Author's response to reviews

Title: Oral administration of PPC enhances antigen-specific CD8+ T cell responses while reducing IgE levels in sensitized mice.

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Author's response to reviews: see over
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To the Editor of BMC Complementary and Alternative Medicine:

Re: Manuscript 9973033993017222; Oral administration of PPC enhances antigen-specific CD8+ T cell responses while reducing IgE levels in sensitized mice, authored by Burrows et al.

Please find attached our response to the three reviewers. The text of our response is indicated by the blue italic lettering under each of the reviewer’s specific points.

Respectfully,

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Reviewer 1: Tong-Rong Jan

Major points:
1. The effective doses of PPC shown in Figures 1-5 are 200 and 2000 microgram/mL, whereas lower doses were used in experiments conducted in aged mice. What is the rationale to compare the effects between different doses under different experimental conditions?

Author’s response: The aged mice studies were performed with B6C3F1 mice that were part of a long-term immunotoxicology study where mice where provided low to moderate doses of PPC continuously for 1 year. This particular strain of mice is recommended for testing of immune modulators by the National Toxicology Program and aged mice of this strain were available from the NIH National Institute of Aging. In our experiments, this particular group of mice was sampled after 25 days of exposure, a period of exposure similar to that used in our IgE and vaccine studies. For this reason (length of exposure) we chose to show that even at a lower dose and in a different strain of mice, PPC was still able to affect the Th1/Th2 balance.

2. Although the authors describe in the Introduction section that systemic absorption may occur after oral administration of PPC. It is unclear if the plasma concentrations reach 200 microgram/mL after taking PPC in humans. The concentrations used in the present studies will have to be justified.

Author’s response: Presently, no dose response studies have been performed in humans and since the specific identity of the active molecules is currently unknown, there is no way to determine plasma levels of the active ingredient. The doses utilized in the mice studies have been based on traditional human use. The levels of PPC contained in the amount of Japanese tea typically consumed per day (calculated as mg/kg body weight) have been determined and was the starting dose in our mice experiments. As demonstrated in Figure 2 (right panel) PPC delivered at 20 ug/mL exhibited no effect on IgE while at 200 and 2000 ug/mL the effect was significant. The difference between the response to 200 and 2000 ug/mL is not significantly different. In numerous experiments this dose response has been quite repeatable.

3. The presented results appear quite diverse, including humoral responses (Figures 1-3), cytokine production (Figures 4-6), and CD8+ T-cell responses (Figures 7 and 8). Although the results of Figures 7 and 8 confirm the enhancing effect of PPC on CD8+ T-cell responses presented in Figure 4, these data are not directly related to allergy, and therefore not to fit with the main idea of conducting the research based on the improvement of allergic symptoms in consumers taking the pine cone extract. The paper would be strengthened if the effect of PPC on allergic inflammatory reactions (i.e. airway hypersensitivity reactions) other than IgE production was studied.

Author’s response: We agree with the reviewer on this point and will be directly examining the effects of PPC on the development of airway hypersensitivity in the OVA induced asthma model. The findings from these studies will be published separately. We are also working with a group of clinical allergist/immunologists at the University of South Florida to undertake a clinical study to determine if PPC can indeed reduce the symptoms and IgE levels associated with perennial allergies.
4. For studying the effect of PPC on the Th1/Th2 immunobalance, an investigation on the production of IgG subtypes (i.e. IgG1 and IgG2a) induced by an allergen (i.e. ovalbumin) would provide a direct evidence to complement the cytokine results. However, Figure 3 shows only IgG2a. It will be more convincing if IgG1 is included in the study.

Author’s response: It is well established that the alterations of the Th1/Th2 balance is most clearly seen in changes in the IgE/IgG2a response in mice, with the Th1 cytokine, IFNγ, inducing production of IgG2a, and the Th2 cytokine, IL-4, inducing the production of IgE and IgG1. Since the presence of IL-4 differentially regulates the production of IgE and IgG1 depending on its concentration (J Exp Med, 1988, 167:183-196; Scand J Immunol 2006, 30:355) the levels of IgE and IgG1 are not always equivalently affected by immune modulators. The publication by Finkleman et al (Intl Immunol 1991, 3(6):599) even reported that while IL-4 is an absolute requirement for a primary IgE response, they found that it played a relatively small role in the induction of IgG1 responses in several in vivo systems. This was demonstrated by blocking IL-4 activity using antibodies against both IL-4 itself and the IL-4 receptor. For these reasons we chose to report only the changes in IgE and IgG2a. However, we did find in our experiments that when PPC suppressed IgE levels it also suppressed IgG1.

