chromosome. Furthermore, altered DNA methylation was observed in both 1d and 21d offspring. To understand the molecular events influenced by DE exposure, differentially methylated genes were bioinformatically categorized using GO terms. This bioinformatic interpretation indicated that differentially DNA methylated genes were enriched in the GO terms related to neuronal differentiation and neurogenesis. These results suggest that aberrant DNA methylation induced by prenatal DE exposure affects neuronal development. The fetal and neonatal period is critical for the development and organization of the neuronal network (Sporns *et al.*, 2004; Smyser *et al.*, 2010). We previously reported that prenatal DE exposure affects spontaneous locomotor activity and monoaminergic system in mice (Suzuki *et al.*, 2010). The genes which aberrant DNA methylation was observed in this study would be associated with development and organization of the monoaminergic system in mice. The detailed analysis about this point is required to clarify the association between aberrant DNA methylation and functional changes in mice.

The regulation of gene expression during fetal and neonatal period is associated with morphological and functional development of the brain (Muotri and Gage, 2006). Given that the established DNA methylation pattern is generally maintained through cell division (Bergman and Cedar, 2013), it is predicted that altered DNA methylation would be partially maintained after development. Several reports suggest a relationship between aberrant DNA methylation and neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's disease (Jakovcevski and Akbarian, 2012). In addition, cortical neuron degeneration has also been observed in canines that inhaled air pollutants containing PM (Calderón-Garcidueñas *et al.*, 2002). When the information from these reports is considered, it would seem that altered DNA methylation induced by prenatal DE exposure would also be associated with the later pathogenesis of neurodegenerative disorder. In the present study, we observed that the genes which showed altered DNA methylation were different between 1d and 21d offspring. These results suggested that the aberrant DNA methylation pattern induced by prenatal DE exposure was partially recovered during growth. Further examinations about DNA methylation in young adult mice are required to clarify the DNA region which shows persistent aberrant DNA methylation.

The authors detected the altered DNA methylation of the genes independently of the presence of CpG islands. Several reports indicate a relationship between reactive oxygen species (ROS) and DNA methylation. Oxidative DNA damage is known to disturb the binding of methyltransferase to the DNA (Valinluck *et al.*, 2004), thus resulting in hypomethylation of cytosine residue. Weitzman *et al.* (1994), showed that DNA methylation can be influenced by free radical adducts on adjacent guanine residues. Taken together, it seems possible that ROS associated with DE inhalation (Li *et al.*, 2010) could disrupt the DNA methylation state in the developing tissues. Since DNA hypomethylation induces genomic instability (Chen *et al.*, 1998), a decrease in genome-wide DNA methylation may lead to an increase in the mutation rate that is induced by prenatal DEP exposure (Ritz *et al.*, 2011).

We previously reported a bioinformatic method for locating candidate brain regions of interest for the effects of nanoparticle exposure using MeSH terms (Umezawa *et al.*, 2012). We applied the method to survey the brain regions that are preferentially affected by prenatal DE exposure. Although several MeSH terms related to brain region were enriched in each experimental group, no common regions were found in the comparisons. With regard to the effects of DE exposure, it therefore seems less likely that any brain region is a specific target for DNA methylation disruption.

In the present study, we interpreted the biological effects caused by differential gene methylation using a bioinformatic method. Further "wet experiments" are required to clarify whether disrupted DNA methylation actually alters the gene expression, neural differentiation, and the function of central nervous systems especially monoaminergic systems which are affected by prenatal DE exposure (Suzuki *et al.*, 2010). Additionally, the molecular mechanisms underlying the effect of prenatal DE exposure on the DNA methylation pattern remain unknown. As indicated above, the disturbance of DNA methyltransferase binding (Valinluck *et al.*, 2004) is potentially involved in the dysregulation of DNA methylation. In addition, the biological systems that determine the DNA regions that are methylated are another possible target of DE exposure. Previous reports indicated some factors essential for the establishment and maintenance of the methylation imprint, including Zfp57 and PGC7/Stella (Li *et al.*, 2008; Nakamura *et al.*, 2007). Shen *et al.* (2013), showed that a dynamic methylation-demethylation cycle occurs at a large number of genomic loci. These molecules and pathways would also be candidate targets of prenatal DE exposure. Recently, a portion of the piRNA, small RNA exclusively expressed in the germ line, was linked to *de novo* DNA methylation (Olovnikov *et al.*, 2012). Wick *et al.* (2010) showed that particles up to a diameter of 240 nm were taken up by

the placenta and, further, were able to cross the placental barrier. The findings in this report suggest that a part of DEP, especially nano-sized particles (diameter < 100 nm), might be transferred to fetus. On the other hand, Weaver *et al.* (2005) showed maternal stress alters the epigenotype in rodent offspring. The analysis about whether DEP or maternal stress disrupts the molecules/pathways which indicated above would help to solve the problem.

Our results showed that altered DNA methylation pattern was different between male and female offspring. Previous reports suggest that steroid hormone and endocrine disruptor change DNA methylation (Jost and Saluz, 1993; Anway *et al.*, 2005; Skinner *et al.*, 2010). Watanabe and Kurita (2001) showed the possibility that prenatal DE exposure alters fetal testosterone levels. Brain sex differences organized by a transient hormone surge may be maintained through epigenetic modification (McCarthy *et al.*, 2009). Our results, combined with these reports, showed the possibility that prenatal DE exposure affects the brain sex difference through alteration of DNA methylation in the developmental stage.

In conclusion, the present study showed that prenatal DE exposure disrupts the genome-wide DNA methylation state in the brain of offspring mice. Bioinformatic GO analysis showed that differentially DNA methylated genes were enriched in neuronal differentiation. These results suggest that disrupted DNA methylation in the infertile mouse brain is involved in neural dysfunctions induced by prenatal DE exposure. Bioinformatic interpretation of the altered DNA methylation data using GO terms may provide clues that lead to the better understanding of the molecular events underlying the effects of prenatal DE exposure in the developmental period. In addition, a decrease in genome-wide DNA methylation may lead to increased mutation rate, which is induced by prenatal DEP exposure. Our results suggest that the early-life social environments in which DE is present could be critical for the construction of the DNA methylation pattern and may be associated with a long-term impact on health.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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Carbon black nanoparticle exposure during middle and late fetal development induces immune activation in male offspring mice

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ABSTRACT

Increasing exposure to nanoparticles (NPs) has raised concerns regarding their health and safety profiles in humans and animals, especially in developing organisms, which may display increased sensitivity to NP toxicity. The present study examined the effects of gestational exposure to carbon black NP (CB-NP) on the development of the offspring immune system. Pregnant mice were exposed to CB-NP (95 μg/kg body weight) by intranasal instillation on gestational days 9 and 15. The thymus and spleen were collected from their offspring mice on postnatal day (PND) 1, 3 and 5. Thymocyte and splenocyte phenotypes were examined by determining the expression of cell-surface molecules using flow cytometry. Gene expression in the thymus and spleen was examined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Prenatal exposure to CB-NP increased total thymocytes and their immunophenotypes ($CD4^-CD8^-$ and $CD4^+CD8^+$ cells). It also induced an increase in total lymphocytes, and $CD4^-CD8^-$, particularly $CD3^-B220^-$cells, at PND 5 in the spleen of newborn male offspring, reflecting the stimulation of immature splenocytes. Furthermore, mRNA expression of genes related to the induction of peripheral tolerance (*i.e.* thymic Traf6) was upregulated. These data suggest that respiratory exposure to CB-NP during middle and late gestation may have allergic or inflammatory effects in male offspring, and may provide initial information on the potential developmental immunotoxicity of nanoparticles.

1. Introduction

The rapid development of nanoscience has been associated with concerns about the possible health impacts of nanoparticles (NPs). The small size of NPs means that they have a larger relative surface area per mass in comparison to bulk-size particles of the same material; this feature often makes NPs more toxic and inflammogenic (Duffin et al., 2007). Their small size also enables certain NPs to cross cell membranes and translocate from the environment into the organism (Stone et al., 2007). The lungs and airways are the most important exposure sites for involuntary exposure to NPs. Respiratory exposure to NPs elicits local pulmonary effects (*i.e.* an inflammatory response) (Brown et al., 2000; Jacobsen et al., 2009; Wilson et al., 2002), and can also translocate from the lungs into circulation and reach secondary target organs (heart, liver, brain, and testicles) (Kreyling et al., 2002; Oberdörster et al., 2002) and the developing fetus (Umezawa and Takeda, 2011). The immunotoxic potential and ability of various NPs to alter immune responses has been documented, including poorly soluble NPs of low toxicity, such as nano-sized titanium dioxide (TiO_2) and carbon black (CB) (Di Gioacchino et al., 2011; Hussain et al., 2012; Tin Tin Win et al., 2006). NP-induced oxidative damage could be one of the leading factors causing an immune imbalance because oxidative stress plays an important role in the pathogenesis of allergies and asthma (Hussain et al., 2009, 2010). Many types of NPs have been shown to produce oxidative stress under *in vivo* (Oberdörster, 2004; Park and Park, 2009; Trouiller et al., 2009) and *in vitro* (Hussain et al., 2009; Park and Park, 2009; Shvedova et al., 2003) conditions.

