

# Journal of Visualized Experiments

## Inhibition of Peanut Allergy with Allergen-Fcg Fusion Protein

--Manuscript Draft--

<b>Manuscript Number:</b>	JoVE50144R2
<b>Full Title:</b>	Inhibition of Peanut Allergy with Allergen-Fcg Fusion Protein
<b>Article Type:</b>	Methods Article - JoVE Produced Video
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<b>Abstract:</b>	<p>Food allergy is a serious health problem of national importance with approximately 1.5 million people in the United States having peanut allergy, 50 to 100 of who die each year from accidental ingestion of peanuts. Currently, the only available treatment is strict avoidance of all peanut-containing food products and timely treatment of accidental ingestion with epinephrine. Peanut-induced allergic reactions are mediated by antigen-specific IgE bound to the high-affinity receptor for IgE (FcεRI) on mast cells and basophils.</p> <p>Previous studies showed that cross-linking FcγRIIb to FcεRI inhibited FcεRI-mediated activation. Here we developed a novel plant-human fusion protein consisting of a major peanut allergen Ara h2 and the human IgG Fcγ1 to extend this approach in peanut allergy.</p> <p>We genetically fused Ara h2 cDNA to genomic DNA of Fcγ1 (hinge-CH2-CH3). Western Blot showed that the fusion protein Ara h2-Fcγ (AHG2) was expressed as the predicted dimer of approximately 88 KD. The purified AHG2 reacted with both specific anti-human IgG Fc and anti-Ara h2 antibodies. Further results showed that AHG2 bound in a fashion similar to native IgG to FcγRIIb expressed on HMC-1 cells.</p> <p>We tested AHG2's function in purified human basophils primed with anti-peanut IgE. We found that AHG2 dose-dependently inhibited peanut-induced histamine release. AHG2 itself did not induce any release in sensitized basophils. AHG2 also inhibited the WPE-induced peanut-specific IgE-mediated degranulation in transgenic mice. Finally, we treated WPE-sensitized mice with AHG2. We found that AHG2 significantly inhibited the WPE-induced acute anaphylactic reaction as well as the prototypical drop in body temperature. Furthermore, we found that WPE-induced inflammatory infiltration in the airway was also decreased.</p> <p>Taken together, these results suggest that AHG2 inhibited mast cell/basophil degranulation and decreased WPE-induced acute anaphylactic reaction and airway inflammation. Linking specific peanut allergen to Fcγ might provide a new approach for allergen immunotherapy of peanut allergy.</p>
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June 8, 2012

Clair Standen, PhD,  
Science Editor,  
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Dear Dr. Standen:

Please find enclosed a manuscript entitled "Experimental Therapy of Peanut Allergy with a Novel Ara h2-Fc $\gamma$  Fusion Protein in Mice" for consideration as an Article in the Journal of Visualized Experiments.

This work describes the conceptualization, design and testing of an entirely new approach to the treatment of peanut allergy. Peanut allergy is frequently fatal and there are no immunotherapy options at present, unlike other allergies. We have produced a major peanut allergen Ara h2-human Fc $\gamma$  chimeric fusion protein and shown that it is able to block whole peanut extract-induced reactivity both *in vitro* and *in vivo*. At the same time, this chimeric protein itself does not act as an allergen. As such, this approach provides an entirely new strategy for immunotherapy of peanut allergy.

We hope you find the submission suitable for publication in the JoVE.

Sincerely yours,

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

## **Inhibition of Peanut Allergy with Allergen-Fc $\gamma$ Fusion Protein**

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### **Keywords:**

Food Allergy, peanut, immunotherapy, anaphylaxis, mast cells, basophils, fusion protein

### **Short Abstract:**

A novel plant-human fusion protein to block peanut allergy is developed. This protein inhibits peanut-induced allergic reactions by indirectly cross-linking inhibitory Fc $\gamma$ RIIb with peanut-specific IgE bound to Fc $\epsilon$ RI. Linking specific peanut allergen to Fc $\gamma$  may provide a new approach for the allergen immunotherapy of peanut allergy.

### **Long Abstract:**

Food allergy is a serious health problem of national importance with approximately 1.5 million people in the United States having peanut allergy, 50 to 100 of who die each year from accidental ingestion of peanuts<sup>1</sup>. Currently, the only available treatment is strict avoidance of all peanut-containing food products and timely treatment of accidental ingestion with epinephrine<sup>2</sup>. Peanut-induced allergic reactions are mediated by antigen-specific IgE bound to the high-affinity receptor for IgE (FcεRI) on mast cells and basophils<sup>3</sup>.

Previous studies showed that cross-linking FcγRIIb to FcεRI inhibited FcεRI-mediated activation<sup>4-6</sup>. Here we developed a novel plant-human fusion protein consisting of a major peanut allergen Ara h2 and the human IgG Fcγ1 to extend this approach in peanut allergy.

We genetically fused Ara h2 cDNA to genomic DNA of Fcγ1 (hinge-CH2-CH3). Western Blot showed that the fusion protein Ara h2-Fcγ (AHG2) was expressed as the predicted dimer of approximately 88 KD. The purified AHG2 reacted with both specific anti-human IgG Fc and anti-Ara h2 antibodies. Further results showed that AHG2 bound in a fashion similar to native IgG to FcγRIIb expressed on HMC-1 cells.

We tested AHG2's function in purified human basophils primed with anti-peanut IgE. We found that AHG2 dose-dependently inhibited peanut-induced histamine release. AHG2 itself did not induce any release in sensitized basophils. AHG2 also inhibited the WPE-induced peanut-specific IgE-mediated degranulation in transgenic mice. Finally, we treated WPE-sensitized mice with AHG2. We found that AHG2 significantly inhibited the WPE-induced acute anaphylactic reaction as well as the prototypical drop in body temperature. Furthermore, we found that WPE-induced inflammatory infiltration in the airway was also decreased.

Taken together, these results suggest that AHG2 inhibited mast cell/basophil degranulation and decreased WPE-induced acute anaphylactic reaction and airway inflammation. Linking specific peanut allergen to Fcγ might provide a new approach for allergen immunotherapy of peanut allergy.

## **Protocol Text:**

### **1.) Peanut allergen Ara h2-Fcγ fusion protein expression and characterization**

- 1.1) Clone cDNA of Ara h2 into the expression vector pSecTag-IgG1, which contains genomic DNA of a human immunoglobulin IgG1 Fc fragment.
- 1.2) Transfect the expression vector into CHO cells with Lipofectamine (Invitrogen).
- 1.3) Select the transfected cells with 500 ug/ml of zeocin.
- 1.4) Collect the supernatant from selected cell culture.
- 1.5) Purify the protein by using a Protein A affinity column.
- 1.6) Run the purified protein on 4.5-15% SDS-PAGE.

- 1.7) Transfer the protein into Immobilon transfer membrane (Millipore).
- 1.8) Detect the blot with anti-Ara h2 antibody or peanut allergic patient serum, overnight.
- 1.9) Wash the blot with 1X PBS-Tween.
- 1.10) Probe the blot with HRP-conjugated goat anti-mouse IgG Fc specific or HRP-conjugated goat anti human IgE Fc specific antibodies (KPL).
- 1.11) Develop by ECL kit (GE Healthcare).

