Author’s response to reviews

Title: MST-4 and TRAF-6 expression in the peripheral blood mononuclear cells of patients with Graves' disease and its significance

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Author’s response to reviews:

Response Letter

Dear Mr./Mrs./Miss Karaca,

We deeply appreciate the time and effort you’ve spent sending the email dated December 22, 2016. We also thank the reviewers for their constructive comments and valuable recommendations. We have carefully revised the manuscript titled “MST-4 and TRAF-6 expression in the peripheral blood mononuclear cells of patients with Graves' disease and its significance” (BEND-D-16-00205) according to reviewers’ suggestions. In addition, we have also provided a point-by-point response to the comments below. We hope that the revised manuscript is acceptable for publication.

Thank you.

Sincerely,

Ai Guo, Chun Liu

Responses to Reviewer 1:

Comment 1: There is no information about the normal range of TRABs.
Reply: we apologize for not including the information concerning the normal range of TRABs. The normal range of TRABs is 0.3-1.8 IU/L. In the Methods section of the revised manuscript (Detection of thyroid function indicators, lines 3-6, page 5), we revise the sentence as follows: “The normal reference values for the thyroid function indicators and antibodies used in our hospital are as follows: FT3: 2.5-3.9 pg/ml; FT4: 0.61-1.12 ng/dl; TSH: 0.35-3.5 IU/ml; TG Ab: < 4 IU/ml; TPO Ab: < 9 IU/ml; and TR Ab: 0.3-1.8 IU/L.”

Comment 2: On Figure 3, where there are the results from Western blot there is visible difference in the expression of both MST-4 and TRAF-6 within the same group of GD samples between two presented blots. There is lower signal from GD samples on Fig3A than on Fig. 3B. This indicate that the SDS-Page and blots were done separately. Authors should place the samples from GD, eGD and normal controls on one blot and then analyze and compare all groups. This will indicate that the conditions for the procedures were the same. I understand that this is only small part of the WB study but it should be presented in more reliable way.

Reply: We agree with the reviewer’s suggestion. During our experiment, the samples from the Graves’ disease (GD) group, the eGD group and the normal controls were run on a single SDS blot. In addition, we also ran separate individual blots from the GD and eGD samples, the GD and normal control samples, and the eGD and normal control samples. In the original manuscript, we improperly showed the expression of both MST-4 and TRAF-6 protein in the GD group and the NC group (Figure 3A), the GD group and the eGD group (Figure 3B), and the eGD group and the NC group (Figure 3C). In the revised manuscript, we updated Figure 3 to show the samples from the GD group, the eGD group and the normal controls on a single blot.

Comment 3: When Authors describe in "Results" thyroid function (first sentence - row 25th) they should mention that thyroid function indicators levels were higher considering only fT3 and fT4 as TSH was suppressed.

Reply: Thank you for this reminder. We apologize for our description of the results of the thyroid function among three groups. We have revised the first sentence of the Results section (Thyroid function and autoantibody levels, line 1-4, page 6) as suggested: “The levels of the indicators of thyroid function (FT3 and FT4) were higher, whereas TSH was lower in the NC group than in the eGD group, and these differences were statistically significant (P < 0.001); however, the differences between the eGD group and the NC group were not statistically significant (P > 0.05).

Replies to Reviewer 2:

Reviewer 2: MST-4 and TRAF-6 expression in the peripheral blood mononuclear cells of patients with Graves' disease and its significance

Comment1: It was a great idea to try to decipher the exact immune mechanisms in Graves' disease. However since the expression of the above receptors in limited in the thyroid gland, I am not sure how much is translated to actual effects within the thyroid gland in Graves disease.
Reply: In our study, we investigated the expression of MST-4 and TRAF-6 in the peripheral venous blood of patients with GD. This suggests that we have only discussed the expression of these substances (MST-4 and TRAF-6) in the human peripheral circulatory system. However, it is well known that GD is organ-specific autoimmune diseases. Therefore, when we study the mechanism of GD, it is necessary to identify the immune status of the thyroid gland. We agree with the reviewer’s opinion that we should observe the expression changes and mechanism of action of these receptors in the thyroid gland because it is the only way to comprehensively evaluate whether changes in the expression of these receptors are translated to actual effects within the thyroid gland and to investigate the immune mechanism of MST-4 and TRAF-6 in GD.

However, it is not easy to obtain the thyroid gland from newly diagnosed GD patients for research. Under normal circumstances in China, endocrinologist will first prescribe medication to the newly diagnosed GD patients or use iodine-131 treatment under certain conditions. Surgical treatment is used only in patients with stage III or above goiters, who undergo anti-thyroid drug treatment after recurrence, and experience thyroid enlargement at stage II or above or who experience goiter oppression symptoms. Therefore, it is difficult to obtain the thyroid gland from newly diagnosed and untreated GD patients. However, this explanation is not meant as an excuse to avoid these experiments. If we are able to collect more thyroid gland samples with enough time, we will not hesitate to improve our research.

