5, representative markers of pneumonia, in the bronchoalveolar lavage fluids of RSV-infected mice had increased significantly in TiO_2-exposed mice compared with the control on day 5 post-infection, but not in uninfected mice. While pulmonary viral titers were not affected by TiO_2 exposure, an increase in the infiltration of lymphocytes into the alveolar septa in lung tissues was observed. Immunohistochemical analysis revealed aggregation of TiO_2 nanoparticles near inflammatory cells in the severely affected region. Thus, a single exposure to TiO_2 nanoparticles affected the immune system and exacerbated pneumonia in RSV-infected mice.

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1. Introduction

Nanomaterials are engineered structures with at least one dimension of 100 nm or less (Nel et al., 2006). Various kinds of nanomaterials are known and have a wide range of applications (Maidalawieh et al., 2014; Nel et al., 2006; Stamatoiu et al., 2012). Titanium dioxide (TiO_2) nanoparticles are used in cosmetics and building materials because they are chemically and thermally stable. When research focused on TiO_2 nanoparticles were in drug delivery systems (Zhang et al., 2012) several findings of toxicity due to exposure to TiO_2 nanoparticles were reported, such as carcinogenesis of the lung in rats (Xu et al., 2010), induction of strong oxidative stress and mitochondrial damage in glial cells (Huerta-Garcia et al., 2014), and inflammatory disorder on the cardiovascular system in ApoE knockout mice (Chen et al., 2013). Although we are exposed to TiO_2 nanoparticles in daily life, the safety of TiO_2 nanoparticles for human health is poorly known.

Human respiratory syncytial virus (RSV), a member of the *Paramyxoviridae* family, is a prevalent infectious agent of acute lower respiratory illness in infants and young children (MacDonald et al., 1982). An initial RSV infection is frequent during the first few years of life, and most children have been infected by 24 months of age (Collins et al., 2001). Clinically severe RSV infection is seen primarily in infants and young children with naïve immune systems and/or genetic predispositions (Holberg et al., 1991) and patients with suppressed T-cell immunity (MacDonald et al., 1982). RSV reinfects adults at a rate of approximately 5–10% per year (Falsey, 2007), and is an important cause of morbidity and mortality in the elderly (Falsey et al., 2005). Thus, because the severity of RSV infection reflects the condition of the host immunity, we established a novel assay system for evaluation of the immunotoxicity of the brominated flame retardants (BFRs) using a murine model of RSV infection (Watanabe et al., 2008a). We subsequently demonstrated that decabrominated diphenyl ether (DBDE) (Watanabe et al., 2008b, 2010a) and tetrabromobisphenol A (TBBPA) (Takeshita et al., 2013; Watanabe et al., 2010b) caused developmental immunotoxicity and irregular production of cytokines in RSV-infected mice, respectively. In addition, we also revealed that perinatal exposure to methamidophos, a representative organophosphate insecticide, suppressed the production of proinflammatory cytokines using this model (Watanabe et al., 2013).

In the present study, we adopted the RSV infection mouse model to evaluate the effects of TiO_2 nanoparticles on the immunotoxicity after a single exposure. Then we investigated the effects of TiO_2 nanoparticles on pneumonia in RSV infection by focusing on the variations in of cytokine and chemokine levels in bronchoalveolar lavage fluid (BALF) and the exacerbation of pneumonia in lung tissues by histopathological assay.

2. Materials and methods

2.1. Animals

Female (5 weeks old) BALB/c mice were purchased from Kyudo Animal Laboratory (Kumamoto, Japan) and housed at $25 \pm 2\,°C$. The mice were allowed free access to the conventional solid diet CRF-1 (Oriental Yeast Co., Chiba, Japan) and water and used in this experiment after 7 d acclimation. The animal experimentation guideline of the Kyushu University of Health and Welfare were followed in the animal studies.

2.2. Cell and virus

The A2 strain of RSV was obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in HEp-2 cell (human epidermoid carcinoma, ATCC CCL-23) cultures. Viral titers of HEp-2 cells were measured by the plaque method (Watanabe et al., 2008a) and expressed as plaque-forming units per milliliter (PFU/mL).