Abbreviations: CB, carbon black; CB-NP, carbon black nanoparticle; cDNA, complementary DNA; GD, gestational days; nm, nanometer; NPs, nanoparticles; PND, postnatal day; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Th, T helper; TiO_2, titanium dioxide.

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Additionally, the immunotoxic effects of NPs include their ability to affect T helper cell type 1 (Th1)/Th2 balance (adaptive immune response) (van Zijverden et al., 2000) and to induce or modify the maturation and differentiation of dendritic cells (Park et al., 2010; Yoshida et al., 2010).

Based on the data collected thus far on different chemicals, drugs and pollutants, the developing immune system can be considered to be significantly more sensitive to xenobiotic insults than the adult immune system (Di Gioacchino et al., 2011). Moreover, there is increasing concern that exposure to NPs during sensitive stages of development (intrauterine life) may predispose the developing organism to diseases later in life. Indeed, experimental studies have revealed that exposure to particulate matter in ambient air is associated with adverse pregnancy outcomes (Hougaard et al., 2008), such as premature birth, reduced birth weight and small size for gestational age (Shah et al., 2011; Takeda et al., 2011), due to intrauterine growth restriction (Xu et al., 2009). It is suggested that the fetus is affected either directly by particles translocating through the placenta (Takeda et al., 2009) and by altered placental function (Yamashita et al., 2011); or indirectly by circulating cytokines or other secondary messengers that are activated in response to inflammation and/or oxidative stress in exposed mothers (Hougaard et al., 2011; Kannan et al., 2006). Maternal exposure to nano-sized TiO_2, CB or diesel exhaust particles seems to promote offspring immune responses to allergens (Fedulov et al., 2008). CB nanoparticles (CB-NP) are attractive benchmark nanoparticles because their toxic effects have been well characterized. In the present study, CB-NP was used as a model nanoparticle to investigate the hypothesis that maternal respiratory exposure to NPs during middle and late pregnancy affects development of lymphoid organs, primarily the offspring's thymus and spleen.

2. Materials and methods

2.1. Carbon-black nanoparticles

PRINTEX 90®, purchased from Degussa Ltd. (Frankfurt, Germany), was used as a CB-NP. CB PRINTEX 90 is a well-characterized carbonaceous core nanoparticle that consists of carbon with less than 1% organic and inorganic impurities (Brown et al., 2000; Jacobsen et al., 2007; Wilson et al., 2002). The primary particle size and surface area of CB-NP are 14 nm and 300 m^2/g, respectively. The particles were suspended at a concentration of 5 mg/ml in distilled water and sonicated for 30 min, followed by filtration through a 450-nm filter (S-2504, Kurabo Co., Ltd. Osaka, Japan) to remove bulk agglomeration. The peak size distribution and concentration of CB-NP in the filtrated suspension were 84.2 nm and 95 μg/ml, respectively (Onoda et al., 2014).

2.2. Animals and treatments

Pregnant ICR mice were purchased from SLC Inc. (Shizuoka, Japan). The mice were housed in a room at a controlled temperature (23 ± 1 °C) and humidity (55 ± 5%), with a 12-h dark/light cycle and *ad libitum* access to food and water. The pregnant mice were put into an anesthesia box filled with halothane and removed from the box when they began to sleep. The mice were immediately laid on their backs and treated with 1 ml/kg body weight of CB-NP suspension (95 μg/ml, for the CB-NP group, $n = 11$) or distilled water (for the control group, $n = 8$) by intranasal instillation into both nostrils. The treatment was performed on gestational days (GDs) 9 and 15, which correspond to the presence of proper embryonic thymus and spleen development (Blackburn and Manley, 2004; Dietert and Holsapple, 2007; Hollander et al., 2006). After treatment of the pregnant mice and the birth of the litters, the thymus and spleen were collected from their offspring on postnatal day (PND) 1, 3 and 5 under sodium pentobarbital anesthesia. The experimental protocol used in this study is summarized in Supplementary Fig. S1. The animal experiments were performed in accordance with the institutional and national guidelines for the care and use of laboratory animals. All efforts were made to minimize the number of mice used and their suffering.

2.3. Flow cytometry

Fluorescein isothiocyanate-labeled anti-CD3 and anti-CD4 antibodies and phycoerythrin-labeled anti-CD8 and anti-B220 antibodies were provided by Abe Laboratory (Division of Immunobiology, Research Institute for Biological Sciences, Tokyo University of Science, Japan). Single-cell suspensions of thymus and spleen in RPMI-1640 (1×10^6 cells/ml) were prepared using frosted glass slides. The suspensions were washed in FACS medium (phosphate-buffered saline containing 1% fetal bovine serum and 0.1% sodium azide) and treated with anti-FcR (2.4G2), followed by staining with fluorescently labeled antibodies. The cells were then washed, resuspended in the FACS medium and subjected to analysis. Dead cells were excluded by forward light scatter gating and propidium iodide staining. The fluorescent data of 10,000 lymphocyte events per sample were acquired on a BD FACS Canto™ II (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo 7.2.2. software (Tomy Digital Biology Co., Ltd Tokyo, Japan).

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from thymus and spleen tissues with Isogen (Nippon Gene Co., Ltd. Tokyo, Japan). Total RNA (1 μg) was used as a template to make the first strand of complementary DNA (cDNA) using M-MLV Reverse Transcriptase (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR Green Real-Time PCR Master Mix (Toyobo Co. Ltd. Osaka, Japan) and primers (Fasmac Co., Ltd. Kanagawa, Japan) for the indicated genes (Table S1). The values of target genes were normalized to the expression level of the housekeeping gene, Gapdh.

2.5. Statistical analysis

All data are expressed as the mean ± standard deviation (SD), and the levels of significance are cited. SPSS statistical package

Table 1
Number and sex ratio of offspring.

Group	Number of dams	Number of offspring/dam[a]			Total offspring	Sex ratio (%) (Male/(males + females) × 100)
		Male	Female	Total		
Control	8	5.75 ± 2.92	5.38 ± 3.66	11.00 ± 5.18	89	51.69
CB-NP	11	6.27 ± 2.57	7.09 ± 2.34	13.36 ± 3.70	147	46.94

[a] Dams were allowed to deliver their pups on gestational day 19, equal to postnatal day (PND) 0. Individual pups were recorded on PND 1, and pups were counted and their sex determined. Values are expressed as mean ± SD. Abbreviation: CB-NP, carbon black nanoparticle.

Table 2
Effects of maternal exposure to CB-NP on the body weight of offspring at PND 1, 3 and 5.

Group	PND 1		PND 3		PND 5	
	Male	Female	Male	Female	Male	Female
Control	2.11 ± 0.41	2.01 ± 0.37	2.25 ± 0.19	2.23 ± 0.26	3.50 ± 0.60	3.60 ± 0.36
CB-NP	2.03 ± 0.21	1.97 ± 0.16	2.54 ± 0.50*	2.35 ± 0.15	4.38 ± 0.74*	3.98 ± 0.35

Three-way ANOVA

	CB-NP main effect	Sex main effect	Age main effect	CB-NP × Sex interaction	CB-NP × Age interaction	Age × Sex interaction	CB-NP × Age × Sex interaction
p-value	<0.001	0.12	<0.001	0.16	<0.01	0.95	0.42

Values are expressed as the mean ± SD. Significantly different from the respective control group within the same PND and offspring sex: $^{*}p < 0.05$. Abbreviations: CB-NP, carbon black nanoparticle; PND, postnatal day.

version 17.0 for Windows (IBM, Armonk, NY, USA) was used for all data analyses. Three-way analysis of variance (ANOVA) was used to determine the effects of CB-NP exposure, sex, and age on body weight. Two-way ANOVA was used to assess the effects of CB-NP exposure and age on the flow cytometry data, and the effects of CB-NP exposure and sex on mRNA expression data. The ANOVA analyses were combined with a *post hoc* least significant difference (LSD) test when appropriated. An independent-sample *t*-test was performed to assess significant differences between the treated and the respective control groups for the number of pups/dam analysis. Significance was determined to be $p < 0.05$.

