



Carbon black nanoparticles promote the maturation and function of mouse bone marrow-derived dendritic cells

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ABSTRACT

Particulate matter including carbon black (CB) nanoparticles can enhance antigen-related inflammation and immunoglobulin production *in vivo*. Dendritic cells (DC) as antigen-presenting cells (APC) are the most capable inducers of immune responses. The present study was designed to determine whether CB nanoparticles affect the maturation/activation and function of DC *in vitro*. DC were differentiated from bone marrow (BM) cells of BALB/c mice by culture with granulocyte macrophage colony stimulating factor (GM-CSF). At day 8 of culture, BM-derived DC (BMDC) were exposed to CB nanoparticles with a diameter of 14 nm or 56 nm for 24 h. The expression of major histocompatibility complex (MHC) class II, DEC205, CD80, and CD86 (maturation/activation markers of BMDC) was measured by flow cytometry. BMDC function was evaluated by an allogeneic mixed lymphocyte reaction (MLR) assay. CB nanoparticles significantly increased the expression of DEC205 and CD86 in BMDC and tended to increase MHC class II and CD80 expression; however, a size-dependent effect was not observed. On the other hand, BMDC-mediated MLR was significantly enhanced by the CB nanoparticles and the enhancement was greater by 14 nm CB nanoparticles than by 56 nm CB nanoparticles. Taken together, CB nanoparticles can promote the maturation/activation and function of BMDC, which could be related to their effects on allergic diseases and/or responses. In addition, BMDC-mediated MLR might be useful assay for *in vitro* screening for adjuvant activity of environmental toxicants.

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1. Introduction

Epidemiological studies have indicated that ambient particulate matter (PM) is associated with the risk of death and hospital admission from pulmonary diseases (Zanobetti et al., 2000; Schwartz et al., 2001; Sunyer and Basagana, 2001). The effects of ultra-fine particles on respiratory health are stronger than those of PM with aerodynamic diameters of less than 10 µm (Peters et al., 1997). Since these reports indicate stronger effects on health from smaller particles, it is important to examine the health effects of nanoparticles and to elucidate their underlying mechanisms.

Our previous study demonstrated that carbon black (CB) nanoparticles with a diameter of 14 nm or 56 nm can aggravate anti-

gen-related airway inflammation and immunoglobulin production *in vivo* and that this is more prominent with smaller particles (Inoue et al., 2005). In general, enhancement of antigen-presenting activity can lead to an increase of antigen-related immunoglobulin production and allergic inflammation through the proliferation and activation of lymphocytes and eosinophils (Wills-Karp, 1999; van Rijt and Lambrecht, 2001; Lambert et al., 2003). Therefore, the activation of antigen-presenting cells (APC) could be important in mechanisms for the aggravation of allergic diseases and/or responses. Our recent study demonstrated that exposure to CB nanoparticles can enhance the expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules as well as the number of APC such as dendritic cells (DC), macrophages, and B-cells in the lung *in vivo* (Koike et al., 2008).

DC as APC are the most capable inducers of primary immune responses and the enhancement of secondary ones. MHC class II molecules (Niederhuber and Shreffler, 1977) and co-stimulatory molecules such as CD80 and CD86 (Freeman et al., 1993; Lenschow et al., 1993) are essential for antigen presentation. DC maturation/activation is defined by enhanced expression of MHC class II and co-stimulatory molecules on their surface, and stimulated T-cell proliferation. Previous *in vitro* studies have reported that PM can up-regulate the expression of HLA-DR and co-stimulatory

Abbreviations: APC, antigen-presenting cells; BM, bone marrow; BMDC, BM-derived DC; BrdU, 5-bromo-2'-deoxyuridine; CB, carbon black; DC, dendritic cells; DEP, diesel exhaust particles; FBS, fetal bovine serum; GM-CSF, granulocyte macrophage colony stimulating factor; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; PBS, magnesium-free, phosphate-buffered saline; PM, particulate matter; R10, RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 50 µM 2-mercaptoethanol.

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molecules in human monocytes (Becker and Soukup, 2003), and that diesel exhaust particles (DEP), which are the main constituents of urban PM can up-regulate the expression of Ia, co-stimulatory molecules, and antigen-presenting activity in rat monocytes (Koike and Kobayashi, 2005). DEP also enhance the expression of co-stimulatory molecules and cytokine production from human monocyte-derived DC (Porter et al., 2007). However, little is known about the effects of nanoparticles on DC maturation and function either *in vivo* or *in vitro*.

