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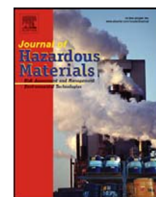

Intratracheal administration of fullerene nanoparticles activates splenic CD11b+ cells

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Fullerene nanoparticles ("Fullerenes"), which are now widely used materials in daily life, have been demonstrated to induce elevated pulmonary inflammation in several animal models; however, the effects of fullerenes on the immune system are not fully understood. In the present study, mice received fullerenes intratracheally and were sacrificed at days 1, 6 and 42. Mice that received fullerenes exhibited increased proliferation of splenocytes and increased splenic production of IL-2 and TNF-α. Changes in the spleen in response to fullerene treatment occurred at different time-points than in the lung tissue. Furthermore, fullerenes induced CDK2 expression and activated NF-κB and NFAT in splenocytes at 6 days post-administration. Finally, CD11b+ cells were demonstrated to function as responder cells to fullerene administration in the splenic inflammatory process. Taken together, in addition to the effects on pulmonary responses, fullerenes also modulate the immune system.

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1. Introduction

As nanomaterials become more widely used in commercial and consumer products, people are continually exposed to their deleterious effects, including inhalation toxicity and inflammogenic effects [1–3]. Fullerene is a generic term for clusters consisting of many carbon atoms in the form of hollow spheres, ellipsoids, tubes or planar structures. A typical fullerene resembles a soccer ball composed of twenty hexagons and twelve pentagons. The first fullerene was discovered in 1985, and fullerenes have been applied in fields such as electronics, environmental science and medical science since that time [4]. The behavior and toxicity of fullerenes correlate with their chemical structure, surface modifications and preparation procedure; differences in these properties can result in drastically different behaviors amongst the fullerenes [5]. Despite these facts, there are limited reports studying the effects of fullerenes on health, especially on the immune system.

NF-κB is one of the most well-studied transcription factors. It regulates the expression of many genes and plays an important role in the process of inflammation [6]. The upstream kinase complex, IKK, regulates NF-κB activation. In resting cells, NF-κB forms a tight complex with inhibitor of NF-κB (IκB), which inhibits the nuclear translocation of NF-κB. Activated IKK triggers the phosphorylation and ubiquitination of IκB. Active NF-κB is then translocated to the nucleus, where it regulates an array of genes responsible for cell proliferation and inflammation [7].

Cell cycle progression is regulated by the activities of cyclin-dependent kinases (CDKs). CDK2 is a catalytic subunit of the CDK complex whose activities are essential for the G1/S transition. Cyclin D-CDK4/6 and cyclin E-CDK2 form complexes with phosphorylate retinoblastoma (Rb) and release E2F, whose target genes are required for the completion of the G1/S phase transition [8,9]. The regulation of the activities of the CDKs is an important mechanism for controlling cell cycle progression. The INK4 family (p16, p15, p18 and p19) and the Cip/Kip family (p21, p27 and p57) are two important families of CDK inhibitors. NFAT proteins, which are phosphorylated and reside in the cytoplasm, play an important role in the transcription of genes, such as IL-2, during the immune response. Stimuli that trigger calcium mobilization induce the dephosphorylation and activation of NFAT proteins, causing their translocation to the nucleus [10].

In this paper, we show that the administration of fullerenes induces pulmonary inflammation and increases the proliferation of splenocytes, with different peak points from each other, suggesting

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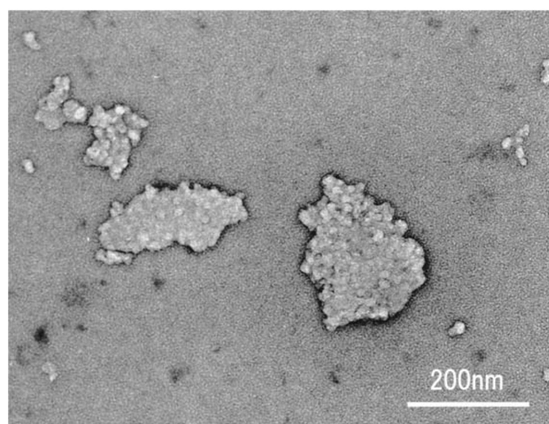

Fig. 1. Characterization of the fullerene suspension in Tween 80. Transmission electron microscopic image of fullerene nanoparticles. Bar = 200 nm.

that fullerenes can potentially influence the immune system. Additionally, we demonstrate the effect of fullerenes on the activation of CD11b$^+$ cells in the splenic inflammatory process.

