

In Vitro Enhancement of Mouse T Helper 2 Cell Sensitization to Ovalbumin Allergen by Carbon Black Nanoparticles

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Agglomerated carbon black nanoparticles (CBNPs) administered via respiratory or subcutaneous routes have been shown to promote allergic sensitization to coadministered ovalbumin (OVA) protein in rodents. In the present study, we aimed to model and elucidate the mechanism of this adjuvant activity using an *in vitro* assay based on T cell sensitization to ovalbumin_{323–339} peptide (OVA_p). CBNP base particles of 22 and 39 nm were characterized and termed CBNP22 and CBNP39 powders. Splenic leukocytes derived from transgenic DO11.10 mice were exposed to suspensions of media alone, concanavalin A mitogen, CBNP agglomerates smaller than 220 nm, OVA_p alone, OVA_p + anti-CD28 costimulant, OVA_p + cyclosporin A immunosuppressant, or OVA_p + CBNPs. Samples were analyzed at 72 h post-exposure. Proliferation rate, a marker of cellular mitosis, was assessed. Polymerase chain reaction arrays were used to assess genes involved in allergic response pathways. The mitogen control, costimulatory control, and immunosuppressive control chemicals modified the T helper cell proliferation rate. CBNP22 mildly reduced proliferation at 12 µg/ml, but CBNP39 did not. Gene expression analysis of cells treated with OVA_p showed that coincubation with 12 µg/ml CBNP22 enhanced gene expression of *interleukin-4* (*IL-4*), *IL-10*, and *IL-13*, all allergy-associated Th2 cytokines. Coincubation of OVA_p with 12 µg/ml CBNP39 significantly enhanced *IL-13* gene expression concurrent with downregulation of the Th1-associated transcription factor *Stat4*. *IL-4* and *IL-13* protein secretion reflected the mRNA trends. The changes were consistently higher in cells exposed to CBNP22 than CBNP39, suggesting that smaller particle size, higher surface area, and higher purity were associated with the direct adjuvant effect on Th2 cells in this genetically susceptible model of OVA allergy.

Key words: nanotechnology; adjuvant; cytokines; immunotoxicology.

Allergy results from the mistaken recognition of an environmental protein by leukocytes (Waserman and Watson, 2011). Immunoglobulin E (IgE)-mediated mechanisms are the most common. Initial sensitization involves presentation of the allergenic protein by antigen-presenting cells and recognition by

T cells in a cytokine microenvironment that promotes allergy pathways. T cells activated in such a microenvironment secrete cytokines such as *IL-4* and *IL-13*, which act in an autocrine manner to promote differentiation to a Th2 phenotype, and act in a paracrine manner to promote B-cell maturation and IgE antibody production (Hunig *et al.*, 2010; Yagi *et al.*, 2011). The exogenous adjuvants that influence the switch from tolerance to sensitization remain largely uncharacterized (Jenerowicz *et al.*, 2012).

Engineered nanomaterials have an internal or external surface structure dimension between 1 and 100 nm and can exhibit unique physical, chemical, and biological characteristics associated with their nanostructure. These chemicals are increasingly used in consumer products. Therapeutic uses take advantage of immunostimulatory or immunosuppressive properties. For example, in vaccine development the elicitation of an inflammatory reaction may be advantageous by conferring immune potentiation to the coadministered vaccine (Fahmy *et al.*, 2008). Humans may be exposed incidentally or occupationally to engineered nanomaterials, and early studies have raised concerns about safety to humans and the environment (Bonner, 2010; Johnston *et al.*, 2013).

Using wild-type animal models, a number of studies have demonstrated an association between exposure to nanomaterials and enhanced allergic reactions, as reviewed by Hussain *et al.* (2012). This outcome appears to depend on physiochemical characteristics and dose. For example, respiratory exposure to ultrafine particulate air pollutants can potentiate immune responses to concomitantly exposed antigens such as pollen (Chang, 2010). Single and multi-walled carbon nanotubes increased lung inflammation and allergen-specific IgE levels in mice sensitized to ovalbumin (OVA) egg allergen (Nygaard *et al.*, 2009). On the other hand, nano-sized silver particles diminished allergic airway reactions in mice sensitized to OVA (Park *et al.*, 2010). Thus, high-throughput studies involving well-characterized nanomaterials are required to determine the

primary target tissues and mechanisms in order to understand if nanomaterials pose a risk to humans.

Carbon black (CB) is a carbonaceous material produced by incomplete combustion of petroleum products. Bulk-sized CB is mainly used as a reinforcing agent for rubber and is also used in printing inks, plastics, and food contact products. Micron-sized bulk CB particles did not modify allergic airway sensitization in wild-type mice, whereas nano-sized carbon black nanoparticles (CBNPs) promoted this adverse reaction (de Haar *et al.*, 2006; Nygaard *et al.*, 2009). However, it was unclear if this was a direct effect of the CBNPs on immune cells or a secondary effect from toxicity to other tissues. *In vitro* assays can answer these mechanistic questions (Pfaller *et al.*, 2010).

DO11.10 mice are transgenic for a T cell receptor that reacts to OVA_{323–339} peptide (OVA_p) and develop allergy symptoms similar to humans (Okamoto *et al.*, 2005). We previously used primary splenic leukocytes from DO11.10 mice to determine the effect of exogenously applied factors on the mechanisms of T cell activation (Chakir *et al.*, 2003). In the present study, we employed this *in vitro* model to determine if CBNPs modulate allergic sensitization to OVA_p by direct modulation of Th1 and Th2 cytokine pathways.