In the present study, we investigated the effects of CB nanoparticles with diameters of 14 nm or 56 nm on the expression of cell surface molecules and the allogeneic T-cell stimulating activity of DC *in vitro*. Here, we demonstrate that CB nanoparticles can promote the maturation/activation and function of DC.

2. Materials and methods

2.1. Animals

Specific pathogen-free, female BALB/c mice and C57BL/6 mice (6 wks of age) were obtained from Japan Charles River Inc. (Yokohama, Japan) and were used at 11–15 wks of age. The mice were given sterile distilled water and CE-2 food (CLEA Japan Inc., Tokyo, Japan) *ad libitum*. The mice were housed in an animal facility that was maintained at 24–26 °C, 55–75% humidity and with a 12-h light/dark cycle.

2.2. CB nanoparticles

CB nanoparticles with diameters of 14 nm (Printex 90) and 56 nm (Printex 25) were obtained from Degussa (Dusseldorf, Germany). The surface area of the 14 nm nanoparticles was $300 \text{ m}^2 \text{ g}^{-1}$ and that of 56 nm nanoparticles was $45 \text{ m}^2 \text{ g}^{-1}$. Nanoparticles were autoclaved at 250 °C for 2 h in an electric heater before use. The nanoparticle suspension was sonicated for 3 min using an ultrasonic disrupter (UD-201; TOMY, Tokyo, Japan).

2.3. Preparation of bone marrow (BM) cells

BALB/c mice were anesthetized with sodium pentobarbital (Dainippon Pharmaceutical Co., Osaka, Japan), given intraperitoneally (50 mg kg^{-1}), and exsanguinated from the abdominal aorta and vein. After removing the surrounding muscle tissue by rubbing it with kleenex tissues, the bones were left in 60 mm dishes with 70% ethanol for 3 min and washed with Dulbecco's calcium and magnesium-free phosphate-buffered saline (PBS; Dainippon Pharmaceutical). Both ends of the bones were cut and then the BM was flushed with PBS using a syringe with a 25 G needle. The BM suspension was passed through a nylon mesh to remove small pieces of bone and debris, and red blood cells were lysed with ammonium chloride. After washing twice with PBS, the cells were resuspended in culture medium R10 [RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Dainippon Pharmaceutical), 100 U ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin (Sigma, St. Louis, MO), and $50 \mu\text{M}$ 2-mercaptoethanol (GIBCO BRL)]. The numbers of viable cells were determined by the trypan blue (Gibco Laboratories, Grand Island, NY) exclusion method.

2.4. Differentiation of BM-derived DC (BMDC)

BMDC were differentiated using a modified protocol of Lutz et al. (1999). Briefly, BM cells (4×10^6) were cultured in 10 ml R10 medium containing 20 ng ml^{-1} recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF; Sigma) in a

100 mm dish. At day 3 of culture, another 10 ml of R10 medium containing 20 ng ml^{-1} GM-CSF was added to the dishes. At day 6 of culture, half of the culture medium was poured into 10 ml of fresh R10 medium containing 20 ng ml^{-1} GM-CSF. At day 8 of culture, non-adherent and loosely adherent cells were collected by gentle pipetting, and centrifuged at $400g$ for 5 min at 20 °C. These cells (immature BMDC) were resuspended in fresh R10 medium.

2.5. Exposure to CB nanoparticles

Immature BMDC were treated with $50 \mu\text{g ml}^{-1}$ mitomycin C (Kyowa, Tokyo, Japan) for 20 min in a water bath at 37 °C in order to prevent the proliferation of the cells. Then, the cells were washed twice with R10 medium. 2.4×10^6 cells were placed in 6-well plates, and exposed to 14 nm and 56 nm CB nanoparticles at various concentrations ($0, 5, 10, 25 \mu\text{g ml}^{-1}$) or $1 \mu\text{g ml}^{-1}$ lipopolysaccharide from *Escherichia coli* (LPS, Sigma) in 2.4 ml of R10 medium containing 10 ng ml^{-1} GM-CSF for 24 h at 37 °C in an atmosphere of 5% CO_2 /95% air. LPS was used as a positive control for DC maturation/activation.