5. A quite extensive discussion on the development and differentiation of Th1 and Th2 cells was included in the Discussion section (page 19). However, the authors did not present data on the influence of PPC on Th cell differentiation, such as the signaling transcription factors T-bet and GATA-3 for Th1 and Th2 cells, respectively.

Author’s response: Great point and will be addressed in a subsequent publication. The purpose of this report is to describe the phenomena while we continue to work on understanding the mechanism.

6. Are the data presented in each figure derived from one single experiment? How many times of each experiment are repeated? The authors should clarify this issue.

Author’s response: All experiments have been performed multiple times and verified in different laboratories. We have included a statement in the Methods section regarding repetition of the experiments.

Minor points:

Author’s response: All of the following minor points have been corrected in the manuscript.

1. For all IgG2a, 2a should change to subscript word.
2. For all CD8+, + should change to superscript word.
3. For the word “ug”, the u should change to the abbreviation of “micro”.
4. For hr, hours and hrs, ml and mL, and ul and uL, standard abbreviations should be followed.
5. For the description of cell concentration (i.e. 3-5x106/mL on page 9), it should change to 3-5 x 106 cells/mL. The entire manuscript has to be checked for consistency.
6. On page 5: 0.2um should change to 0.2 um.
7. On page 5: 11.3k should change to 11,300.
8. On page 6: “+/- either” should be rewritten.
9. On pages 6, 7 and 9: Day should change to day.
10. On page 10: Avidin-HRP should change to avidin-HRP.
11. On page 10: PBST and AEC substrate should be specified.
12. On page 12, line 3 from the bottom: were should change to was.
13. For all figures: do error bars represent standard deviation?
14. Statistic significance should be indicated in all figures.
15. Figure 2 legend: Day should change to day.
16. Figure 2 legend: 100ug should change to 100 ug.
17. Figure 2 legend: CPG should change to CpG.
18. Figure 5: the IL-4 level in the OVA+PPC group is less than that in the non-sensitized Naïve group. This will have to be explained.
19. Figure 7 legend: ovalabumin should change to ovalbumin or OVA.

Reviewer 2: Kouya Yamaki

Reviewer's report:

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

Description about Figure 2 in results and discussion sections:
The experimental condition of Figure 2 is unclear. In page 11, the authors described that C57Bl/6 is immunized with IFA for Figure 2 experiment. However, in page 15, they mentioned that balb/c was immunized with Alum for Figure 2 experiment. Authors must clarify the strains of mice used (C57l/6 or balb/c) and change the explanations about Figure 2 in discussion section according to modifications of results section.

Author’s response: This was a mistake. The strain of mice used was indeed C57Bl/6. This has been corrected in the manuscript

Results:
Authors should comment (or examine) about the effects of PPC on Th1 and Th2 cytokines productions and other (e.g. CD4+) T cell functions in experiments with C57Bl/6 mice, and IgG2a production in experiments with balb/c mice.

Author’s response: These types of experiments are underway and will be reported in a subsequent manuscript. The purpose of this report is to describe the phenomena while we continue to work out the mechanism of action.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Text:
Standardize the unit (micro, mu or u; liter, l or L; rpm should be changed to x g).

Author’s response: Corrected.

Describe the company data (e.g. “Source” and “Pierce”) in methods section more precisely.

Author’s response: This has been corrected.
Information about B16-OVA cells should be included.

*Author’s response: B16-OVA were not used so all references to these cells have been removed from the paper.*

In page 26, IgGa should be corrected.

*Author’s response: Done*

Discretionary Revisions (which the author can choose to ignore) Figures:

To improve appearance:

Please indicate statistical significance (e.g. “*”) in Figures as well as in text.

*Author’s response: This has been done, revised figures will be sent.*

In figure 2, it is unclear that PPC-treated groups were immunized or not.

*Author’s response: Clarification has been included in the Figure legend. All mice receiving PPC were immunized.*

In figure 3, include the word “IgG2a” in the title of Y axis.

