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August 27, 2012

Claire Standen, Ph.D.
Science Editor,
Journal of Visualized and Experiments

RE: JoVE50144R1

Dear Dr. Standen,

Thank you again for the opportunity to resubmit this work. We have revised the manuscript extensively in light of the reviewers' comments. We have also provided below a point-by-point response to those comments.

Reviewers' comments:
Reviewer #1:
Summary:
This manuscript describes the use of a reagent, AHG2, that supposedly co-aggregates IgG and IgE receptors on basophils (and mast cells) by directly binding the FcgRIIb and indirectly with FceRI by interacting with peanut specific IgE bound to FceRI. Such an interaction is thought to initiate inhibitory signals that prevent mediator release mediated through FceRI alone and thus the ensuing inflammatory reactions associate with peanut allergy. AHG2 could therefore be a potentially new and important therapeutic reagent to prevent allergic reactions to peanut.

Major Concerns:
1) Regarding the Passive Cutaneous Sensitization protocol. It is currently not clear to this reviewer whether AHG2 is added along with serum during PCS or whether the serum (or AHG2) is added first. Please clarify. How effective is the AHG2 reagent if added after PCS but before allergen challenge?

Answer: We clarified in the manuscript that AHG2 was added with the peanut allergic patient serum immediately after the mice were sensitized. We have also tested different time administration of AHG2. For instance, mice were sensitized first with serum, and after two hours, we injected AHG2 at the same spot sensitized with serum. After the mice were kept idle for two more hours, we challenged the mice with peanut allergen. In this experiment, we obtained the same results as the original, same-time administration test.

2) It is not clear to this reviewer the need to purify human basophils when only conducting histamine release assays. Purification may actually be problematic. For example, the purification method involves the use of

FcR blocking reagent. Is there an outside chance that this step could be influencing the activity of the AHG2 reagent, since the latter supposedly works through FcR receptors?

Answer: The purified human basophils will be stripped with lactic acid before peanut-specific IgE sensitization and AHG2 treatment. Thus, the FcR blocking reagent could not influence AHG2 function.

3) In addition, the histamine release assays appear to all be done using target cells passively sensitized with serum/plasma from peanut allergic subjects. Assays should also be done using basophils directly obtained from peanut allergic subjects in order to better assess whether the AHG2 reagent does not induces histamine release.

Answer: The reason we used passive sensitization for basophils is that it is difficult to obtain an acceptable amount of human basophils from a peanut-allergic patient because only 1% of peripheral blood monocytes are basophils. In addition, concerning optimization of experimental condition and repeatability, it is also difficult to acquire basophils from a single peanut allergic patient multiple times.

4) Are any of the data in Figure 3 significant? It's stated in the text that AHG2 significantly inhibited basophil histamine release, but statistics are not shown and the inhibition is, at best, modest at the highest concentration of AHG2 tested.

Answer: We demonstrated that AHG2 inhibited histamine release in purified human basophils in dose-dependent manner in Figure 3. We have clarified this in text.

Minor Concerns:
2nd sentence of long abstract needs the word "with" before the word "epinephrine"
2nd paragraph of long abstract. "Previous studies showed?" remove the first word "The" from this sentence.

Answer: We have incorporated these corrections into the manuscript.

Reviewer #2:
Summary:
The paper from Liu et al. entitled "Inhibition of Peanut Allergy with Allergen-Fcg Fusion Protein" described the construction of a chimeric molecule (Ara h 2 to human IgG Fc)to inhibit peanut allergy. The authors describe the construction of the molecule and check the immunoreactivity of coupled Ara h 2 by western blot analyses. The in vivo immunoreactivity was checked in murine models using 2 different backgrounds: hFcepsilonRIa transgenic mice (for PCA) and C57BL/6 (for peanut allergy model). Using the 2 models, the authors illustrate the safety and the efficiency of the administration of their biomolecule. A dose-effect study (using 2 dosages) was done and showed the effect.

The results from in vivo studies were completed by in vitro analysis of human basophil stimulation and measure of histamine release. Results demonstrated the safety and efficacy of administration. It was not really clear how many different peanut allergic serum were used and if non allergic sera were used as negative controls. Results (figures and tables) were well explained and illustrated but some experiments are missing (more information of the status of sensitization for mice).
Major Concerns:
The measurement of histamine release were done from purified basophils. Major studies in this fields used

RBL-SX38, a mast-cell line well characterized. Why the authors did not used this cell line and how the relevance of the use of basophils instead of RBL-SX38?

Answer: RBL-SX38 is a rat basophilic leukemia cell line, transfected to express a human FcεRI. We believe that human basophils are much more natural than RBL-SX38 in histamine release experiments.

Concerning the mouse model of peanut sensitization. No information on the level of production of specific IgE, IgG1 and IgG2a were done as well as the profile of secretion of cytokines by in vitro stimulated spleen cells. The authors have to give this information to justify the relevance of their model.
Then, when using injecting their biomolecule in one shot to sensitized mice, the authors evaluate the effect of the treatment administrated just before the challenge. Is the effect maintained over time? how long?
The impact of the treatment on antibody levels was not evaluated. It should be. Developing a treatment for peanut allergy should not be a treatment with short time effect... This has to be demonstrated.

Answer: The peanut allergy mouse model we used was developed by Manel Jordana. We have cited his paper in our manuscript. We followed his protocol and confirmed peanut-specific IgE level. We agree that the half-life of AHG2 is an important point and will plan future murine in vivo studies, using longer time periods between injection of the fusion protein and administration of the antigen to determine how long the inhibition lasts. However, we hope that the reviewer agrees that the duration of the effect is not of central importance for the present concept that we advance in this study.

It is not clear if the analysis of the lung tissue was done after aerosol/intranasal challenge or not. If it is not the case, it should be done, to be sure to target the organ. This analysis could be completed by an analysis of airway hyperreactivity (AHR) by in vivo measurement by whole body plethysmography (noninvasive methods) or flexivent (invasive method for resistance-compliance measurement.

Answer: We did not test aerosol/intranasal challenge in this model. Since peanut allergy is a type of food allergy, we ever tested oral WPE challenge for the peanut-sensitized mice. In addition, because the histological change in the airway of peanut-sensitized mice did not show airway remodeling, we did not check airway hyper-reactivity at this time. However, a future analysis of AHR in murine studies is planned.

Minor Concerns:
Typographical errors were found. The authors have to correct them all over the manuscript.
The use of ketamine alone to anesthetize mice is not recommended. A mix of ketamine/xylazine is more adapted.

Answer: We have carefully looked over our manuscript and corrected all errors. In our experiments, we did use a mix of ketamine and xylazine to anesthetize mice, following the standard procedure. The manuscript was not clear, and we have since clarified this detail in the manuscript.

Thank you very much!

Sincerely yours,

Daocheng Zhu, M.D., Ph.D.