2.3. Chemical compound

TiO_2 nanoparticles were kindly provided by Tayca Corp. (Osaka, Japan). The particles form ultra-fine rutile crystals primarily 35 nm in diameter. TiO_2 nanoparticles readily aggregate to form microparticles in phosphate-buffered saline (PBS). To avoid aggregation, the suspension of TiO_2 nanoparticles in PBS was dispersed using a portable ultrasonic disruptor just before treatment of mice. Then the mean secondary diameter of the particles was 913 nm, ranging from 804 to 1022 nm, as measured by a Zetasizer Nano (Malvern Instruments, Worcestershire, UK).

2.4. Animal tests

Six-week-old mice were intranasally administered 0.1 mL of a suspension of TiO_2 nanoparticles at 0.25 or 2.5 mg/kg of body weight one time under anesthesia for histological assays or 0.5 mg/kg for measuring cytokines in BALF and pulmonary viral titers. In control group, mice were given PBS intranasally under anesthesia.

The RSV infection test was performed as reported previously (Watanabe et al., 2008a). Briefly, 5 d after from TiO_2 exposure, mice were infected intranasally with 3.5×10^5 PFU of the A2 strain of RSV under anesthesia. In a mock-infected group, mice were given PBS intranasally. On day 5 after infection, blood samples were prepared from RSV-infected mice under anesthesia and BALF was obtained from the mice under anesthesia by instilling 0.8 mL of cold PBS into the lungs and aspirating it from the trachea using a tracheal cannula. Following the acquisition of BALF, the lungs were removed, immediately frozen in liquid N_2, and stored at $-80\,°C$ until virus titration. Ice-cold BALF was centrifuged at $160 \times g$ at $4\,°C$ for 10 min. After centrifugation, the supernatant was stored at $-80\,°C$ until to use. The cell pellet was suspended in 0.3 mL of cellbanker-1 (Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan) as bronchoalveolar lavage cells, and then stored at $-80\,°C$ prior to use. Frozen lung tissue was homogenized with cold quartz sand in a homogenizer. After centrifugation at $480 \times g$ at $4\,°C$ for 15 min, the supernatants of the homogenates were used for a plaque assay. Viral titers in lungs of mice were expressed as PFU/mL.

2.5. ELISA

Interleukin (IL)-2, IL-4, IL-10, and interferon (IFN)-γ levels in BALF were measured using specific ELISA kits (Ready-set-go, eBioscience Inc., San Diego, CA) according to the manufacturer's instructions. Levels of CCL5 (RANTES) in BALF and serum and CCL3 (MIP-1α) in BALF and the culture supernatant of bronchoalveolar lavage cells were measured using specific ELISA kits (Quantikine, R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. The lower limits of detection of the kits are 2 (pg/mL) for IL-2, 4 (pg/mL) for IL-4, 8 (pg/mL) for IL-10, 15 (pg/mL) for IFN-γ, 2 (pg/mL) for CCL5, and 1.5 (pg/mL) for CCL3. The intra- and interassay coefficients of variation for the ELISA results were less than 10%.

2.6. Flow cytometric analysis of bronchoalveolar lavage cells

Flow cytometric analysis was performed according to our previous report (Takeda et al., 2014). Briefly, bronchoalveolar lavage cells were stimulated with BD GolgiStop (BD PharMingen, San Diego, CA) at 1 μL/mL for 6 h at 37 °C. After incubation, the cells were washed twice and stained for intracellular IFN-γ (FITS Rat Anti-Mouse IFN-γ, BD PharMingen, San Diego) and IL-4 (PE Rat Anti-Mouse IFN-γ, BD PharMingen, San Diego), according to the manufacturer's instructions. The cells were washed twice and analyzed on an FACS Calibur 35 flow cytometer (Becton Dicknson, Sunnyvale, CA).