2. Materials and methods

2.1. Animals

Male BALB/c mice (8 weeks) were purchased from Charles River Japan, Inc. (Yokohama, Japan). They were housed in plastic cages and bred in rooms kept at a temperature of 23 °C ± 2 °C and a relative humidity of 55% ± 10%, under a 12-h, light–dark cycle. They were allowed free access to tap water and food. Animal experiments were performed according to the guidelines for the care and use of animals, which are approved by the University of Occupational and Environmental Health, Japan.

2.2. Preparation and intratracheal administration of fullerenes

The test substance, nanom hybrid MA310-10 (LOT No. 7B0166-A), consisted of a fullerene nanoparticles mixture provided by Frontier Carbon Corporation (Tokyo, Japan) with a purities of 60% C60 and 25% C70 and a high fullerene content (<C76, 15%). The test sample contained 9.1 wt% fullerene mixture and 3.9 wt% Tween 80. Brix was approximately 13%. The central diameter of the fullerenes (d50) was 0.16 μm, which was analyzed by laser diffraction. The fullerenes can easily aggregate, and after preparation in Tween 80, the average diameter was 160 nm. As shown in Fig. 1, negative stain electron microscopy images were taken using a JEM-1200EX microscope (JEOL Ltd., Tokyo, Japan). Sample grids were prepared as described [11]. After dilution of the original fullerenes or Tween 80 with physiological saline, 0.1 ml of material suspension or vehicle Tween 80 was intratracheally administered once into BALB/c mice (8 weeks old) at a concentration of 0.2 or 2 mg/kg. The animals were dissected post administration at days 1, 6 and 42. We confirmed that the fullerene suspension did not contain endotoxin (<0.0001 μg/ml) using a turbidity LAL assay. LAL was purchased from Associates of Cape Cod, Inc. (Cape Cod, MA).

2.3. Preparation of bronchoalveolar lavage fluid (BALF)

The tracheas of mice were cannulated and lavaged with 1.0 ml cold PBS. After centrifugation at 5000 rpm for 5 min, the supernatant was used as the BALF.

2.4. Proliferation assay

Cell proliferation was measured using the Cell Titer-Glo luminescent cell viability assay system (Promega, Madison, WI). Single-cell suspensions of splenocytes were prepared using gentle MACS Dissociator (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions and previously published reports [12]. The cells were suspended at 1×10^6 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), L-glutamine and penicillin–streptomycin. Splenocytes were plated into 96-well plates at 200 μl per well and then stimulated with PBS, ConA or LPS for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. After culture, 20 μl of the assay reagent was added to each well, and fluorescence was measured using a Luminescencer-JNR-II (ATTO, Tokyo, Japan).

2.5. ELISA

BALB/c mice were intratracheally administered fullerenes and were sacrificed at days 1, 6 and 42. Splenocytes were prepared and suspended in medium at a concentration of 1×10^6 cells/ml and stimulated with PBS, ConA or LPS in 24-well plates. After incubation for 6 h, the culture supernatant was harvested for the analysis of the IL-2 and TNF-α concentrations. Cytokine production in the culture supernatant or BALF was measured using ELISA kits (IL-1β, TNF-α (PEPROTECH, Rocky Hill, NJ) and IL-2 (BioLegend, San Diego, CA)). The measurement of IgG production was performed as previously reported [13]. Stimulators, such as ConA, LPS, coated anti-CD3 and anti-CD40, were purchased from EY Laboratories (San Diego, CA), Sigma, eBioscience (San Diego, CA) and R&D system (Minneapolis, MN), respectively.