MATERIALS AND METHODS

Materials. Printex 90 CB nanomaterial powder from Evonik/Degussa (Frankfurt, Germany) was a kind gift from Dr. Håkan Wallin of the National Research Center for the Working Environment, Denmark, and was termed CBNP22 because the primary particles were a mean of 22 nm. Carbon nanopowder (Sigma-Aldrich, St. Louis, MO) was termed CBNP39 because the primary particles were 39 nm. Standard safety precautions were used (Howard, 2012). Fetal bovine serum was from HyClone Laboratories (Logan, UT). RPMI 1640 medium, penicillin, streptomycin, L-glutamine, and β -mercaptoethanol were from Invitrogen (Burlington, ON, Canada).

Surface area and pore volume of CBNP powders. Nanomaterial characterization was carried out at Intertek ASA (Allentown, PA). Surface area and pore volume were characterized by Brunauer Emmett Teller analysis (Nygaard *et al.*, 2009). Briefly, nitrogen adsorption isotherms were measured on a Micromeritics ASAP unit to a relative pressure of 1.0 at 77 K. Samples were activated by degassing in stages up to 300°C under dynamic vacuum. Temperatures were ramped at $\sim 10^\circ\text{C}/\text{min}$ to preset temperatures and held until a vacuum pressure of 10^{-3} torr was achieved. The activation procedure used temperatures of 80°C, 100°C, 150°C, 200°C, 250°C, and 300°C. The samples were held at 300°C until a vacuum of $\sim 10^{-4}$ torr was obtained. Cumulative pore volume was quantified assuming applicability of the Kelvin equation and a thickness curve represented by the Harkins and Jura model.

Composition of CBNP powders. The elemental composition was analyzed on a PANalytical PW4400R Axios wavelength dispersive X-ray fluorescence spectrometer in a helium atmosphere. A carbon matrix correction was applied and elemental concentrations were calculated as the weight percentage of each oxide, normalized to 100% using the program UNIQANT. Elements representing greater than 0.1 weight percentage were reported.

Surface composition and functionality of CBNP powders. X-ray photoelectron spectroscopy was performed on a PHI 5000VersaProbe Spectrometer equipped with multiple channel plates and a focused Al monochromatic X-ray source. Briefly, high-resolution multiplex scans were performed at 23.50 eV pass energy, 0.100 eV/step, and a 100 ms/step dwell time. CasaXPS software used a transmission function corrected Area Sensitivity Factor. The atomic percentage of surface elements was calculated, omitting hydrogen, and was normalized to 100%. Curve fitting for chemical functionality was charge corrected to $C_{\text{amorphous}}$ 283.7 eV and functionalization of the surface carbon was quantified.

CBNP primary particle and agglomerate size before and after sonication. Scanning electron microscopy was performed with the CBNP powders before and after suspension and sonication in ethanol. Images were obtained with a Hitachi S-4800 field emission electron microscope at 5 kV accelerating voltage and analysis was performed using AnalySIS software. The longest particle dimension was measured.

Zeta potential. Zeta charge potential analysis was performed with a Malvern ZetaSizer following sonication of the CBNPs in water at pH 7.1. A wavelength of 632 nm was used along with a relative refractive index of 1.50–0.10 and a liquid refractive index of 1.33 for water.

Suspension of CBNPs in cell culture medium. Fetal bovine serum was heat inactivated at 56°C for 30 min. Complete RP-10 cell culture medium at pH 7.1 consisted of RPMI 1640, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine, and 25 μM β -mercaptoethanol (Chakir *et al.*, 2003). Suspensions of the CBNP powders were prepared in this RP-10. A Q700 sonicator with a microtip probe (Qsonica, Newtown, CT) was used to sonicate for 15 effective min at 10% amplitude on ice, with 30 s of manual mixing by inversion every 5 min (Jacobsen *et al.*, 2009). An aliquot was retained for a standard concentration curve, and the remainder was filtered with a 220 nm Steriflip (Millipore, Billerica, MA) to sterilize the suspension and purify agglomerates smaller than 220 nm—reflecting a defined size range from a specific processing condition (Bourdon *et al.*, 2012a).

Characterization of CBNPs in cell culture medium. The mass concentration of CBNPs in the Steriflip filtrate used for cell

culture was quantified by spectrophotometry at 470 nm using a Powerwave XS plate reader and KC4 software (BioTek, Winooski, VT) against a standard curve of unfiltered CBNPs. The filtrates were then adjusted for final concentrations of 0.0125, 0.125, 1.25, and 12.5 $\mu\text{g/ml}$ and frozen at -80°C until equilibration to 37°C for cell exposure (Jacobsen *et al.*, 2009). Nanomaterial surface area per volume was calculated as previously described (Pfaller *et al.*, 2010).

Endotoxin in the filtered CBNP suspensions was measured using the Limulus amoebocyte lysate kinetic-QCL assay (Lonza, Allendale, NJ) as in Neun and Dobrovolskaia (2011). Briefly, CBNP aliquots were centrifuged six times for 10 min at $18,000 \times g$ to remove nanomaterials before quantification of free endotoxin (Nygaard *et al.*, 2009).

A similar approach was used to determine if the nanomaterials sequestered a significant amount of protein from the medium (de Haar *et al.*, 2006). Briefly, the RP-10 media and CBNP suspensions in RP-10 were centrifuged four times at 22°C for 15 min at $8000 \times g$. Protein level in the supernatants was quantified using the BCA Protein Assay kit (Thermo Scientific, Rockford, IL).

Animal care. Animal handling complied with the guidelines of the Canadian Council on Animal Care, and protocols were approved by the Health Canada Animal Care Committee (Ottawa, ON). D011.10 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Equal numbers of 7 week old male and female mice were pair housed and acclimated for 1 week with *ad libitum* access to Harlan 2014 diet and drinking water. These 8 week old mice were then anesthetized with isoflurane, cardiac puncture was performed, and the spleen was excised.

