Author's response to reviews

Title: Targeting insulin resistance in type 2 diabetes via immune modulation of cord blood-derived multipotent stem cells (CB-SCs) in stem cell educator therapy: phase I/II clinical trial

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Author's response to reviews: see over
Dear Dr. Denyer:

Please find enclosed the revision of manuscript (MS: 8902020692081360) “Targeting insulin resistance in type 2 diabetes via immune modulation of cord blood-derived multipotent stem cells (CB-SCs) in stem cell educator therapy: phase I/II clinical trial”.

We have addressed the reviewers’ comments through a point-by-point response. Please see the Letter-Answers to Reviewers. Based on their constructive critiques and guiding, we have revised our manuscript. All changes have been highlighted in the revision.

Thank you for your kind consideration of our paper.

Sincerely yours,

Yong
Answers to reviewer’s comments:

We appreciate the reviewers’ very constructive critiques of our manuscript. We found them to be extremely useful in guiding our revisions to improve the manuscript. We have followed each of the suggestions and comments.

Answers to reviewer #1:

Major compulsory Revisions:

2) Major comment: This is a very interesting observation; however, the weakness is that the authors can not really provide a mechanistic explanation for this improvement. In particular it is not clear, how and in which concrete way this Stem Cell Educator causes this reversal of immune cell dysfunction. This must be explained better in the discussion section.

We agree with the reviewer that it is important to provide the detailed \textit{in vivo} mechanisms underlying the reversal of immune cell dysfunction by Stem Cell Educator therapy. Based on our previous works [1-6], we have added following mechanisms and revised the Discussion. We understand there are additional mechanisms needed to be explored in our future studies.

Stem Cell Educator therapy functions as “an artificial thymus” that circulates a patient’s blood through a blood cell separator [1], briefly co-cultures the patient’s blood mononuclear cells (such as T cells, B cells, Tregs, monocytes, and neutrophils) with CB-SCs \textit{in vitro}. These mononuclear cells play key roles in insulin resistance and the development of diabetes [7-11]. During the \textit{ex-vivo} co-culture in the device, these mononuclear cells can be educated by the favorable microenvironment created by CB-
SCs through: a) the action of autoimmune regulator (Aire) expressed in CB-SCs [6]; b) the cell-cell contacting mechanism via the surface molecule programmed death ligand 1 (PD-L1) on CB-SCs [2]; c) the soluble factors released by CB-SCs. Previous work [2] and current data demonstrate that CB-SC-derived nitric oxide mainly contributes to the immune modulation on T cells and monocytes. d) correcting the functional defects of regulatory T cells (Tregs) [3]; e) directly suppressing the pathogenic T cell clones [4]. During this procedure, both peripheral and infiltrated immune cells in visceral adipose tissue (VAT) can be isolated by a blood cell separator and treated by CB-SCs, leading to the correction of chronic inflammation, the restoration of the immune balance, and clinical improvements in metabolic control via increasing of insulin sensitivity.

In particular the authors should explain why it is apparently sufficient to treat the mononuclear cells in the blood only to obtain the therapy success. Why is it not necessary to modulate the behaviour of the immune cells which reside in the tissues, for example in the adipose tissue? Is it not that such untreated immune cells from tissues constantly migrate into the circulation and decrease the therapy success with time?

We appreciate these important comments. During the procedure of Stem Cell Educator therapy, the mononuclear cells circulating in a patient’s blood are collected by a Blood Cell Separator and briefly co-cultured with adherent CB-SCs before returning to the circulation. Additionally, patients are required to move their hips, legs, and turn to one side every 15 – 30 minutes during the treatment, in order to mobilize the immune cells from peripheral tissues (include adipose tissues) and organs entering into the blood
circulation to be processed by a Blood Cell Separator. Thus, the immune cells both in peripheral blood and in adipose tissues can be isolated by a Blood Cell Separator and treated by CB-SCs.

Yes. There are some pathogenic immune cells remaining in tissues and lymph nodes which fail to enter into the blood circulation during the procedure and may escape from the treatment by CB-SCs. These immune cells may migrate into the blood circulation and decrease the therapeutic effectiveness. Therefore, we plan to give patients additional treatment 6 - 9 months later after receiving the 1st treatment in our ongoing Phase II clinical trial.

Further comments:

3) Results, Fig. 1: How many of the patients in each group showed an improvement of HbA1C and how many did not? This should be mentioned additionally in the results section.

We appreciate your clarification. We have added this information in the Results Section.

The primary efficacy end point was the change in HbA1C between baseline and follow-up, with an absolute difference in HbA1C level of at least 0.5% from baseline. Based on this efficacy criteria, 11 of 18 (61.1%) subjects in Group A, 8 of 11 (72.7%) subjects in Group B, and 4 of 7 (57.1%) subjects in Group C with reduction of A1C value (> 0.5%) at 4 weeks post treatment.
Figure 1A shows the A1C data at 12 weeks post treatment: 13 of 18 (72.2%) subjects in Group A, 9 of 11 (81.8%) subjects in Group B, and 6 of 7 (85.7%) subjects in Group C with reduction of A1C value (> 0.5%).