2.7. Histological methods and evaluation

For histological examination of RSV-infected lungs, 3–5 mice per group of infected mice were sacrificed by cervical dislocation on day 5 after infection, and the lungs were removed and placed in buffered formalin for a minimum of 24 h. The tissue was then embedded in low-melting point paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin. After taking two pictures randomly of each pulmonary lobe using a microscope ($\times$100), the pictures were analyzed for the proportion of alveolar septa and infiltration of the inflammatory cells into the tissues per unit area by Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

2.8. Immunohistochemical evaluation

The lung tissue sections were deparaffinized and hydrated through xylenes and graded alcohols. After washing with water, they were incubated in unmasking solution (Vector Laboratories, Inc., Burlingame, CA) at 90 °C for 30 min. Then, the sections were incubated in the 0.3% H_2O_2 in PBS for 30 min to quench the endogenous peroxidase activity and treated with blocking serum (Vector Laboratories, Inc.) for 30 min. The lung tissues were stained with a goat polyclonal antibody against RSV protein (1:250, Acris Antibodies GmbH, Inc., San Diego, CA) for 90 min. Then, RSV proteins were detected using a VECTASTAIN ABC kit (Vector Laboratories, Inc.) according to the manufacturer's instructions. The sections were faintly counterstained with hematoxylin.

2.9. Culture of bronchoalveolar lavage cells

Culture of bronchoalveolar lavage cells obtained from RSV-infected mice on day 1 post-infection was performed according to our previous report (Watanabe et al., 2010a). Briefly, 200 μL of bronchoalveolar lavage cells suspension (2.5 $\times$ 10^5 cells/mL) was seeded on each well in a 96-well microtiter plate and incubated at 37 °C for 24 h in a humidified air with 5% CO_2. After incubation, the culture medium was removed by aspiration and replaced in fresh RPMI medium with or without 0.1 mg/mL of TiO$_2$ nanoparticles. Following 24 h further incubation, the culture medium was removed by aspiration and replaced in fresh RPMI medium with or without 100 ng/mL of lipopolysaccharide (W E. coli O127: B8, Difco, Detroit, MI; LPS) for 24 h. The culture supernatant was harvested from each well and the amount of CCL3 was measured by ELISA.

2.10. Statistical analysis

Comparisons between the pulmonary viral titers and the levels of cytokines and chemokines of the control and TiO$_2$-treated groups were carried out using Student's t-test. A P value of 0.05 or less was considered to be significant.

3. Results

3.1. Effects of TiO$_2$ nanoparticles on RSV infection in mice

To investigate the effects of TiO$_2$ nanoparticles on the immune response to RSV infection, six-week-old female BALB/c mice were exposed intranasally to 0.1 mL of TiO$_2$ suspension at 0.5 mg/kg of body weight under anesthesia. No abnormal behavior or dystrophy due to the stress of TiO$_2$ exposure was observed compared to the control (0 mg/kg) in the mice, and the mice were infected intranasally with the A2 strain of RSV at 3.5 $\times$ 10^5 PFU five days after TiO$_2$ exposure. The levels of IFN-γ, a representative marker of pneumonia in RSV infection, in BALF were measured on day 5 post-infection (Table 1). The IFN-γ levels of RSV-infected mice treated with TiO$_2$ were significantly (P < 0.05) higher than those in the control. In mock-infected mice treated with or without TiO$_2$, the levels of IFN-γ in BALF were under the limit of detection. These results indicated that pneumonia in RSV-infected mice was exacerbated by TiO$_2$ exposure. To investigate further effects of exposure to TiO$_2$ on the immune system of RSV-infected mice, the levels of Th1 cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-4 and IL-10) in BALF were also measured on day 5 after infection (Table 1). The levels of IL-10 in BALF were significantly (P < 0.05) increased by approximately 92% compared with the control. No significant increase of IL-2 in BALF was found after TiO$_2$ treatment, and the levels of IL-4 in BALF were under the limit of detection. In mock-infected mice treated with or without TiO$_2$, the levels of cytokines in BALF were under the limit of detection. To reveal effects of exposure to TiO$_2$ on the Th1/2 immune balance of RSV-infected mice on day 5 post-infection, intracellular IFN-γ and IL-4 productions by the bronchoalveolar lavage cells were examined by flow cytometry (Table 2).