*Author’s response: This has been done.*

**Reviewer 3: Shuichi Segawa**

**Reviewer’s report:**

In this study, the authors clearly demonstrated that the oral administration of PPC significantly inhibited IgE production by skewing the Th1/Th2 balance toward Th1 dominant. PPC might be promising material to alleviate human allergic symptom. However, there are several issues that should be attended by the authors.

**Discretionary Revisions**

1. Page 5, line 14. It is preferable to describe a precise condition for preparation of PPC. A description such as elevated pH and high temperature is ambiguous.

   *Author’s response: The production of the commercial product is via a proprietary method and the manufacturer is reluctant to describe the exact conditions of production. However, the manufacturer will make their product available upon request.*

2. In this study, positive control CpG strongly inhibited the increase in serum IgE level (Figure 2) and promoted allergen-specific IgG2a in comparison with 200 or 2000 ug/ml of PPC administered group (Figure 3). Nevertheless, the administration of 200 or 2000 ug/ml of PPC strongly enhanced the antigen-specific CD8+/IFN-gamma+ T cells in comparison with positive control CpG group (Figure 4). It is commonly thought that IFN-gamma+ Th1-type T cells induce the production of IgG2a antibody and suppress the production of IgE antibody. You had better discuss the reason why the ability of PPC to suppress IgE production and promote IgG2a production was lower than CpG in
spite of its higher ability to proliferate the CD8+/IFN-gamma+ T cells.

Author’s response: Since PPC is a crude extract likely to contain hundreds or even thousands of different molecules, the ability to produce CpG-like activity (suppression of IgE and enhancement of IgG2a) while boosting the CD8+/IFNg+ response to levels higher than CpG is not surprising. We have included these comments in the discussion (5th paragraph in the discussion).

3. In Figure 6, IL-12p70 production from PPC-treated mouse splenocytes was significantly increased compared to those from untreated mouse splenocytes. However, IFN-gamma production from PPC-treated mouse splenocytes was not enhanced, rather, suppressed compared to those from untreated mouse splenocytes. It is well known that IL-12 stimulates the production of IFN-gamma.

You need to discuss the reason for this result.

Author’s response: Based on the variations between the splenocytes cultures’ production of IFNγ there was no significance in the amount of IFNγ produced by naïve mice, DHEAS-treated, or PPC-treated mice. The following text was inserted into the 4th paragraph in the Discussion section.

“Interestingly, even though IL-12p70 levels were elevated in ConA-stimulated splenocytes cultures from the PPC- and DHEAS-treated mice, we failed to detect a significant enhancement of IFNγ in these cultures. The finding that splenocytes from the DHEAS treated mice also failed to induce IFNγ in the presence of elevated IL-12p70 levels suggests that the lack of IFNγ response was not specifically associated with PPC. In a parallel experiment (not shown), young B6C3F1 mice were also treated with DHEAS and PPC. The ConA-stimulated splenocyte cultures from these mice were found to produce significantly more IFNγ than the cultures from the untreated mice. Also, the levels of IFNγ in the cultures from the young naïve mice were much higher than that found in the cultures prepared from the older naïve mice. This suggests that some age-related phenomenon was preventing the splenocytes from secreting IFNγ. Based on these findings, further experiments will be performed to determine if the suppression of IL-4 and IL-10, concurrent with the enhancement of IL-12p70 production in the aged mice translates into an increased cellular response to vaccines, resistance to infections, and reduction of IgE. The findings from such experiments could be significantly beneficial to the aging population of humans.”

4. What kind of components contained in PPC is responsible for the improvement of Th1/Th2 balance? In the background section, you mentioned that PPC contains poly-phenylpropanoid-polysaccharide complex. Does this polysaccharide complex have an adjuvant activity and contribute to the improvement of Th1/Th2 balance?

Author’s response: Experiments are in progress to identify the active fractions associated with the in vivo responses and will be presented in a subsequent manuscript.

Minor Essential Revisions

1. Page 19. The delivery of either IFN-gamma or IFN-gamma lead to ~. I cannot understand this.

Author’s response: This was meant to read, The delivery of either IFN-alpha or IFN-gamma lead
to ... This correction has been made in the manuscript.