3. Results

3.1. Number, sex ratio and body weight of offspring

No deaths or changes related to CB-NP intranasal instillations in pregnant ICR mice were observed during the exposure period. There were no significant differences in the number of offspring per dam or the sex ratio of live pups at birth between the control and the CB-NP groups (Table 1). Three-way ANOVA showed the significant main effects for CB-NP exposure [$F(1, 119) = 11.92$; $p < 0.001$] and offspring age [$F(2, 119) = 178.56$; $p < 0.001$] with CB-NP/offspring age interaction [$F(2, 119) = 5.74$; $p < 0.01$] on the body weight of neonates. A *post hoc* LSD test showed that the body weight of male neonates was significantly increased ($p < 0.05$) at PND 3 and 5 (Table 2).

3.2. Total count and immunophenotypes of lymphocytes in the thymus and spleen

To investigate the postnatal immunotoxic effects of CB-NP exposure, we examined the total lymphocyte count and the immunophenotyping of lymphocytes within the thymus and spleen of male offspring. A significant main effect of CB-NP exposure was detected on the total number of splenocytes with CB-NP exposure/age interaction, where the total number of splenocytes was significantly increased ($p < 0.05$) at PND 5 (Fig. 1, Table S2). Additionally, CB-NP exposure significantly increased the $CD3^+$ (T lymphocytes), $B220^+$ (B lymphocytes), $CD3^-B220^-$, $CD4^+$ (helper T cells) and $CD4^-CD8^-$ phenotypes, with significant CB-NP exposure/age interaction on the $CD3^-B220^-$ and $CD4^-CD8^-$ phenotypes that were significantly increased ($p < 0.05$) at PND 5 in the spleen of neonates whose dams were exposed to CB-NP (Fig. 2, Table S2). Maternal exposure to CB-NP, however, did not affect splenic $CD8^+$ population. Moreover, CB-NP exposure significantly increased ($p < 0.05$) the total lymphocyte count, their immunophenotypes of $CD4^-CD8^-$ and $CD4^+CD8^+$ cells in the thymus of offspring whose dams were exposed to CB-NP (Figs. 1 and 3, Table S2).

3.3. Quantitative analysis of thymic and splenic mRNA expression

To clarify the observed changes in the number of T cells, gene expression profiles in the thymus (PND 1) and spleen (PND 3) were

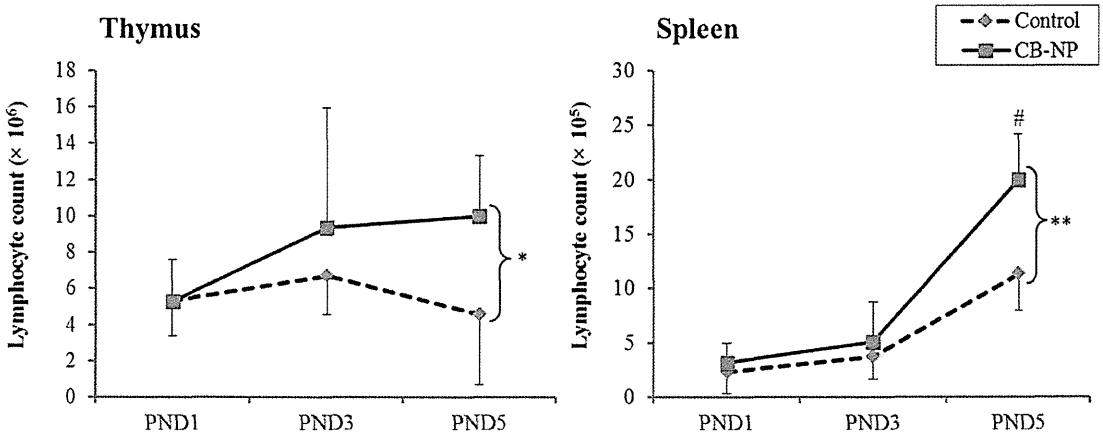

Fig. 1. Effect of prenatal exposure to CB-NP on the total lymphocyte count in the thymus and spleen of male offspring at PND 1, 3 and 5. Values are expressed as mean ± SD. Two-way ANOVA showed a significant main effect of CB-NP exposure on the total lymphocyte count in the thymus [$F(1, 29) = 4.39$; $^{*}p < 0.05$] without CB-NP exposure/age interaction; and in the spleen [$F(1, 27) = 11.52$; $^{**}p < 0.01$] with CB-NP exposure/age interaction [$F(2, 27) = 5.30$; $p < 0.05$]. *Post hoc* LSD test showed that the total lymphocyte count in the spleen was significantly increased ($^{\#}p < 0.05$) at PND 5. Abbreviations: CB-NP, carbon black nanoparticle; PND, postnatal day.

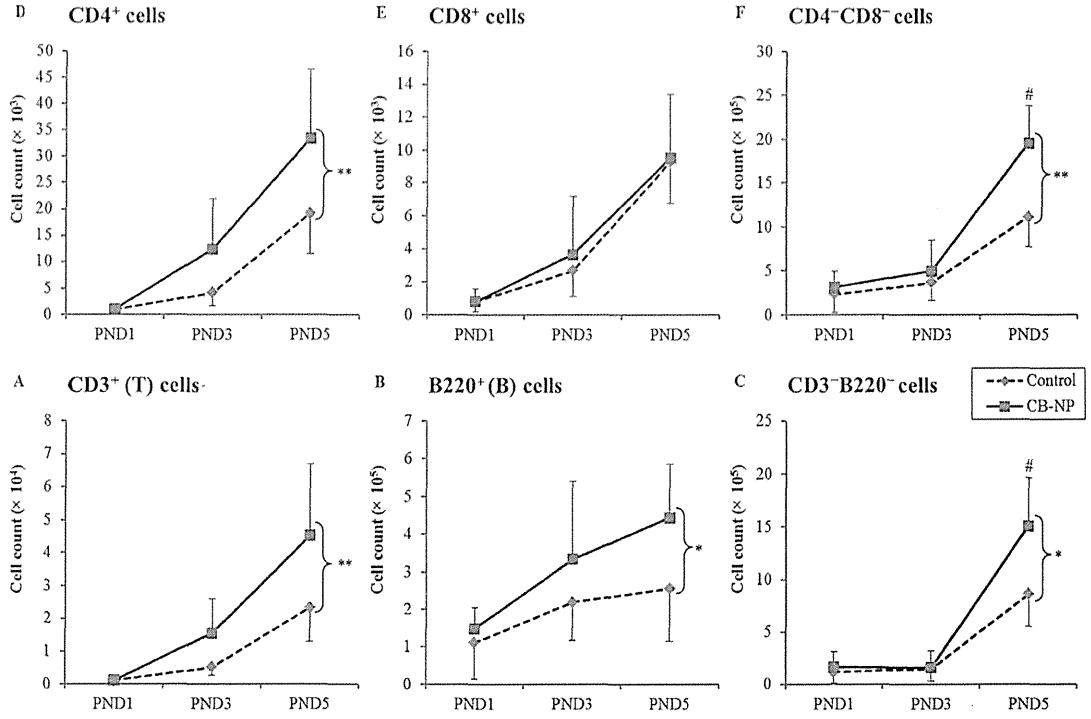

Fig. 2. Effects of prenatal CB-NP exposure on the number of CD3, B220, CD4 and CD8 cells in the spleen of male offspring at PND 1, 3 and 5, as determined by flow cytometry. Values are expressed as mean ± SD. Two-way ANOVA showed significant main effects for CB-NP exposure on (A) $CD3^+$ [$F(1, 27) = 8.44$; $^{**}p < 0.01$], (B) $B220^+$ [$F(1, 27) = 6.10$; $^{*}p < 0.05$], (C) $CD3^-B220^-$ [$F(1, 27) = 7.36$; $^{*}p < 0.05$], (D) $CD4^+$ [$F(1, 27) = 8.84$; $^{**}p < 0.01$], and (F) $CD4^-CD8^-$ [$F(1, 27) = 11.16$; $^{**}p < 0.01$] cell number, with significant CB-NP exposure/age interaction on $CD3^-B220^-$ [$F(2, 27) = 5.12$; $p < 0.05$], and $CD4^-CD8^-$ [$F(2, 27) = 5.20$; $p < 0.05$] cell numbers. *Post hoc* LSD test showed that the $CD3^-B220^-$ and $CD4^-CD8^-$ cells were significantly increased ($^{\#}p < 0.05$) in PND 5 offspring. Abbreviations: CB-NP, carbon black nanoparticle; PND, postnatal day.

examined by qRT-PCR in male and female offspring. Two-way ANOVA showed significant main effects for CB-NP exposure on offspring thymic mRNAs encoding Traf6, and for offspring sex on thymic mRNAs encoding IL-7 and Themis genes, without CB-NP exposure/sex interaction. Two-way ANOVA did not show any significant main effects for CB-NP exposure or offspring sex on the mRNA expression levels of thymic IL-7, Ccl19, Ccr7, Themis, Runx3 and Aire genes (Fig. 4, Table S3), and of splenic IL-7, IL-15, Ccl19, T-bet, Stat4, Stat6, Gata3, Socs5 and Runx3 genes (Fig. 5, Table S3).