Table 1 – Effects of TiO₂ on levels of cytokines in BALF of RSV-infected mice on day 5 post-infection.

TiO₂ exposure (mg/kg)	Concentration (ng/mL)[a]							
	RSV-infected				Mock-infected			
	IFN-γ	IL-2	IL-4	IL-10	IFN-γ	IL-2	IL-4	IL-10
0	8.59 ± 3.44	0.02 ± 0.01	<0.01	1.65 ± 0.71	<0.01	<0.01	<0.01	<0.01
0.5	12.90 ± 2.10*	0.03 ± 0.01	<0.01	3.17 ± 1.15*	<0.01	<0.01	<0.01	<0.01

[a] Concentration (ng/mL) of each cytokine in BALF from RSV-infected mice treated with or without TiO₂ (0.5 mg/kg) was measured by ELISA for each specific cytokine. Data represents mean values of 3–6 mice. Numbers in parentheses indicate standard deviation.
* Statistically different from control at $P < 0.05$ (Student's t-test).

Table 2 – Effects of TiO₂ on intracellular cytokine levels in bronchoalveolar lavage cells on day 5 post-infection from RSV-infected mice.

TiO₂ exposure (mg/kg)	% of total population[a]			
	IFN-γ⁻IL-4⁻	IFN-γ⁺IL-4⁻	IFN-γ⁻IL-4⁺	IFN-γ⁺IL-4⁺
0	98.7	1.1	0.1	0.1
0.5	98.9	0.8	0.1	0.2

[a] Bronchoalveolar lavage cells were collected from RSV-infected mice treated with or without TiO₂ (0.5 mg/kg) on day 5 post-infection. The pooled bronchoalveolar lavage cells were stimulated BD GolgiStop for 6 h at 37 °C and stained intracellular IFN-γ and IL-4. The stained cells were analyzed by flow cytometry.

There was not a significant change in the population of IFN-γ-positive cells and IL-4-positive cells due to TiO₂ treatment. These results suggested that TiO₂ exposure should affect the immune response to RSV infection.

Chemokine CCL5 is a common marker of the severity of inflammation in the lungs due to RSV infection (Lambert et al., 2003) and chemokine CCL3 also is an inflammatory marker. Therefore, we measured the levels of CCL5 and CCL3 in BALF on day 5 after infection (Table 3). The levels of CCL5 in BALF were significantly ($P < 0.05$) increased by approximately 36% compared with the control, but there was no significant increase of CCL3 levels in TiO₂-exposed mice. In mock-infected mice treated with or without TiO₂, the levels of chemokines in BALF were under the limit of detection. The levels of CCL5 in serum were significantly ($P < 0.05$) increased by approximately 31% compared with the control (Table 3). Thus, these results strongly suggested that TiO₂ exposure exacerbated the pneumonia due to RSV infection.

To evaluate the effects of TiO₂ exposure on the growth of RSV in mice, pulmonary viral titers were measured by plaque assay (Fig. 1). Viral titers of mice exposed to TiO₂ were not elevated significantly compared with those of control. Thus, TiO₂ exposure did not enhance proliferation of RSV in mice.