2.6. FACS analysis

For FACS analysis, the following monoclonal antibodies were used. MHC class II molecules: I-A/I-E (2G9, FITC-conjugated, BD Biosciences Pharmingen, CA); DC marker: DEC205 (NLDC-145, PE-conjugated, Cedarlane Labs, Ontario, Canada); co-stimulatory molecules: CD80 (16-10A1, PE-conjugated, BD Biosciences Pharmingen), CD86 (GL1, PE-conjugated, BD Biosciences Pharmingen). BMDC ($3\text{--}5 \times 10^5$) were resuspended in $100 \mu\text{l}$ PBS with 0.3% BSA and 0.05% sodium azide (Wako Pure Chemical Industries, Osaka, Japan) and were stained with $10 \mu\text{g ml}^{-1}$ antibodies for 30 min on ice. After incubation, the cells were washed with the buffer, and the fluorescence was measured by a FACSCalibur (Becton, Dickinson and Company, NJ). Fluorescence data were expressed as a percentage of positive cells.

2.7. Allogeneic mixed lymphocyte reaction (MLR)

Allogeneic T-cells were isolated from pooled C57BL/6 spleen cells. Briefly, the spleen was pushed through a sterile stainless wire mesh (200 mesh) and red blood cells were lysed with ammonium chloride. T-Cells were isolated from the spleen cells by using a nylon fiber column (Wako Pure Chemical Industries). T-cells (2×10^5) were co-cultured with BMDC (2.5×10^3 or 5×10^3) in 96-well flat-bottom plates. The MLR was performed in $200 \mu\text{l}$ of R10 medium in triplicate for 3 d at 37 °C in a 5% CO_2 /95% air atmosphere. BMDC-stimulated T-cell proliferation was measured after the culture period.

2.8. Measurement of T-cell proliferation

T-cell proliferation was measured with a Cell-Proliferation-ELISA Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. This technique is based on the incorporation of the pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. BrdU incorporated into DNA is measured by a sandwich-type enzyme immunoassay using monoclonal anti-BrdU antibody. Cell proliferation was measured by adding BrdU to each well 18 h before measurement. The absorbance of the samples was measured in an ELISA reader (ImmunoMini NJ-2300, Inter Med, Tokyo, Japan) at a wavelength of 450 nm (reference wavelength: 620 nm).

2.9. Statistical analysis

Data were represented as the mean $\pm$ SEM of three animals. Differences between groups were determined using ANOVA by Fisher's LSD (StatView J-4.5; Abacus Concepts, Inc., Berkeley, CA). A p -value of <0.05 was considered to indicate a significant difference between pairs of groups.

3. Results

3.1. Effect of CB nanoparticles on the expression of cell surface molecules in BMDC

Immature BMDC were exposed to CB nanoparticles or LPS (positive control) for 24 h. The expression of MHC class II, DEC205, CD80, and CD86 in the cells was analyzed to investigate the dose-dependent effect of CB nanoparticles on the maturation/activation of BMDC. The percentage of DEC205⁺ cells (Fig. 1b) and CD86⁺ cells (Fig. 2c) was significantly increased by addition of 25 $\mu\text{g ml}^{-1}$ of 14 nm CB nanoparticles, or 10 or 25 $\mu\text{g ml}^{-1}$ of 56 nm CB nanoparticles. Also, the percentage of MHC class II⁺DEC205⁺ cells (Fig. 1c) and MHC class II⁺CD86⁺ cells (Fig. 2d) was significantly increased by addition of 10 or 25 $\mu\text{g ml}^{-1}$ of 56 nm CB nanoparticles. The expression of MHC class II and CD80 tended to increase after addition of both sizes of CB nanoparticles. Conversely, a significant size-dependent effect on the expression of these molecules was not observed. The percentage of MHC class II⁺DEC205⁺ cells in the control, 25 $\mu\text{g ml}^{-1}$ of 14 nm CB nanoparticles, 25 $\mu\text{g ml}^{-1}$ of 56 nm CB nanoparticles, and the positive control (LPS) was approximately 31%, 40%, 43%, and 50%, respectively (Fig. 1c). The percentage of MHC class II⁺CD80⁺ cells

