June 28 2016

Dear Dr. Roberts,

We thank you and the reviewers for reviewing our manuscript titled, “Immunomodulation by gastrointestinal carbon black nanoparticle exposure in ovalbumin T cell receptor transgenic mice” (TNAN-2016-0031). This point-by-point letter addresses the comments. We also attached the manuscript with ‘Tracked Changes’.

*REVIEWER 1*

*The immunological effects of oral exposure to carbon black nanoparticles (CBNPs) with and without co-administration of OVA were examined in allergy-prone mice. DO11.10 mice were exposed to CBNPs +/- OVA via oral gavage every other day for two weeks and analysis of systemic immune parameters was performed at necropsy. The authors found that OVA exposure alone increased serum anti-OVA IgG1, anti-OVA IgM, and anti-OVA IgA antibodies relative to vehicle control. Treatment with OVA+CBNPs reduced OVA-specific CD4+ T helper cells in the spleen but increased secretion of IL-4, IL-9, and IL-13 by splenocytes from exposed mice. Small increases in IL-4 and STAT6 mRNA and decreases in CSF3R and Retnlg mRNA were also observed in splenocytes from mice exposed to OVA+CBNPs. The manuscript was very well written, however, there are concerns regarding the experimental methods and the evidence supporting the author’s objectives:*

Thank you for your constructive review. Below we have replied to your comments and indicated where they have been addressed in the track changes manuscript.

*1) Additional characterization data would have been helpful to better understand the CBNPs utilized in the study, in particular the size of the agglomerates. Nanoparticle size plays a significant role in the effects induced by exposure, and more information beyond “smaller than 220 nm” would have been useful. Similarly, the characterization described in the Methods was presumed to have been done with CBNPs in a dry powder form. As the CBNPs were delivered to the mice in suspension, it would have been beneficial to determine if characteristics of the CBNPs were changed in the suspension media.*

We agree that it would have been ideal to be able to get more information on the CBNP agglomerates and their characteristics in the gavage solution. You’re correct that our characterization of the primary particle size of 10-35 nm was obtained after suspension in ethanol, sonication and drying in our 2014 Toxicological Sciences paper (Lefebvre et al., 2014). In the Materials section of the present manuscript we have now indicated that the primary particle diameter was measured, “after sonication in ethanol and drying for characterization”. In that Toxicological Sciences paper with the same batch of nanoparticles, we also made attempts to characterize complex suspensions in cell culture media that also contained salts, proteins, etc. However, we were not confident with the reliability of the data due to crystallization of the vehicle salts in electron micrographs, and interference from vehicle components including protein stabilizer in dynamic light scattering analyses. This has also been observed with CBNP suspensions in the literature (Jacobsen et al., 2009, Lefebvre et al., 2014). We also reviewed the lack of validated methods (Lefebvre et al., 2015). Although we can’t comment whether characteristics of the CBNPs changed in suspension, we felt it was important to report how we created the suspensions to allow future studies to replicate, or add on to our findings. The comment “smaller than 220nm” refers to the fact that the suspensions were passed through a 0.22 µm filter. What we were able to indicate, thanks to our filtration, is that the CBNPs were in agglomerates smaller than 220 nm in the gavage suspension that was administered to the mice. In the methods section, we have added the line “We were not able to characterize aggregates or agglomeration of the carbon-based nanoparticles in these suspensions containing salts and proteins.”

*2) Clarification in the Study Design section of the Methods is needed to differentiate between the doses of CBNPs and the concentration of OVA used in the exposures. It is difficult to interpret if the 0.03 and 0.3 mg/kg/day doses are referring to CBNPs or OVA.*

To clarify the study design we inserted the acronym to give the following sentence.