2.6. Western blotting

Freshly isolated splenocytes were lysed with RIPA lysis buffer [14] for the preparation of whole cell extracts. Equivalent amounts of protein (10 μg) were resolved on SDS-PAGE gels, transferred and then immobilized on nitrocellulose membranes (Amersham, Buckinghamshire, U.K.). Membranes were probed with the appropriate primary and secondary antibodies. Immunodetection was performed using a chemiluminescence detection system (Alpha Innotech, San Leandro, CA). The intensity of the bands that were detected was quantified by densitometric analysis with an ATTO Densitograph 4.0 (ATTO). The expression of proteins was normalized to the expression of β-actin. Antibodies for CDK2 (sc-163), IκBα (sc-371), p65 (sc-109) and p-NFAT (sc-32993) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for p-IκBα (#9241), p-p65 (#3031) and NFAT1 (#4289) were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin antibody was purchased from Sigma (St. Louis, MO).

2.7. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described [15]. Splenocytes were lysed with 100 μl of RIPA lysis buffer and used as whole cell extracts. The murine NF-κB oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, derived from the Igκ enhancer region, was purchased from Promega (Madison, WI).

2.8. Cell separation

The CD4$^+$, CD11b$^+$ and CD45R$^+$ cells (purity > 95%) were isolated from splenocytes using the IMag beads separation system according to manufacturer's instructions (BD Biosciences, San Jose, CA).

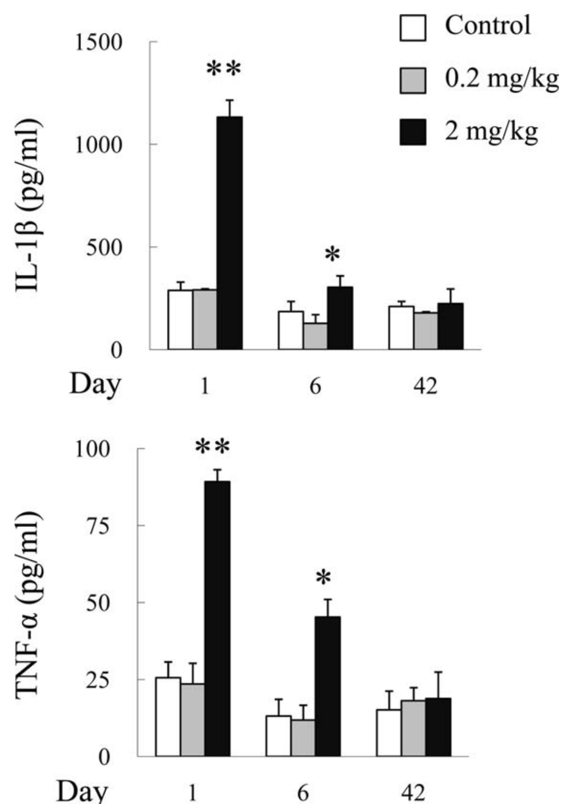

Fig. 2. Fullerene administration induces the production of inflammatory cytokines in BALF. BALB/c mice were intratracheally administered a dose of 0, 0.2 or 2 mg/kg fullerenes and were sacrificed at days 1, 6 and 42, as indicated. The levels of IL-1β (upper) and TNF-α production (lower) in BALF were analyzed by ELISA. The values are shown as mean ± SD ($n = 6$ mice). $^{**}p < 0.01$ vs. control mice. $^{*}p < 0.05$ vs. control mice. The white column represents the control mice. The gray and black columns represent 0.2 mg/kg and 2 mg/kg fullerenes administered, respectively; this color code is also used in subsequent figures. The results are representative of two similar experiments.