in the control, 25 $\mu\text{g ml}^{-1}$ of 14 nm CB nanoparticles, 25 $\mu\text{g ml}^{-1}$ of 56 nm CB nanoparticles, and the positive control was approximately 28%, 35%, 34%, and 63%, respectively (Fig. 2b). The percentage of MHC class II⁺CD86⁺ cells in the control, 25 $\mu\text{g ml}^{-1}$ of 14 nm CB nanoparticles, 25 $\mu\text{g ml}^{-1}$ of 56 nm CB nanoparticles, and the positive control was approximately 24%, 28%, 32%, and 59%, respectively (Fig. 2d).

3.2. Effect of CB nanoparticles on allogeneic T-cell stimulating capacity of BMDC

BMDC function was assessed via their capacity for stimulating allogeneic T-cells. The proliferation of T-cells (responder cells) was increased by addition of BMDC (stimulator cells). The reaction was significantly increased by exposure to CB nanoparticles with a diameter of either 14 nm (Fig. 3a: $p < 0.05$ for 2.5% BMDC exposed to 5 or 10 $\mu\text{g ml}^{-1}$; $p < 0.01$ for 1.25% or 2.5% BMDC exposed to 25 $\mu\text{g ml}^{-1}$) or 56 nm (Fig. 3b: $p < 0.05$ for 1.25% or 2.5% BMDC exposed to 25 $\mu\text{g ml}^{-1}$). The function of BMDC exposed to 14 nm CB nanoparticles was more effective than that of those exposed to 56 nm CB nanoparticles, in particular at a concentration of 5 $\mu\text{g ml}^{-1}$ ($p = 0.01$; Fig. 3c).

4. Discussion

In the present study, we demonstrated that CB nanoparticles can promote the maturation/activation and function of BMDC *in vitro*.

We have previously demonstrated that CB nanoparticles can aggravate a murine model of antigen-related airway inflammation characterized by infiltration of inflammatory leukocytes (including