## **2.) Passive cutaneous anaphylaxis**

- 2.1) Anesthetize the hFcεRIα transgenic mouse with Ketamine/xylazine at the dose of 100mg/kg.
- 2.2) Shave the back skin of mouse.
- 2.3) Inject 50 µL of peanut allergic patient serum intradermally.
- 2.4) Add the different doses of AHG2 from 0.1 to 10 ug to the patient serum prior to passive sensitization.
- 2.5) Four hours later, challenge the mice intravenously with a solution of 100 ul of WPE (100 ug) and 200 ul of 1% Evan blue.
- 2.6) Sacrifice the mice in 30 minutes after the intravenous challenge. Separate the skin and measure inside blue color density.

## **3.) Human basophil purification**

- 3.1) Obtain 54 ml of cell concentrated peripheral blood from the blood bank. Add 27 ml of blood and 15 ml of Ficoll-Paque (GE Healthcare) into a 50ml tube.
- 3.2) Spin at 2000rpm, 40min at room temperature, no brake.
- 3.3) Collect the peripheral blood mononuclear cells (PBMC) from interphase. Add 20 ml of HBSS medium and mix very well.
- 3.4) Spin at 1200rpm, 10min.
- 3.5) Wash the cells with HBSS medium again.
- 3.6) Remove supernatants and suspend cells with 40ml of HBSS medium. Count the cell number.
- 3.7) Spin at 1200 rpm, 10min. Remove supernatants completely.
- 3.8) Resuspend the cells in 1200 ul of AutoRun buffer (provided by Miltenyi Biotec) for  $9.6 \times 10^8$  cells.

3.9) Add 400 ul of FcR Blocking Reagent and 400 ul of basophil Biotin-Antibody Cocktail. Mix very well and keep at 4°C for 10min.

3.10) Add 1200 ul of AutoRun buffer and 800 ul of anti-biotin MicroBeads. Mix very well and keep at 4°C for 15min.

3.11) Wash the cells with 60ml of AutoRun buffer.

3.12) Spin at 1200rpm, 10min.

3.13) Aspirate supernatants completely and resuspend the cells in 6ml of AutoRun buffer.

3.14) Aliquot the cells into 1 ml in 15 ml sterile tubes. Run AutoMACS separator according to manufacturer's instruction. Choose negative selection program.

3.15) Collect depleted cells. Determine basophil purity by using both Acid Toluidine Blue staining and FACS analysis (BD LSRII) with CD203c and CD123 antibodies.

#### **4.) Histamine release**

4.1) Culture purified basophils with RPMI 1640 and 10% of peanut allergic serum in 37°C, 5 % CO<sub>2</sub> incubator, overnight.

4.2) Wash the sensitized cells with 1XHBSS without Ca<sup>2+</sup>.

4.3) Spin at 1200 rpm, 5 min.

4.4) Remove the supernatants and resuspend the cells with 1XHBSS with Ca<sup>2+</sup>. Add cells into a 24 well plate, 1X10<sup>5</sup> cells/well.

4.5) Add different doses of AHG2 into the cells.

4.6) 2 hours later, challenge the cells with 5 ul of 1ug/ml WPE.

4.7) Collect the supernatants in 30min.

4.8) Measure histamine concentrations by using the Histamine ELISA Kit (Geneway).

#### **5.) Peanut allergy mouse model**

5.1) Sensitize C57BL/6 mice orally with 500 µg of WPE along with 10 µg of cholera toxin once a week for four weeks.

5.2) Challenge the mice intravenously with 100 µg of WPE two weeks after the last sensitization.

5.3) Treat mice subcutaneously with 1mg/kg and 10mg/kg of AHG2 protein before the challenge.

5.4) Evaluate each mouse's anaphylactic symptom 30 minutes after the challenge utilizing a defined scoring system (Table I). Perform scoring of symptoms in a blinded manner by 3

independent investigators. Meanwhile, measure each mouse's body temperature every 10 min with a rectally inserted thermal probe for 40 min.

5.5) Collect all mice lung tissues two days after the challenge for histological H&E staining.

**Representative Results:** AHG2 fusion protein is composed of major peanut allergen Ara h2 and human immunoglobulin IgG Fc fragment (**Figure 1A**). PCA experiment confirmed that AHG2 blocked Ara h2-induced degranulation in transgenic mice (**Figure 2D**). But AHG2 itself did not induce any degranulation as allergen Ara h2 (**Figure 2C**). AHG2 also inhibited WPE-induced histamine release in human basophils (**Figure 3A**) and degranulation in transgenic mice (**Figure 3B**) in a dose-dependent manner. We further tested AHG2 protein in a mouse peanut allergy model. We found that AHG2 significantly inhibited WPE-induced acute anaphylactic reaction and the drop of body temperature in sensitized mice upon WPE challenge (**Figure 4**). This inhibition is FcγRIIb-dependent (**Figure 5**). In addition, AHG2 also inhibited WPE-induced airway inflammation (**Figure 6**). AHG2 itself did not induce anaphylaxis and inflammation in WPE-sensitized mice. These results suggested that AHG2 is safe and may be used in allergen immunotherapy for peanut allergy.

#### Tables and Figures:

**Figure 1:** Diagram of AHG2-induced inhibition. (A) Computerized 3D structure of dimerized AHG2. (B) Proposed mechanism by which AHG2 inhibits FcεRI-mediated degranulation.

**Figure 2:** AHG2 blocked Ara h2-induced vascular leak in transgenic mice. (A) WPE induced peanut specific IgE-mediated PCA in transgenic mice. PS: Peanut allergy patient serum; NS: Non allergic serum. (B) Ara h2 induced PCA mediated by different concentration of peanut specific IgE from peanut allergy patient serum. (C) AHG2 did not induce peanut specific IgE-mediated PCA in transgenic mice. (D) AHG2 inhibited Ara h2-induced peanut specific IgE-mediated PCA in a dose-dependent manner. Each experiment was repeated with three transgenic mice.

**Figure 3:** AHG2 inhibited WPE-induced allergic reactions *in vitro* and *in vivo*. (A) AHG2 inhibited WPE-induced histamine release in human basophils. Results are representative of 3 separate experiments, each done in duplicate. (B) AHG2 inhibited peanut-specific IgE-mediated PCA reaction in a dose-dependent manner. (C) The average blue density of five mice for each of the above tests.

**Figure 4:** AHG2 inhibited systemic anaphylaxis caused by the peanut extract in mice. Four groups of mice were sensitized with the peanut antigen. Group 1(n=5) was a control; Group 2(n=9) was challenged with WPE; Group 3 (n=9) was treated with 1mg/kg of AHG2 (AHG2-L) and then challenged with WPE; Group 4 (n=9) was treated with 10mg/kg of AHG2 (AHG2-H) and then challenged with WPE. Each mouse was evaluated for their symptoms score (A), body

temperature (B), and histamine level in blood (C). Data are representative of two separate experiments. \*,  $P < 0.05$ .