4. Discussion

Developmental immunotoxicity has gained increasing recognition as a significant factor for influencing the risk of disease in later life (Dietert and Holsapple, 2007). Immunosuppression, which is associated with an elevated risk of infectious diseases and cancer, is not only the concern. The immunotoxic changes that increase the risk of autoimmune, inflammatory or allergic responses have also been considered (DeWitt et al., 2012; Dietert, 2011; Dietert and Holsapple, 2007). It has been reported that CB-NP administered intratracheally can partially pass the air-blood barrier (Shimada et al., 2006), cause pulmonary inflammation and translocate to the mediastinal lymph nodes (Tin Tin Win et al., 2005). The intranasal co-administration of particles with an antigen is reported to be a more effective way to stimulate an immune response in mice than separate particle and antigen dosing (de Haar et al., 2005; van Zijverden et al., 2001). The present study was motivated by concerns related to the adverse effects of CB-NP on the developing immune system of infant mice that may result from exposure during the prenatal period. The thymus is a primary lymphoid organ in which bone marrow-derived T cell precursors undergo differentiation, leading to the migration of $CD4^+$ and $CD8^+$ selected thymocytes to the T cell-dependent areas of peripheral lymphoid organs as the naïve T cells (Savino, 2006; Zlotoff and Bhandoola, 2011). Additionally, evaluating the expression of regulatory genes during intrathymic T cell development is critical for understanding the cellular and molecular interactions that constitute the immune response (Anderson and Jenkinson, 2000). The spleen is one of the most important lymphoid organs involved in the initiation of the immune response (Mebius and Kraal, 2005). It has a central function in the immune system and is highly sensitive to damage by xenobiotics (de Visser et al., 2006; Son et al., 2010). Lymphocyte proliferation is an important phase in the immune response (Lee et al., 2013).

In newborns, migrated thymic cells account for a major portion of the total lymphocyte populations in the lymph nodes and spleen (Weissman, 1967). Therefore, the increased thymocytes and their immunophenotypes of $CD4^-CD8^-$ and $CD4^+CD8^+$ T cells seem to be responsible for the overall high numbers of increased populations of splenic cells, particularly at PND 5, in the spleen of offspring that were prenatally exposed to CB-NP; reflecting stimulated immature splenocytes. The increased population of $CD3^-B220^-$ splenic cells and total number of splenocytes is generally known to be associated with allergic and/or inflammatory response (von Freeden-Jeffry et al., 1998; Walker et al., 2013; Wolterink et al., 2012). $CD3^-B220^-$ splenic cells "non-T/non-B lymphocytes" include the cell population representing group 2 innate lymphoid cells (ILC2s, also known as natural helper cells, noucytes or innate helper cells), which promptly produce large amounts of the Th2 cytokines in response to IL-7, IL-25 or IL-33 stimulation (Koyasu and Moro, 2011; Licona-Limon

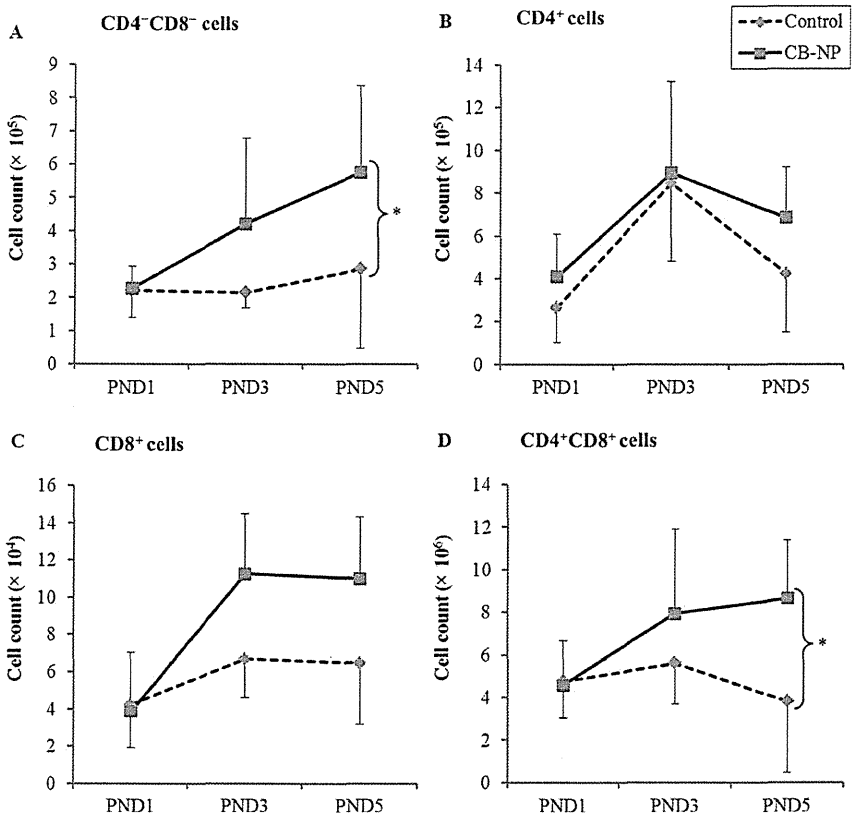

Fig. 3. Effects of prenatal CB-NP exposure on the number of CD4 and CD8 cells in the thymus of male offspring at PND 1, 3 and 5, as determined by flow cytometry. Values are expressed as mean ± SD. Two-way ANOVA showed significant main effects for CB-NP exposure on (A) $CD4^-CD8^-$ [$F(1, 29) = 4.34$; $^*p < 0.05$] and (D) $CD4^+CD8^+$ [$F(1, 29) = 4.20$; $^*p < 0.05$], without significant effects on (B) $CD4^+$ or (C) $CD8^+$ cell numbers. Non-significant main effects for CB-NP exposure/age interaction were observed on $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+$ and $CD8^+$ cell numbers. Abbreviations: CB-NP, carbon black nanoparticle; PND, postnatal day.