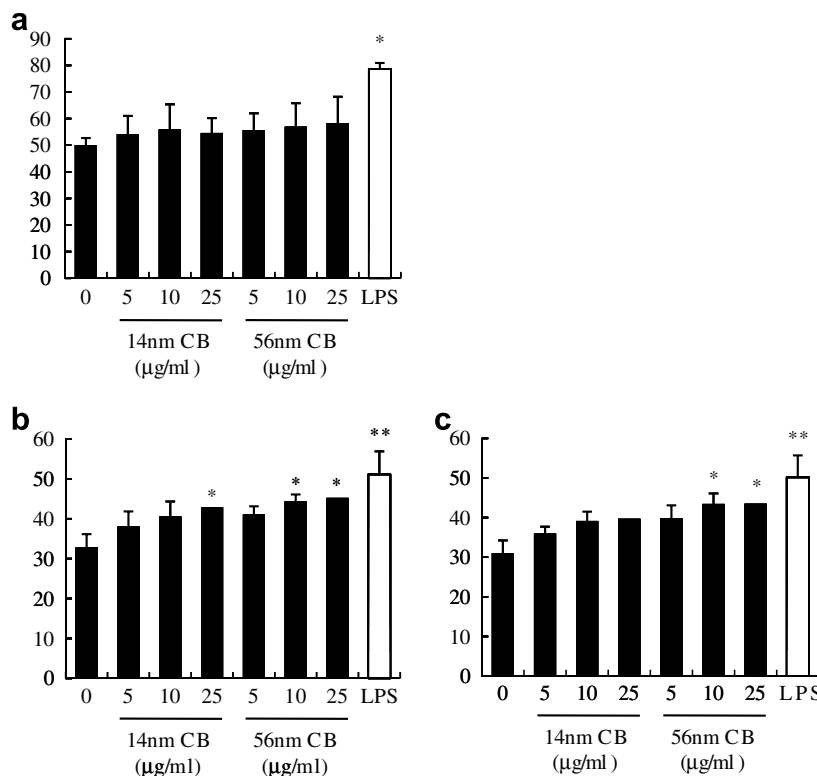


Fig. 1. Effects of CB nanoparticles on the expression of MHC class II and DEC205 in BMDC. Immature BMDC ($1 \times 10^6 \text{ ml}^{-1}$) from BALB/c mice were exposed to 14 nm or 56 nm CB nanoparticles (0, 5, 10, 25 $\mu\text{g ml}^{-1}$) or LPS (1 $\mu\text{g ml}^{-1}$, positive control) in the presence of 10 ng ml^{-1} GM-CSF for 24 h. After exposure, the expression of MHC class II and DEC205 as markers of dendritic cells was analyzed by flow cytometry. Percentages of each cell type in the BMDC population are shown. (a) MHC class II⁺, (b) DEC205⁺, (c) MHC class II⁺DEC205⁺. Data represent the mean $\pm$ SEM of three animals from one experiment representative of two experiments ($^*p < 0.05$, $^{**}p < 0.01$ compared with the corresponding control).

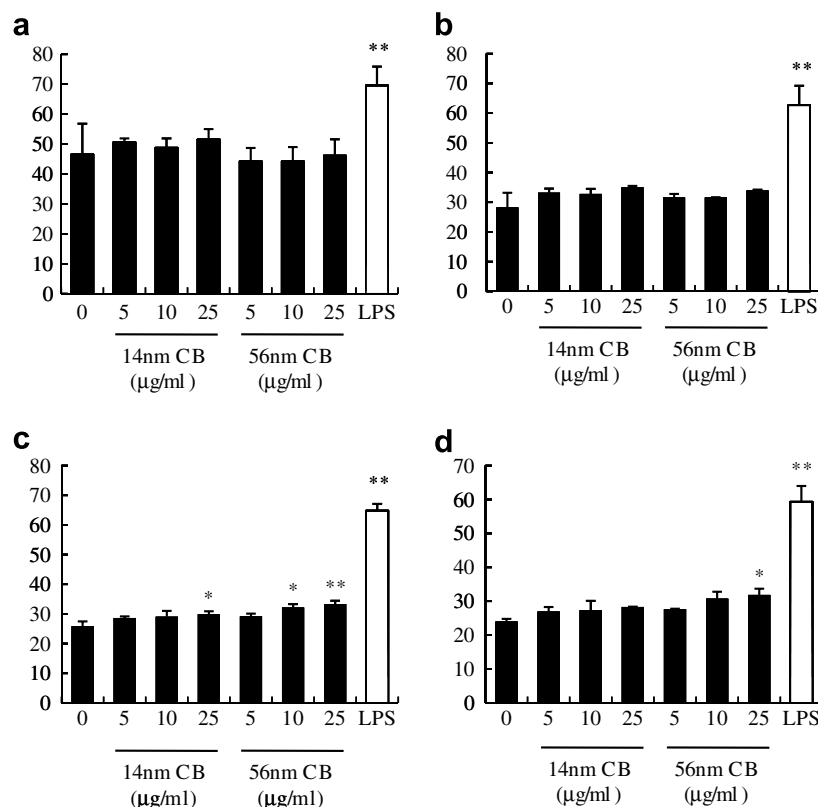


Fig. 2. Effects of CB nanoparticles on the expression of MHC class II and co-stimulatory molecules in BMDC. Immature BMDC from BALB/c mice were exposed to CB nanoparticles or LPS in the same manner as shown in Fig. 1. After exposure, the expression of MHC class II and co-stimulatory molecules was analyzed by flow cytometry. Percentages of each cell type in the BMDC population are shown. (a) CD80⁺, (b) MHC class II*CD80⁺, (c) CD86⁺, (d) MHC class II*CD86⁺. Data represent the mean $\pm$ SEM of three animals from one experiment representative of two experiments (* $p < 0.05$, ** $p < 0.01$ compared with the corresponding control).

