Peer Review File
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Reviewer A

This is an interesting study that evaluates the role and molecular mechanism of allergen immunotherapy (AIT) with alutard SQ in the asthma rat model. This present study provides additional support for AIT in asthma, particularly about its molecular mechanism. As we know according to the GINA guideLine, AIT may be a treatment option where allergy plays a prominent role and the most common studies have been conducted for house dust mites and grass pollens. GINA states that they will review evidence about AIT for asthma and will update its advice based on the findings.
This present study used asthma rat model divided into 7 groups (n=6 in each group) : normal (saLine sensitized) ; asthma group (HDM sensitized); asthma + alutard SQ low dose; asthma + alutard SQ medium dose; asthma + alutard SQ high dose; asthma + alutard SQ high dose + HMGB1 lentivirus; asthma + alutard SQ high dose + AMGZ (HMGB1 inhibitor). Using a one-way analysis of variance followed by Tukey's multiple comparison test authors noted that AIT alutard SQ ameliorates Th2 and the expression of total and specific IgE and enhances Th1 response in HDM induces asthmatic rat by blocking HMGB1/TLR-4/NF-kB signal pathway.

MAJOR COMMENTS

1. There was no mention of the limitation of the study Also. the author should compare this present study with another study that was published about other mechanisms of allergen immunotherapy particularly in asthma and the recent clinical recommendation of allergen immunotherapy in clinical practice.
Reply: Thank you for your suggestion. We have added the mention of the limitation of the study, and compared this present study with another study that was published about other mechanisms of allergen immunotherapy particularly in asthma and the recent clinical recommendation of allergen immunotherapy in clinical practice in the discussion.
Changes in the text: Line 323-328, and 264-270.

2. What type of cells are the most important source of HMBG1 after alutard SQ administration in this model?
Reply: Thank you for your suggestion. Airway epithelium are the most important source of HMBG1 after alutard SQ administration in this model.
Changes in the text: Line 304.

MINOR COMMENTS

1. In methods (on page 4, Line 88) groups of HDM + AHD + HMGB1 inhibitor ammonium glycyrrhizinate (AMGZ) were written into HDM+AHD+AMGZ but figures 5 and 6 (Page 18 and 19) didn't mention the name of that groups. In figures 5 and 6 mention about HDM+AHD+GA group, whether that is the same group or not, but it needs consistently written about the name of the groups because this can be helpful to the reader.
Reply: Thank you for your suggestion. We have changed HDM+AHD+GA group to HDM+AHD+AMGZ group in the revised figures 4, 5 and 6.
Changes in the text: The revised figures 4, 5 and 6.

2. The abbreviation of AHR should be "airway hyperresponsiveness".
Reply: Thank you for your suggestion. We have modified it.
Changes in the text: Line 255-256, and Line 345.

3. On page 6, Line 143-145, the authors stated that the number of differential counts (eosinophil, monocyte, lymphocyte) in HDM induces rats were significantly lower as compared with the normal group (Figure 1A), but in figure 1A, the authors describe that differential count in HDM is higher than normal (page 14 Figure 1A)
Reply: Thank you for your suggestion. We have modified it.
Changes in the text: Line 161-162.

4. On page 7, Line 165, the authors stated that alutard SQ inhibits IFN-gamma expression. It seems contradictory with the statement on page 6, on Line 160 that stated alutard SQ enhanced IFN-gamma expression. It seems that an ambiguous conclusion and they should make clear whether alutard SQ inhibits or increases IFN-gamma expression.
Reply: Thank you for your suggestion. We have made clear alutard SQ increases IFN-gamma expression.
Changes in the text: Line 180.

5. On page 7, Line 169, the authors cited the INITIAL study, which is an observational study. This reference is not related to HMGB1.
Reply: Thank you for your suggestion. We have modified it.
Changes in the text: Line 189-190.

6. On page 8, Line 205, the authors stated that alutard SQ downregulation of IFN-gamma expression in the BALF, serum, and lung tissues (figure 5). This conclusion needs to be evaluated again because in Figure 5 (page 18) the expression of IFN-gamma in HDM+AHD is higher than HDM group.
Reply: Thank you for your suggestion. We have modified it.
Changes in the text: Line 229.