**Figure 5:** AHG2 lost its inhibitory effect in Fc $\gamma$ RIIb deficient mice. Three groups of Fc $\gamma$ RIIb KO mice were sensitized and challenged with WPE. Group 1 (n=6) was control, challenged with saline; Group 2 (n=6) was not treated with AHG2; Group 3 (n=7) was treated with 10mg/kg of AHG2 before WPE challenge. Each mouse was evaluated for their symptoms score (A) and body temperature (B). Data are representative of two separate experiments.

**Figure 6:** AHG2 inhibited WPE-induced inflammation in the airway of WPE-sensitized mice. (A) Differential cell counts in BAL. The BAL was collected from each mouse of the above groups. The results are representative of the average of each group. \*,  $P < 0.05$  (B) Histological changes of the airway. Representative histological sections of the lung tissues from the mice in Figure 5 were stained with hematoxylin and eosin. Bar, 100  $\mu$ m.

**Figure 7:** Specific IgE levels to WPE and the peanut components in three peanut allergic sera we used in this study.

**Discussion:** Subcutaneous immunotherapy (SIT) is an effective therapy for allergies<sup>7</sup>. However, SIT requires numerous administrations of allergen and may cause severe adverse events that range from local allergic reactions to fatal anaphylaxis<sup>8,9</sup>. This study is the first report on the peanut allergen-Fc $\gamma$  fusion protein's ability to block severe peanut-induced anaphylaxis. We demonstrated that this fusion protein induced cross desensitization in the murine model of peanut allergy. The fusion protein itself does not cause any allergic reaction in sensitized subjects. This approach of using the major allergen in conjunction with Fc $\gamma$  may provide a novel immunotherapy against allergies induced by multiple allergens.

In the systemic model, we found that AHG2 did not completely block the WPE-induced anaphylactic reaction, unlike the previous tests in skin. It is possible that AHG2 is inactive in the systemic model due to the interference of mast cell independent or IgE independent mechanisms. Previous studies demonstrated that there are two pathways involved in systemic anaphylaxis in this model: the classic IgE-Fc $\epsilon$ RI-mast cell-mediated pathway and the alternate IgG-Fc $\gamma$ RIII-macrophage-mediated pathway<sup>10</sup>. When the amount of antigen is small, the IgE-dependent pathway is favored. However, larger amount of antigens readily induces the alternative pathway<sup>11</sup>. Therefore, we may increase AHG2-induced inhibition by further decreasing the challenge allergen amount.

There are as many as eleven proteins from peanuts that are identified as allergens. More than 90% of peanut hypersensitive individuals react to three main allergens—Ara h1, Ara h2, and Ara h3<sup>12</sup>. Here we found that a fusion protein composed of the major peanut allergen Ara h2 and Fc $\gamma$  inhibited WPE-induced anaphylaxis and airway inflammation. Future studies will be required to determine whether the WPE-induced reaction may be further reduced—or even completely

inhibited—if we were to use three fusion proteins in conjunction—Ara h1-Fcγ, Ara h2-Fcγ, and Ara h3-Fcγ.

In conclusion, our results demonstrate that this fusion protein dose-dependently inhibited WPE-induced peanut-specific IgE-mediated histamine release in human basophils as well as allergic responses in transgenic mice. Through the inhibitory receptor FcγRII, AHG2 partially inhibited WPE-induced acute anaphylactic reaction and airway inflammation in the peanut allergy murine model. AHG2 itself did not induce anaphylaxis and inflammation in WPE-sensitized mice, suggesting that all properties of the allergen were mitigated by the presence of the FcγRIIb binding moiety. This approach of using the allergen in conjunction with Fcγ may provide the first step toward a therapy that will induce an allergen specific immunotherapy response.

### **Acknowledgments:**

The authors would like to thank Andrew Saxon for experiment design. This work was funded by NIH grant R21 AI088808, the Food Allergy & Anaphylaxis Network, AAAAI, the Northwestern Memorial Foundation and the Food Allergy Initiative.