“Per mouse this translated to 0.03 and 0.3 mg/kg bw/day CBNPs, in the LOW and HI groups respectively, using the 24.5 mg average starting body weight”

*3) The addition of BSA to the suspensions used to deliver the CBNPs and OVA was intriguing. BSA can act as an immunogen on its own, and therefore this reviewer was curious about the potential for cross-sensitization between the OVA and BSA.*

Although BSA can act as an immunogen on its own in wild-type mice the likelihood for cross-sensitization in our model is minimal, or non-existent for these reasons:

* Most T cells in DO11.10 mouse express a T cell receptor that is specific for the ovalbumin peptide 323-339 when presented by MHC II molecules. This peptide sequence is ISQAVHAAHAEINEAG
* The bovine serum albumin protein does not harbour any homology to the chicken egg ovalbumin peptide that is bound by MHC II.
* The BSA concentration in all of the dose groups is identical, thus if sensitization due to BSA occurred we would likely have observed a greater serum antibody response in the PBS and PBS+CBNPs groups.

We added a sentence to the materials section: “It is important to note that BSA does not share homology to the chicken egg ovalbumin peptide 323-339 recognized by DO11.10 mice”.

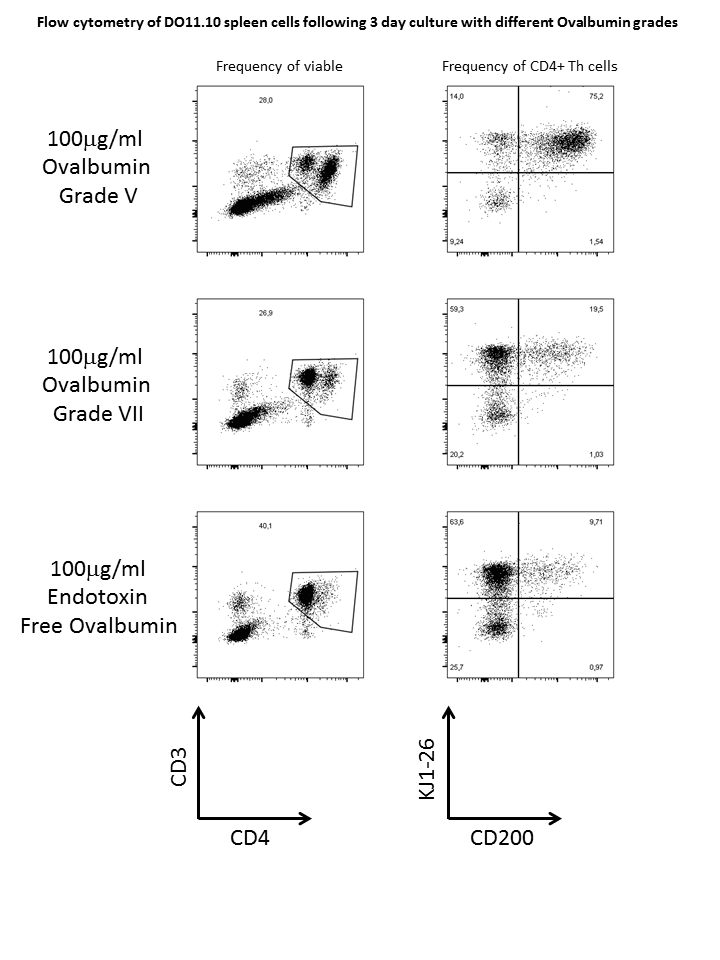
*4) The reviewer had concerns about the high levels of endotoxin found in the OVA and how this could affect the interpretation of the results. Is it possible that increased endotoxin could lead to leakiness in the gut and alter subsequent immune responses?*