3.2. Effects of TiO₂ nanoparticles on severity of pneumonia in RSV infection

To clarify the effects of TiO₂ nanoparticles on the severity of pneumonia in RSV infection, a histopathological assay was performed. In this experiment, 3–5 mice in each group were treated with TiO₂ as follows: a control group at 0 mg/kg, low-dose group at 0.25 mg/kg, and high-dose group at 2.5 mg/kg. These mice were infected with or without RSV 5 days after TiO₂ exposure. On day 5 post-infection, the mice were sacrificed, and their lung tissues were analyzed histopathologically. Representative results and changes in severity are presented
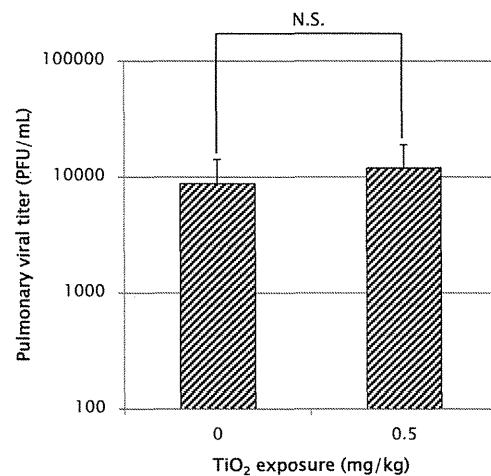
Fig. 1 – Effect of exposure to TiO₂ on pulmonary viral titers of RSV-infected mice on day 5 post-infection. The data represents mean ± standard deviation of values of 7 control or 5 TiO₂-treated mice. N.S., not significant.

in Fig. 2 and Table 4, respectively. In mock-infected mice, no obvious change in the lung tissues due to TiO₂ exposure was observed compared with the control (Fig. 2A-a, -c, and -e). In RSV-infected mice, typical features of pneumonia due to RSV infection, such as degeneration of the bronchial epithelium and infiltration of lymphocytes and neutrophils, were observed in mice treated with or without TiO₂ (Fig. 2A-b, -d, and -f). Severity of pneumonia was assessed as the proportion of alveolar septum tissue in RSV-infected mice (Table 4). In the control group at 0 mg/kg, the proportion of alveolar septa of all mice was less than 60%. On the other hand, two mice in the TiO₂ (0.25 mg/kg)-treated group had more than 60% alveolar septa, and one mouse in the TiO₂ (2.5 mg/kg)-treated group had more than 70%. The unit area means were 51.8%, 60.6%,

Table 3 – Effects of TiO₂ on levels of chemokines in BALF and serum of RSV-infected mice on day 5 post-infection.[a]						
TiO₂ exposure (mg/kg)	Concentration in BALF (ng/mL)				Concentration in serum (ng/mL)	
	RSV-infected		Mock-infected		RSV-infected	Mock-infected
	CCL3	CCL5	CCL3	CCL5	CCL5	CCL5
0	0.10 ± 0.04	0.24 ± 0.06	<0.01	<0.01	0.14 ± 0.01	0.10 ± 0.01
0.5	0.11 ± 0.03	0.32 ± 0.07*	<0.01	<0.01	0.18 ± 0.03*	0.09 ± 0.03

[a] Concentration (ng/mL) of each chemokine in BALF and serum from RSV-infected mice treated with or without TiO₂ (0.5 mg/kg) was measured by ELISA for each specific chemokine. Data represents mean values of 3–6 mice. Numbers in parentheses indicate standard deviation.
* Statistically different from control at $P < 0.05$ (Student's t-test).

(A)