Preparation of primary splenic leukocytes. The spleen was placed in sterile RPMI medium at 4°C and single cell suspensions were prepared as described previously (Chakir *et al.*, 2003). Briefly, segments of spleen were crushed between frosted glass slides and filtered through a $70 \mu\text{m}$ nylon strainer. Erythrocytes were eliminated with ACK lysis buffer (Invitrogen). The remaining splenic leukocytes were washed in RPMI and counted by trypan blue exclusion.

OVA peptide and immunomodulatory control chemicals. OVA_{323–339} peptide (Anaspec, Fremont, CA) was resuspended in RP-10 and sterile filtered with a prerinsed Millex-GP filter (Millipore). A pilot study determined that D011.10 splenic leukocytes treated with $0.4 \mu\text{M}$ OVA_p had a half-maximal proliferation and cytokine response (data not shown). Thus, a final culture concentration of $0.4 \mu\text{M}$ OVA_p was used. Purified NA/LE hamster antimouse CD28 antibody (clone 37.51, BD Pharmingen) was used at $10 \mu\text{g/ml}$ (Blankson and Morse, 1994). Solid cyclosporin A from *Tolypocladium inflatum* (Sigma) was resuspended in dimethyl sulfoxide, sterilized with a Millex-LG syringe filter (Millipore), and diluted 1/10,000 to a final culture concentration of $1 \mu\text{g/ml}$ (Shimoda *et al.*,

1998). Aseptically processed concanavalin A (Con A) Type IV-S (Sigma) was used in RP-10 at $2 \mu\text{g/ml}$.

Flow cytometry phenotyping and proliferation assay. On day 0, splenic leukocytes were stained with $5 \mu\text{M}$ CellTrace Violet (Invitrogen) and 1×10^6 stained cells were cultured in 1 ml RP-10 in a 24-well plate (BD Falcon, Mississauga, ON) with or without control chemicals at 37°C in a humidified atmosphere and 5% CO_2 in air. At 72 h the nonadherent cells were harvested, washed, and stained with V500 anti-mouse CD45, PE anti-mouse KJ1–26 T cell receptor, and APC anti-mouse CD4 (BD). Cells were fixed with formalin and cell surface phenotype was analyzed on an LSR Fortessa flow cytometer with FACS Diva software (BD). Briefly, after gating on CD45⁺ leukocytes, the percentage of OVA_p-specific KJ1–26⁺CD4⁺ T helper cells among that population was determined. The percentage of cells that proliferated was also assessed with ModFit LT, version 3.2 (Verity Software House, Topsham, ME). Briefly, gated leukocytes were further gated on KJ1–26⁺CD4⁺ cells to generate a CellTrace Violet histogram for that population. The parent generation and spacing of the daughter generations in the histograms was set on the cells treated with OVA_p. The percentage of cells in daughter generations was quantified. Cells from individual mice were excluded from the analysis if the proliferation of the OVA_p-treated cells was within one standard deviation (SD) of the mean proliferation of the OVA_p + anti-CD28 group or within one SD of the mean of the OVA_p + cyclosporin A group.

To rule out nanomaterial interference, wells of cells that had not been treated were spiked with CBNP, before harvesting at 72 h, to ensure that the nanomaterials did not interfere with antibody binding or fluorescence compared with nonspiked cells.

Real-time qPCR array plate analysis. Nonstained splenic leukocytes were treated for 72 h as described above, harvested, washed with PBS, and stored at -70°C . Total RNA was isolated using an miRNeasy Mini kit (Qiagen, Toronto, ON). cDNA was synthesized using an RT² First Strand kit (Qiagen). Briefly, 500 ng of total RNA was treated with genomic DNA elimination mix. Reverse transcription was carried out for 15 min at 42°C followed by 5 min at 95°C . RT² profiler polymerase chain reaction (PCR) array plates for mouse Th1–Th2–Th3 (SA Biosciences, Mississauga, ON) were used on a ViiA7 real-time PCR system (Applied Biosystems, Burlington, ON). One 10 min cycle at 95°C was followed by 40 cycles consisting of 15 s at 95°C and 1 min at 60°C . Threshold cycle (Ct) values were determined for each gene following the establishment of an automated baseline and a defined threshold. Targets *IL23a*, *IL17a*, *Ccl11*, *Ccl7*, *Ccr10*, *IL12b*, *IL1r1*, *IL7*, and *Sftpd* were excluded because their melting curve peak had high SD between plates, indicating non-specific PCR priming. The remaining 75 genes were analyzed using the SA Biosciences RT² Profiler PCR Array Data Analysis Template v3.3 by a comparative Ct method (Lewkowicz *et al.*, 2011). *Gusb*, *Hprt*, and *Hsp90ab1* were se-

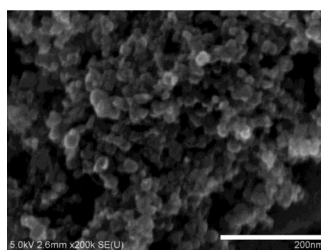
lected by the RT² software as the reference housekeeping genes. The $2^{-\Delta Ct}$ values were used to calculate fold change for each treatment group relative to the OVA_p control group. A fold-change cut-off of ≥ 4 or ≤ 0.25 , along with a significant difference $p < 0.05$ from the OVA_p control group, was used specifically for this model because it reproducibly flagged CBNP concentrations at which mRNA gene expression changes corresponded with significant protein changes—a key functional indicator of effects. The GeneBank Refseq for the genes with changes which met the cut-off requirements are NM.021283 for *IL-4*, NM.010548 for *IL-10*, NM.008355 for *IL-13*, and NM.011487 for *Stat4*. A clustergram analysis was performed using the RT² software (Lu *et al.*, 2011).