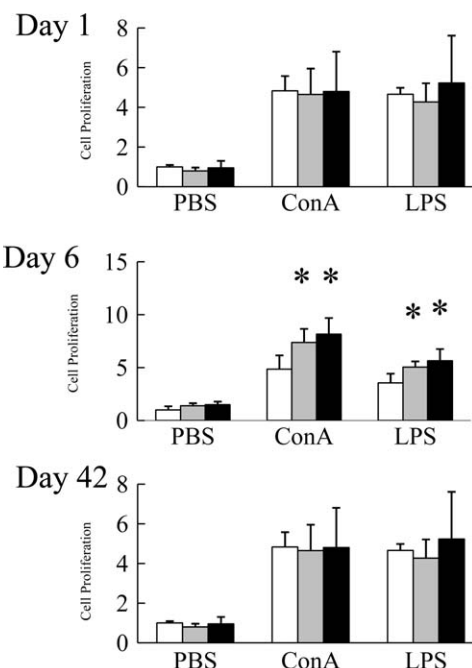

Fig. 3. Proliferation of splenocytes is increased by fullerene administration. A dose of 0, 0.2 or 2 mg/kg of fullerenes was administered intratracheally to BALB/c mice, which were sacrificed at days 1, 6 and 42. Freshly isolated splenocytes (2×10^5/well) from these mice were stimulated with PBS, ConA (5 μg/ml) or LPS (1 μg/ml) for 72 h. The proliferation of splenocytes was determined using the Cell Titer-Glo luminescent cell viability assay. The values are shown as the mean of relative activity ± SD ($n = 6$ mice). $^{*}p < 0.05$ vs. control mice. The results are representative of two similar experiments.

2.9. Statistics

Values are given as the mean ± SD. Comparisons between two values were performed using ANOVA, and then, statistical significance was assessed by conducting a post hoc Scheffe test. A confidence level of $p < 0.05$ was considered significant.

3. Results

3.1. Fullerene administration induces inflammatory cytokines in BALF

We first investigated the cytokine production in BALF after fullerene administration. Fig. 2 shows that inflammatory cytokines, such as IL-1β and TNF-α, were dramatically induced after high-dose (2 mg/kg) fullerene administration at day 1 but not after control administration, which is similar to previous reports [16]. The production of inflammatory cytokines decreased in a time-dependent manner and returned to normal levels by 42 days post-fullerene treatment. Fullerene administration induced an increase of $CD4^+$ (control administration: 0.49 ± 0.19, fullerenes; 0.2 mg/ml administration: 30.3 ± 4.95) and $CD11b^+$ (control administration: 0.83 ± 0.30; fullerenes, 0.2 mg/ml administration: 13.8 ± 0.14) cells in the BALF at day 1. Overall, these results suggest that fullerene administration induces acute inflammation in the lungs.

3.2. Proliferation of splenocytes is increased by fullerene administration

To clarify whether fullerene administration affects immune cells, the proliferation of splenocytes was measured using the Cell Titer-Glo luminescent cell viability assay system. As shown in Fig. 3, the proliferation of splenocytes induced by ConA and LPS was enhanced at day 6 post-fullerene treatment (both 0.2 and 2 mg/kg) but not at day 1. Furthermore, this increase in the proliferation of splenocytes returned to the levels of control mice at 42 days after fullerene treatment, which indicated that the fullerenes triggered inflammation in the spleen with different timing than the lungs. The populations of splenocytes were not changed by fullerene treatment, as analyzed by flow cytometry (data not shown).

3.3. IL-2 and TNF-α production from splenocytes are increased by fullerene treatment

To further characterize splenic inflammation induced by fullerene administration at day 6, the production of IL-2 and TNF-α in splenocytes was measured. As shown in Fig. 4, IL-2 and TNF-α production were increased by both 0.2 and 2 mg/kg of fullerene treatment. IL-2 and TNF-α production were not affected by fullerene administration at days 1 and 42 (data not shown). Importantly, Figs. 3 and 4 demonstrate that fullerene administration not only triggered pulmonary inflammation but also splenic inflammation; however, pulmonary and splenic inflammation occurred at different times and doses.

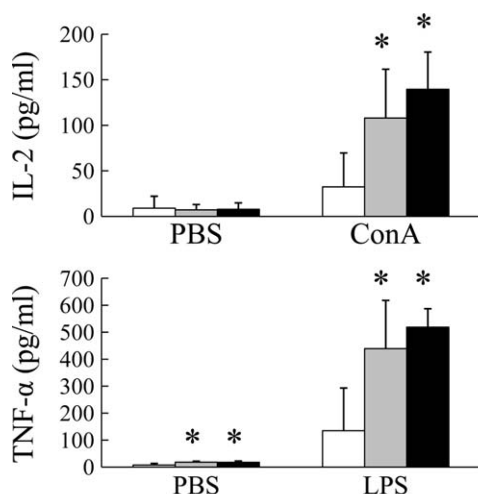

Fig. 4. IL-2 and TNF-α production from splenocytes are increased by fullerene treatment. BALB/c mice received an intratracheal dose of 0, 0.2 or 2 mg/kg fullerenes and were sacrificed at day 6. Freshly isolated splenocytes (1×10^6/well) from these mice were stimulated with PBS, ConA (5 μg/ml) or LPS (1 μg/ml) for 6 h. The IL-2 and TNF-α production from these splenocytes were measured by ELISA. The values are shown as the mean ± SD ($n = 6$ mice). $^*p < 0.05$ vs. control mice. The results are representative of two similar experiments.