lymphocytes and eosinophils) and by the enhanced lung expression of interleukin (IL)-5, IL-13, eotaxin, monocyte chemoattractant protein-1, and RANTES (regulated on activation and normal T cells expressed and secreted) (Inoue et al., 2005, 2006). These results suggest that CB nanoparticles have enhancing effects on antigen-related airway inflammation possibly via Th2 dominant allergic responses. APC-mediated antigen presentation for T-cells is the first step in both primary and secondary immune responses. Thus, recruitment and/or maturation/activation of APC could be important in the underlying mechanisms of the exacerbation of allergic responses and/or diseases. Our recent study showed that pulmonary exposure to CB nanoparticles enhances the expression of cell surface molecules related to antigen presentation such as MHC class II, CD80, and CD86 and increases the number of APC including DC in the murine lung (Koike et al., 2008). Also, DC as APC are the most capable inducers of the activation and differentiation of T-cells. Maturation of DC is an important step for polarized antigen presentation, resulting in either a Th1 or Th2 response (de Jong et al., 2005). Therefore, the enhancement, not only in the cell numbers but also in the maturation/activation of DC, is essential in allergic reactions and/or diseases. The present *in vitro* study demonstrated that exposure to CB nanoparticles significantly increased the percentage of DEC205⁺ cells (Fig. 1b), CD86⁺ cells (Fig. 2c), MHC class II*DEC205⁺ cells (Fig. 1c), and MHC class II*CD86⁺ cells (Fig. 2d). These results suggest that CB nanoparticles can promote maturation/activation of DC.

Also, DC are efficient stimulators of both allogeneic and syngeneic MLR (Inaba and Steinman, 1986). It has been demonstrated that allogeneic MLR and specific antigen presentation show similar tendencies, which include APC activation and enhanced expression of MHC class II and/or co-stimulatory molecules (van den Heuvel

et al., 1998; Koike and Kobayashi, 2004). Therefore, in the present study, the alteration of BMDC function by CB nanoparticle-exposure was assessed by allogeneic MLR assay. As a result, it was found that CB nanoparticles could amplify BMDC-mediated allogeneic MLR (Fig. 3a and b) in both of the BMDC:T cells ratios used. In this experiment, however, the allogeneic T-cell stimulating capacity ratio of 1.25% BMDC was relatively weak. In addition, the smaller (14 nm) CB nanoparticles had more prominent effects in the higher BMDC population (2.5%). Therefore, taking our previous *in vivo* findings (Inoue et al., 2005; Koike et al., 2008) into consideration, 2.5% BMDC might be a suitable percentage for evaluating the effect on T-cell stimulating capacity. In other words, the current data may partially support our previous observations that smaller (14 nm) CB nanoparticles lead to a migration of larger numbers of DC into the lung (Koike et al., 2008) than larger (56 nm) ones and subsequent worsened Th2-mediated airway inflammation (Inoue et al., 2005). In any case, our previous *in vivo* studies and the present *in vitro* study suggest that CB nanoparticles can increase the number of APC in the lung, and can also enhance maturation/activation and the functions of DC, which may lead to the consequent augmentation of antigen-related inflammation and immunoglobulin production.

In the present study, we used CB as a source of elemental carbon. Since CB is often a core of ambient environmental particles and is relatively inert, the effects of particle size can be elucidated without confounding factors (Lambert et al., 2003). Exposure to ultrafine but not to fine CB particles alone caused an increase in airway inflammation (Gilmour et al., 2004). Similarly, our previous *in vivo* studies have also demonstrated that aggravation of antigen-related airway inflammation is more prominent with 14 nm CB nanoparticles than with 56 nm CB nanoparticles (Inoue et al.,