Quantification of intracellular and secreted proteins. Non-stained splenic leukocytes were treated for 72 h as described above. The cells were used for Western blot quantification of intracellular STAT4. Cell-free culture supernatants were also used for analysis of secreted proteins following centrifugation at $12,000 \times g$ for 10 min at 4°C to remove particles. IL-4 was quantified with an OptEIA enzyme-linked immunosorbent assay (ELISA) kit as well as a Cytometric Bead Array kit for mouse Th1/Th2 cytokines (Goulaouic *et al.*, 2008; Val *et al.*, 2009). IL-10 and IL-13 were assessed with Flex Set kits (BD). False positives from nanomaterial interference were ruled out before culture by analyzing media compared with CBNP suspensions. False negatives were ruled out by comparing non-spiked and CBNP-spiked cytokine standard (Neun and Dobrovolskaia, 2011).

Intracellular STAT4 was also quantified *in situ* by cell-based ELISA. Briefly, on day 0, 96-well culture plates were pre-coated with 10 $\mu\text{g}/\text{ml}$ poly-L-lysine (Sigma) for 30 min at 37°C, washed with sterile water, and dried. Cells were cultured in triplicate at $1 \times 10^5/\text{well}$ in 200 μl and treated as above. At 72 h, supernatants were harvested and the cells were fixed with 8% formaldehyde in PBS for 20 min at 22°C. Intracellular STAT4 was quantified by Fast Activated Cell-based ELISA for nonadherent cells (Active Motif, Carlsbad, CA). To ensure that residual nanomaterials did not interfere with the assay, control wells containing CBNPs were developed without the primary antibody.

Statistical analysis. All statistical analyses were performed with SigmaPlot version 12.0. A Shapiro-Wilk normality test and an equal variance test were performed, followed by one-way analysis of variance (ANOVA) (Rieu and Powers, 2009). The parametric Holm-Sidak *post hoc* test was run versus the control. For nonparametric data, the Kruskal-Wallis one-way ANOVA on Ranks was run and followed by the Dunnett's *post hoc* test versus the control if treatment group sizes were equal or followed by the Dunn's *post hoc* test versus the control if treatment group sizes were unequal.

CBNP22



CBNP39

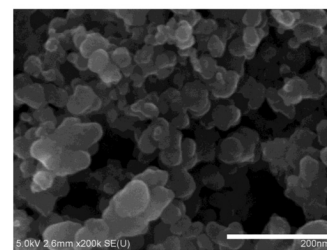


FIG. 1. Scanning electron micrographs of agglomerates composed of primary carbon black nanoparticles (CBNPs). The images were obtained following suspension and sonication of the agglomerated source powders in ethanol. The magnification was $\times 200,000$ and the white scale bars represent 200 nm.

RESULTS

Particle Characterization

The surface area and total pore volume of the CBNP22 powder were approximately six and four times greater than the CBNP39 powder, respectively (Table 1). The CBNP22 was composed of 99.77% carbon, containing sulfur as the greatest contaminant. CBNP39 was less pure consisting of 83.06% carbon containing the notable contaminants silicon, zinc, and sulfur (Table 2). The surface of the CBNP22 particles was 98.4 ± 0.1 relative atomic percent carbon with the remainder oxygen. Carbon represented $93.8 \pm 0.4\%$ of the CBNP39 surface. The remainder was $4.7 \pm 0.5\%$ oxygen, $0.6 \pm 0.1\%$ silicon, $0.6 \pm 0.1\%$ sulfur, and $0.5 \pm 0.1\%$ zinc, the latter three present as silicate and zinc sulfide. The functionalization of the surface carbon of both materials was predominantly amorphous (Table 3).

Particle shape and size was characterized before and after sonication. The CBNP22 and CBNP39 powders were highly agglomerated (Table 4). Following suspension in ethanol, sonication, and drying, the primary nanoparticles making up the powders were visible as distinct primary structures on the agglomerate surface (Fig. 1). Sonication reduced the mean size and size distribution of these amorphous primary particles (Table 4). The CBNP22 primary particles were a mean of 22 nm and those making up CBNP39 were 39 nm. Peak zeta surface charge was -31.0 mV for CBNP22 and -31.1 mV for CBNP39.

For cell culture, the CBNPs were dispersed by sonication in complete cell culture medium and sterilized by filtration through a 220 nm filter to purify this size range. Mass per volume concentration in the filtrate was quantified and normalized. At the highest dose of 12 $\mu\text{g}/\text{ml}$ CBNP22, the particle surface area per volume was $0.0041 \text{ m}^2/\text{ml}$. The same concentration of CBNP39 was $0.0006 \text{ m}^2/\text{ml}$. The nanomaterials did not significantly alter the concentration of free protein in the cell culture medium. Free endotoxin level was less than 0.005 EU/ml, or less than 0.4 EU/mg nanomaterial, and it was less than 0.005 EU/mg OVA_p; well below immunomodulatory concentrations.

TABLE 1
Surface Area and Pore Volume of the Nanomaterial Powders Before Suspension

Parameter	CBNP22	CBNP39
Surface area (m ² /g)	341.0	54.4
Total pore volume (cm ³ /g)	1.316	0.330

Note. Precision data indicated an uncertainty of 5%.