(B)
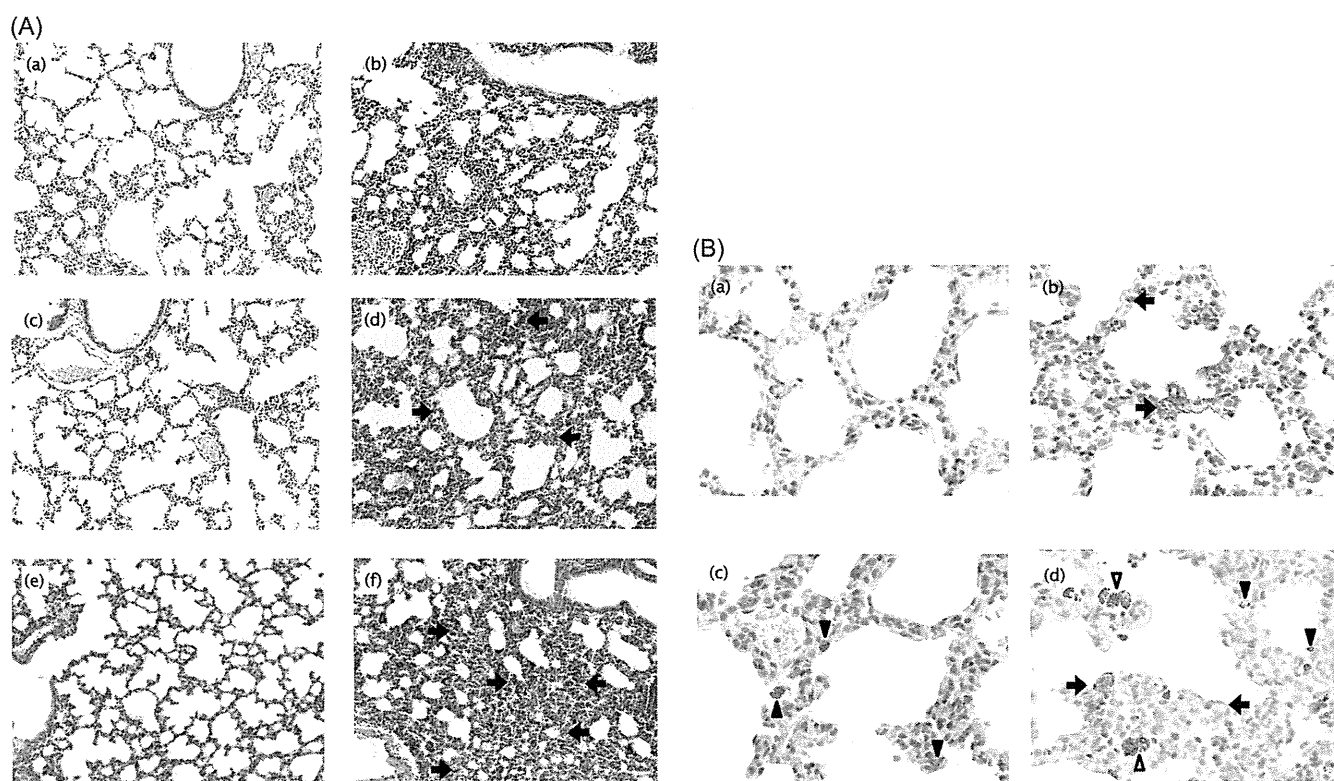
Fig. 2 – Lungs of mice 5 days after RSV-infection. (A) Hematoxylin and eosin staining (×100). (a) Control mouse with mock infection. (b) Control mouse with RSV infection. (c) TiO₂-treated (0.25 mg/kg) mouse with mock infection. (d) TiO₂-treated (0.25 mg/kg) mouse with RSV infection. (e) TiO₂-treated (2.5 mg/kg) mouse with mock infection. (f) TiO₂-treated (2.5 mg/kg) mouse with RSV infection. Arrows indicate infiltration of lymphocytes in alveolar septa. (B) Immunostained with anti-RSV protein antibodies (1:250) and counterstained with hematoxylin (×400). (a) Control mouse with mock infection. (b) Control mouse with RSV infection. (c) TiO₂-treated (2.5 mg/kg) mouse with mock infection. (d) TiO₂-treated (2.5 mg/kg) mouse with RSV infection. Closed arrows indicate RSV-positive cells, closed arrowheads indicate TiO₂ nanoparticles, and open arrowheads indicate aggregation of TiO₂ nanoparticles in inflammatory cells.

Table 4 – Effects of TiO₂ on proportion of tissue of alveolar septa in RSV-infected mice on day 5 post-infection.					
TiO₂ exposure (mg/kg)	% of alveolar tissues[a]				% of mean alveolar tissues
	>40	>50	>60	>70	
0	2*	2	0	0	51.8 (3.7)
0.25	0	2	2	0	60.6 (1.6)
2.5	0	0	1	1	68.6 (3.1)

[a] The proportion of tissue in alveolar septa per unit area by Adobe Photoshop.
* Number of mice. Numbers in parenthesis indicate the standard error.