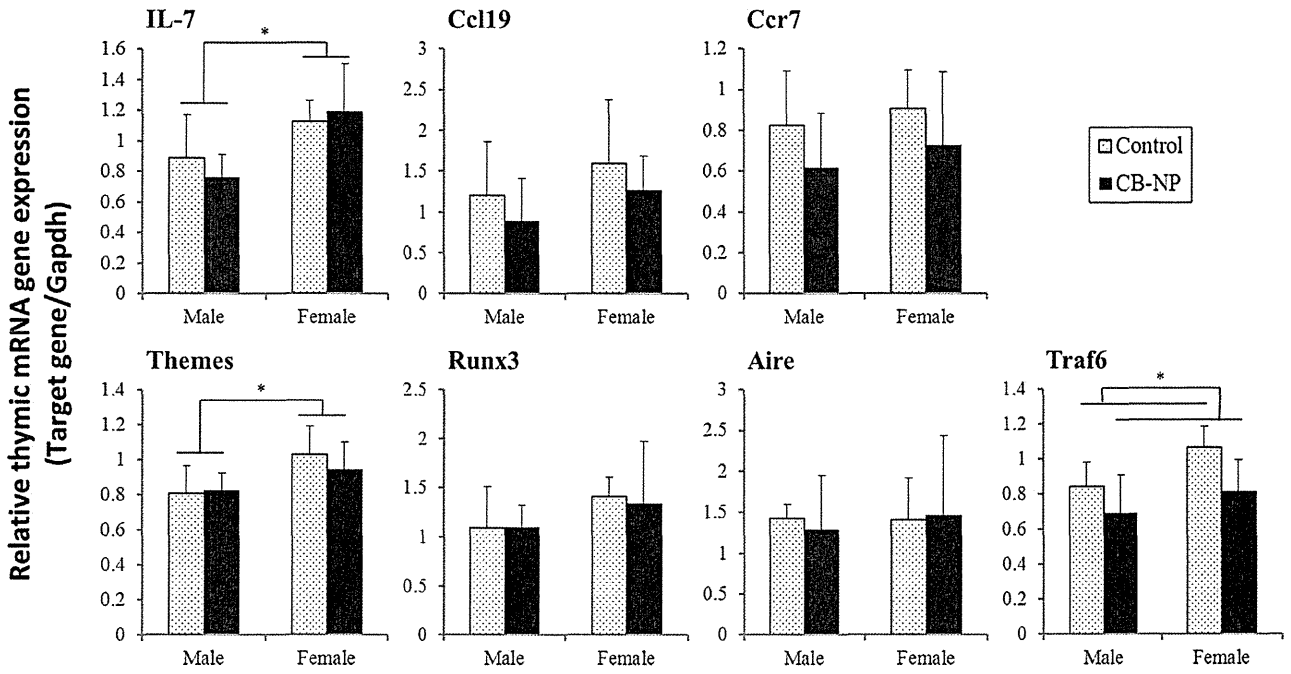

Fig. 4. mRNA expression levels of IL-7, Ccl19, Ccr7, Themis, Runx3, Aire and Traf6 in the thymus of postnatal day 1 offspring as examined by qRT-PCR. Values are expressed as mean ± SD. Two-way ANOVA showed significant main effects for CB-NP exposure on Traf6 [$F(1, 17) = 5.17$; $^*p < 0.05$], and for offspring sex on IL-7 [$F(1, 17) = 8.03$; $^*p < 0.05$] and Themis [$F(1, 17) = 6.58$; $^*p < 0.05$] genes. Abbreviation: CB-NP, carbon black nanoparticle.

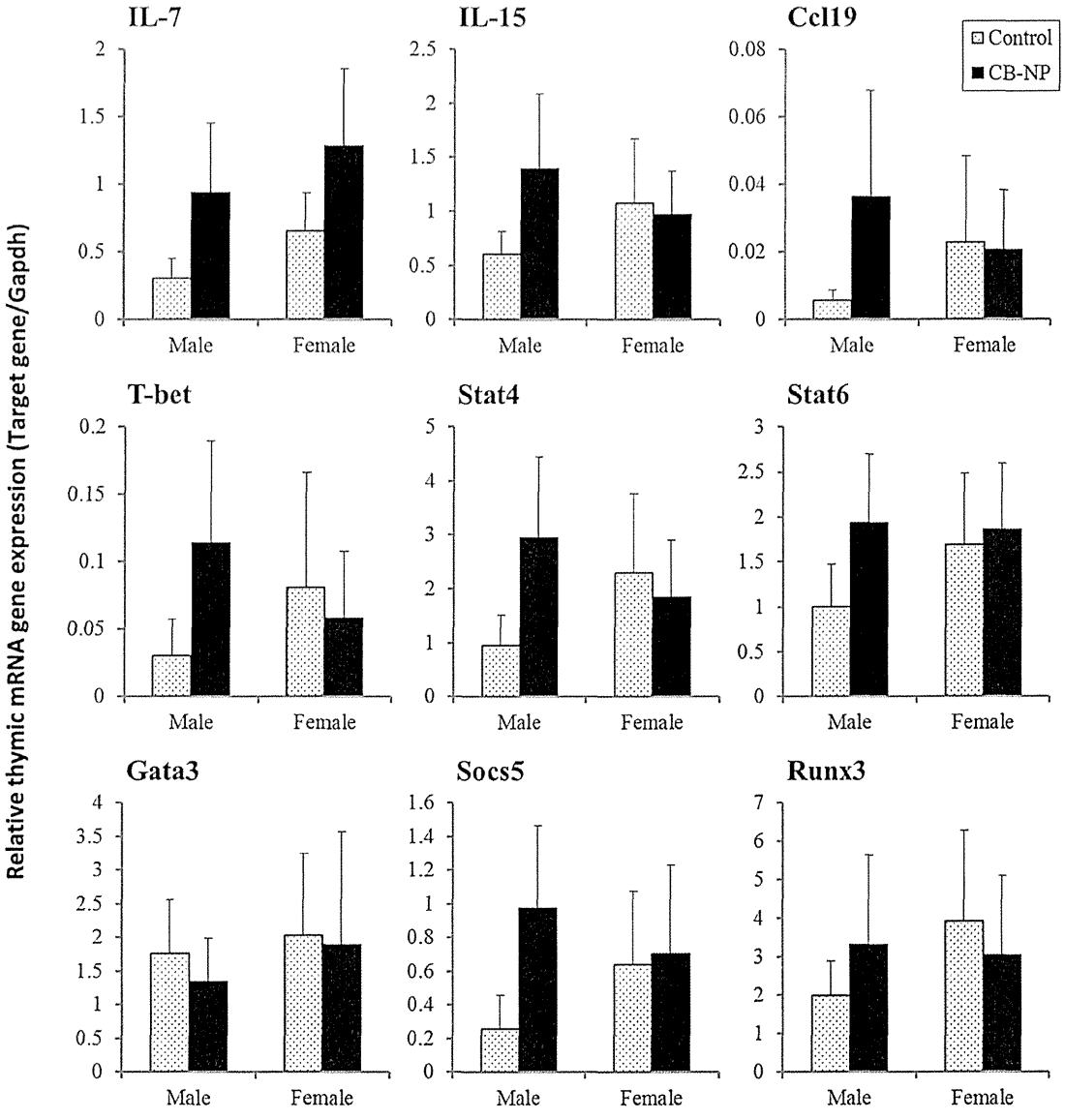

Fig. 5. mRNA expression levels of IL-7, IL-15, Ccl19, T-bet, Stat4, Stat6, Gata3, Socs5 and Runx3 in the spleen of postnatal day 3 offspring as examined by qRT-PCR. Values are expressed as the mean ± SD. Abbreviation: CB-NP, carbon black nanoparticle.

et al., 2013; Walker et al., 2013). ILC2s are responsible for the immunopathology that develops in association with allergic inflammatory diseases and asthma (Licona-Limon et al., 2013; Walker et al., 2013). $CD4^-CD8^-$ cell subsets originate in the thymus by escaping negative selection followed by migration in the periphery (Priatel et al., 2001), and are present in the peripheral lymphoid organs of mice at PND 3 (Duncan et al., 2010). They belong to the T cell compartment and carry the ability to inhibit inadequate immune response and promote peripheral immune tolerance in various autoimmune settings (Hillhouse and Lesage, 2013).

Studying the gene expression profiles in the thymus and spleen may provide insight into the mechanisms governing the development, proliferation and migration of T cells (Anderson and Su, 2011; Bosselut, 2004). The lack of severe change in the thymic phenotypes, which are sensitive to maternal stress (Moore et al., 2009; Park et al., 2008), may mean that the prenatal CB-NP exposure in the present study was not particularly stressful to the fetus or offspring. Meanwhile, the qRT-PCR assay confirmed a significant main effect for CB-NP on the mRNA expression of thymic Traf6 gene in newborn mice. Traf6 is a member of the Traf (TNF receptor-associated factor) family of proteins, which have been characterized as adaptor molecules that mediate signals induced by the TNFR superfamily (Arch et al., 1998; Chung et al., 2002). Traf6 is required for the induction and maintenance of peripheral T-cell self-tolerance (Akiyama et al., 2005). Interestingly, the thymic Traf6 results confirm the finding of an increased $CD4^-CD8^-$ splenocyte population. Taken together, these data suggest that the expression level of Traf6 might be an important factor for extrathymic $CD4^-CD8^-$ cluster development. Moreover, the lack of changes in the Runx3 gene in the spleen is consistent with the $CD8^+$ T cell-related results. Runx3 is expressed in mature $CD8^+$ T cells (Egawa et al., 2007) but not in naïve $CD4^+$ T cells; however, its expression is upregulated during Th1 cell differentiation (Djuretic et al., 2007). The sex difference in the developmental toxicity of NPs is important for understanding their risk to

human and animal health. Our data only showed significant main effects for offspring sex on thymic IL-7 and Themis mRNA expression in newborn offspring. No significant interaction between maternal exposure and offspring sex was found. Jackson et al. (2012) observed that mRNA expression of hepatic genes related to inflammatory disease was altered in male offspring prenatally exposed to CB-NP via maternal pulmonary exposure, while altered genes were mainly associated with metabolic and endocrine disorders in female offspring.