TABLE 2
Contaminants in the Carbon Nanomaterial Powders

Contaminant	CBNP22 (weight%)	CBNP39 (weight%)
Si	bl	6.2 ± 0.13
Zn	bl	3.2 ± 0.11
S	0.210 ± 0.0100	2.0 ± 0.07
Ca	bl	1.5 ± 0.06
Al	bl	1.4 ± 0.06
Fe	bl	1.1 ± 0.05
Na	bl	0.66 ± 0.033
K	0.012 ± 0.0006	0.20 ± 0.010
Mg	bl	0.20 ± 0.010
Ti	bl	0.12 ± 0.006

Notes. Data represent mean ± standard error of two separate scans by wavelength dispersive X-ray fluorescence spectrometry. bl denotes below the selected inclusion limit of 0.1 weight percent.

TABLE 3
Surface Chemical Functionality of the Carbon Nanomaterial Powders

Surface functionality	CBNP22 (atomic%)	CBNP39 (atomic%)
C _{amorphous} 283.7 C1s	64.9 ± 0.8	71.6 ± 1.3
CH _x 285.5 C1s	9.8 ± 0.6	6.9 ± 0.4
C-O 287.1 C1s	7.0 ± 0.6	3.8 ± 2.4
O-C-O 288.2 C1s	5.7 ± 0.0	4.8 ± 0.0
O-C = O 289.5 C1s	6.7 ± 0.8	4.5 ± 0.1
CO ₃ 291.2 C1s	4.6 ± 1.0	3.0 ± 0.1

Note. Data represent mean ± SD of X-ray photoelectron spectroscopy measurements made at two sample locations.

TABLE 4
Primary Particle and Agglomerate Size Distribution

Analyte	Observed component of the analyte	Parameter	CBNP22 (nm)	CBNP39 (nm)
Source powder	Primary particles	Mean size	66.1	55.8
		Minimum size	38.0	29.8
		Maximum size	120.7	104.7
	Agglomerates	Mean size	5500	12,800
		Minimum size	1300	2900
		Maximum size	21,200	54,100
Following sonication	Primary particles	Mean size	22.1	38.8
		Minimum size	9.6	14.7
		Maximum size	34.6	67.9
	Agglomerates	Mean size	3900	7200
		Minimum size	1400	600
		Maximum size	11,000	21,400
Manufacturer's reported size	Primary particles	Mean size	14	<50

Effects on T Cell Proliferation and Phenotype

Following 72 h culture in media alone, the percentage of OVA_p-specific T helper cells that completed mitosis was $23.4 \pm 4.9\%$ (Figs. 2A and B). The Con A positive control promoted significant T cell proliferation. In cells cultured with the filtered CBNPs alone there was a statistically significant suppression of cellular division at the highest dose of CBNP22, which was a relatively small fold-change from the media control (Fig. 2B). CBNP39 had no effect on proliferation. An MTT metabolic assay demonstrated that the nanomaterials did not reduce cell viability (data not shown). Culture of the splenic leukocytes with OVA_p resulted in a cell division level of $62.6 \pm 9.9\%$ (Figs. 2A and C). The T cell-specific anti-CD28 agonist enhanced proliferation to OVA_p. The cyclosporin A immunosuppressant inhibited the response. Coculture of the cells with OVA_p in combination with the carbon nanomaterial suspensions did not alter the proliferative response in comparison to OVA_p alone (Fig. 2C). Flow cytometric phenotyping confirmed that the final percentage of KJ1-26⁺CD4⁺ T helper cells at 72 h corresponded with the proliferation rate (data not shown).

Effects on T Cell Cytokine Pathways

Upregulation of cytokines can occur in the absence of changes in T cell proliferation (Rojo *et al.*, 1989). Thus, we selected a Th1-Th2-Th3 PCR array to screen for effects on a panel of 84 genes consisting of transcription factors, cytokines, and chemokines. As described below, cytokine and chemokine secretion was very low in cells treated with CBNPs alone. Thus, we focused the gene expression analysis screening on the OVA_p treatment groups to identify genes of interest for further protein analysis (Fig. 3). Among the studied genes, there was a significant increase in gene expression of the Th2 cytokines *IL-4*, *IL-10*, and *IL-13* above the fold-change threshold in the cells treated with OVA_p in combination with the highest dose of CBNP22, compared with OVA_p alone (Table 5). *IL-13* transcript was also upregulated in the cells treated with OVA_p in combination with the highest dose of CBNP39. Expression of the *Stat4* transcription factor, which is downregulated during differentiation of Th2 cells, was significantly reduced following coincubation with OVA_p and 12 $\mu\text{g/ml}$ CBNP39. Although only these four genes met the cut-off criteria, the clustergram revealed dose-dependent trends in other functionally related genes, which provides useful subclinical mechanistic data (Fig. 3). With regard to CBNP dose, the cut-off was not surpassed for any genes at or below the nanomaterial dose of 1.2 $\mu\text{g/ml}$.

In order to determine if transcriptional changes translated to significant changes at the protein level, intracellular STAT4 was measured by cell-based ELISA. The protein showed a 25% reduction at the highest concentration of CBNP39 during the response to OVA_p (data not shown). However, there were no statistically significant differences, as confirmed by Western blotting (data not shown). Secreted cytokine proteins were also quantified. The splenic leukocytes cultured in me-

dia without antigenic stimulation secreted low baseline levels of IL-4, IL-10, and IL-13 (Fig. 4) and the filtered carbon suspensions alone did not induce these cytokines. Anti-CD28 significantly enhanced the OVA_p-induced production of these Th2 cytokines, and cyclosporin A was inhibitory. Culturing the cells with OVA_p combined with 12 $\mu\text{g/ml}$ CBNP22 significantly enhanced IL-4 and IL-13 protein levels compared with cells treated with OVA_p alone. Similar, but nonsignificant trends were observed following coincubation of CBNP39.