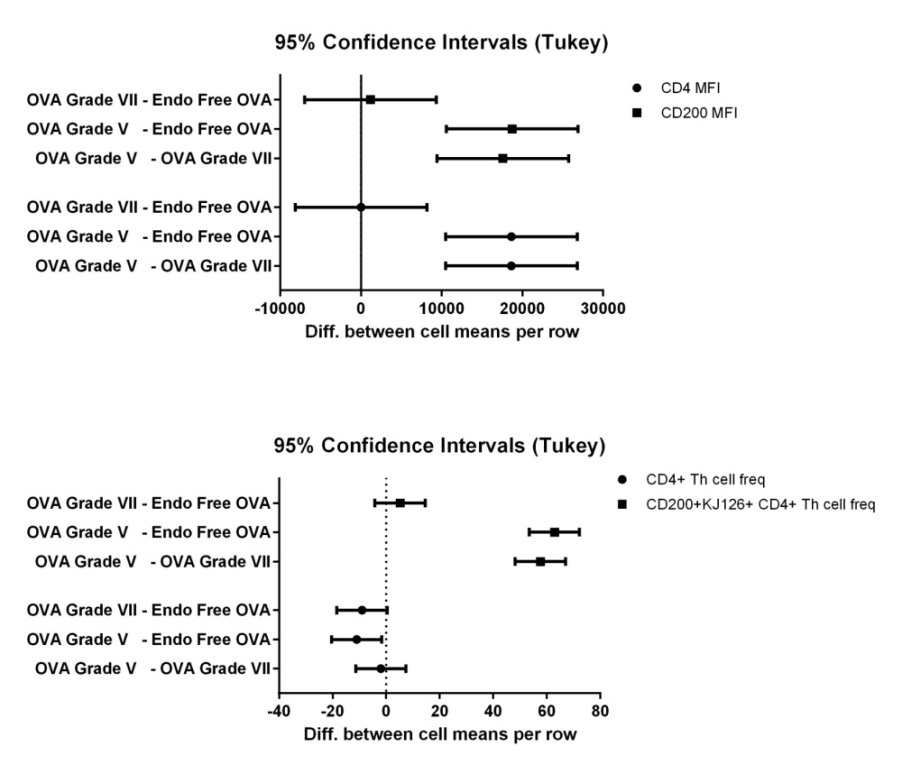
Thank you for your comment. We explored this issue prior to conducting the study because we also had similar concerns. If the amount of endotoxin contained in the ovalbumin were to be injected by IP or IV, we certainly would expect an effect on the results. However in previous studies when mice were dosed orally with 1,000,000 endotoxin units (3.3mg/kg bw/day in a 30g mouse), no signs of acute toxicity were observed (Harper et al., 2011). We used grade VII ovalbumin which had the least amount of endotoxin when tested by Kinetic QCL-LAL assay, compared to grade V and grade VI.

In splenocyte cultures endotoxin can interact directly with the immune cells, yet we observed only small changes in the magnitude of expression of CD4 (T helper cell marker), KJ1-26 (Ovalbumin peptide specific TCR), and CD200 (expression increased on activated T cells) when comparing the proliferative effects of grade VII and endotoxin free ovalbumin. In contrast, grade V ovalbumin caused considerable changes, likely due to the presence of endotoxin (Figure 1). Statistically (Figure 2), there was no difference in the median fluorescence intensity (MFI) of CD4 (increased on activated T cells) on T cells, and CD200 (increased on activated T cells) on Th cells as well as the relative abundance of CD4+ Th Cells and CD4+KJ126+CD200+ T cells between the OVA grade VII and endotoxin free ovalbumin treated spleen cells. Experimental data was generated from the spleen from 3 individual mice over 2 different days. However, in this model, the cells were exposed directly.

As you have proposed, gut leakiness is a possibility. With the *in vivo* model, the endotoxin travels through the gut before being processed (e.g. neutralized/sequestered/inactivated) in the liver. Following a blinded analysis by our pathologist, no signs of inflammation were observed in the duodenum or jejunum sections of the small intestine when comparing the control and OVA treated groups. This was also the case with liver, the primary site for endotoxin detoxification. Nonetheless, to address your concern we have now acknowledged the potential for gut leakiness and our expectations in the following paragraph of the discussion. “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 (Munford, 2005, Heymann and Tacke, 2016). Thus, despite exposure to equivalent endotoxin levels splenocytes from OVA+CBNP treated mice achieved statistical increases in cytokine mRNA expression compared to mice treated with OVA alone (**Table 1**). “

