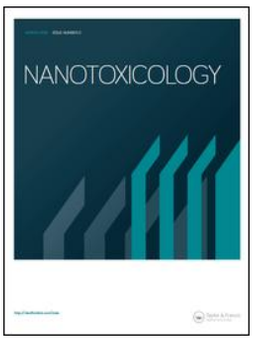

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ORIGINAL ARTICLE

Immunomodulation by gastrointestinal carbon black nanoparticle exposure in ovalbumin T cell receptor transgenic mice

Jason H. Fine[1], Genevieve S. Bondy[1], Laurie Coady[1], Bevan Pearce[1], Nikia Ross[1], Azam F. Tayabali[2], Sabina Halappanavar[2], Don Caldwell[1], Ivan Curran[1], and David E. Lefebvre[1]

[1]Regulatory Toxicology Research Division, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch, Health Canada, Ottawa, ON, Canada and [2]Environmental Health Science and Research Bureau, Environmental and Radiation Health Sciences Directorate, Health Environments and Consumer Safety Branch, Health Canada, Ottawa, ON, Canada

Abstract

Humans could become exposed to carbon black nanoparticles (CBNPs) in consumer products or an occupational setting. In rodent models, acute respiratory, subcutaneous, and direct immune cell exposure to CBNPs has been shown to enhance allergic sensitization to co-administered ovalbumin (OVA) protein from chicken egg. However, little is known about the effects of ingested CBNPs on immunological responses and oral tolerance to food antigens. We hypothesized that ingestion of CBNPs would enhance the development of food allergy to OVA. Allergy prone DO11.10 mice were orally exposed to CBNPs every second day for 2 weeks (total dose 10.8 (LOW) or 108 μg (HI)), with and without (±) co-administered OVA. Systemic immune parameters were measured at necropsy. Exposure to OVA resulted in significant increases in serum anti-OVA IgG1, anti-OVA IgM, and anti-OVA IgA antibodies relative to vehicle control. Immunophenotyping revealed a reduction in the number of OVA-specific $CD4^+$ T helper cells upon OVA ± $CBNP^{HI}$ treatment in the spleen. Yet, secretion of the allergy-associated Th2 cytokines IL-4, IL-9, and IL-13 was greater in $OVA_{323\text{-}339}$ peptide-pulsed splenocytes from OVA + $CBNP^{HI}$-treated mice compared with control. Transcriptome analysis at necropsy of splenocytes from OVA + $CBNP^{HI}$ dose mice compared with OVA mice revealed increases in the allergy associated genes *Il4* and *Stat6* and decreases in *Csf3r* and *Retnlg*. Although oral exposure to high-dose CBNPs did not impact OVA-specific antibody production relative to OVA, we did observe increased expression of genes and cytokines associated with allergy in peripheral splenocytes. This work suggests that CBNP gastrointestinal exposure may potentiate allergy pathways.

Keywords

Allergy, egg, immunotoxicology, nanotechnology, oral sensitization

History

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Introduction

The apparent prevalence of food allergy and other allergic diseases has increased over the last two decades and the reasons are not clearly known (Tan et al., 2012). It is well known that certain chemical contaminants in food and the environment can suppress immune responses (Campbell et al., 2004; Rier, 2008). However, recently there has been increased consideration that stimulation of IgE antibody production by foreign chemicals could influence the development of allergy in susceptible individuals (Schutze et al., 2010).

Conventional bulk carbon black is used primarily as a filler and ink in industrial polymer matrices (Long et al., 2013). The emergence of nanotechnology has allowed the scaling down of particles making up carbon black powders to the 1–100 nanometer (nm) diameter range. Humans may be exposed to carbon black nanoparticles (CBNPs) additives or contaminants through consumer products such as cosmetics, foods, and food packaging (Bott et al., 2014; Sk et al., 2012), as well as through occupational exposure (Long et al., 2013; Zhang et al., 2014). In a regulatory context, it is important to have data on the threshold dose at which adverse health effects manifest, in order to be able to derive a safe maximum exposure level.

Previous work has shown that exposure to high-dose CBNPs by certain routes can contribute to immunological responses, including allergy. In rodents, airway exposure to CBNPs induces pulmonary inflammation, affects allergy associated T helper (Th2) cytokine bias (Saputra et al., 2014; de Haar et al., 2005), and induces genotoxic damage and hepatic oxidative stress (Bourdon et al., 2012a,b). Subcutaneous administration of CBNP suspensions has adjuvant activity similar to diesel exhaust particles in the generation of an allergic response (Lovik et al., 1997). Workers occupationally exposed to carbon black nanomaterials had reduced pulmonary function and increased proinflammatory cytokines in their serum (Zhang et al., 2014). In animal models, acute respiratory, subcutaneous, and direct immune cell exposure to CBNPs enhances allergy to chicken egg ovalbumin (OVA) protein (Lefebvre et al., 2014; Nygaard et al., 2009). However, little is known about the effects of orally ingested CBNPs on gastrointestinal (GI) and systemic immunological responses and oral tolerance to food antigens *in vivo*. This is important not only for safety assessment of oral applications but also for understanding the contribution of gut responses to

Correspondence: Jason Fine, Regulatory Toxicology Research Division, Bureau of Chemical Safety, Food Directorate, Health Canada, 251 Sir Frederick Banting Driveway, Ottawa, ON, Canada K1A 0K9. Fax: +1 613 946 5161. E-mail: jason.fine@canada.ca

exposures via other routes. For example, up to 50% of intratracheally instilled particles are cleared through the gut (Kuehl et al., 2012), highlighting a potential unappreciated role for oral responses following inhalation. Via oral gavage, a fraction of CBNPs are absorbed through the GI epithelium and gain exposure to immune cells in the lamina propria, Peyer's patches, and mesenteric lymph nodes (Joel et al., 1978). We aimed to determine if gastrointestinal exposure to CBNPs is associated with immunostimulation or increased sensitization to co-administered OVA allergen in genetically susceptible animals.