4) Discussion, Fig. 1: The authors should discuss why the improvement of insulin sensitivity and beta cell function progresses slowly over weeks. Rather one would expect that the observed improvement vanishes again with progression of time after treatment?

We appreciate your suggestion and have added this information in the Discussion section. We observed that the improvement of beta cell function (C-peptide levels in Figure 1C) progresses slowly over weeks after receiving Stem Cell Educator therapy, not vanishing again with progression of time after treatment. We reported the similar data in previously published type 1 diabetic trial [1,6].

If Stem Cell Educator therapy only temporarily corrects the immune dysfunctions, the clinical efficacy in metabolic control should be vanished soon after receiving the Stem Cell Educator therapy, because of the short lifespans of the most immune cells (e.g., 5.4 days for neutrophils [12], 3 months for lymphocytes, 1 - 3 days for bone marrow-derived monocytes existing in blood and then migrating into tissues). Thus, we think that Stem Cell Educator therapy can fundamentally modulate the immune system and correct the immune dysfunctions, and result in lasting reversal of immune dysfunctions. Previous work demonstrated that CB-SCs showed the marked modulation of Th1-Th2-Th3 cell-related genes including multiple cytokines and their receptors, chemokines and their receptors, cell surface molecules, along with signaling pathway molecules and
transcription factors, as indicated by quantitative real time PCR array [3]. Due to these fundamental immune modulations and induction of immune balance [1], a single treatment with Stem Cell Educator therapy displays the long-lasting effectiveness in reversal of immune dysfunction that allows regeneration of islet β cells and improvement of metabolic control in individuals with T1D [1,6] and current T2D subjects.

5) Results, page 13: The authors found after Stem Cell Educator therapy no change in such important cytokines like IL-1, IL-6 and TNF. The only major improvement they found was a suppression of the cytokine TGF. Can this suppression explain the complete therapy success or is the therapy success also the result of reduction of other cytokines such as IL-17, IL-12, IL-4 and IL-5? This should be discussed properly in the discussion section and put in to relation to references from the literature. It is unlikely that it is only a result of a change of CD-86.

We appreciate the reviewer’s comments. Using ELISA kits, we found that interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNFα) were all at background levels in these long-standing T2D subjects and failed to show changes after Stem Cell Educator therapy ($p = 0.557$, $p = 0.316$, $p = 0.603$ respectively), probably because metabolic inflammation is a chronic sub-degree inflammation [13] and the serum samples which were directly collected from the blood of T2D patients, not from the lipopolysaccharide (LPS)-activated monocytes of T2D subjects [14]. Monocytes can produce high levels of IL-1, IL-6, TNFα and other proinflammatory cytokines in the presence of LPS stimulation (Figure 3F). Blocking experiments with iNOS-specific inhibitor 1400W demonstrated
that CB-SC-derived NO could markedly suppress the expressions of these cytokines (Figure 3F).

TGF-β1 is a well-recognized cytokine with a pleiotropic role in immune modulation on multiple immune cells such as the differentiation and function of Th1/Th2 cells and Tregs, as well as B cells, monocytes/macrophages, dendritic cells, granulocytes, and mast cells [15,16]. These immune cells have involved in the inflammation-induced insulin resistance in T2D [7-11,17-21]. Therefore, together with our previous mouse studies [3,22] and human clinical trial in type 1 diabetes [1,6], we think that the up-regulation of TGF-β1 level is one of the major mechanisms underlying the immune modulation in T2D subjects after receiving Stem Cell educator therapy.

We agree with the reviewer’s comments on the molecular mechanisms underlying the complete therapy which are more complicated than what we have found. We are exploring other potential mechanisms which may contribute to the immune modulation and the improvement of metabolic control after receiving Stem Cell Educator therapy. We have added above information in the Discussion section.

6) Results, page 16: The changes in NO production and its relation to the observed effects should be properly discussed in a mechanistic perspective in the discussion section.

We appreciate your suggestion. Based on current data and previous works [1-3,6], we have added information regarding the relationship between NO production and the observed effects in the Discussion section.
7) Discussion, page 17, 2nd para: Is there experimental evidence that the Stem Cell Educator therapy also reduced chronic inflammation of VAT?

We appreciate your suggestion. Currently, we do not have experimental evidence to show that Stem Cell Educator therapy can reduce the chronic inflammation of VAT. We will perform this study in the near future.

8) Discussion, page 17, 2nd para: The authors consider abnormalities in monocytes/macrophages as of crucial importance for chronic inflammation and insulin resistance in T2DM and they cite in this context references 27 and 30. If this is really the case, these cells should show signs of activation, i.e. expression of proinflammatory cytokines and others mediators. Can the authors provide experimental evidence for this?