**Disclosures:** We have nothing to disclose

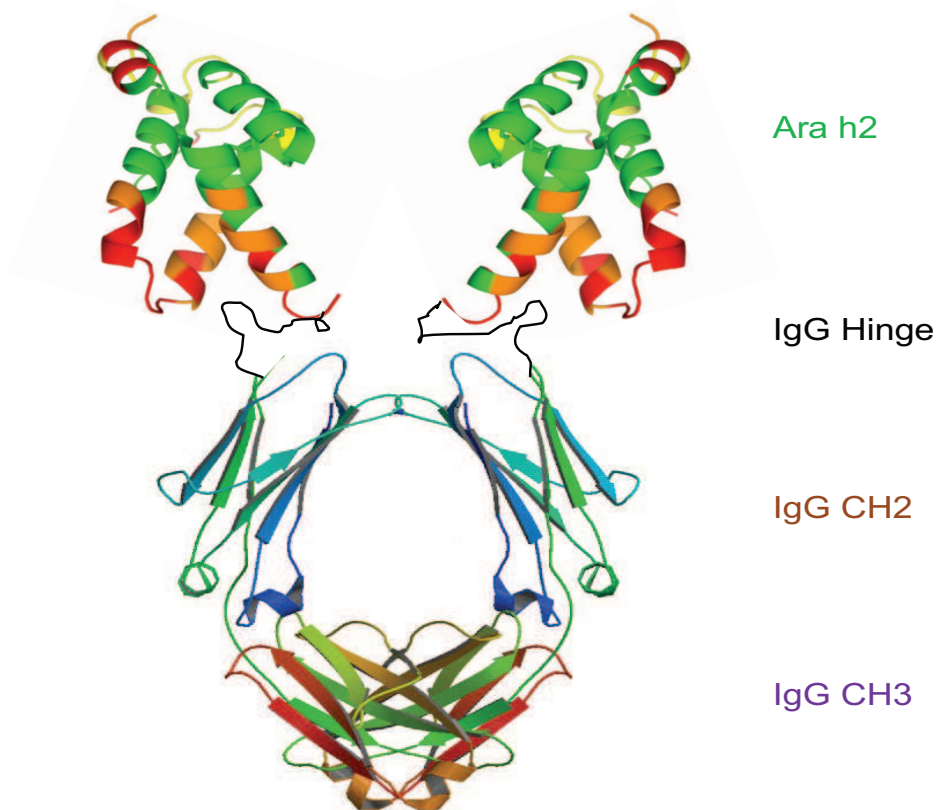
### **References:**

1. Leung DY, Sampson HA, Yunginger JW, et al. Effect of anti-IgE therapy in patients with peanut allergy. *N Engl J Med* 2003;348:986-93.
2. Blumchen K, Ulbricht H, Staden U, et al. Oral peanut immunotherapy in children with peanut anaphylaxis. *J Allergy Clin Immunol* 2010;126:83-91 e1.
3. Burks AW. Peanut allergy. *Lancet* 2008;371:1538-46.
4. Daeron M, Malbec O, Latour S, Arock M, Fridman WH. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *J Clin Invest* 1995;95:577-85.
5. Zhu D, Kepley CL, Zhang M, Zhang K, Saxon A. A novel human immunoglobulin Fc gamma Fc epsilon bifunctional fusion protein inhibits Fc epsilon RI-mediated degranulation. *Nat Med* 2002;8:518-21.
6. Zhu D, Kepley CL, Zhang K, Terada T, Yamada T, Saxon A. A chimeric human-cat fusion protein blocks cat-induced allergy. *Nat Med* 2005;11:446-9.
7. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol* 2011;127:18-27; quiz 8-9.
8. Uermosi C, Beerli RR, Bauer M, et al. Mechanisms of allergen-specific desensitization. *J Allergy Clin Immunol* 2010;126:375-83.
9. Thyagarajan A, Varshney P, Jones SM, et al. Peanut oral immunotherapy is not ready for clinical use. *J Allergy Clin Immunol* 2010;126:31-2.
10. Arias K, Chu DK, Flader K, et al. Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice. *J Allergy Clin Immunol* 2011;127:1552-61 e1.
11. Finkelman FD. Peanut allergy and anaphylaxis. *Curr Opin Immunol* 2010;22:783-8.

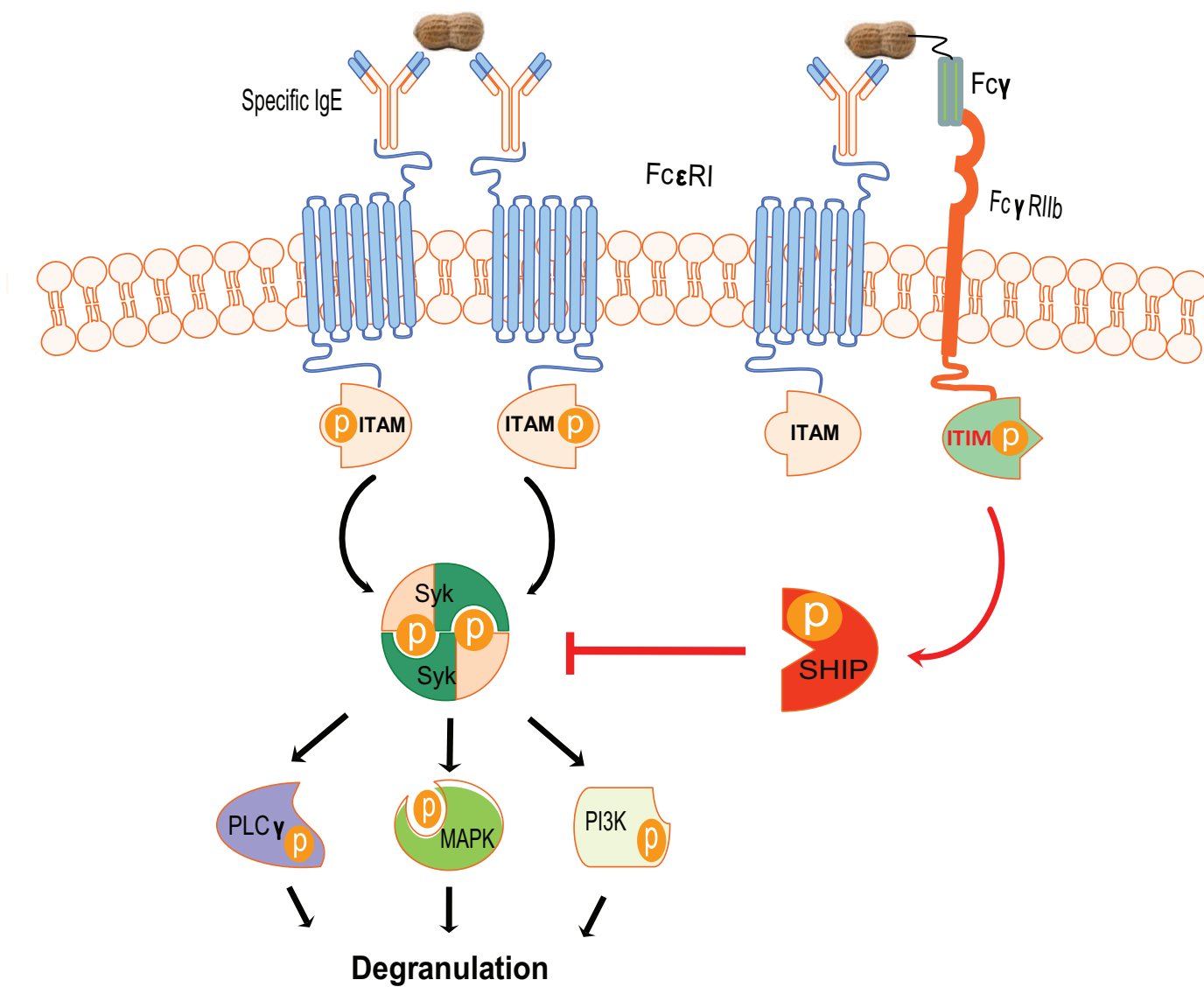
12. McDermott RA, Porterfield HS, El Mezayen R, et al. Contribution of Ara h 2 to peanut-specific, immunoglobulin E-mediated, cell activation. Clin Exp Allergy 2007;37:752-63.