The DO11.10 mouse expresses T cells that are MHC Class II restricted for chicken egg OVA peptide 323–339 (Murphy et al., 1990). It can serve as an effective model of food-allergic sensitization due to its specificity for OVA and its allergy susceptible Balb/cJ genetic background (Okamoto et al., 2005; Simioni et al., 2004; Whitehead et al., 2003). Egg allergy is one of the most common food allergies in young children (Tan and Joshi, 2014), and, therefore, there is a great deal of interest in determining external factors that influence its development. Under normal conditions, intragastric administration of antigenic foods results in tolerance in mammals. However, under certain environmental conditions, orally ingested antigens can begin to elicit an allergic response (Strobel & Mowat, 2006). Using the DO11.10 transgenic model, allergic sensitization to OVA can be achieved orally with adjuvants, by intraperitoneal or subcutaneous injection, or by modulating dose (Marth et al., 2000; Nygaard et al., 2009; Shindo et al., 2012). In adjuvant-free oral feeding regimens, a dose of 10 or 100 mg OVA every other day caused antigen-specific leukocyte proliferation and the secretion of IL-2, IL-4, and IFN-γ upon *ex vivo* stimulation of splenocytes (Marth et al., 2000). In a similarly designed study, feeding 100 mg OVA every other day for six total doses was sufficient to generate an anti-OVA IgG1 and anti-OVA IgE response (Okamoto et al., 2005). In contrast, feeding 250 mg every other day or daily feeding of 100 µg–10 mg OVA to DO11.10 mice induced a suppressive immune response via clonal deletion of OVA-specific T cells, T cell anergy, and decreased cytokine release upon restimulation (Marth et al., 2000; Omata et al., 2005; Wu et al., 1998).

In this work, we used the DO11.10 mouse model to investigate the immunostimulation and adjuvant capacity of CBNPs following intragastric administration with OVA, and to establish potential correlations to *in vitro* data previously generated in our lab (Lefebvre et al., 2014). We gavaged transgenic DO11.10 mice with CBNPs in combination with OVA for 2 weeks. At necropsy, we characterized blood and immune tissues for changes in serum antibodies, immunophenotype, allergy associated transcriptome, and cytokine secretion. This study highlights baseline effects of ingested CBNPs on the immune system and the role of this chemical in modulating systemic primary immune sensitization to a food allergen.

Materials and methods

Materials

Printex 90 carbon black powder (Carbon black nanoparticles: CBNPs) from Evonik/Degussa (Frankfurt, Germany) was a kind gift from Dr. Håkan Wallin (National Research Center for the Working Environment, Denmark). Purity was $>99\%$ carbon (Lefebvre et al., 2014). Average primary particle diameter after sonication in ethanol and drying for characterization was 22 nm (10–35 nm range). The surface area was 341 m^2/g, with surface chemical functionality being $>60\%$ amorphous. Zeta potential was -31 mV (Lefebvre et al., 2014). In this study, the powder was suspended, sonicated, and filtered as previously described to obtain agglomerates smaller than 220 nm (Lefebvre et al., 2014), with the exception that the suspension was generated in Dulbecco's phosphate-buffered saline (D-PBS) containing 0.5% bovine serum albumin (BSA) as a stabilizer to improve CBNP dispersion (Fleischer & Payne, 2014) and create a protein corona mirroring biological exposure (Bellmann et al., 2015). It is important to note that BSA does not share homology to the chicken egg OVA peptide 323–339 recognized by DO11.10 mice. We were not able to characterize aggregates or agglomeration in suspensions containing salts or proteins. After the addition of OVA or vehicle described below, the final concentration of BSA in each dose group was 0.25%.

Study design

Animal handling and procedures complied with Canadian Council on Animal Care guidelines, and the protocol was approved by the Health Canada Animal Care Committee (Ottawa, ON). Male 7–8-week old D011.10 mice (Jackson Laboratory) were pair housed with *ad libitum* access to Harlan 2014 diet and drinking water. Following 1 week acclimation, the mice were weighed, on the designated study day 0, to establish the baseline mean body weight, which was 24.5 ± 0.2 g. The mice were dosed (200 µL) by oral gavage using a 20 G feeding needle every second day for 2 weeks (on days 2, 4, 6, 8, 10, and 12), using the regimen of Okamoto et al. (2005). The total cumulative CBNP dose per mouse over the 2 weeks was 10.8 µg (LOW) dose or 108 µg (HI) dose, in the presence and the absence of a cumulative mass of 120 mg of OVA Grade-VII (Sigma Aldrich, St. Louis, MO). Per mouse, this translated to 0.03 and 0.3 mg/kg bw/d CBNPs, in the LOW and HI groups, respectively, using the 24.5 mg average starting body weight. There were six test groups: PBS, PBS + $CBNP^{LOW}$, PBS + $CBNP^{HI}$, OVA, OVA + $CBNP^{LOW}$, and OVA + $CBNP^{HI}$. The CBNP gavage masses of 1.8 µg or 18 µg (9 and 90 µg/ml CBNP in gavage solution), delivered every second day, were approximately equal to the mass of conventional carbon black that would be cleared into the GI tract after inhalation of 0.2 and 2 working day equivalents, respectively. This was extrapolated from the exposure limit for conventional carbon black established by the Occupational Safety and Health Administration and the National Institute for Occupational Safety (3.5 mg/m^3 per 8 h work shift), factoring in lung deposition [33% of inhaled mass from 1.8 L/h for 8 h (Bourdon et al., 2012a; Jacobsen et al., 2009)], and evidence that 50% of inhaled particles are cleared through the GI tract (Kuehl et al., 2012).

Mice were weighed and observed for outward signs of morbidity daily throughout the study. On day 14 (2 days after the final dose), mice were sacrificed by exsanguination via cardiac puncture under isoflurane anesthesia. Blood was aliquoted into SST Vacutainers (BD Biosciences, San Jose, CA). After gross inspection, selected organs were harvested. The spleen and mesenteric lymph nodes (MLN) were harvested for downstream processing. The liver and the small intestine were placed in 10% neutral buffered formalin, fixed for at least a week, trimmed, and embedded into paraffin blocks. Sections of liver and small intestine were cut at 5 µm from these blocks, and stained with hematoxylin and eosin (H&E) for light microscopy examination for lesions and deposited particle aggregates.

Endotoxin testing

Endotoxin levels in the gavage solutions containing BSA with CBNP and/or OVA Grade VII were determined using the Limulus Amebocyte Lysate (LAL) Kinetic-QCL™ (Lonza) assay following the instructions of the manufacturer. Per gavage, mice in each group received PBS (249 EU), PBS + $CBNP^{LOW}$ (392 EU), PBS + $CBNP^{HI}$ (392 EU), OVA (1.7×10^4 EU),

OVA + CBNP^{LOW} (1.7×10^4 EU), and OVA + CBNP^{HI} (1.7×10^4 EU).

Mesenteric lymph node processing

Nodes were gently mashed through a 70 µm strainer in a 50 ml polypropylene (PP) tube using a 1 cc syringe plunger and rinsed with D-PBS (GIBCO, Life Technologies, Carlsbad, CA). Cells were centrifuged at $340 \times g$ for 6 min at 4 °C. Pellets were resuspended in 1 ml of D-PBS and counted using a Coulter counter prior to labeling for flow cytometric analysis.