Numerous studies have employed lower grades (with respect to endotoxin content) of ovalbumin (Simioni et al., 2004, Whitehead et al., 2003, Shindo et al., 2012), or not mentioned it at all (Marth et al., 2000, Omata et al., 2005, Wu et al., 1998, Yoshida et al., 2007, Miyajima et al., 1997). Considering the results of our *in vitro* work and based on the route of administration, grade VII ovalbumin had the greatest cost to benefit and it does not add appreciably to background levels of endotoxin in the gut.

Figure 1. Immunophenotyping of spleen cell cultures 3 types of ovalbumin. Left panel is gated on CD3+ CD4+ Th cells and the right side panels are gated on KJ1-26 (OVA specific TCR) and CD200 expression on TH cells.  
  
Figure 2. 2-way ANOVA-Row comparison of the effects of 3 types of Ovalbumin on CD4 and CD200 expression levels and abundance in T cells



*5) The authors did not observe any significant changes in absolute spleen cell number between test groups, but is calculation of this parameter a predictive measure of systemic immunostimulation?*

Counting the absolute spleen cell number is on its own not a predictive measurement of systemic immunostimulation. It was merely a quantification of the harvested splenocytes. In the field of immunology it is a common number that is reported, along with relative numbers. The rationale is that if two groups have an identical % T cells, but have different absolute spleen cell number, there may be an effect on overall cellular proliferation that may require further investigation. We found no statistically significant treatment related effects on absolute cell numbers from isolated spleens and from the 3 day cultures (1 million cells per well on Day 0). To address your concern and to avoid over-interpreting the results of this endpoint we removed the following words from the results section: “as an additional measure of systemic immunotoxicity”. In the conclusions we also removed the word “systemic” and instead used the term “peripheral splenocytes”.

*6) The reviewer is concerned about the lack of an allergic phenotype induced by OVA. OVA exposure would have been expected to significantly increase serum IgE and levels of IL-4 and IL-13. While there was a slight increase in IL-4 mRNA and secreted IL-4 and IL-13 protein in mice exposed to OVA+CBNPs, the levels were fairly low and it was surprising that no changes were observed in mice exposed to OVA alone.*

Thank you for the comment. It’s a good observation. In fact it was our intention to have only a mild threshold allergic response to the OVA alone so that we could see if the CBNPs increased the reaction. In the Introduction we have added a sentence: “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”. To the discussion we added: “to be at the sensitization threshold”. To the conclusions we added “signs of threshold allergic sensitization”. Here in the rebuttal letter we have included a more detailed explanation for you. While it is more challenging to sensitize wild-type Balb/c mice by oral feeding of ovalbumin, even with the use of a strong adjuvant, oral feeding ovalbumin in the DO11.10 mouse has been shown to be sufficient for sensitization and the induction of an allergic response (Simioni et al., 2004). Addition of an adjuvant or priming event in these types of studies can induce anaphylaxis (Shindo et al., 2012). Using a model that would induce anaphylaxis in the mice would likely be insensitive to mild or moderate adjuvant effects of the CBNPS. So we used a slightly lower OVA dose than previous studies to avoid an extensive immune response. We observed anti-OVA IgG1 production, which precedes IgE in the isotype switching reaction. The objective of the study was to determine if there were any interactive adjuvant effects of the CBNPs by the oral administration route. In the conclusions section we have now highlighted “via the gastrointestinal route … by gavage”, to highlight that the results via this route are the unique focus of the paper. The CBNPs themselves didn’t have an effect on most key parameters, suggesting safety by that route. In the conclusion we mentioned that lengthier *in vivo* experiments may result in a more distinct response; by giving additional time for antibody affinity maturation and isotype switching.