3.4. Fullerenes induce the expression of CDK2 and activation of NF-κB and NFAT

To study the mechanisms of inflammation and proliferation, the expression of key molecules such as CDKs and several transcription factors that relate to cell cycle progression and regulation of proliferation were analyzed by western blotting. The administration of fullerenes strongly increased CDK2 expression and total NF-κB p65 while also activating NFAT, as shown by the dephosphorylation of NFAT (Fig. 5A). These data may relate to the enhancement of the proliferation of splenocytes. Because NF-κB is known to be important for IL-2 induction, the entire NF-κB pathway was examined. Phosphorylated and total IκBα was also induced by fullerene administration. Furthermore, results from EMSA showed that fullerene administration induced the activation of NF-κB, as shown in Fig. 5B. Overall, the expression of CDK2 and activation of NF-κB and NFAT were enhanced by fullerene administration.

3.5. CD11b$^+$ cells function as responder cells in fullerene-induced splenic inflammation

To further investigate the responder cells during the splenic inflammatory process at day 6, the CD4$^+$, CD11b$^+$ and CD45R$^+$ cells were purified from splenocytes, and the production of cytokines, such as IL-2 and TNF-α, and IgG were analyzed by ELISA. As shown in Fig. 6A, TNF-α production in CD11b$^+$ cells was increased by 2 mg/kg fullerene treatment. However, the IL-2 production in CD4$^+$ cells and IgG production in CD45R$^+$ cells were not affected by fullerene administration. Thus, these results indicate that fullerenes may directly activate CD11b$^+$ cells, such as macrophages other than T cells or B cells, during splenic inflammation. Next, the CDK2 expression and activation of NF-κB in CD11b$^+$ cells were analyzed by western blotting. Fig. 6B shows that fullerene administration enhanced the phosphorylation of p65 and CDK2 expression in CD11b$^+$ cells. In total, these results indicate that fullerenes induced the activation of NF-κB and enhancement of CDK2 expression in CD11b$^+$ cells, which function as responder cells during the splenic inflammatory process.

4. Discussion

As fullerenes have gained enormous popularity for their antioxidant properties [17], there are growing investigations regarding their pro-inflammatory characteristics. Previous studies have indicated that intratracheal instillation of fullerenes induced temporary pulmonary inflammatory responses [1,16]. Fujita et al. have also reported that fullerenes remained deposited in lung tissue at 1 week post-instillation [18]. The deposition of fullerenes in the lungs contributes to acute-phase inflammatory responses in the lung tissue. Consistent with these reports, mice treated with fullerenes displayed a transient increase of pro-inflammatory cytokines in BALF at day 1, which returned to normal levels within 6 weeks. It seems that pulmonary responses induced by fullerenes are not prolonged in lung tissue.

In contrast to pulmonary studies, there are few reports concerning whether lymphoid organs, such as the spleen, are affected by fullerenes. As shown in Figs. 3 and 4, our study demonstrates, for the first time to our knowledge, that the proliferation of and inflammatory cytokine production by splenocytes were significantly increased by both low and high doses of fullerenes. It is well known that IL-2 production is important for the differentiation and proliferation of T cells [19]. TNF-α has also been demonstrated to activate the NF-κB and MAPKs signaling pathways, which are involved in cell survival and proliferation [20]. Liu et al. have reported that treatment with $C_{60}(OH)_{20}$ nanoparticles or polyhydroxylated metallofullernol enhanced TNF-α levels in serum [21,22]. Thus, this increased production of inflammatory cytokines induced by fullerenes may contribute to the enhancement of the proliferation of splenocytes.