Spleen processing

Spleens were gently mashed through a 70-µm strainer using a 3 cc syringe plunger and rinsed with RPMI-1640 unsupplemented media (Gibco, Life Technologies, Carlsbad, CA). Red blood cells were lysed using ACK lysis buffer (Gibco, Life Technologies, Carlsbad, CA) following the directions of the manufacturer. Splenocytes were resuspended in complete media [RPMI-1640 with phenol red without HEPES or L-glutamine; supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamax (Gibco, Life Technologies, Carlsbad, CA), 50 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin and 55 µM beta-mercaptoethanol]. Cells were counted using a Moxi Z cell counter (Orflo Technologies, Ketchum, ID) and aliquoted for flow cytometry, RNA preparation, and *in vitro* culture.

Serum preparation

Vacutainer tubes containing whole blood collected at necropsy were inverted three times and incubated for 30 min at room temperature to allow clotting. The tubes were centrifuged at $2600 \times g$ for 10 min, and then aliquots were stored at −80 °C.

Total serum IgE and anti-OVA antibody detection

Antibodies were detected using mouse serum anti-OVA IgG1, anti-OVA IgA, anti-OVA IgE, and anti-OVA IgM antibody assay kits and the mouse total IgE detection kit (Chondrex, San Diego, CA). Serum samples were diluted using diluent provided at 1/20 (total IgE); 1/2 (anti-OVA IgE); 1/500 and 1/5000 (anti-OVA IgG1); 1/10, 1/50, and 1/500 (anti-OVA IgM); 1/5 and 1/10 (anti-OVA IgA). The chromogenic readout at OD_{450} and OD_{630} (reference) was performed using a Biotek Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT).

Flow cytometry

Mesenteric lymph node cells, splenocytes harvested at necropsy, or splenocytes from subsequent 3 day culture (1×10^6 cells/ml) were centrifuged at $400 \times g$ for 8 min at 4 °C. All labeling was performed on ice and in the dark. Following aspiration, cells were labeled with 1 µl/10^6 cells of LIVE/DEAD® Fixable Aqua dye (Life Technologies, Carlsbad, CA) for 30 min, then washed with D-PBS. Cells were blocked with 1 µl/sample of Mouse BD Fc Block™ (BD Biosciences, San Jose, CA) for 10 min. Cells were then stained for 25 min with either Panel 1 [anti-CD3ε FITC, anti-CD4 PE-CF594, anti-CD8α APC-H7, and KJ1-26 PE from BD Biosciences, San Jose, CA; anti-CD200 eFluro660, anti-CD40L PerCp eFluor710, and anti-CD25 Pe-Cy7 from eBioscience, San Diego, CA], or Panel 2 [anti-CD3ε FITC, anti-CD19 PE-CF594, and anti-CD138 APC from BD Biosciences, San Jose, CA; Gr-1 APC-eFluor780, anti-CD11c PE-Cy7, anti-CD11b PerCp-Cy5.5, and anti-CD205 PE from eBioscience, San Diego, CA]. Cell stain dilutions and cell washes were performed with Flow Buffer [HBSS no Ca^{+2}, no Mg^{+2}, no phenol red, supplemented with 0.5% BSA and 0.09% sodium azide]. Compensation controls were prepared with OneComp Beads (eBioscience, San Diego, CA), BD™ CompBead anti-rat/hamster Ig^K (BD Biosciences, San Jose, CA) and ArC™ Amine Reactive Compensation Bead Kit for the LIVE/DEAD® cell stain. Cells were analyzed on a BD LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA) using FacsDIVA software (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Palm Springs, CA). A minimum of 50 000 events were collected. All plots were gated for viable single cells, as determined by live-cell gating by forward scatter, side scatter, and lack of viability dye uptake.

RNA extraction, cDNA synthesis, and PCR array

RNA was extracted from 10^7 splenocytes (snap frozen at necropsy) using the miRNeasy kit (Qiagen, Valencia, CA) following instructions of the manufacturer. Homogenization was achieved with a Qiashredder column (Qiagen, Valencia, CA). RNA was DNAse I digested on column and quantified using a Nanodrop-1000 (Fisher Scientific, Waltham, MA). cDNA was synthesized following instructions of the manufacturer for the RT_2 profiler first strand synthesis kit (Qiagen, Valencia, CA) using 500 ng of total splenocyte RNA. PAMM-067Z Mouse Allergy & Asthma 96 well PCR Arrays (Qiagen, Valencia, CA) were conducted following instructions of the manufacturer. Plates were run on a Viia7 Real Time PCR System as follows: 1 × 10 min at 95 °C [40 × (15 s at 95 °C; 1 min at 60 °C)]. Threshold cycle (*Ct*) was calculated for each gene following the establishment of an automated baseline and identical defined threshold value. The data were analyzed using web-based RT_2 profiler PCR Array data analysis software V4 (BD Biosciences, San Jose, CA) using *Actb* and *GAPDH* as housekeeping genes.

Splenocyte culture

Splenocytes harvested at necropsy were plated at 10^6 cells/ml in RPMI-1640 complete media in a 24-well plate and incubated at 37 °C 5% CO_2 for 1–2 h prior to addition of $OVA_{323\text{-}339}$ peptide (OVAp; 0.4 µM final, Invivogen, San Diego, CA) for 3 days. Cells were then harvested and culture supernatant was collected after spinning the cells down at $1900 \times g$ for 5 min at 4 °C. Supernatants were stored at −80 °C. The cells were processed for flow cytometry as described above and stained with Panel 1.

Cytokine detection

A 10-plex Cytometric Bead Array [CBA Flex Sets: IL-2 (A5), IL-3 (A8), IL-4 (A7), IL-5 (A6), IL-6 (B4), IL-9 (B9), IL-10 (C4), IL-13 (B8), GM-CSF (B9), and IFN-γ (A4) (BD Biosciences, San Jose, CA)] was performed on supernatants from 3 day splenocyte culture, following the instructions of the manufacturer using the Mouse/Rat Soluble Protein Master Buffer Kit (BD Biosciences, San Jose, CA) with 96-well Millipore $MultiScreen_{HTS}$-BV 1.2 µm clear non sterile filter plates. Aspirations were performed using a Biotek ELx50 plate washer (BioTek Instruments, Inc., Winooski, VT). Plate mixing was performed using a Titer Plate Shaker 4265 (Barnstead Lab-Line, Melrose Park, IL). Data were acquired on a BD LSR Fortessa flow cytometer and analyzed using FCAP Array V3 (Soft Flow Inc., Burnsville, MN).

Statistics

All statistical analyses were performed with Microsoft Excel or Graphpad Prism (GraphPad Software, Inc., La Jolla, CA). Normality was analyzed with a Shapiro–Wilk normality test. Variance was analyzed with an *F* test. Comparison between test groups was conducted using a two-tailed equal variance *t*-test.