Table 5 – Effects of TiO_2 on CCL3 production from bronchoalveolar lavage cells on day 1 post-infection in RSV-infected mice.

TiO_2 exposure (mg/mL)	CCL3 (ng/mL)[a]	
	−LPS	+LPS
0	<0.01	0.17 (0.12–0.23)*
0.1	<0.01	0.15 (0.13–0.16)

[a] Bronchoalveolar lavage cells were collected from RSV-infected mice and cultured for 48 h with or without TiO_2 (0.1 mg/mL).
* Data represent mean of values of two separate experiments. Numbers in parentheses indicate the range of values.

and 68.6% in each group, respectively. Thus, we confirmed exacerbation of the pneumonia due to RSV infection by TiO_2 exposure.

To investigate whether the distribution of RSV-infected cells was changed qualitatively due to TiO_2 (2.5 mg/kg) exposure, sections of the lung tissues of RSV-infected mice were stained immunohistochemically with a goat-polyclonal antibody against RSV protein (Fig. 2B). There was no significant change in the localization of RSV-positive cells, but the TiO_2 nanoparticles were not close to RSV-positive cells (Fig. 2B-b and -d). Similar results were observed for TiO_2 (0.25 mg/kg)-treated mice (data not shown). However, there was aggregation of TiO_2 nanoparticles near inflammatory cells in the severe region (Fig. 2B-d). Because these results suggested that TiO_2 nanoparticles might influence the function of macrophage/monocyte in an early phase of RSV infection, bronchoalveolar lavage cells on day 1 post-infection from RSV-infected mice were incubated for 48 h with or without 0.1 mg/mL of TiO_2, corresponding to a dose of 0.5 mg/kg *in vivo*. After incubation, the levels of CCL3 in the culture supernatant of the cells were measured by ELISA (Table 5). Although the cells were stimulated with LPS, there was no significant change in the production of CCL3 from bronchoalveolar lavage cells due to TiO_2 treatment.

4. Discussion

We assessed the effects of TiO_2 nanoparticles on the immune response using a mouse model of RSV infection (Watanabe et al., 2008a) and found that prior exposure to TiO_2 nanoparticles exacerbated pneumonia in the lungs of mice.

Various studies concerning risk assessment of TiO_2 nanoparticles in murine models have been reported (Chen et al., 2013; Kwon et al., 2012; Lindberg et al., 2012; Xu et al., 2010). In many cases, TiO_2 exposure was performed at a high dosage and/or multiple times. For example, to evaluate the carcinogenesis of TiO_2 nanoparticles in lung tissues, female (10 weeks old) SD rats were subjected to intra-pulmonary spraying five times with 0.5 mL suspensions of TiO_2 nanoparticles at 500 μg/mL in saline (Xu et al., 2010). In our assay, female (6 weeks old) BALB/c mice were exposed once intranasally to 0.1 mL of a suspension of TiO_2 nanoparticles at 50 μg/mL or 100 μg/mL, corresponding to a dose of 0.25 mg/kg or 0.5 mg/kg of body weight. The levels of TiO_2 exposure in our assay are much lower than those in previous studies (Chen et al., 2013; Kwon et al., 2012; Lindberg et al., 2012; Xu et al., 2010), and

suppression of body weight, abnormal behavior, or dystrophy due to the stress of TiO_2 exposure was not observed compared with the control mice (data not shown). Furthermore, in the TiO_2-treated mice without RSV infection, no significant enhancement of cytokines or chemokines in BALF and any obvious changes in lung tissues were observed (Table 1, 3, Fig. 2A-a, -c, -e, B-a, and -c). Based on these experiments, we are confident that RSV infection is a useful tool for evaluation of the effects of low-level exposure to TiO_2 nanoparticles on the immune system, which was not reported previously.