Taken together, our results suggest that maternal exposure to CB-NP can induce dysregulation of lymphocyte populations and that the spleen and thymus are target organs in offspring. In addition, the changes in the lymphocyte population representative of immunostimulation may be mediated via allergic reactions and inflammatory responses. In contrast, a previous study showed that prenatal intranasal instillations of CB-NP suspension on GD5 and 9 induced a phenotype similar to immunosuppression in newborn mice, which was characterized by the depletion of splenic $CD3^+$, $CD4^+$ and $CD8^+$ T cells in newborn mice (Shimizu et al., 2014). However, the neonates of mothers that were intranasally exposed to a single dose of 250 μg CB-NP on GD14 developed a more pronounced asthmatic phenotype than did the sham-exposed control offspring (Fedulov et al., 2008). Thus, the dose level of maternal exposure to NP and the route of exposure during later-stage gestation seemed to promote offspring immune responses to allergen sensitization. Increased reactivity of the immune system has also been reported in response to other prenatal particulate exposure, including diesel exhaust exposure during mid-gestation (Watanabe and Ohsawa, 2002), leachate of residual oil fly ash during later-stage gestation (Hamada et al., 2007), and tobacco smoke (Penn et al., 2007; Singh et al., 2003) and exhaled nitric oxide (Latzin et al., 2009) throughout gestation, which were linked to asthma or allergies. These reports support our suggestion that *in utero* CB-NP exposure after thymic and splenic development induces immune-activating effects in newborn offspring, such as allergic or inflammatory responsiveness, as evidenced by alteration in lymphocytic phenotyping and other effects, which were dependent on the stage of gestation in which exposure occurred. NPs may activate systemic immune responses through several possible mechanisms, including the absorption of blood proteins (such as complement) to induce phagocyte activation (Salvador-Morales et al., 2006), an increase in cytokine expression (Dobrovolskaia and McNeil, 2007) and the induction of exosomes (extracellularly secreted membrane nanovesicles) (Zhu et al., 2012) as signaling mediators in the induction of dendritic cell maturation and splenic T cell immune activation.

In summary, maternal respiratory exposure to CB-NP during critical periods of development induced alteration in lymphocytic phenotyping and gene expression related to the induction of peripheral tolerance that may be a predictive of allergic or inflammatory responsiveness in the early life of newborn offspring. The magnitude and nature of the neonatal host immune response and the allergic/inflammatory response elicited by NPs were based on the stage of gestation in which they were exposed.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2014.11.005.

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トキシコロジーからナノ規制ガバナンスへの提言 - 予防原則の最適化

Proposal of regulation and governance of nanomaterials from toxicology for optimizing the precautionary principle

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Abstract. Over the last decade, rapid progress has been made in the field of nanotechnology, especially regarding knowledge of health issues pertaining to toxicity of nanomaterials. This progress can be attributed to the increase in concerns regarding the risks associated with nanomaterials, increase in the research data available for discussion, and implementation of regulations based on reported data. The toxicity characteristics of nanoparticles are under investigation. However, advances have been made in research on design of nanomaterials with low toxicity, and limiting exposure to these materials to avoid health risks. In addition to these developments, regulations for a "Nano Labeling" and "Nano Product Registration" is also being established. Taking a comprehensive view of the current situation, effective policies for avoiding such risks in the future will be discussed.

Key Words: Nanomaterial, Toxicology, Regulation, Definition, Measurement

1．はじめに

ナノ材料による潜在的な健康影響の問題は、ハザード懸念の勃興、基礎研究データの蓄積から、それに基づいた規制への動きまでが急速に進められてきた。物質がナノサイズであるために生じる有害性の特徴も明らかになりつつある。そのリスク回避のために、曝露の抑制と有害性の小さいナノ材料設計のための研究が進められている。その過程で「ナノ表示」や「ナノ製品の登録」といった規制も並行して進められている。これらの現状を総括し、将来にわたる効果的なリスク回避の方策のあり方を考えたい。

2．ナノ規制の対象の定義

規制の対象になるナノ材料の定義は、従来は「少なくとも一次元が1～100 nmである」物体というものであった。この定義の下で、わが国では2009年3月に、厚生労働省労働基準局通達「ナノマテリアルに対するばく露防止等のための予防的対応について」が出され、ナノ材料の曝露がヒトの健康に及ぼすリスクを予防する観点から、曝露抑制のための方法が示された。一方で、現実のナノ材料が粒子径分布に一定の幅を示す粒子の集合体である（単一のサイズ（径）を持たない）ために、従来の定義ではナノ材料の規制が運用しづらいこ

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とが指摘されていた。

その指摘の中で、欧州委員会 (2011) がナノ材料の定義の勧告を発表した。そこでの定義は、“個数基準の粒子径分布で、少なくとも一次元が 1～100 nm である粒子が 50%以上（50%という閾値は、1～50%の間で置き換えてもよい）の材料とし、凝集した粒子もそれを構成する一次粒子まで数える」というものであった。

この定義は、リスクの予防原則とナノ材料の測定の観点から大きな問題を提起している。まず、この定義では一次粒子径が 1～100 nm の物質が、当該ナノ材料（集合体）の 50%未満（個数ベース）であればナノ材料の規制を受けないが、それで問題がないのかという指摘である。毒性学は、この線引きが種々のナノ材料の安全性の担保につながっているかを検証し、さらには対案を出すことが求められていると言えよう。

さらに、欧州委員会の勧告はナノ材料の定義に一次粒子径を含めたが、その測定は現状不可能と言わざるを得ない。一次粒子径の計測は現状、透過型もしくは走査型電子顕微鏡（TEM、SEM）に頼ることになるが、この技術では粒子径分布を定量的に計測することが困難である。一方で、粒子の沈降速度や光散乱などの物理的な現象から粒子径分布を測定する方法では、凝集した粒子が一体となった挙動を示すために、凝集粒子を構成する一次粒子の径を測定できない。リスクをもたらす物質をどう定義し、そのリスク管理の枠組みを計測手法とあわせてどのように提示すべきかという点が、ナノ材料のリスク管理の大きな課題である。

3．ナノ粒子の毒性メカニズム

ナノ粒子による健康影響は従来、大気汚染のうち超微小な浮遊粒子が健康影響に大きく寄与する可能性の懸念から、注目されるようになった。まず、米国での疫学（ハーバード六都市）研究により PM2.5 の健康影響に注目が集まった（Dockery ら、1993）。さらに、径の異なる微小粒子（難溶解性）をそれぞれ同じ質量、動物に気管内投与した場合に、粒子径の小さい粒子の方が誘導される炎症の程度が大きいことが報告された（Oberdörster ら、2000）。ここから、環境中ナノ粒子 (UFPs: ultrafine particles)、さらには工業ナノ材料の持つハザードに対する懸念が起こった。

ナノ粒子特有の毒性メカニズムは主に、①大きな比表面積に起因する高い反応性と、②小さな動力学的粒子径にに起因する独特の体内動態の 2 つである。ナノ粒子の高い反応性は、例えば粒子表面で酸化還元反応を起こしやすく、生体に大きな酸化ストレスを誘導できることによる毒性が指摘されてきた（Nel ら、2006）。体内動態については、吸入した際に肺の深部にまで到達して蓄積しやすいこと（Oberdörster ら、2005）、肺から肺外臓器に少量ながら移行すること（Kreyling ら、2002；Oberdörster ら、2002）、胎盤通過能があり（Wick ら、2010）妊娠動物に投与したナノ粒子が出生仔の主要臓器に移行・蓄積すること（Takeda ら、2009）などが報告されている。

ナノ粒子が示す胎盤通過能などの血液組織関門の透過性は、受動拡散が起こりやすいことに起因するわけではないようである。体内に入った「10～100 nm 程度の径を持つ」ナノ粒子は、クリアランス（排出）されにくく体内に長く留まりやすいことが報告されている（Choi ら、2010）。血液組織関門の本態は、細胞がある物質を取り込まない性質を持つことでなく、取り込んだ物質を排出する系が働いていることである。ナノ粒子が細胞に容易に取り込まれるサイズであり、かつクリアランスを受けにくいという特徴を持つという事実は、物質を「ナノ」というサイズの特徴から規制すべきという根拠の一つになると考えられる。