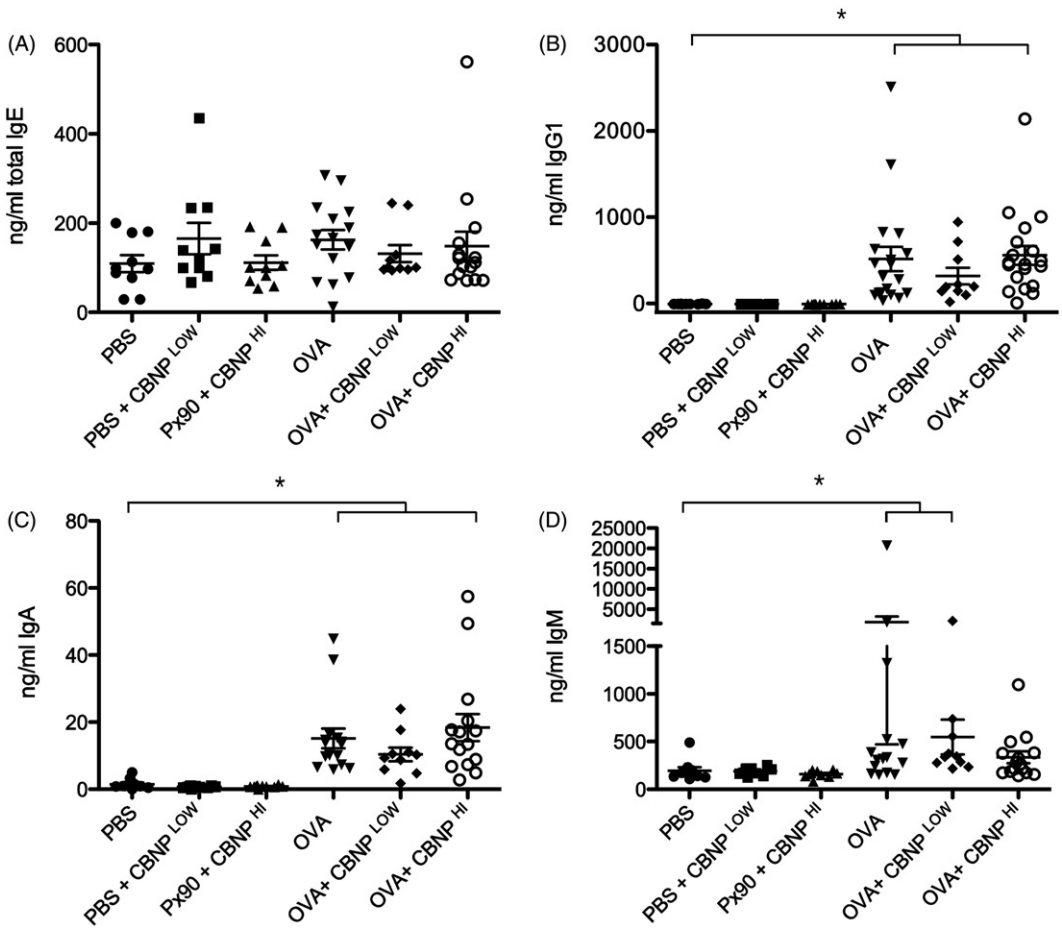


Figure 1. Serum immunoglobulin profile was determined at necropsy for (A) total IgE, (B) anti-OVA IgG1, (C) anti-OVA IgA, and (D) anti-OVA IgM by ELISA (± SEM) where $*p < 0.05$.

The ELISA results were analyzed using the Kruskal–Wallis test with Dunn's post hoc multiple comparison.

Results

Mouse weight, intestinal and liver histology, and splenocyte number did not change following oral CBNP exposure

During the 2 week study, the mice exhibited normal behavior. There were no significant fluctuations in average mouse weight in all dose groups. No histological lesions and no nanoparticle aggregates were observed by light microscopy in the duodenal and jejunal sections from mice which were necropsied 2 days after the final dose. Only mild incidental non-treatment-related lesions were seen microscopically in livers from all groups (data not shown). CBNP aggregates were not observed

Immune cells from the GI tract circulate through spleen. Therefore, at necropsy, splenocytes were prepared and counted. Average absolute splenocyte cell numbers did not differ significantly between the test groups (data not shown). This was also the case following 3 day culture of splenocytes with $OVA_{323–339}$.

Oral CBNP exposure did not stimulate an increase in anti-OVA antibodies

Oral administration of OVA (20 mg per dose) in the absence of prior immunization resulted in no detectable anti-OVA IgE and no difference in total IgE, irrespective of CBNP exposure (Figure 1A and data not shown). Anti-OVA IgG_1, which precedes IgE in the switching reaction, was statistically increased ($p < 0.05$) in mice exposed to OVA ± CBNPs compared with the PBS control group (Figure 1B). This was also the case for anti-OVA IgA (Figure 1C). However, the low or high dose of CBNP with OVA did not cause an increase in anti-OVA IgG_1 or IgA compared with OVA treatment alone (Figure 1B and C). Anti-OVA IgM was also increased in the OVA and OVA + $CBNP^{LOW}$ groups, but not the OVA + $CBNP^{HI}$ group compared with PBS (Figure 1D).

Transcriptome analysis of allergy-associated genes revealed changes in the OVA + CBNP groups compared to the PBS and to OVA groups

Statistically significant changes in splenocyte gene expression at necropsy are reported, with no screening cutoff for fold change (Table 1). Compared with PBS, *Ccr3*, *Cma1*, *Il4* (1.92-fold), and *Ms4a2* (2.05-fold) were upregulated and *Ccr4*, *CD40lg*, *Gata3*, *Il1rl1*, and *RetnIg* were downregulated in the OVA + $CBNP^{HI}$ group (Table 1A). There were fewer genes with significant changes at the OVA + $CBNP^{LOW}$ dose. Compared with PBS, *Ccr3* was upregulated and *Ccr4*, *Cd40lg*, *Il4ra*, *Pparg*, and *Stat6* were downregulated in the OVA group (Table 1A). Compared with PBS, the CBNPs alone had no statistically significant effects. In comparison with OVA, *Il4* (2.29-fold) and *Stat6* were upregulated and *Csf3r* and *RetnIg* were downregulated in the OVA + $CBNP^{HI}$ test group (Table 1B).

Immunophenotyping at necropsy and subsequent 3 day cultures revealed changes in distinct cell populations

The relative abundance of $CD4^+$ $KJ1\text{-}26^+$ ($OVA_{323–339}$ specific T cell receptor) T helper cells was lower in the OVA ± CBNP groups compared with PBS in the spleen but not the mesenteric

Table 1. Transcriptome analysis of allergy-associated gene expression in splenocytes that revealed statistically significant fold changes in the (A) OVA ± CBNP groups compared with PBS and (B) OVA + CBNP groups compared with OVA- genes where at least one treatment group had a statistically significant difference from the control group (*$p < 0.05$). Data that were not statistically significant are denoted as N.S.