Previously, Naota et al. have reported that fullerenes immediately translocate into the systemic circulation through diffusion and caveolae-mediated pinocytosis [23]. Similarly, in an instillation study of carbon fullerenes carried out by Park et al., inflammatory cytokine production in the blood was increased after treatment, which reached a maximum level at day 1 and decreased in a time-dependent manner [24]. These reports may demonstrate that in addition to pulmonary inflammation, fullerenes also trigger systemic inflammation by intratracheal administration. Our results from splenic responses may relate to the circulation of fullerenes after intratracheal administration. We did not observe lung inflammation after low-dose fullerene administration, in contrast to the splenic responses. This result may also relate to the circulation of fullerenes. After circulation, accumulation of fullerenes in the spleen might cause splenic responses to a low-dose treatment, although this mechanism is still unclear.

Previously, Kleinman et al. showed that inhaled ultrafine particulate matter affected activation of the JNK and NF-κB pathways in vivo [25]. The transitions of the cell cycle are associated with the activation of CDKs [26]. CDK2 has been considered to be a key downstream target for the S-phase checkpoint [27]. It is also well known that the transcription factor NF-κB is a major regulator of inflammatory responses and cell proliferation [28]. Here, we found that fullerenes activated CDK2 and NF-κB p65, which may contribute to the elevated proliferation of splenocytes. Some previous reports have shown that nanomaterials activate NF-κB and increase CDK2 and IκBα production in human keratinocytes, human skin fibroblast cells and murine macrophages in vitro [29–31]. Our study demonstrates, for the first time, that fullerenes influence the expression of CDK2 and activation of NF-κB in vivo. Additionally, levels of phosphorylated IκBα and total IκBα also increased after fullerene administration. Meanwhile, we found that NFAT was slightly activated after fullerene treatment for 6 days. NFAT served as representative marker for the activation of T cells, and these activated T cells may assist in the development of splenic inflammation at day 6 post-administration [32].