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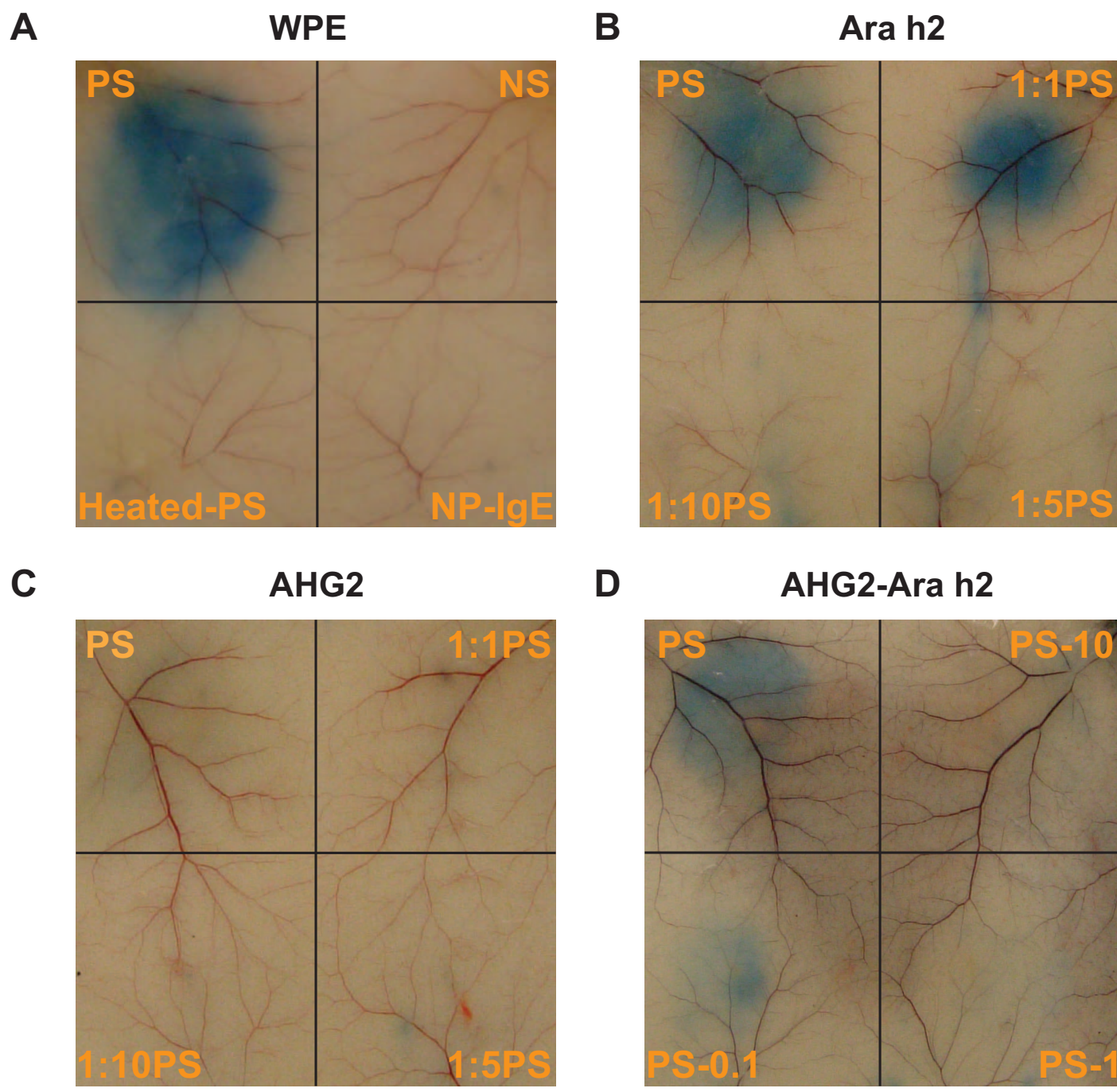
**A**



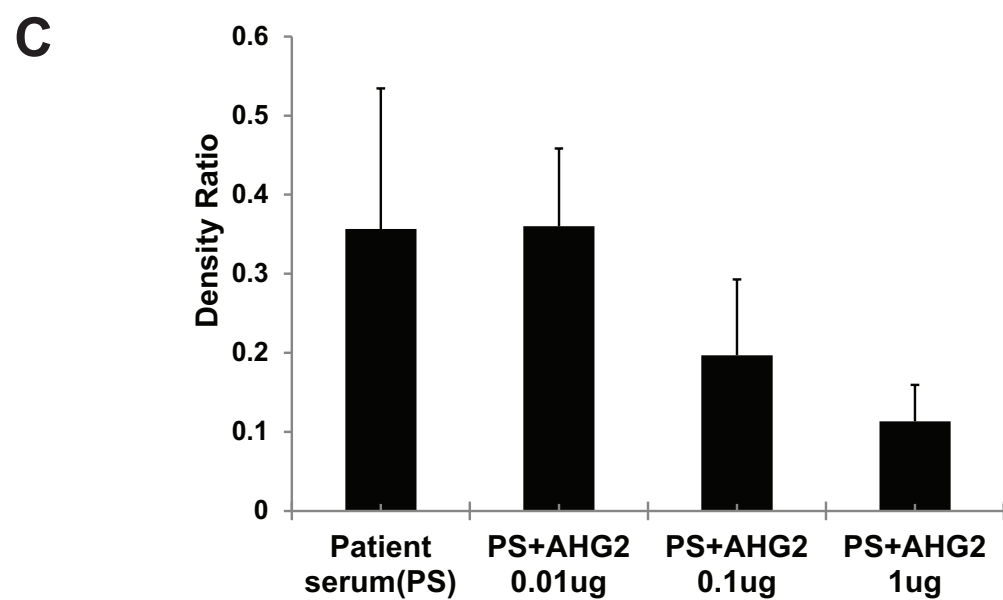
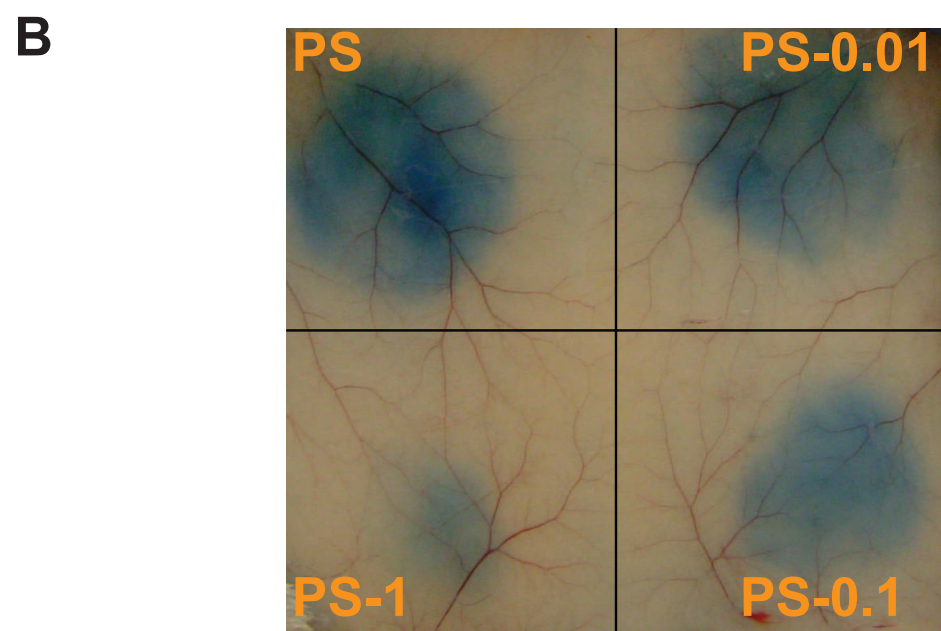
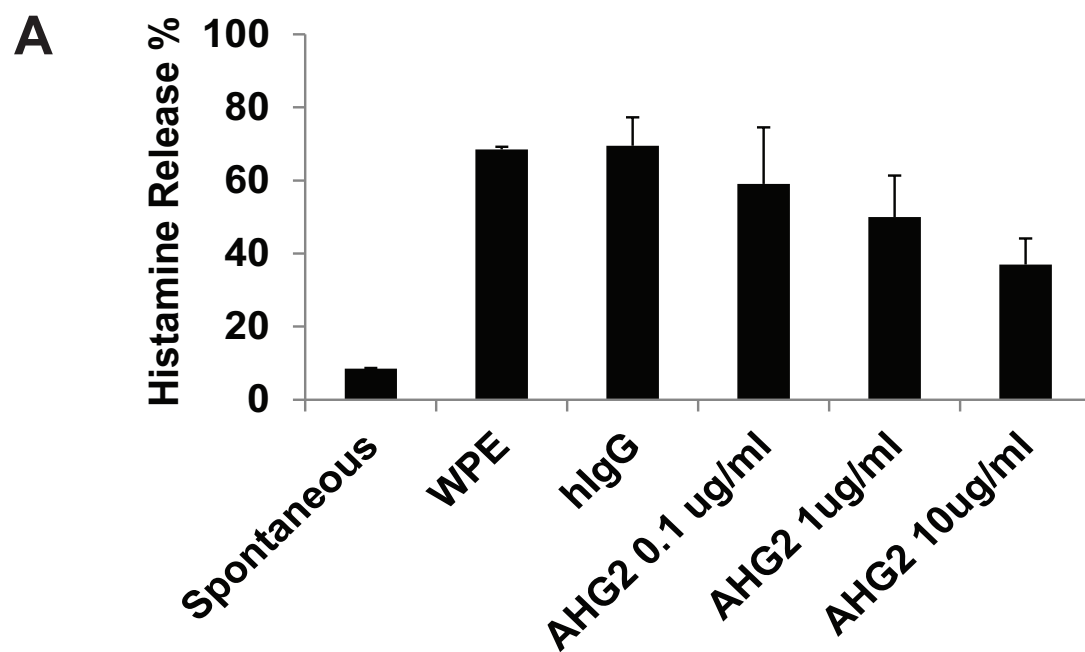
**B**