A	Gene name	PBS	$CBNP^{L0W}$	$CBNP^{HI}$	OVA	$OVA + CBNP^{L0W}$	$OVA + CBNP^{HI}$
	Ccr3	1	N.S.	N.S.	**1.72***	**1.56***	**1.57***
	Ccr4	1	N.S.	N.S.	**0.64***	N.S.	**0.65***
	Cd40lg	1	N.S.	N.S.	**0.45***	**0.49***	**0.54***
	Cmal	1	N.S.	N.S.	N.S.	N.S.	**1.65***
	Gata3	1	N.S.	N.S.	N.S.	N.S.	**0.68***
	Ifng	1	N.S.	N.S.	N.S.	**0.59***	N.S.
	Illrl	1	N.S.	N.S.	N.S.	N.S.	**0.56***
	114	1	N.S.	N.S.	N.S.	N.S.	**1.92***
	II4ra	1	N.S.	N.S.	**0.68***	N.S.	N.S.
	Ms4a2	1	N.S.	N.S.	N.S.	N.S.	**2.05***
	Pparg	1	N.S.	N.S.	**0.69***	**1.49***	N.S.
	Retnlg	1	N.S.	N.S.	N.S.	N.S.	**0.56***
	Stat6	1	N.S.	N.S.	**0.71***	N.S.	N.S.

B	Gene name	OVA	$OVA + CBNP^{L0W}$	$OVA + CBNP^{HI}$
	Ccr4	1	**1.85***	N.S.
	Csf3r	1	N.S.	**0.64***
	114	1	N.S.	**2.29***
	Retnlg	1	N.S.	**0.62***
	Stat6	1	N.S.	**1.43***

lymph nodes (Figure 2A). However, within the $CD4^+KJ1\text{-}26^+$ subset, the abundance of $CD200^+CD25^-$ T cells was higher in the OVA + CBNP groups compared with PBS in the spleen (Figure 2B). It was also higher in the OVA ± CBNPs in the MLN. There were no significant differences between the OVA and OVA + CBNP groups.

The abundance of $CD3^+CD8^+$ cytotoxic T cells was higher than PBS treatment in the $OVA + CBNP^{LOW}$ and $OVA + CBNP^{HI}$ groups in the spleen but not in the mesenteric lymph nodes (Figure 3A). The abundance of activated $CD3^+Gr\text{-}1^+(Ly6c^+)$ T cells was higher in the $OVA + CBNP^{HI}$ group compared with PBS in the spleen but not the mesenteric lymph nodes (Figure 3B). In contrast, the abundance of activated $CD4^+CD25^+$ T cells (activated and/or T regulatory) was higher in the $OVA + CBNP^{HI}$ group compared with PBS in the mesenteric lymph nodes but not the spleen (Figure 3C). CBNPs themselves did not modify the abundance of B cells ($CD3^-$ $CD19^+$) and there were no significant changes in other cell populations investigated, including $CD19^-$ $CD138^+$ (plasma cells), $CD3^-$ $CD11c^+$ (dendritic cells), $CD11b^+Gr\text{-}1^+$ (myeloid derived suppressor cells and granulocytes), and $CD3^-$ $CD11b^+$ (macrophages) (Figure 3D and data not shown).

Due to limited numbers of cells from the MLN at necropsy, only splenocytes were cultured for 3 days with $OVA_{323–339}$. The relative abundance of $CD4^+KJ1\text{-}26^+$ T helper cells trended in the same manner as the splenocytes at necropsy; however, it was not statistically significant (Figure 4).

Splenocytes from mice treated with $OVA + CBNP^{HI}$ exhibited significant increases in allergy-associated cytokines following culture with $OVA_{323–339}$ peptide

In order to determine if the changes in transcriptome profile and immunophenotype (Table 1 and Figures 2 and 3) translated into functional changes, we measured cytokine secretion of *ex vivo* stimulated splenocytes (Figure 5). No significant changes in IL-6 and IL-10 were observed, and no IL-5 was detected (not shown). Compared with the PBS treated group, the allergy-associated cytokines IL-4, IL-9, and IL-13 were statistically increased in splenocytes from the $OVA + CBNP^{HI}$ group (Figure 5A–C), IL-2 was decreased in the OVA, $OVA + CBNP^{LOW}$, and $OVA + CBNP^{HI}$ groups (Figure 5D) and IFN-γ and GM-CSF were increased in the OVA and $OVA + CBNP^{HI}$ groups (Figure 5E and F). When compared with the OVA group IFN-γ, GM-CSF and IL-3 were significantly lower in the $OVA + CBNP^{LOW}$ group but not the $OVA + CBNP^{HI}$ group (Figure 5E–G).

Discussion

With allergy prevalence increasing, there is growing interest in understanding the possible role of chemicals in the development of this broken tolerance reaction. While pulmonary effects of CBNPs in skewing immune responses toward allergy have been documented (de Haar et al., 2005; Nygaard et al., 2009; Saputra et al., 2014), the potential impact of oral exposure remained largely unknown.

In this study, we sought to test the immunostimulatory and adjuvant capacity of CBNPs using a model prone to food allergy. Previous work with transgenic DO11.10 mice demonstrated that continuous feeding of OVA resulted in sensitization to antigen as opposed to the tolerance observed in its wild-type background strain, the Balb/cJ mouse (Simioni et al., 2004). We observed that feeding DO11.10 mice every other day for 2 weeks was sufficient to increase the frequency of $CD4^+KJ1\text{-}26^+CD200^+CD25^-$ T cells in the mesenteric lymph nodes and generate systemic anti-OVA IgM, anti-OVA IgG1, and anti-OVA IgA antibodies. The latter two allergen specific antibodies are known to be involved in anaphylaxis (Frossard et al., 2004; Miyajima et al., 1997), and the titers observed are in support of sensitization to OVA. We did not detect any anti-OVA IgE. Previously, doses of 100 mg OVA per gavage have been shown to generate an anti-OVA IgE response, but no dose–response test was performed (Okamoto et al., 2005). We used a 20 mg per gavage dose to be at the sensitization threshold and allow the detection of any adjuvant effects of the added CBNPs, since that dose was sufficient to induce an immune response (Marth et al., 2000) while maintaining a reasonable gavage volume to minimize stress and reduce the chance of aspiration into the lungs. Overall, CBNPs did not enhance OVA-specific antibody titers relative to OVA alone. This suggests a lack of overt allergy induction risk at the CBNP gavage doses used and is key information in regulatory safety considerations for additives or contaminants. However, there were other effects by the