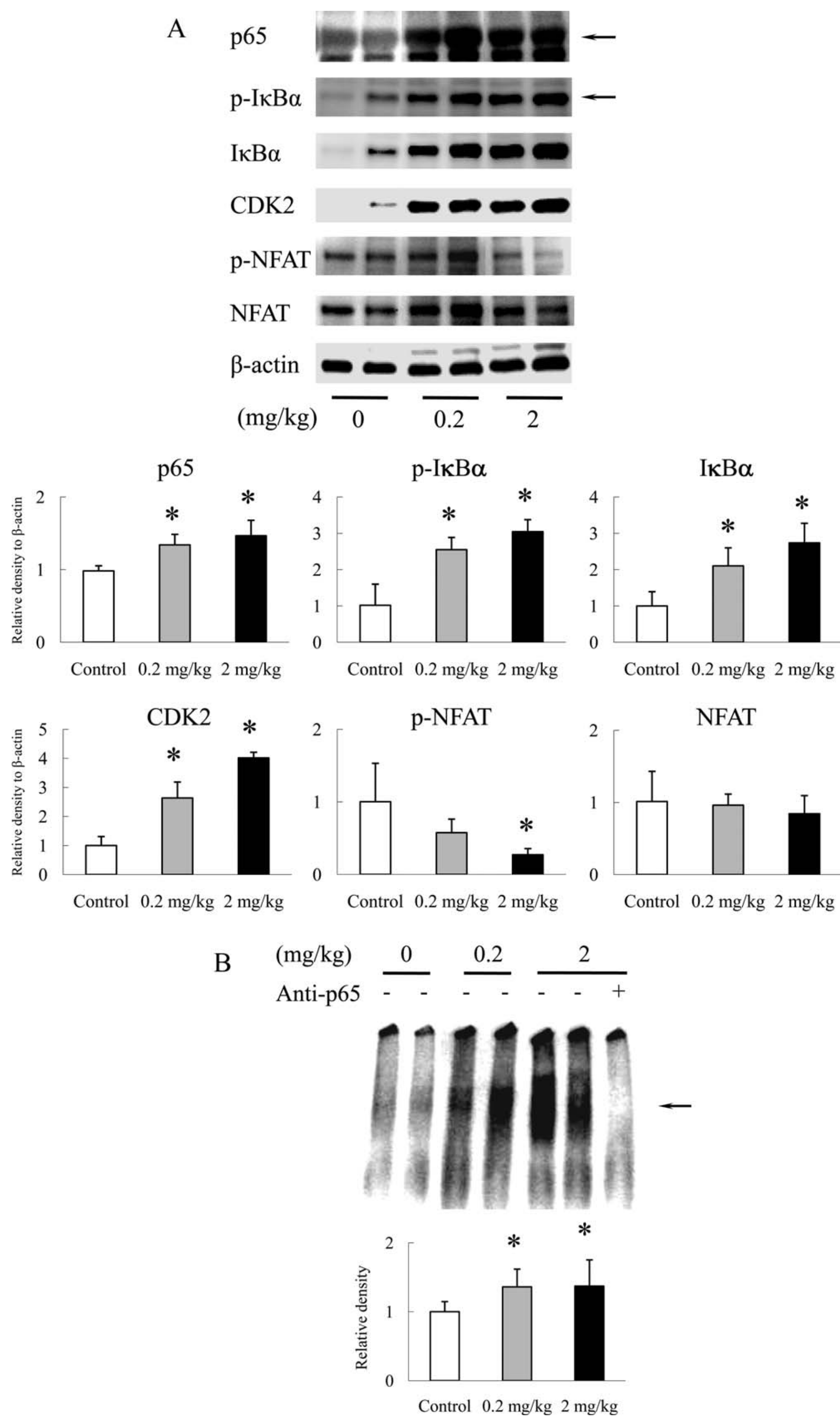

Fig. 5. Fullerenes induce expression of CDK2 and activation of NF-κB and NFAT. (A) A dose of 0, 0.2 or 2 mg/kg fullerenes was administered intratracheally to BALB/c mice, which were sacrificed at day 6 as indicated. Whole cell lysates of freshly isolated splenocytes (1×10^6) were prepared and probed by western blot analysis to measure the levels of p65, phospho (p)-IκBα, IκBα, CDK2, p-NFAT and NFAT. β-Actin served to demonstrate equal protein loading. The quantification of western blot data is shown at the bottom. The values are shown as the mean of relative density to β-actin ± SD ($n = 6$ mice). $^*p < 0.05$ vs. control mice. (B) Whole cell lysates of freshly isolated splenocytes (1×10^6) were prepared and applied for EMSA using a radiolabeled oligonucleotide, which contains the NF-κB DNA binding site of immunoglobulin κ enhancer (Ig-κB). The arrow indicates a protein–DNA complex. The quantification of EMSA data is shown at the bottom. The values are shown as the mean of relative density ± SD (each group contained 6 mice). $^*p < 0.05$ vs. control mice. The results are representative of two similar experiments.