The IFN-γ levels of RSV-infected mice treated with TiO_2 were enhanced significantly ($P < 0.05$) compared with the control (Table 1). These results suggest that pneumonia in RSV-infected mice was exacerbated due to TiO_2 exposure. However, viral titers in lung tissues of RSV-infected mice exposed to TiO_2 were not elevated significantly compared with the control (Fig. 1), and there was no significant increase of the number of RSV-positive cells in lung tissues due to TiO_2 exposure (Fig. 2B). Previous studies concerning evaluation of immunotoxicity of BFRs such as DBDE and TBBPA have shown that these compounds clearly increased both the levels of IFN-γ and viral titers in RSV-infected mice, resulting in the exacerbation of pneumonia (Watanabe et al., 2008b, 2010a,b). Therefore, we speculated that the mechanism of action of TiO_2 nanoparticles was different from that of BFRs on the immune system (Watanabe et al., 2008b, 2010a,b). We investigated further effects of exposure to TiO_2 on the Th1/2 balance of RSV-infected mice. The levels of IL-10, a Th2 cytokine, in BALF were increased significantly ($P < 0.05$) compared with the control (Table 1). IL-10 is produced mainly by T cells and acts by inhibiting the production of pro-inflammatory cytokines (Cavalcanti et al., 2012). Remarkably, although enhancement of the IL-10 levels should alleviate immune response, exacerbation of pneumonia was conversely exhibited (Fig. 2A). On the other hand, enhancement of IL-4 production due to TiO_2 exposure was not observed (Table 1). However, the levels of chemokine CCL5, an inflammatory marker called RANTES produced mainly by T cells and basophils (Schall et al., 1988), in BALF were also increased significantly ($P < 0.05$) compared with the control (Table 3). Thus, this irregular activation of T cells due to TiO_2 exposure might induce a Th1/2 imbalance. However, there was not a significant change in the population of IFN-γ-positive cells and IL-4-positive cells due to TiO_2 treatment by flow cytometric analysis (Table 2). These results suggested that TiO_2 exposure probably affected the secretion of the cytokines rather than differentiation of $CD4^+$ cells, responding to RSV infection.

In the histopathological analysis, the infiltration of lymphocytes in alveolar septa in RSV-infected mice tended to be increased by TiO_2 exposure, particularly in wide areas of lung tissues (Fig. 2A and Table 4). Because chemokine CCL5 promotes the migration of T cells and basophils (Schall et al., 1988), enhancement of it should contribute to the infiltration (Table 3). Moreover, in an immunohistochemical analysis, we observed TiO_2 nanoparticles explicitly because the sections were faintly counterstained with hematoxylin (Fig. 2B). It became evident that TiO_2 nanoparticles were aggregated by lymphocytes/macrophages but were not close to RSV-positive cells. It has been reported that TiO_2 nanoparticles were engulfed in alveolar macrophages and involved in the

inflammation in lung tissues of the TiO₂-treated rodents (Kwon et al., 2012; Xu et al., 2010). Then, to evaluate whether TiO₂ exposure influence the function of macrophages/monocytes in lung tissue of RSV-infected mice, the bronchoalveolar lavage cells on day 1 post-infection, consist mainly of the macrophage/monocyte-like cells (Watanabe et al., 2010a), were cultured with or without TiO₂ (Table 5). The *in vitro* experiment using a LPS showed that TiO₂ treatment did not affect the production of chemokine CCL3 from the bronchoalveolar lavage cells, although the ingestion of TiO₂ nanoparticles in the macrophage/monocyte-like cells was observed under a microscope. Therefore, further studies are needed to understand whether macrophages are involved in the immune response in an early phase of RSV infection.

TiO₂ nanoparticles are used in various products, and we may be exposed to them at various times of life. We have already confirmed that anatase crystals of TiO₂ nanoparticles used in building materials induced the elevation of CCL5 in RSV-infected mice (data not shown). In this study, we used TiO₂ nanoparticles that form rutile crystals and are used in cosmetics. Further studies are required to investigate the effects of TiO₂ nanoparticles of different forms and sizes on immune response. These studies should provide useful information to manage their effects on health, including methods of skin care.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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