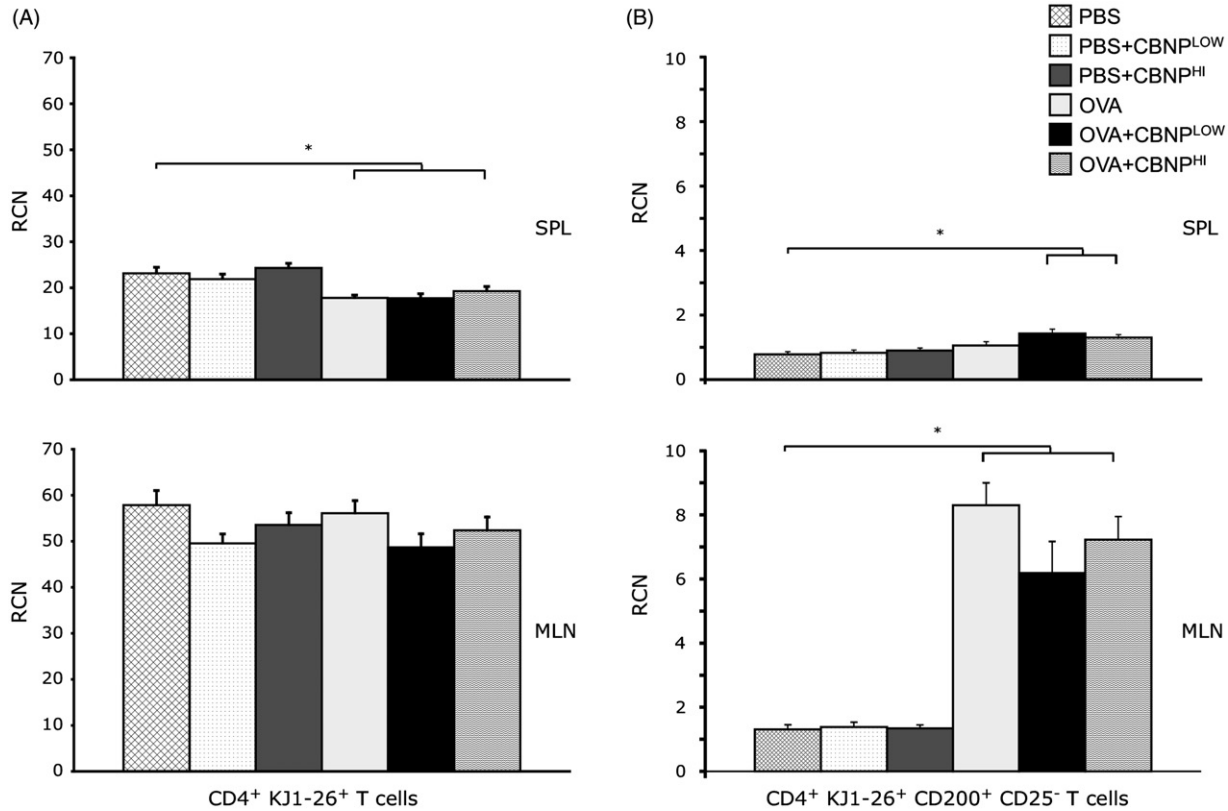


Figure 2. The relative abundance of T helper cell subsets is different between the spleen (upper panel; SPL) and mesenteric lymph nodes (lower panel; MLN) as determined by immunophenotyping at necropsy. The abundance (A) $CD4^+KJ1\text{-}26^+(OVA_{323–339}$ specific T cell receptor) T helper cells and (B) $CD4^+KJ1\text{-}26^+CD200^+CD25^-$T helper cells were analyzed in the two compartments. Data represent relative cell number (RCN;%) from live cells from a total of 10 mice per group ± SEM where $*p<0.05$ relative to PBS.

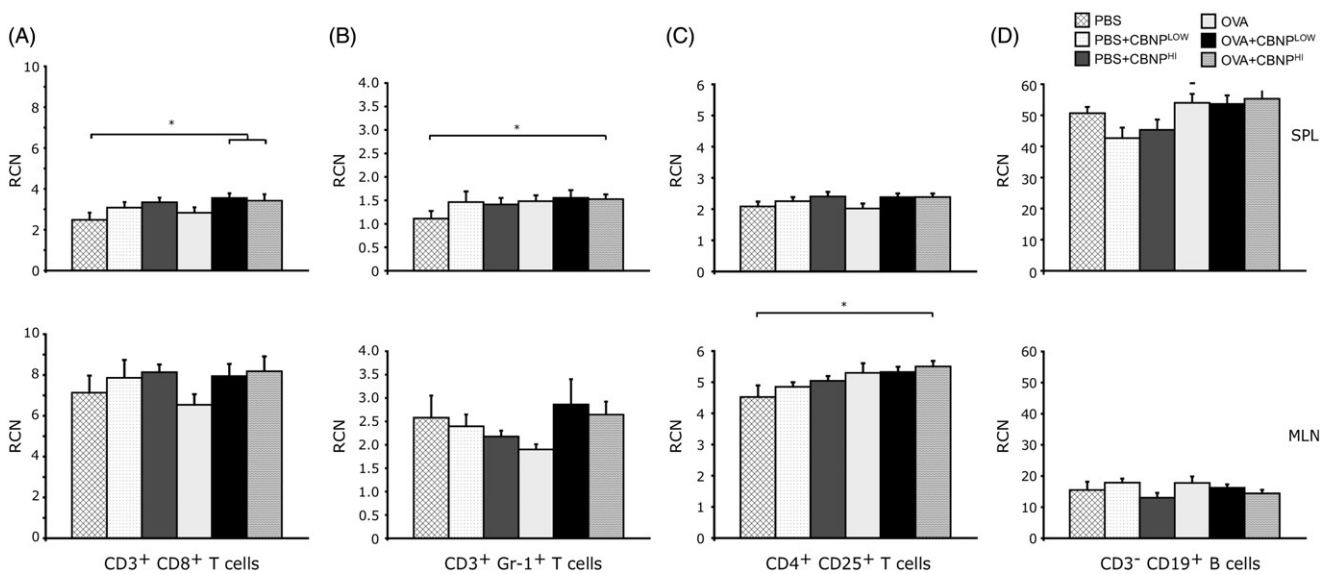
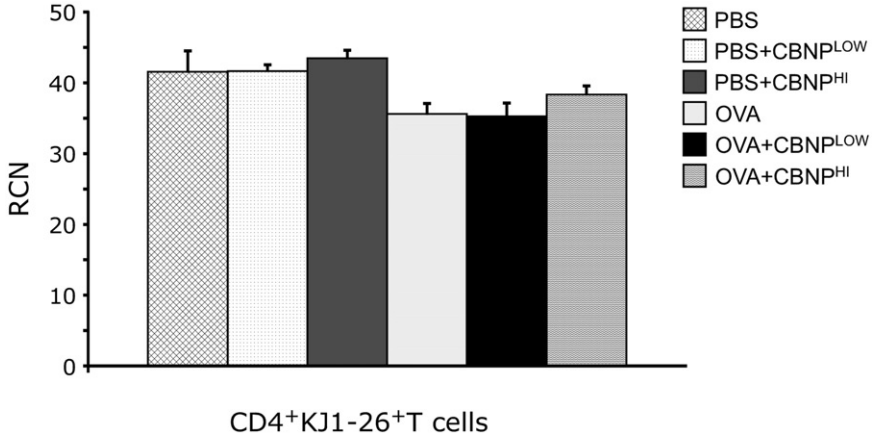