In addition, we should mention that some endocrinologists at our hospital are conducting thyroid needle aspiration cytology, which may provide the opportunity to obtain thyroid gland cells for our study. Therefore, if we are able to obtain enough tissue samples, we will conduct these experiments and share our results. Finally, we added the sentence “It is well known that GD is an organ-specific autoimmune disease. Therefore, it is important to comprehensively evaluate the influence of MST-4 and TRAF-6 in the thyroid gland to fully understand the effects of MST-4 and TRAF-6 in GD.” to the Discussion section (line 3-6, paragraph 10, page 11).

Comment2: As expected there is a similar expression as would be seen in sepsis in infection which is a generalised inflammatory state. Since active Graves' disease in the hyperthyroid state is a generalised inflammatory state the response may be similar. We cannot decipher much as to the exact roles of these in Graves' disease in the current study.

Reply: Our study is based on the research by Jiao et al. (Jiao S, Zhang Z, Li C, Huang M, Shi Z, Wang Y, Song X, Liu H, Li C, Chen M et al: The kinase MST4 limits inflammatory responses through direct phosphorylation of the adaptor TRAF6. NAT IMMUNOL 2015, 16(3): 246-257), which discusses the immune inflammation mechanism of sepsis in infections. Through our experiments, we found that the expression of MST-4 mRNA and protein in the GD group was lower than that in the NC group, which was consistent with the results observed in sepsis due to infection. However, the levels of TRAF-6 mRNA and protein were lower than normal, which is not consistent with a generalized inflammatory state. However, we agree with the reviewer’s comment that we cannot deny that there is a possibility of a relationship between the generalized inflammatory state and the abnormal expression of MST-4 and TRAF-6. Nevertheless, the lower TRAF-6 expression may indicate that there are additional causes for the generalized inflammatory state. The altered expression may also be associated with an imbalance of immune...
tolerance as we mentioned in our manuscript. Consequently and according to the reviewer’s comments, continued efforts should be given to study the immune pathogenesis of GD.

Comment3: Another limitation of this study was that the confounder graves' ophthalmopathy was not described as all. The presence of GO in the euthyroid Graves would still constitute an inflammatory state and be a likely cause of why no significant differences were seen between active Graves and euthyroid Graves. Since inflammation and the immune system play a major role we need to elicit the difference in GO as well.

Reply: This point from the reviewer is something that we neglected. When recruited the GD patients, we observed the patients for eye lesions, but this was not a prerequisite for our inclusion criteria; therefore, only some of the GD patients had ophthalmopathy. Even if our GD patients had ophthalmopathy, their eye symptoms had improved and even recovered after treatment for more than 1 year. Therefore, the euthyroid GD patients we include have few ophthalmopathy. As for representing an inflammatory state in the euthyroid GD patients with ophthalmopathy, which is probably why there were no significant differences between the GD group and the euthyroid GD group, perhaps GO indicates an inflammatory state in euthyroid GD; however, unfortunately, this possibility cannot explain the results of our study. In addition, we have added the following sentence: “(4) anterior tibial mucinous edema; (5) eye bulging and other infiltrative ophthalmopathy; and (6) positive for TR Ab, TS Ab, TPO Ab, and Tg Ab. For the above criteria, (1)-(3) are a prerequisite for the diagnosis of GD and (4)-(6) are the diagnosis of auxiliary conditions.” to the Methods section (Study subjects, lines 9–12, page 4).

We think that the immune inflammatory status in the euthyroid GD patients may be between that of the GD patients and the normal controls; as a result, there were no significant differences in the expression of MST-4 and TRAF-6 between the GD group and the eGD group, or between the eGD group and the NC group. To directly identify the degree of the immune inflammatory status, we could also investigate inflammatory cytokines in addition to fully investigate ophthalmopathy. Because of the reviewer’s suggestion, which expands the range of our research, we will continue to investigate the immune mechanism of GD by paying more attention to GO. According to above statement, we have revised the sentence (Discussion section, lines 4-7, paragraph 4, page 9) “However, the expression of MST-4 was not significantly different between the GD group and the eGD group, or between the eGD group and the NC group, suggesting that the immune inflammatory status in the eGD group is probably between that of the GD group and the normal controls, which contributed to the above results.”, and the sentence (Discussion section, lines 8-11, paragraph 5, page 9) “However, there were no significant differences in the expression of TRAF-6 between the GD group and the eGD group or between the eGD group and the NC group, suggesting that the immune inflammatory status in the eGD group was remission after treatment, but it did not return to the normal level.”

In addition, we have been modified the language of the whole manuscript which expressed not clearly, and inappropriate or incorrect words. However, we did not change the format of the manuscript, which meets the requirements of the journal.

PS: The authors' response letter has been included as a supplementary file.