DISCUSSION

The present work used an *in vitro* assay with cells from transgenic DO11.10 mice to mechanistically determine the threshold concentration of nanomaterials that directly modulate sensitization to OVA protein from chicken egg white. To validate the approach, we demonstrated that Con A, a T cell mitogen, enhanced proliferation of OVA-specific T cells as well as the secretion of IL-10 and IL-13. Furthermore, signaling through the CD28 costimulatory receptor on activated T cells synergized with the OVA_p-specific T cell receptor to enhance proliferation as well as IL-4, IL-10, and IL-13, consistent with previous reports (Blankson and Morse, 1994; Hunig *et al.*, 2010; Suntharalingam *et al.*, 2006). Lastly, cyclosporin A, an immunosuppressant to T cells (Shimoda *et al.*, 1998), reduced the proliferative response to OVA_p and suppressed cytokine production.

In this work, two carbon-based model nanomaterial powders were characterized. Comparably, a previous study with sonicated 14 nm Printex 90, termed CBNP22 in the present study, confirmed that nonfiltered Printex 90 was agglomerated in suspension (Bourdon *et al.*, 2012b). Dynamic light scattering hydrodynamic volume-size distribution analysis showed a peak size of 3100 nm. In the present study, the sonicated, nonfiltered CBNP22 showed agglomerates with a mean diameter of 3900 nm consisting of primary particles with a diameter of 22 nm. CBNP39 agglomerates were a mean of 7200 nm and consisted of 39 nm base particles. The CBNP22 surface charge was similar to previous reports (Bourdon *et al.*, 2012b). Bourdon *et al.* (2012b) used filtration to confirm the presence of free Printex 90 nanoparticles as small as ~ 7 nm in a saline suspension containing 10% bronchoalveolar lavage fluid. Another report demonstrated that Printex 90 in 2% fetal bovine solution maintained particle size peaks at 98 and 153 nm, indicating small agglomerates (Jacobsen *et al.*, 2008). To study this size range, we used a CBNP suspension filtrate of ≤ 220 nm for cell exposure.

To determine if the mechanism by which CBNPs promote allergy to OVA involves direct effects on immune cells, the DO11.10 mouse splenic leukocytes were treated for 3 days in culture. The CBNPs alone did not induce proliferation or cytokine secretion. Coincubation of the CBNPs with OVA_p did not alter T cell proliferation to OVA_p, however, CBNP22 at 12 $\mu\text{g/ml}$ significantly increased IL-4 and IL-13, indicating that