**Fig. 2**

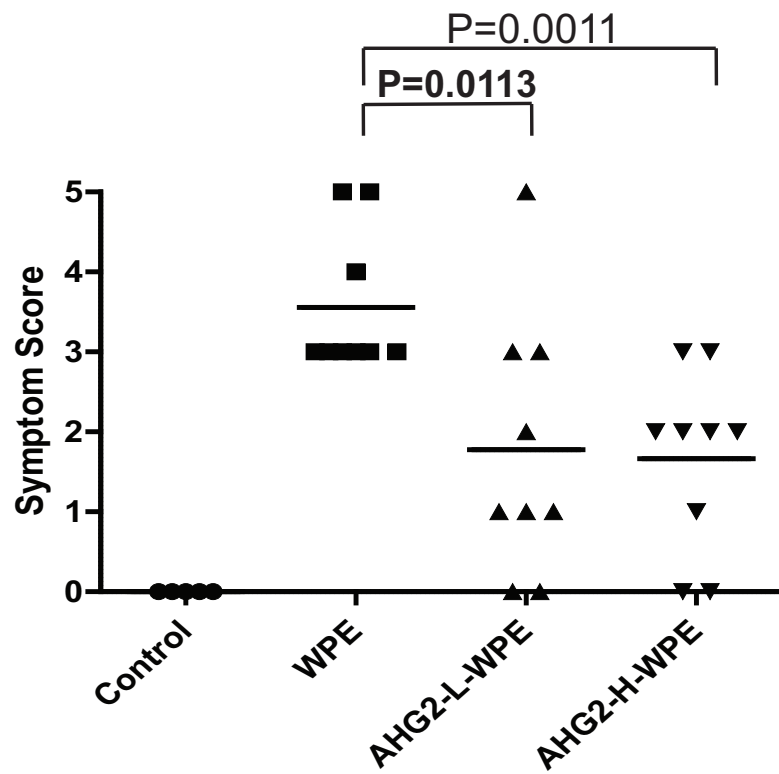


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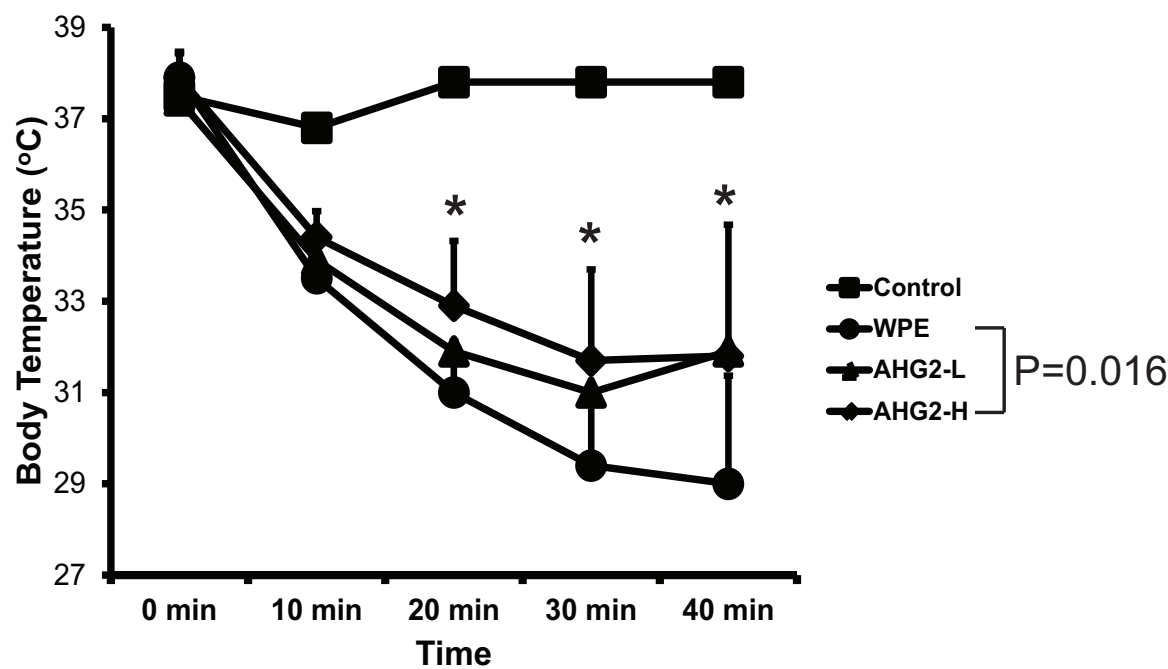