ナノ粒子の毒性が表面積に依存する反応性の高さであるとすれば、そのリスクを計る上で、表面積の大きさに寄与し得る一次粒子径が重要であると言える。一方で、ナノ粒子の毒性が独特の体内動態によるとすれば、考慮すべきは凝集体としての二次粒子径だけであると言える。ナノ粒子のトキシコロジー研究により毒性メカニズムの本質を明らかにし、それを踏まえたナノ粒子の規制のあり方を、規制対象の定義とあわせて最適化してい

く必要があろうと思われる。

4．ナノ粒子は“未知の遭遇”か

ナノ粒子は産業の発展後に曝露の機会が増えたと考えられている一方で、実際には古代から環境中に存在していたことから、現代の人に新しいリスクをもたらしたわけではないという意見もある。例えば、フラーレンやカーボンナノチューブは燃焼により生成し、太古から自然界・環境中に存在していたという事実がある。また、溶解性のある銀ナノ粒子の表面に水が存在すると、銀が溶解（イオン化）し、元の銀ナノ粒子より小さな銀粒子（直径約 10 nm）が自然に生じる「再粒子化」が報告されている（Glover ら、2011）。これは、粗大な（非“ナノ”の）材料が溶解性を持つ場合に、自然発生的にナノ粒子が生じる例を示した知見であり、これも「ナノ粒子は生体にとって古くから触れてきたものであり、現代になって曝露量が増えてきたわけでもリスクが増大したわけでもない」という考えの根拠になっている。

ナノ粒子は我々人間にとって、さらには生物・生体にとって“未知の遭遇”なのであろうか。果たしてナノ規制の必要性は、これらの問いにどう答えるべきであろうか。

注意を払うべきは、ナノ粒子は液相においても気相においても、容易に凝集して大きな二次粒子径を持つという事実である。これは、環境中微小粒子の濃度と発生源からの距離との相関関係に明瞭に反映されている。環境中微小粒子の発生源の一つにディーゼルエンジンがあり、幹線道路上でのナノ粒子 UFPs や PM2.5 排出量は大きい。米国の研究機関 Health Effects Institute（HEI：大気汚染の健康影響の研究をあらゆる角度からサポートする機関）は、PM2.5 についてはその量（質量濃度）と発生源（幹線道路）からの距離との間に逆相関が認められないのに対し、UFPs の濃度については発生源からの距離との間に有意な逆相関が認められることを報告している。我々も、CPC3007（TSI 社製、気相中に浮遊する二次粒子径 20～1000 nm の粒子の個数濃度の計測機）で大気環境中の超微小粒子の濃度の測定を進めてきた。その結果、幹線道路の歩道上では認められる高濃度が、そこから障害物なしに 300 m 離れた後背地では認められないことを確認している（梅澤ら、2012）。なお、粒子径 20～1000 nm の個数濃度の大部分は、径が 100 nm 以下のいわゆるナノ粒子が占めていることも分かっている。

この事実は、環境中ナノ粒子の局所的な高濃度地点の存在を示唆するものである。これを考慮に入れると、幹線道路至近での業務の従事者に高いリスクが局在していると言える。同様に工業ナノ材料についても、生産、分析、廃棄の多様な現場における曝露の程度を評価し、職業環境における局所的高濃度から生じ得る高リスクに注意を払う必要がある。逆に言えば、環境中に常に存在するレベルよりも高濃度でナノ粒子が存在し得る地点に焦点を絞り、ここにリスク管理の高い優先順位を付けて対応することが必要であろう。

5．結論

ここまで述べたように、ナノ規制を実効性のあるものにするためには、まず規制対象を定量的に計測できる形で定義する必要がある。また、ナノ粒子の凝集性を考慮し、高濃度にナノ粒子が局在する地点に優先順位を付けることが、実効性のある施策につながるであろう。トキシコロジー研究は現在、既存ならびに新規のナノ材料を簡便にハザードレベルで分類する技術や知見を創出しようとしているところである。このハザード分類は、各ナノ材料について取るべき許容曝露量や用途の明示に貢献するであろう。こういった情報を、今後も新規に創出されていくナノ材料のリスク管理にどのように活かすべきかについて、トキシコロジーならびにリスク研究者の工夫や働きかけが今後いっそう求められると考えられる。

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Original Article

Effect of maternal exposure to carbon black nanoparticle during early gestation on the splenic phenotype of neonatal mouse

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ABSTRACT — Maternal exposure to environmental factors is implicated as a major factor in the development of the immune system in newborns. Newborns are more susceptible to microbial infection because their immune system is immature. Development of lymphocytes reflects an innate program of lymphocyte proliferation. The aim of this study was to investigate the effects of maternal exposure to carbon black nanoparticle (CB-NP) during early gestation on the development of lymphoid tissues in infantile mice. Pregnant ICR mice were treated with a suspension of CB-NP (95 μg kg^{-1} $time^{-1}$) by intranasal instillation on gestational day 5 and 9. Spleen tissues were collected from offspring mice at 1, 3, 5, and 14 days post-partum. Splenocyte phenotypes were examined by investigating the pattern of surface molecules using flow cytometry. Gene expression in the spleen was examined by quantitative RT-PCR. $CD3^+$ (T), $CD4^+$ and $CD8^+$ cells were decreased in the spleen of 1-5-day-old offspring in the treated group. Expression level of *Il15* was significantly increased in the spleen of newborn male offspring, and *Ccr7* and *Ccl19* were increased in the spleen of female offspring in the CB-NP group. Splenic mRNA change profiles by CB-NP were similar between male and female offspring. This article concluded that exposure of pregnant mothers to CB-NP partially suppressed the development of the immune system of offspring mice. The decrease in splenic T cells in the treated group recovered at 14 days after birth. This is the first report of developmental effect of nanoparticle on the lymphatic phenotype.

Key words: Carbon black, Maternal exposure, Newborn, Spleen, T lymphocyte

INTRODUCTION

Newborns are more susceptible to microbial infection than adults because their immune system is immature (Levy, 2007). Lymphoid tissue develops dynamically in the first few days post-partum (Fagoaga *et al.*, 2000), reflecting an innate program of lymphocyte proliferation, which is independent of pathogen stimulation (Forni *et al.*, 1988). Lymphocytes in secondary lymphoid organs define host defense capabilities and thus immune activity status. Maternal exposure to environmental factors has been implicated as a major factor influencing newborn immune system. For example, maternal exposure to dioxin induced atrophy of thymus and decrease of thymocytes in the offspring (Camacho *et al.*, 2004; Mustafa *et al.*, 2009). Recently, maternal exposure to nanoparticles (defined as substances measuring 1-100 nm in at least one dimension) in nanotechnology (Kessler, 2011) or to those suspended in the air (Fukuhara, *et al.*, 2008), has become a major focus in research on environmental effects on human health.

Previous studies have suggested that exposure of preg-

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nant mice to nanomaterials affects various organs in their offspring (Ema *et al.*, 2010). Transfer of titanium dioxide (TiO_2) nanoparticles administered subcutaneously to pregnant mice (treated on gestational days 6-18; total dose: 400-500 μg/mouse) to the body of their offspring was demonstrated by elemental analysis with energy dispersive X-ray spectroscopy (EDX) (Takeda *et al.*, 2009). Subsequent studies indicated that maternal exposure to TiO_2 nanoparticle altered gene expression related to brain development (Shimizu *et al.*, 2009) and mainly affected the function of prefrontal region and dopaminergic neuronal systems (Takahashi *et al.*, 2010; Umezawa *et al.*, 2012). Inhalation of TiO_2 nanoparticle to pregnant mouse (on gestation days 8-18; 1 hr/day to 42 mg/m^3 aerosolized powder) also affected moderately behavior of offspring mouse (Hougaard *et al.*, 2010). Transmission electron microscopy also showed that TiO_2 and silica nanoparticles had passed from pregnant mice to the liver of their offspring (Yamashita *et al.*, 2011). Maternal exposure to carbon or TiO_2 nanoparticles also altered genes expression in the lung of pregnant mothers (Lamoureux *et al.*, 2010), increased allergic susceptibility in airways (Fedulov *et al.*, 2008), altered the phenotype of perivascular cells in the brain of offspring (Onoda *et al.*, 2014), and affected behavior and sexual development of female offspring (Jackson *et al.*, 2011) and male offspring (Takeda *et al.*, 2009; Yoshida *et al.*, 2010; Kubo-Irie *et al.*, 2014). The effects on renal *Col8a1* expression (Umezawa *et al.*, 2011) and hepatic gene expression profile (Jackson *et al.*, 2012a, 2013) in their offspring were also reported. Furthermore, in human placental perfusion model showed that nano- and submicro-sized particles (< 240 nm, polystyrene beads) can cross the placental barrier (Wick *et al.*, 2010). Suppressive effects of a carbon nanomaterial, fullerene, on an allergic hypersensitivity of adult mouse was also shown (Yamashita *et al.*, 2009). Although the transport of nanoparticles to offspring after pulmonary exposure may be low proportion (Sadauskas *et al.*, 2007), nanoparticles may also affect developing fetus indirectly by circulating cytokines or other secondary messengers that are activated in response to inflammation and/or oxidative stress in the exposed mothers (Kannan *et al.*, 2007; Jackson *et al.*, 2012a). We hypothesized that nanoparticles may influence systemic biological systems as well as the immune system. Splenic phenotypes determined by flow cytometry and mRNA expression analyses provide indices of the immunological status under infectious (Tasker *et al.*, 2008) and immunosuppression disorders (Clouser *et al.*, 2012). Here, we investigated the effects of carbon black nanoparticle (CB-NP) administered to pregnant mice, during early gestation (gestational days 5-9), via the airway on the splenic phenotypes in infantile mice.