Reviewer B

This is interesting work on animal model of airway allergic inflammation exploring possible mechanism by which allergen-specific immunotherapy inhibits allergen-induced airway inflammation. Authors use rats sensitized peritoneally with HDM followed by airway allergen challenges. Sensitized animals were subsequently subjected to subcutaneous immunotherapy with Alutard SQ. Multiple indices of allergic inflammation were collected including cellular composition of BAL fluid, cytokine levels in BAL, and immunocytochemistry of the lung tissue. All observations strongly indicated TH2-type airway inflammation supporting reliability of used animal model of Th2-type airway inflammation. Moreover, they convincingly showed the effectiveness of allergen-specific immunotherapy with Alutard SQ. However, the major hypothesis tested in the paper was targeting HMGB1/TLR4/NFkappaB pathway(s) by Alutard SQ; inhibition of this pathways was considered to play significant role in alleviating symptoms of allergic inflammation. While observed correlation of changes in HMGB1 and TLR4 expression with effect of Alutard SQ treatment suggest association between immunotherapy and HMGB1/TR4 levels, it does not necessarily indicate causation. They do show (he most valuable and novel part of the study) the effect of treatment with HMGB1 inhibitor that potentiated effectiveness of Alutard SQ treatment which support causative relationship though while adding more HMGB1 to the airways (by lentivirus) seemed to reverse the effect. However, authors need to address correctly what it means that they observed more HMGB1 in the lung tissues of sensitized animals. Since HMGB1 is ubiquitously, increased HMGB1 expression would simply reflect increased number of cells; however, GAPDH suggested same level of protein tested. This it is also possible there is more extracellular HMGBS detected in this study. Since authors did not test posttranslationaly ymodified forms of HMGB1 (acetylated or disulfide or redox forms) they should offer some interpretations of the observed increased HMGB1 levels. Furthermore, connection of HMGB1 to NFkappaB while quite possible and predictable is not sufficiently supported by difficult to interpret western blots of phosphorylated p65 (left panel of the blots seem to have misplaced p65 and p-p656 legend). These major queries need to be addressed.
Reply: Thank you for your suggestion. We have offered some interpretations of the observed increased HMGB1 levels. HMGB1 levels were increased in HDM-induced the lungs and BALF. Please see the results and discussion.
Changes in the text: Supplementary Fig 1B, D, and Fig 4A, B. Line196, 212, 314-315.

There are several minor comments:
1. Alutard SQ is commercial name and should be written with capital A.
Reply: Thank you for your suggestion. We have modified it.
Changes in the text: The full text.

2. Line 29, word “nevertheless” perhaps should be replaced with additionally or

moreover.
Reply: Thank you for your suggestion. We have modified it.
Changes in the text: Line 39.

3. Line 91-92:" Anaphylactic response was assessed by changes in rat behavior"; more details needed here.
Reply: Thank you for your suggestion. We have added the more details.
Changes in the text: Line 105-106.

4. Term of "asthmatic rats" should be avoided; use allergic or sensitized as this animal model of allergic airway inflammation is often disputed as not real asthma.
Reply: Thank you for your suggestion. We have modified it.
Changes in the text: Line 2, 4, 200, 272, 303.

Reviewer C

Zhai et al presented a manuscript focusing on SQ extract therapy in asthmatic rats. The author should state in the title that the SQ extract was based on HDM and therefore change the title accordingly.
Reply: Thank you for your suggestion. We have modified it.
Changes in the text: Line 2.

The abstract is very short and should be extended, especially on the methods section as well as in the results and discussion.
The authors shoud compare the effect of SQ with standard AIT, as it was shown that IL-10 producing B-cells and coinciding Th/Tr17 shift during three year grass-pollen AIT. As the authors investigated the effects of SQ on the allergic inflammation, they did not assess the effects on the airway epithelium, however described none of the known mediators of airway epithelial cell induced in allergic conditions. They should include the role of respiratory epithelial cells in allergic diseases at least in the discussion. As the it is more than just a barrier, the immune functions of the airway epithelium in asthma pathogenesis should be introduced. Further, the ability of Interleukin-4 and interferon-γ to orchestrate an epithelial polarization in the airways should be included in the dicussion. Also comparable results in human beings as shown in a biomatrix for upper and lower airway biomarkers in patients with allergic asthma should be discussed. Furthermore, the lung epithelial CYP1 activity, which regulates Aryl Hydrocarbon Receptor dependent allergic airway inflammation should be included. Moreover, the authors should measure the levels of TGF-β1 in local samples to include the effect of TGF-β1 drives Inflammatory Th cell but not Treg cell compartment upon allergen exposure and include it in the discussion. This would be important as HMGB1 is known to attenuate e.g. the TGF-β1-induced epithelial-mesenchymal transition. Moreover, the authors need at least to discuss the anti-

inflammatory potential of the airway epithelium as it was shown that allergen-specific immunotherapy induces the suppressive secretoglobin 1A1 in cells of the lower airways. This would be of importance as there is a known central role of the NF-kappa B pathway in the Scgb1a1-expressing epithelium. But not only SCGB1A1 is of importance but also IL-37, which regulates allergic inflammation by counterbalancing pro-inflammatory IL-1 and IL-33. The authors should at least extend their introduction section and discussion for these points. Last, the authors should discuss their results with respect to current and future biomarkers in allergic asthma.
Also of interest would be, whether the SQ-related effects on NFkB-pathway and HMGB1 would influence the constitutive immune activity, which promotes JNK- and FoxO-dependent remodeling. At least, the authors sould speculate about these interlinks if they are not able to show this on an experimental level.
Reply: Thank you for your suggestion. We have extended the methods section in the abstract, and the results and discussion.
Changes in the text: Line 26-42, 168-186, 229, 233, 264-288, 303-306, 323-328.