**Fig. 4**

**A**



**B**



**C**

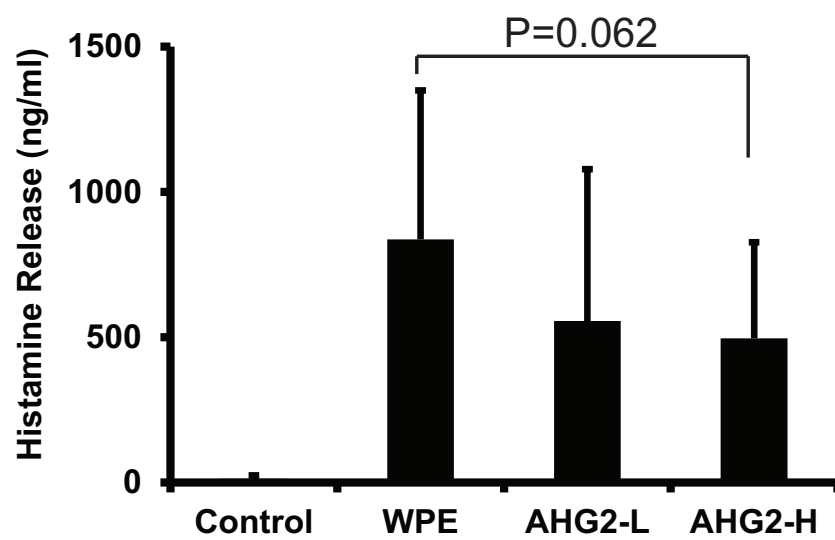
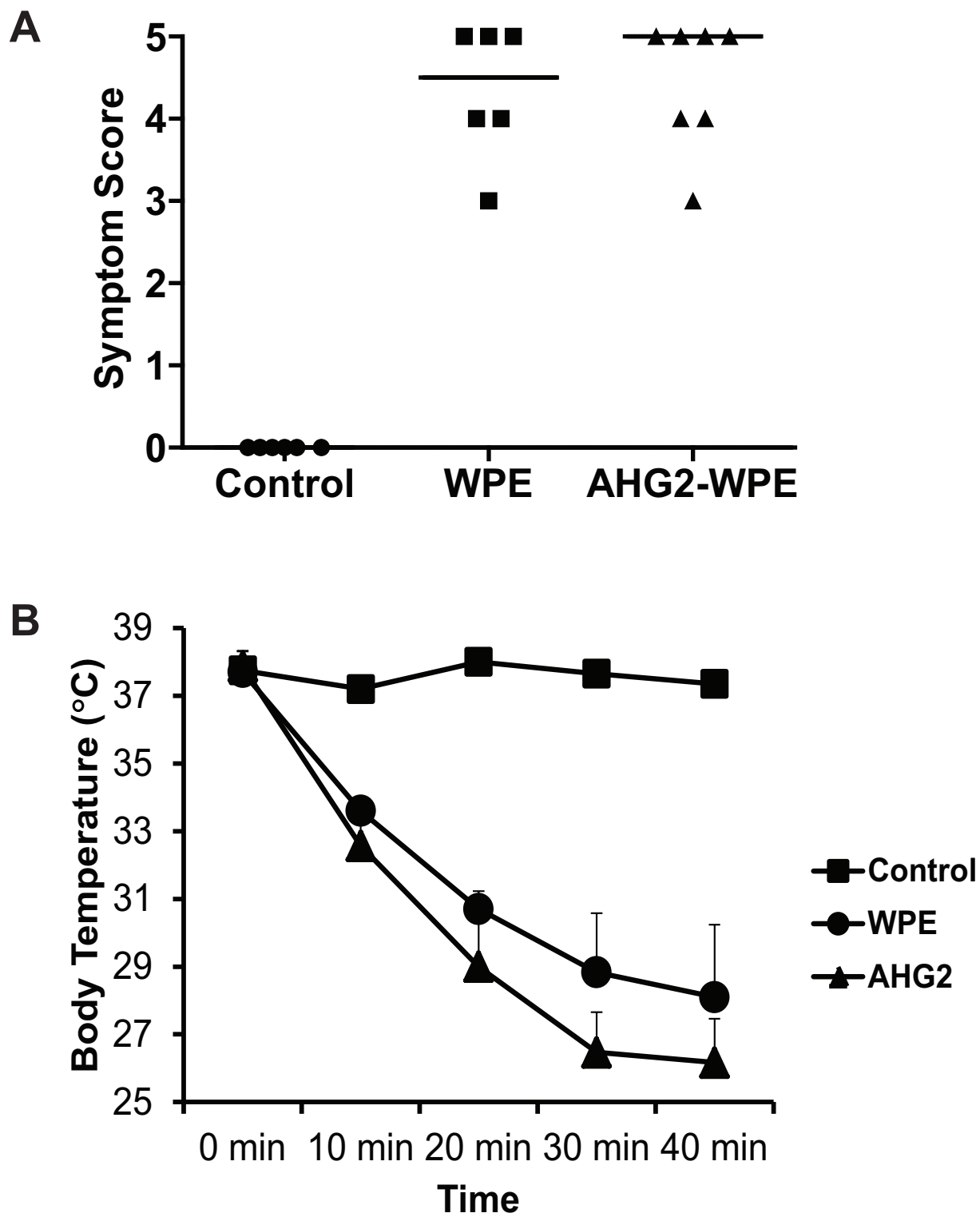
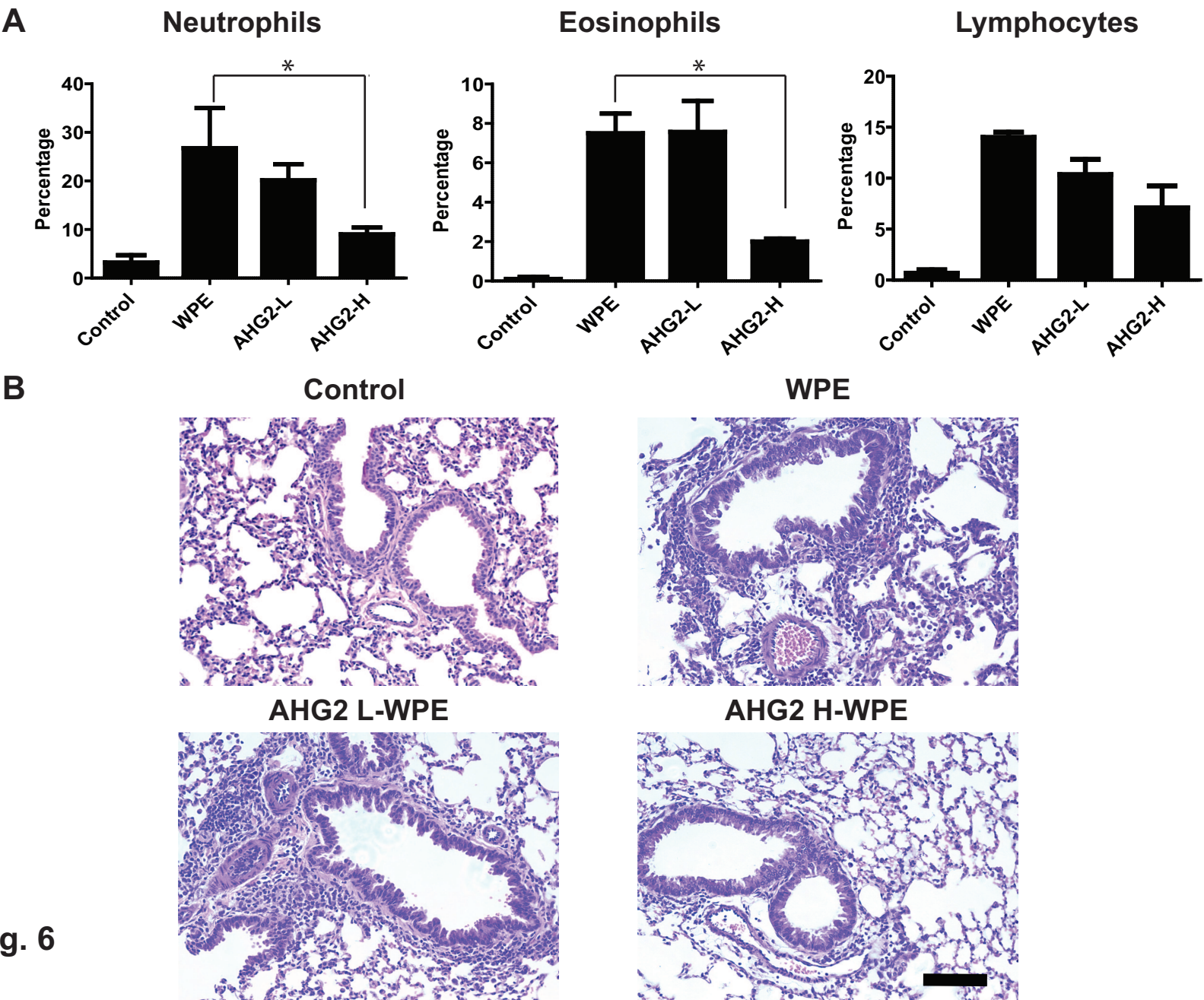