Figure 3. The relative abundance of cytotoxic T cells, activated T cells, and B cells are different between the spleen (upper panel; SPL) and mesenteric lymph nodes (lower panel; MLN) as determined through immunophenotyping at necropsy. The abundance of (A) $CD3^+CD8^+$ cytotoxic T cells, (B) $CD3^+$ Gr-1 (Ly-6c)$^+$-activated T cells, (C) $CD4^+CD25^+$ T regulatory cells, and (D) $CD3^-CD19^+$ B cells were analyzed in the two compartments. Data represent an average relative cell number (RCN;%) from live cells from a total of 10 mice per group ± SEM where $*p<0.05$ relative to PBS.

CBNPs on the cytokines secreted in response to OVA, as discussed later.

Most of the significant immunophenotype changes in the study were between the OVA ± CBNPs versus the PBS group, thus negating any major toxicity or immunomodulation by the CBNPs alone. This is expected since the CBNPs were to act as an adjuvant, but not a direct stimulator of OVA-targeted responses. For instance, the abundance of $CD4^+KJ1\text{-}26^+$T cells, which are specific for $OVA_{323–339}$ were lower in the OVA ± CBNPs groups compared with PBS in the spleen. The decrease in abundance is

Figure 4. The relative abundance of CD4 T helper cell subsets in splenocytes from treated mice does not change following 3 days of *in vitro* culture with $OVA_{323–339}$ as determined by immunophenotyping of $CD4^+KJ1\text{-}26^+$ T helper cells. Data represent an average relative cell number (RCN; %) from live cells from a total of 10 mice per group ± SEM.



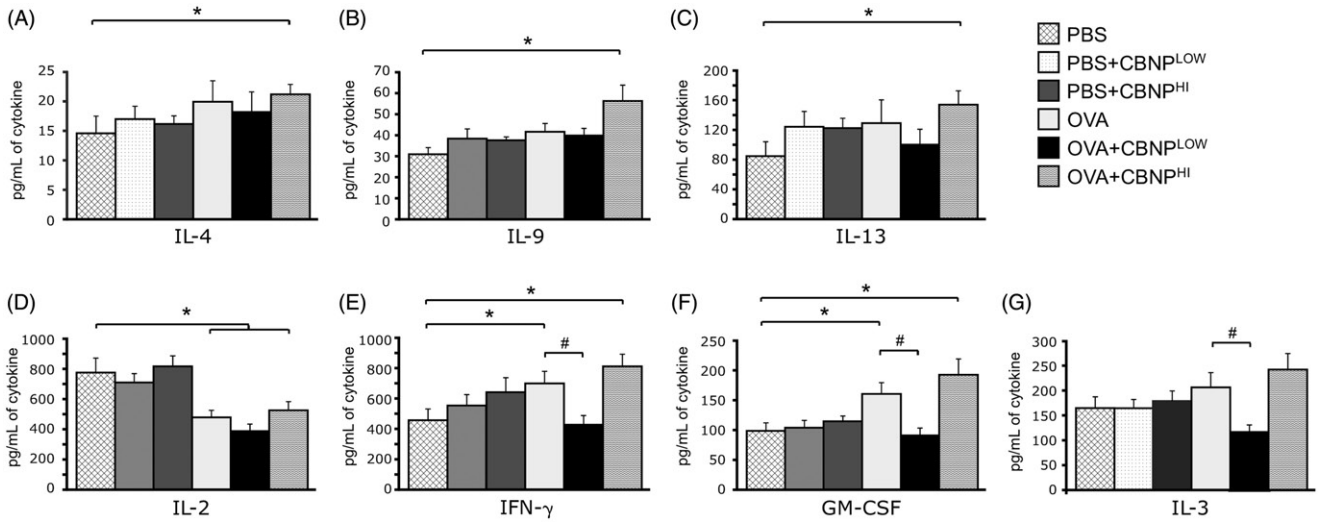


Figure 5. Splenocytes incubated with $OVA_{323–339}$ for 3 days from mice treated with OVA^+CBNP^{HI} exhibit significant increases in the secretion of allergy associated cytokines. Cell culture supernatants were subjected to a 10-plex cytometric bead array for IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IFN-gamma, and GM-CSF. Data represent average cytokine concentration from a total of 10–15 mice per group (±SEM). Only cytokines showing statistically significant changes are presented. Significant changes are denoted as * $p<0.05$ relative to PBS (vehicle); # $p<0.05$ relative to OVA.

likely due to clonal deletion of these food-reactive T cells, a hallmark of oral tolerance (Chen et al., 1995). Although there was no change in the abundance of $CD4^+KJ1\text{-}26^+$ T cells in the MLN, $CD4^+KJ1\text{-}26^+CD200^+$ T cells were enriched in the OVA ± CBNPs groups compared with PBS. CD200 is a surface glycoprotein found on numerous cell types of myeloid and hematopoietic origin and serves as a marker of immune activation (Rygiel et al., 2012). It also serves as a marker of T follicular helper cells, which aid in B cell antibody switching reactions (Crotty, 2011), correlating well with the increased anti-OVA IgG1 and IgA antibody production in these groups. In some cases, feeding of $OVA + CBNP^{HI}$ elicited changes relative to PBS reflective of immune activation that was not observed in the OVA group. For example, while biologically small in magnitude, the abundance of activated T cells ($CD3^+Gr\text{-}1^+(Ly6c^+)$) in the spleen and activated $CD4^+CD25^+$ T cells (activated and/or T regulatory) in the MLN were statistically higher. These changes are not simply due to the presence of endotoxin in the OVA, as endotoxin level was identical in the OVA and OVA + CBNP groups. Previously, mice treated with 1 000 000 endotoxin units (EU) in a single oral dose showed no signs of acute toxicity (Harper et al., 2011). In our study, endotoxin was less than 20 000 EU per gavage in all the dose groups. We cannot rule out whether endotoxin influenced gut leakiness, consequently enhancing the adjuvant effect of the CBNPs. However, we did not observe any signs of inflammation in the duodenum, jejunum, or the liver, the primary site of endotoxin detoxification. In addition, endotoxin is constitutively present in the gastrointestinal tract due to the gut microflora (Heymann & Tacke, 2016; Munford, 2005). Thus, despite exposure to equivalent endotoxin levels, splenocytes from OVA + CBNP-treated mice achieved statistical increases in cytokine mRNA expression compared with mice treated with OVA alone (Table 1).