*7) There is a lack of focus in the discussion on the effects of CBNPs in the experimental model used. While this is likely because much of what was observed was not different in the OVA and OVA+CBNPs groups, this is important to capture since that is the focus of this journal.*

Yes, the similarity of the response to OVA and OVA+CBNPs was a reason for the limited discussion on their effects. To focus the discussion more on the nanoparticle effects we have added the following sentences to the discussion and conclusions:

“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”.

“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…”.

“This is expected since the CBNPs were to act as an adjuvant, but not a direct stimulator of ovalbumin targeted responses”.

“Increased Th2 cytokines were also observed in rodents intratracheally exposed to CBNPs (de Haar et al., 2005, Saputra et al., 2014).”

“Either due to a lack of effect or lack of sensitivity, no increase in cytokine secretion was observed when comparing the OVA and OVA+CBNP groups.”

“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 systemic allergy-associated biomarkers in peripheral splenocytes compared to vehicle and to a lesser extent (Il4 transcript only) compared to OVA alone”.

Thank you again for your helpful input.

*REVIEWER 2*

*0. The authors examined the effect of ingestion of carbon black nanoparticles on allergic sensitization to OVA antigen. Many parameters of sensitization (such as Th2 cytokines) were increased by CBNP. The studies are well designed and described.*

Thank you for your constructive review. Below we have replied to your comments and indicated where they have been addressed in the track changes manuscript.

*1. Abstract. It is not clear what “systemic allergy associated biomarkers” refers to. This seems to be an important point since these are increased by CBNP and it is the final result mentioned before the conclusion. Does this refer again to the cytokine results? Please specify.*

Good point. By “systemic” we were referring to the observed immune activation changes in splenocytes, which are peripheral to the primary point of gastrointestinal food antigen and CBNP encounter. The systemic allergy associated biomarkers we referred to were both the splenocyte cytokine results and the splenocyte transcriptional changes. We made changes to the text to reflect this: “we did observe increased expression of genes and cytokines associated with allergy in peripheral splenocytes”.

*2.Methods, Study Design. What starts the timeline (day 0)? Mice are dosed with CBNP on even numbered days (2 through 12), but it is not clear what occurs on day 0.*

On Day 0, the mice were weighed to establish a baseline at the beginning of the study. The numbering convention was utilized to mirror a previously published study (Okamoto et al., 2005). I have added text to the methods to clarify this: “the mice were weighed, on the designated study day 0, to establish the baseline mean body weight, which was …”, and “The mice were dosed … (on days 2, 4, 6, 8, 10, 12), using the regimen of Okamoto et al., 2005”.

*3. Results, Immunotyping at necropsy, second paragraph. It is stated “CBNPs themselves did not modify the abundance of B cells (CD3- CD19+) and there were no significant changes in other cell populations investigated, including CD3- CD19+ (B cells).” The authors probably intended to mention a different cell type at the end of the sentence rather than repeating B cells.*

Thank you for pointing this out. This was an error introduced when the structure of the sentence was changed and did not remove the second mention of B cells. This error has been removed in subsequence revisions.

*4. Discussion, end of second paragraph (p. 12). The authors state “splenocytes from OVA+CBNP treated mice achieved statistical increases in cytokine production compared to mice treated with OVA alone (Fig.5A-C)”. The cytokine levels were only significant compared to PBS alone, as noted correctly in the next paragraph.*

Thank you for pointing this out as well. This was a wording error. I have changed the wording of this sentence from “cytokine production” to “cytokine mRNA expression … (Table 1)” to reflect this intended message.

*5.Discussion, last paragraph, last sentence. Should be “an increase in allergy”.*

Thank you this change been added, with a minor revision. The new sentence ends with “with regard to the risk of increasing allergic sensitization”.

*6. All figures are small with small font size, but Figures 3 and 5 are especially small with tiny font. These should be scaled up in size.*

I spoke to the editor, and this is likely a scaling issue during the upload. The figures have been reformatted so they upload in a legible format.

Thank you for your helpful input.

Best regards,

Jason Fine, PhD

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