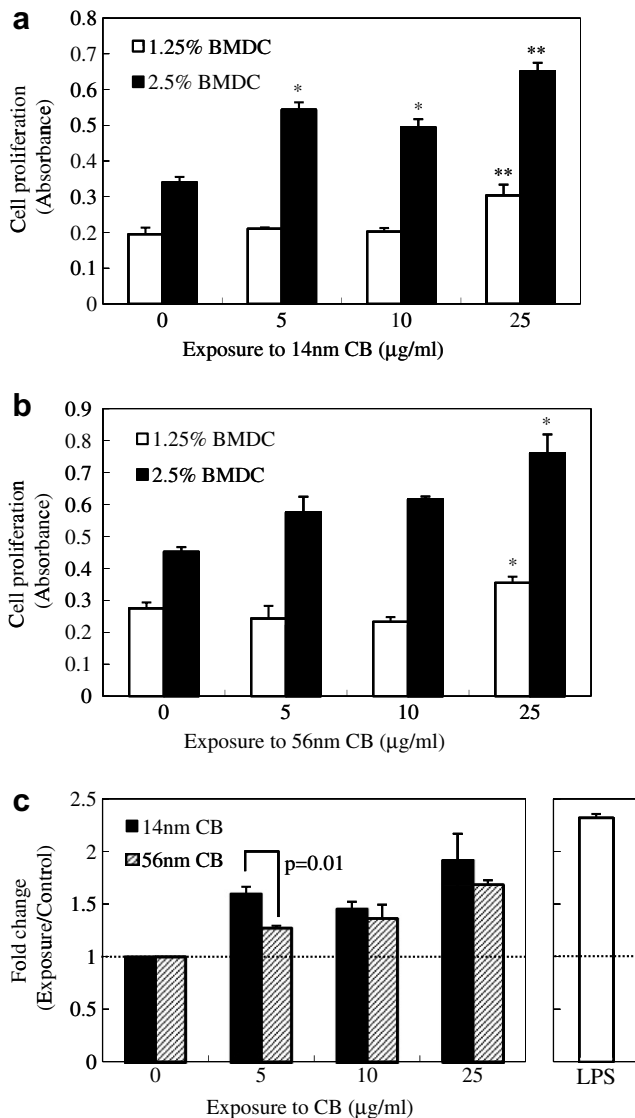


Fig. 3. Effects of CB nanoparticles on allogeneic T-cell stimulating capacity of BMDC. Immature BMDC from BALB/c mice were exposed to CB nanoparticles or LPS in the same manner as shown in Fig. 1. T-cells (2×10^5) from C57BL/6 mice were co-cultured with BMDC (2.5×10^3 for 1.25%, or 5×10^3 for 2.5%) from BALB/c mice for 3 d and pulsed with BrdU for the last 18 h. The MLR was performed in triplicate and T-cell proliferation was evaluated by BrdU incorporation into proliferating cells (a, b). Fold change in allogeneic T-cell stimulating capacity of 2.5% BMDC exposed to 14 nm nanoparticles was compared with that of 56 nm CB nanoparticles (c). Data represent the mean $\pm$ SEM of three animals from one experiment representative of two experiments (* $p < 0.05$, ** $p < 0.01$ compared with the corresponding control).

2005). Further, the number of APC in the lung is increased by 14 nm CB nanoparticles in the presence or absence of antigen. In contrast, 56 nm CB nanoparticles do not show significant effects (Koike et al., 2008). Meanwhile, the present *in vitro* results were a little different from the above mentioned previous *in vivo* ones in that the induction of BMDC maturation/activation by 14 nm CB nanoparticles was not significantly different from that by 56 nm CB nanoparticles, whereas, the function of BMDC exposed to 14 nm CB was partially but significantly greater than of those exposed to 56 nm CB. These slightly conflicting results between the *in vivo* and *in vitro* studies may have resulted from the difference in the *in vivo* system and the *in vitro* simple cell culture system. Direct or indirect interaction with other cells such as epithelial cells, endothelial cells, and lymphocytes may be essential

for the maturation/activation of BMDC *in vivo*. Consistent with this, it has been reported that DEP do not affect DC phenotype and subsequent T cell activation, whereas co-culture with DEP-treated bronchial epithelial cells and DC results in both phenotypic and functional enhancement of DC (Bleck et al., 2006). Therefore, we will investigate cell to cell interactions on the effects of CB nanoparticles on DC maturation/activation in a future study.

Finally, CB nanoparticles with a diameter of 14 nm and 56 nm were used in our studies. The surface area of 14 nm CB nanoparticles is 6.7-fold larger than that of 56 nm CB nanoparticles of the same weight ($300 \text{ m}^2 \text{ g}^{-1}$ versus $45 \text{ m}^2 \text{ g}^{-1}$). It is possible that nanoparticles with a larger surface area attach/affect larger numbers of immune cells than those with a smaller surface area. Nonetheless, we did not examine the effects of CB nanoparticles with the same surface area in the *in vitro* study or in the *in vivo* study. Thus, further studies are necessary to determine the effects of CB particles with uniform surface areas on immune networks both *in vivo* and *in vitro*.

In conclusion, our present results are the first *in vitro* demonstration that CB nanoparticles can promote DC maturation/activation and function, which could be one of the mechanisms of CB nanoparticle-enhanced allergic and inflammatory responses. Furthermore, BMDC-mediated MLR might be useful assay *in vitro* screening system for adjuvant activity of environmental toxicants.

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