We observed increases in the Th2 response-associated *Il4* cytokine transcript in splenocytes from the $OVA + CBNP^{HI}$ group when compared with either the PBS group or the OVA group. At the protein level, significant production of IL-4 was noted in *ex vivo* stimulated splenocyte cultures from the $OVA + CBNP^{HI}$ group, when compared with PBS, but not OVA. Moreover, an increase in *Stat6* transcript was observed in the $OVA + CBNP^{HI}$ group compared with OVA at necropsy. Stat6 is a transcription factor that is predominantly activated by IL-4 and is necessary for Th2 T helper differentiation in the allergic asthma response (Wurster et al., 2000). Overall, IL-4 transcript and protein levels were increased in the $OVA + CBNP^{HI}$ group. Importantly, this occurred despite the fact that the relative abundance and absolute

numbers of $CD4^+KJ1\text{-}26^+T$ cells were lower in the OVA ± CBNPs groups compared with the PBS ± CBNPs groups (statistically different at necropsy, remaining a trend after *ex vivo* culture). Normally, this would cause a reduction in the total potential for IL-4 production. This is likely the rationale for the downregulation of other Th2 cell-associated genes, *Ccr4*, *CD40lg*, and *Gata3* in the OVA ± CBNP groups (Table 1A) and the decrease in IL-2 production from the 3 day cultures from the OVA ± CBNP groups (Figure 5D). Alternatively, an explanation for the increase in IL-4 production is that there are other cellular sources of this cytokine, accounting for the discrepancy between different Th2 biomarkers (Chakir et al., 2003). Increased Th2 cytokines have also been observed in rodents intratracheally exposed to CBNPs (de Haar et al., 2005; Saputra et al., 2014).

Basophils, eosinophils, and mast cells are myeloid cells of the innate immune system that have overlapping and distinct characteristics in the promotion of allergic responses (Stone et al., 2010). All three cell types have the capacity to produce IL-4. Basophils function as antigen-presenting cells and produce IL-4 early in the allergic immune response, which contributes to the Th2 phenotype (Yoshimoto, 2010). We did not stain for any markers of basophils. However, based on the current convention for the identification of basophils (Lee & McGarry, 2007), we were able to determine that in the spleen, the relative proportion of $CD19^-$ $CD3^-$ $CD11b^{lo}$ Gr-1^- $CD11c^-$ FSC^{lo}/SSC^{lo} basophils did not change between the treatment groups. In contrast, we did see an increase in this population in the MLN for the OVA versus the PBS group (Supplementary Figure 1). This is consistent with an increased Th2 allergy phenotype resulting from the OVA exposure. However, due to the low abundance of these cells, their significance is unknown. Eosinophils, mast cells, human basophils, and specialized Th2 cells are known to express the chemokine receptor CCR3 (Griffith et al., 2014; Sallusto et al., 1997). *Ccr3* transcripts were significantly increased over 1.5-fold in splenocytes from the OVA and OVA + CBNP groups compared with PBS. Like the changes in *Il4*, this increase could be explained by increased expression of *Ccr3* per T cell or increased numbers of the other cell types expressing *Ccr3*.

As mentioned above, serum anti-OVA IgE was below the limit of detection of the assay owing to low amounts of total circulating IgE (Figure 1A). Nonetheless, we observed transcriptional changes relevant to an allergic phenotype resulting from OVA exposure with some additional effects from $CBNP^{HI}$ inclusion. The FcεRI beta subunit *Ms4a2* was increased over 2-fold in the OVA + $CBNP^{HI}$ group relative to PBS. Additionally, increased levels of *Cma1*, a chymase secreted by mast cells, were observed. We cannot comment on the abundance of eosinophils or mast cells, since we did not stain for the high affinity IgE receptor (FcεRI) found on these cell types.

The $OVA_{323–339}$ peptide that we used to re-stimulate splenocytes *ex vivo* has been shown to induce a Th1 or Th2 T cell response in culture depending on the conditions (Nakajima-Adachi et al., 2012). Either due to a lack of effect or lack of sensitivity, no difference in cytokine secretion was observed when comparing the OVA and OVA + CBNP groups. The increase in IFN-γ and GM-CSF production in the 3 day splenocyte cultures from the OVA and OVA + $CBNP^{HI}$ groups is suggestive of Th1 pathway activation. However, reports indicate that IFN-γ is also important in the pathogenesis of allergy (Gaudieri et al., 2012) and GM-CSF is an immunomodulatory cytokine implicated in numerous processes, including allergic sensitization (Cates et al., 2004; Stampfli et al., 1998). In support of a Th2 response, statistical increases in IL-4, IL-13, and IL-9 cytokine production were observed in the OVA + $CBNP^{HI}$ group but not the OVA group. This also corroborates our previous *in vitro* DO11.10 splenocyte exposure work (Lefebvre et al., 2014). Like IL-4, IL-13 can be produced by Th2 cells, mast cells, eosinophils, and basophils (Schuh et al., 2003; Stone et al., 2010). IL-9 is known to be produced by T helper cells (Th2 or Th9), mast cells, and eosinophils (Soroosh & Doherty, 2009). It also plays a role in oral antigen hypersensitivity by triggering intestinal permeability (Forbes et al., 2008). Thus, despite the decreased number of $CD4^+KJ1\text{-}26^+T$ cells in the cultures relative to control groups, an increase in allergy-associated cytokines was produced owing to either their increased production by activated $CD4^+KJ1\text{-}26^+T$ cells or by other cell types in the OVA + $CBNP^{HI}$ dose group.

Conclusions

Following up on our previous *in vitro* work (Lefebvre et al., 2014), we sought to investigate the immunomodulatory and adjuvant capacity of CBNPs via the gastrointestinal route in a model of food allergy. Phenotypically, DO11.10 mice treated by gavage with a low dose OVA exhibited signs of threshold allergic sensitization measured through anti-OVA Ig serological markers. While the effects of CBNPs in combination with OVA allergen were not as marked as in previous pulmonary exposure papers, this combination did enhance the expression of several allergy-associated biomarkers in peripheral splenocytes compared with vehicle and to a lesser extent (*Il4* transcript only) compared with OVA alone. This was a potentiation effect, as the CBNPs alone did not significantly modify any of the measured parameters. In addition to the cytokine biomarker data, changes to several cell types of interest were identified, warranting further consideration in developing *in vitro* and *in vivo* models to investigate the adjuvant capacity of test substances. This work will aid in creating a more comprehensive screening method for the evaluation of adverse adjuvant effects. In the future, lengthier *in vivo* experiments may result in a more distinct response in determining the adjuvant capacity of manufactured nanoparticles with regard to the risk of increasing allergic sensitization.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online