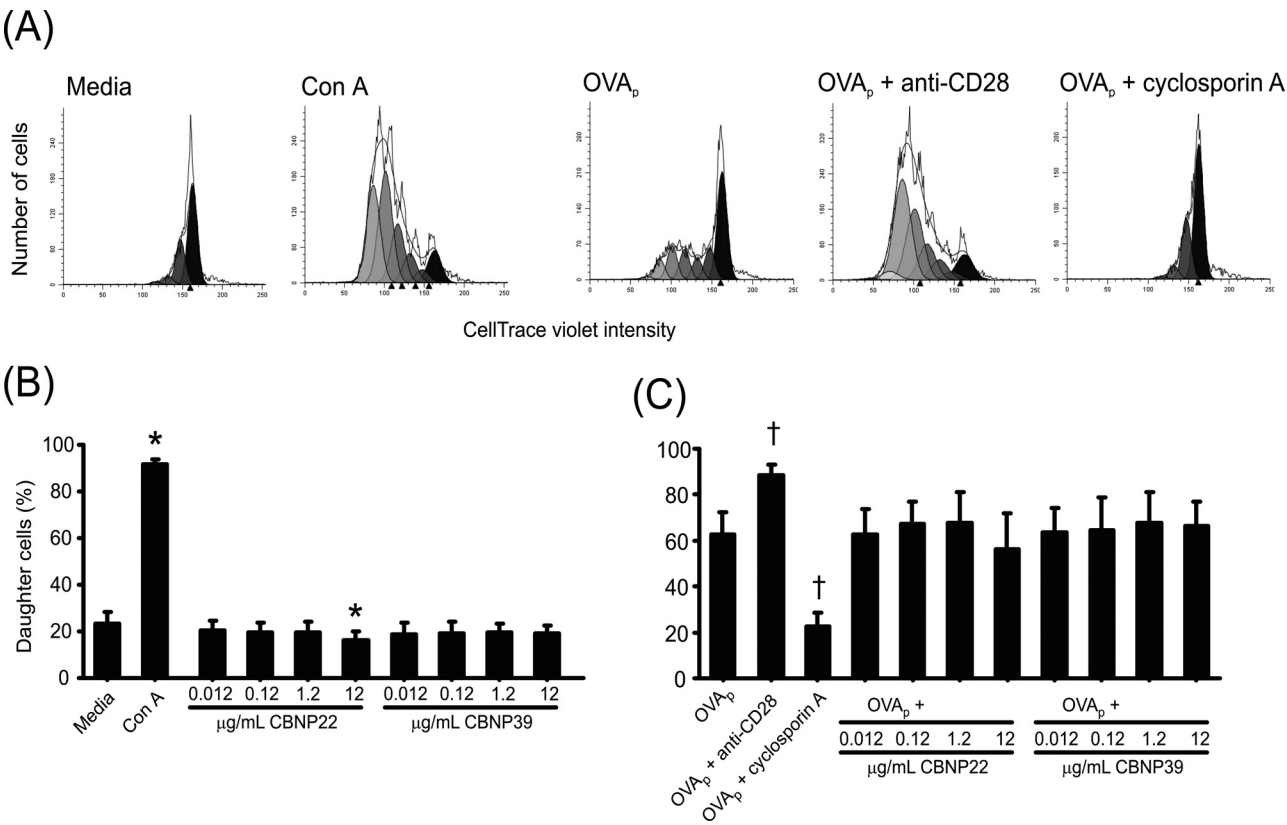


FIG. 2. The proliferation of OVA-specific T helper cells in culture. The DO11.10 splenic leukocytes were stained with CellTrace Violet dye and cultured in complete media, media containing concanavalin A (Con A), filtered carbon black nanoparticles (CBNPs), ovalbumin_{323–339} peptide (OVA_p), OVA_p + anti-CD28 antibody, OVA_p + cyclosporin A, or OVA_p + filtered CBNPs. At 72 h, KJ1–26⁺CD4⁺ T helper cell division was quantified by flow cytometry. (A) Representative proliferation histograms. The parent nondivided population is black. The gray populations represent daughter generations in which the CellTrace Violet fluorescence is diluted. (B) and (C) Cell proliferation was quantified as the percentage of cells in daughter generations. Data represent the mean ± SD for cells from *n* = 6 mice. **p* < 0.05 compared with media alone in (B); †*p* < 0.05 compared with OVA_p alone in (C).

TABLE 5
Normalized Gene Expression Levels in DO11.10 Splenic Leukocytes Following 72 h Treatment in Culture

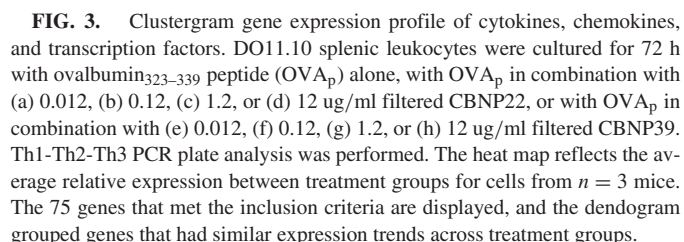
Gene	OVA _p	OVA _p + 0.012 µg/ml CBNP22	OVA _p + 0.12 µg/ml CBNP22	OVA _p + 1.2 µg/ml CBNP22	OVA _p + 12 µg/ml CBNP22	OVA _p + 0.012 µg/ml CBNP39	OVA _p + 0.12 µg/ml CBNP39	OVA _p + 1.2 µg/ml CBNP39	OVA _p + 12 µg/ml CBNP39
<i>IL-4</i>	1.00 ± 0.00	2.04 ± 0.49	2.26 ± 0.51	2.15 ± 0.19	4.94 ± 1.93 ^a	2.54 ± 0.60	2.30 ± 0.36	2.28 ± 0.60	1.79 ± 0.24
<i>IL-10</i>	1.00 ± 0.00	1.91 ± 0.78	1.67 ± 0.57	2.06 ± 0.34	4.23 ± 1.19 ^a	1.79 ± 0.31	2.93 ± 0.74	2.50 ± 0.70	3.87 ± 2.63
<i>IL-13</i>	1.00 ± 0.00	2.85 ± 0.73	2.99 ± 0.42	3.52 ± 0.71	6.92 ± 1.06 ^a	3.29 ± 0.35	3.64 ± 0.18	3.79 ± 0.60	4.25 ± 0.50 ^a
<i>Stat4</i>	1.00 ± 0.00	0.72 ± 0.36	0.63 ± 0.21	0.60 ± 0.13	0.36 ± 0.15	0.47 ± 0.24	0.57 ± 0.38	0.51 ± 0.22	0.20 ± 0.02 ^a

Notes. Data represent the mean ± SD of PCR fold-change values relative to the ovalbumin_{323–339} peptide (OVA_p) group for cells from *n* = 3 mice. The table displays the 4 out of 84 genes for which a treatment group showed a fold change ≥4 or fold change ≤0.25, along with a significant difference *p* < 0.05 compared with the OVA_p alone group. ^aindicates the groups that met these criteria.

the nanomaterials modified cytokine production during sensitization. These cytokines act in common in an autocrine manner on the IL-4 receptor-α signaling pathway to promote naïve T helper cell differentiation to a Th2 phenotype, driving the mechanism of allergic sensitization (Chakir *et al.*, 2003; Hunig *et al.*, 2010; Paul and Zhu, 2010). They also promote B-cell activation and IgE antibody production. Thus, modulation of these

cytokines is a mechanism by which agglomerated CBNPs act as an adjuvant to promote subcutaneous and respiratory allergy (Nygaard *et al.*, 2004, 2009).

In comparison with the present study, the *in vivo* effect of CBNPs on the proliferation and cytokine production of adoptively transferred DO11.10 mouse OVA-specific CD4 T cells in the lung of recipient Balb/c mice was previously studied (de



CBNP22 had substantially lower levels of immunomodulatory silicate, zinc sulfide, and other impurities than CBNP39,

Although the present study demonstrates that high concentrations of CBNPs can promote allergic sensitization, this cannot be generalized to other nanomaterials, as immune effects are dependent on the unique physico-chemical structure and properties of each type of nanomaterial. For example, carbon-based fullerene structures have been shown to act as an antioxidant and inhibit mast cell-mediated allergic responses (Ryan *et al.*, 2007). High-throughput methodologies are useful for screening nanomaterials to identify potential risks.