Fig. 5



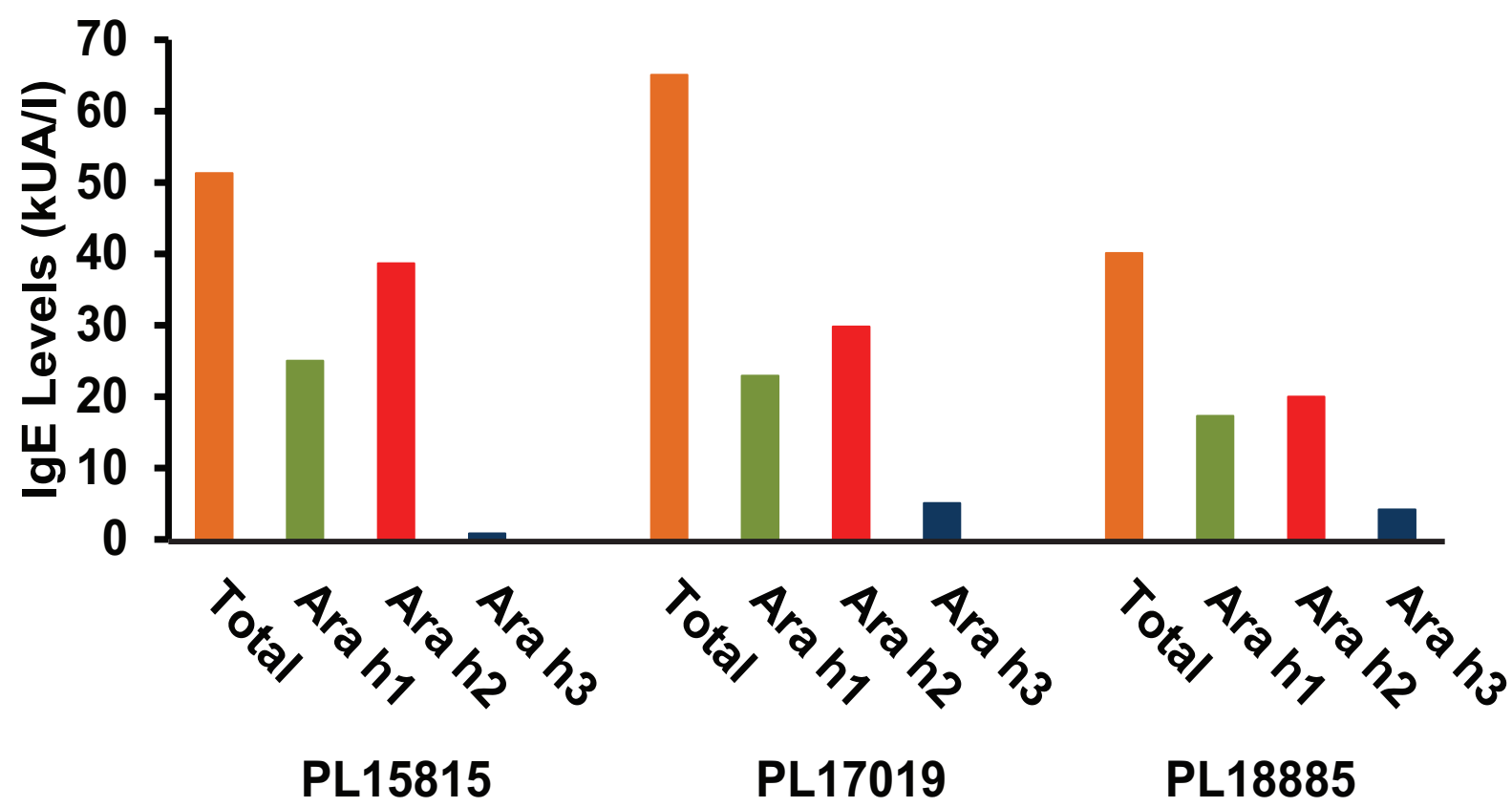


**Fig. 6**

**Table 1.** Anaphylactic symptom score.

Score	Symptoms
0	No clinical symptoms
1	Repetitive mouth/ear scratching and ear canal digging with hind legs
2	Decreased activity; self isolation; puffiness around eyes and/or mouth
3	Periods of motionless for more than 1 min; lying prone on stomach
4	No response to whisker stimuli; reduced or no response to prodding
5	Endpoint: tremor; convulsion; death

Fig. 7



Name of Reagent/Material		Company	Catalog Number	Comments
Cholera Toxin		List Biological Laboratories	100B	
Whole peanut extract		GREER Laboratories	F171	
Ara h2 antigen		INDOOR Biotechnologies	NA-AH2-1	
Evans Blue		Sigma-Aldrich	E2129-10G	
Histamine ELISA		GenWay Biotech	40-371-25010	
RPMI1640		Life Technologies	A1049101	
HBSS		Life Technologies	14170120	
Fetal Bovine Serum		Life Technologies	10438-018	
Ficoll-Paque PLUS		GE Healthcare	17-1440-02	
Basophil Isolation Kit II		Miltenyi	130-092-662	
NP(23)-BSA		Biosearch Technologies	N-5050H-10	
Chimaeric human IgE anti-NP		AbD Serotec	MCA333S	
BD Fastimmune CD63/123/Anti-HLA-DR		BD Biosciences	341068	
Human IgE		CalbioChem	401152	
Mouse Anti-Human IgE		CalbioChem	411507	
<b><u>Equipments</u></b>				
autoMACS Pro Separator		Miltenyi		
Microplate Reader		Bio-Rad		
Thermal probe		Physitemp Instruments		
Invertoskop		Zeiss		
AXIOStar Microscope		Zeiss		
Inverted Microscope (IX71)		Olympus		
Flow cytometry (BD LSR II FACS)		BD		
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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 FcγRIIb and indirectly with FcεRI by interacting with peanut specific IgE bound to FcεRI. Such an interaction is thought to initiate inhibitory signals that prevent mediator release mediated through FcεRI 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: hFcεpsilonRIα 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 use 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:*

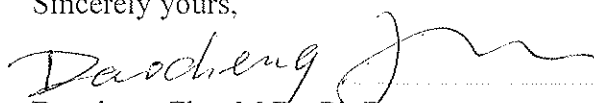
Typographical errors were found. The authors have to correct them all over the manuscript.

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.