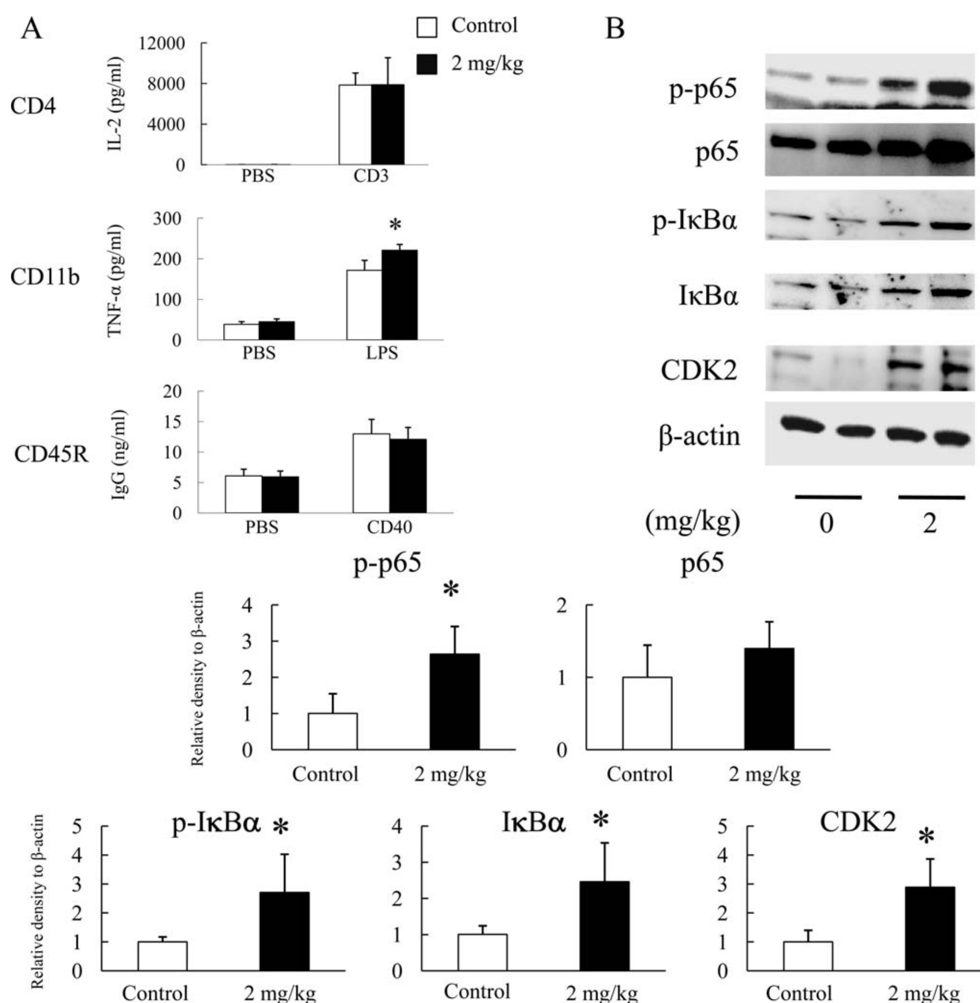

Fig. 6. CD11b+ cells function as responder cells in fullerene-induced splenic inflammation. (A) BALB/c mice were administered intratracheally a dose of 0 or 2 mg/kg fullerenes and were sacrificed at day 6. The CD4+, CD11b+ and CD45R+ cells were isolated from splenocytes, as shown in Section 2. Separated CD4+ and CD11b+ cells (1×10^6/well) were stimulated with PBS, coated anti-CD3 (2 μg/ml) or LPS (1 μg/ml) for 12 h. Separated CD45R+ cells were stimulated with PBS or anti-CD40 (1 μg/ml) for 5 days. The IL-2, TNF-α and IgG production levels of these cells were measured by ELISA. The values are shown as the mean ± SD ($n = 6$ mice). $^*p < 0.05$ vs. control mice. (B) Whole cell lysates of CD11b+ cells (1×10^6) were prepared and probed by western blot analysis to measure the expression levels of p-p65, p65 and CDK2. β-Actin served to demonstrate equal protein loading. The quantification of western blot data is shown at the bottom. The values are shown as the mean of relative density to β-actin ± SD ($n = 6$ mice). $^*p < 0.05$ vs. control mice. The results are representative of two similar experiments.

Previously, Manna et al. demonstrated that fullerenes could activate macrophages in vitro [29]. Moreover, Morimoto et al. and Sayes et al. have shown the infiltration of alveolar macrophages in alveoli after the intratracheal instillation of fullerenes [1,16]. These studies mentioned the activating effect of fullerenes on pulmonary macrophages in vivo and with in vitro assays. Here, our study is the first to demonstrate that CD11b+ cells, other than T cells and B cells in the spleen, could be directly activated in vivo at day 6 after fullerene administration. As discussed in the previous paragraph, CDK2 expression was affected by fullerenes. Macrophages seem to be targeted by fullerenes, as evidenced by CDK2 expression being affected in these non-proliferating cells. Fullerenes may activate CD11b+ cells, T cells and whole splenocytes, which exhibited increased proliferative activity and IL-2 production, although fullerenes do not directly activate T cells. Considering our results and those from previous reports, macrophages activated by fullerenes may trigger CD11b+ cell activation.

Taken together, it seems that the effects of fullerenes on the spleen are observed at both the transcriptional level and the cell cycle level. It is important to take into account that fullerenes triggered inflammation in the spleen, which may introduce possible adverse effects during subsequent contact with fullerenes. Further studies are needed to fully understand the possible effects of fullerene administration in the lungs and spleen.

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