Cell culture can provide a useful reflection of leukocytes in the apical fluids lining mucosal tissues. In the present study, exposure to 12 $\mu\text{g}/\text{ml}$ CBNPs was the threshold concentration that had an adverse effect by enhancing allergy-associated Th2 cytokines during exposure to OVA_p. This mass concentration was present in 1 ml and represented 12 μg total. There were no observed effects at the 1.2 $\mu\text{g}/\text{ml}$ concentration or below. Cell culture conditions may also approximate tissue exposure to a localized injection. Nygaard *et al.* (2009) sensitized mice with a single subcutaneous injection of OVA alone or OVA plus Printex 90 CBNPs. Following an OVA booster challenge, they quantified serum IgE antibodies specific for this protein. The lowest Printex 90 dose that significantly increased OVA-specific IgE was 50 μg delivered in a 20 μl volume. There were no observed adverse effects from the 12 μg dose. Thus, the lack of adjuvanticity from 1.2 $\mu\text{g}/\text{ml}$ Printex 90 in the present assay is consis-

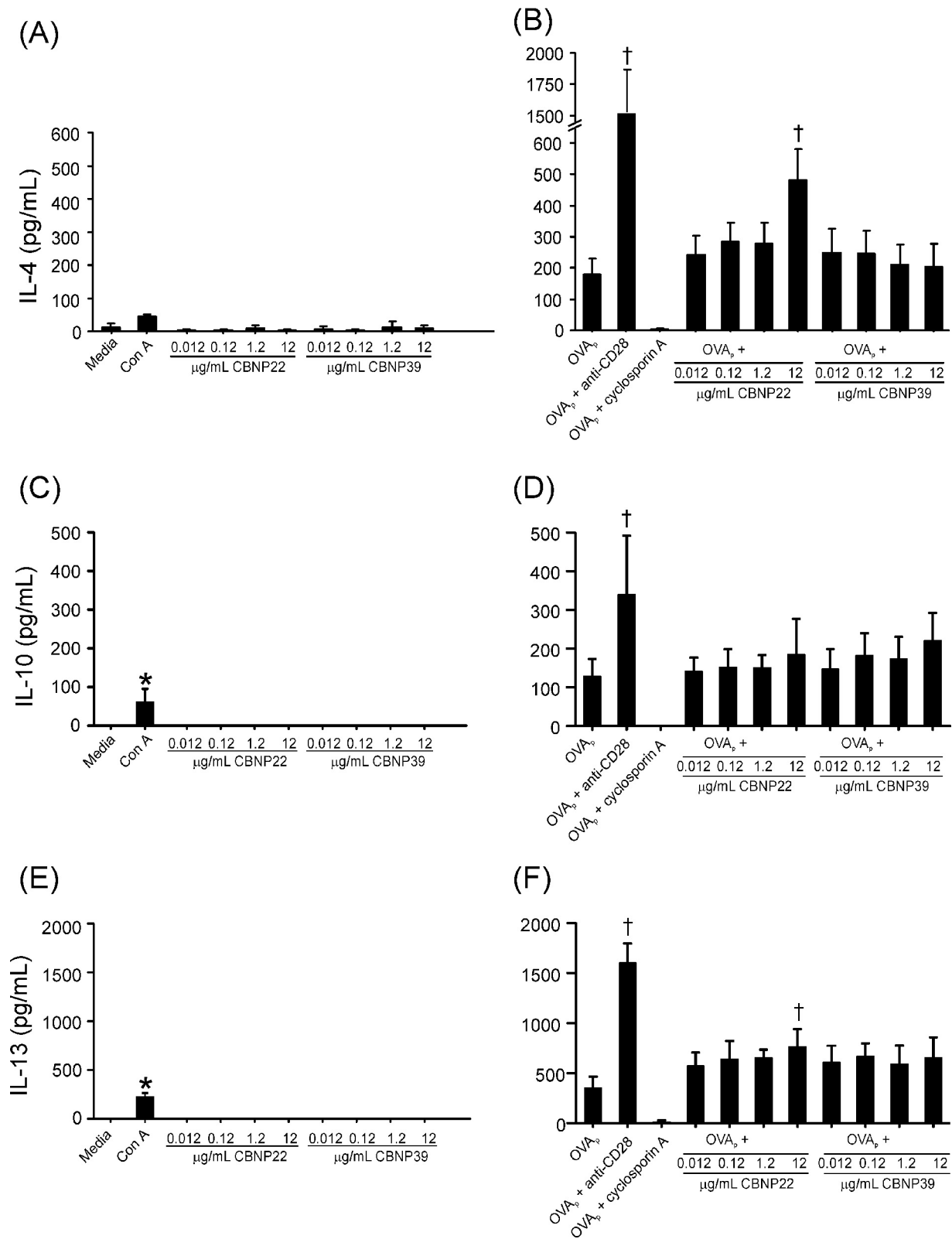


FIG. 4. Cytokine proteins secreted from the cultured cells. DO11.10 splenic leukocytes were cultured with complete media, concanavalin A (Con A), filtered carbon black nanoparticles (CBNPs), ovalbumin_{323–339} peptide (OVA_p), OVA_p + anti-CD28, OVA_p + cyclosporin A, or OVA_p + filtered CBNPs. At 72 h, cell-free culture supernatants were harvested to measure (A,B) interleukin-4 (IL-4), (C,D) IL-10, and (E,F) IL-13. Data represent the mean ± SD for cells from *n* = 5 mice. **p* < 0.05 compared with media alone in (A), (C), and (E); †*p* < 0.05 compared with OVA_p in (B), (D), and (F).

tent with a subcutaneous NOAEL of 12 µg for that endpoint in wild-type mice (Nygaard *et al.*, 2009).

The present work demonstrated that 12 µg/ml CBNPs directly enhanced T helper 2 allergy pathways in OVA-specific leukocytes responding to their cognate antigen, which corresponded with *in vivo* adjuvanticity in wild-type mice at a comparable dose. Further work will be required to determine the effects of other categories of nanomaterials.

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