MATERIALS AND METHODS

Carbon black nanoparticle

PRINTEX90, purchased from Degussa Ltd. (Frankfurt, Germany), was used as CB-NP. The primary particle size and surface area of CB-NP are 14 nm and 300 m^2/g, respectively. CB-NP was suspended at 5 mg/ml in distilled water, sonicated for 30 min, and then filtrated through a 450-nm filter (S-2504; Kurabo Co. Ltd., Osaka, Japan) immediately before administration. It was characterized by field emission-type scanning electron microscopy (FE-SEM; JSM-6500F, JEOL Ltd., Tokyo, Japan) on a silicon wafer. The size distribution of filtrated CB-NP in suspension was determined by dynamic light scattering (DLS) measurement using a NANO-ZS (Sysmex Co., Hyogo, Japan) and the Rayleigh-Debye equation. The CB-NP concentration in the filtrated suspension was calculated to be 95 μg/ml by peak area of carbon signal (2.77 keV) by energy dispersive X-ray spectroscopy under the FE-SEM (JSM-6500F) (Onoda *et al.*, 2014).

Animals and treatments

All animals were treated and handled in accordance with the national guidelines for care and use of laboratory animals and with the approval of the Tokyo University of Science Institutional Animal Care and Use Committee. Fifty-four pregnant ICR mice were purchased from SLC Inc. (Shizuoka, Japan) and were randomly divided into CB-NP-treated (n = 26) and control (n = 28) groups. The mice were housed under controlled temperature (23 ± 1°C) and humidity (55% ± 5%), with a 12-hr dark/light cycle and *ad libitum* access to food and water. Pregnant ICR mice were intranasally treated on gestational days (GD) 5 and 9 with CB-NP (95 μg/kg body weight each time), in order to examine the effect of maternal exposure to CB-NP during early gestation. The day the plug was detected was considered GD 0. The total dose of CB-NP (190 μg/kg body weight) was lower than the doses used in many earlier studies of nano-sized particle effects. Control animals were treated with distilled water (1 ml/kg body weight) each time. After parturition, spleen tissues were removed from the offspring mice at 1, 3, 5 and 14 days after birth (postnatal days [PNDs] 1, 3, 5 and 14), for investigating the effect of CB-NP on the immune system of infantile mice, under anesthesia by sodium pentobarbital.

Flow cytometry

Anti-CD3 (2C11) and anti-CD4 (GK1.5) antibodies were prepared and purified from hybridoma culture supernatants and labeled with fluorescein isothiocyanate (FITC) at the Division of Immunobiology, Research Institute for Biomedical Sciences, Tokyo University of Science (Chiba, Japan) (Watanabe *et al.*, 2012). Phycoerythrin (PE)-labeled anti-CD8 (53-6.7) and anti-B220 (RA3-6B2) antibodies were purchased from BD Bioscience Co. (San Jose, CA, USA).

Spleen cell suspensions from individual male offspring mice (PND 1: CB-NP, n = 11; control, n = 10; PND 3: CB-NP, n = 10; control, n = 9; PND 5: CB-NP, n = 11; control, n = 12; PND 14: CB-NP, n = 8; control, n = 8) were prepared in FACS medium (PBS containing 1% FBS and 0.1% sodium azide), treated with anti-FcR (2.4G2) to block nonspecific binding (Watanabe *et al.*, 2012), and then stained with fluorescently labeled antibodies. The cells were then washed, resuspended in wash buffer, and subjected to analysis. Dead cells were excluded by forward light scatter gating and propidium iodide staining. Fluorescent data of 10,000 lymphocyte events per sample were acquired on a FACS Canto II (BD Biosciences) and were analyzed using the FlowJo software (Tomy Digital Biology Co., Ltd., Tokyo, Japan).

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from spleen tissues of PND5 offspring mice (male: CB-NP, n = 6; control, n = 8; female: CB-NP, n = 8; control, n = 9) with Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). Total RNA (1 μg) was treated with M-MlV reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA) to obtain first-strand complementary DNA (cDNA). Quantitative PCR was performed with SYBR Green Realtime PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan) and primers (Fasmac Co. Ltd., Kanagawa, Japan) for the indicated genes. Values were normalized to those of the housekeeping gene, *Gapdh*.

Statistical analysis

Values are given as mean ± S.D. Data on the number of offspring per dam were analyzed by Student's *t* test. Body weight of offspring mouse and flow cytometry data were analyzed using two-way, repeated-measures analysis of variance (ANOVA), with exposure and age as factors, followed by *post hoc* Tukey-Kramer's test. Data on mRNA expression level were analyzed by unpaired *t* test to compare the means of control and CB-NP groups for each sex, and corrected with Bonferroni's method. The level of significance was set at $P < 0.05$.

RESULTS

CB-NP in filtrated suspension

FE-SEM showed secondary particles, approximately 50-500 nm in diameter, in the filtered CB-NP suspension (Fig. 1A). The mode value of the aerodynamic diameter distribution of CB-NP in the suspension was 68 nm (Fig. 1B).

Litter size and body weight of offspring

There was no significant effect of CB-NP exposure on litter size (CB-NP: 13.7 ± 1.9; control: 13.7 ± 2.1) ($P = 0.91$) or body weight of male offspring between

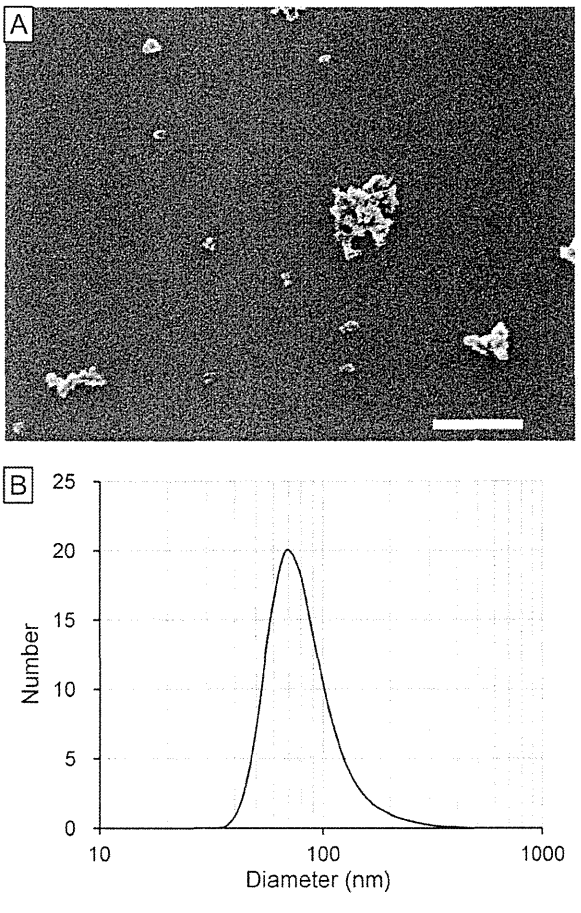

Fig. 1. Characterization of ultrafine carbon black in suspension. (A) An image of carbon black nanoparticle (CB-NP) in a filtered suspension analyzed by scanning electron microscopy. The scale bar represents 500 nm. (B) Particle diameter distribution of filtrated CB-NP, as determined by dynamic light scattering (DLS).