Based on current reports, we have updated that neutrophils [19], Eosinophils [20], mast cells [17], and dendritic cells (DCs) [18,21] also contribute to the extensive repertoire of immune cells which participate in inflammation-induced insulin resistance and T2D.

Growing evidence strongly demonstrated that an accumulation of macrophages by metabolic stress in the sites of affected tissues (such as vasculature, adipose tissue, muscle and liver) has emerged as a key process in the chronic metabolic-stress-induced inflammation [23]. Due to the destructive effects of lipid influx (e.g. fatty acids and cholesterol) in those tissues, these persistent proinflammatory stimulants causes macrophage dysfunction (including defective efferocytosis and unresolved inflammation), resulting in recruitment and activation of more monocytes/macrophages via monocyte chemoattractant protein 1 (MCP-1) and its receptor CCR2 [23]. Consequently,
inflammatory cytokines (e.g., IL-6 and TNFα) produced by activated macrophages induce insulin resistance in major metabolic tissues [23-25]. To prove the action of macrophage in chronic inflammation and insulin resistance in T2DM, conditional depletion of CD11c⁺ macrophages or inhibition of macrophage recruitment via MCP-1 knockout in obese mice resulted in a significant reduction in systemic inflammation and an increasing in insulin sensitivity [26-28].

9) Page 18, last line: Can the authors please specify, to which restoration of monocyte function they specifically refer here with respect to the therapy success?

We appreciate the reviewer for clarification. Restoration of monocyte function includes the expression of CD86, cytokine productions, and chemokine productions.

10):

Page 16, line 5: Typo: co-cultured.

Page 18, line 6: Typo: unique therapy success.

We have corrected these mistakes in typing.
Answers to reviewer #2:

Major Compulsary Revisions

1. Introduction:

a) Type 2 diabetes starts with a dysfunction and later leads to a loss of pancreatic beta cells. Please clarify this in the introduction as well as in the discussion sections.

We appreciate the reviewer’s clarification. We have revised the Introduction and Discussion, with more detailed information on how the immune dysfunctions lead to the insulin resistance in Introduction, and with more detailed information on the dysfunction of islet β cells leading to the loss of pancreatic islet β cells in Discussion.

2. Materials and Methods:

Please introduce all methods used for the present study, especially the in vitro studies with mononuclear cells using, for example, Western Blots and ELISA for the patient groups.

We apologize for the simplicity regarding these Methods. We have updated the Methods section to include the detailed descriptions.

3. Results:

a) Please make statements about the importance of the different cytokines. The cytokine, IL-17A is very important for the ongoing autoimmune process in T1DM as well as in other autoimmune diseases. IL-12 is important for IFN-gamma production. These cytokines are primarily not involved in T2DM.
We appreciate the reviewer’s suggestions. We agree with the reviewer that IL-17A is a well-known proinflammatory cytokine involved in the autoimmune diseases. Importantly, mounting evidence collected over the past decade indicates that the etiology of T2D includes an autoimmune component that initiates an inflammation affecting pancreatic islet β cells [13,29-33], which provides new insight into the mechanism and potential treatment of insulin resistance through immune modulation. Additionally, recent clinical studies showed the increasing of circulating Th17 cells and IL-17 production in T2D patients [34] and obese patients [35].

We agree with the reviewer that IL-12 is a potent inducer of IFN-γ production, leading to the differentiation of Th1 cells. Both IL-12 and IL-17A are not primarily involved in T2D. Interestingly, recent studies showed that the level of IL-12 is increased in T2D subjects [36,37]. Therefore, we explored the changes in the expression of IL-17A and IL-12 in current clinical trial.

b) The analysed panel of cytokines is not a biomarker panel for T2DM which uses more TNF-alpha, IL-6 and others.

We agree with the reviewer that it is of interest to evaluate the conventional panel of cytokines (e.g., IL-1, IL-6, TNFα) for T2DM. However, using ELISA kits, we found that interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNFα) were all at background levels in these long-standing T2D subjects and failed to show changes after Stem Cell Educator therapy ($p = 0.557$, $p = 0.316$, $p = 0.603$ respectively), probably because the metabolic inflammation is a chronic sub-degree inflammation [13] and the serum samples which were directly collected from the blood of T2D patients, not from the
lipopolysaccharide (LPS)-activated monocytes of T2D subjects [14]. Therefore, we focused on the changes of T cell 1 (Th1) and Th2-related cytokines by using flow cytometry after intra-cellular staining.

c) The in vitro analyses with the co-culture needs longer exposure than performed in the in vivo situation. This is a barrier for a comparative view.

We agree with the reviewer that there was time difference between in vitro analysis with the co-culture and in vivo treatment with Stem Cell Educator therapy. To address this concern, we provide following reasons.

1) For in vitro blocking experiment with iNOS inhibitor 1400W (Figure 3F), the LPS-stimulated monocytes were used to co-culture with CB-SCs. It may need longer exposure with CB-SCs for the immune modulation of these activated monocytes. It is different from the in vivo treatment with Stem Cell Educator therapy, which monocytes and other mononuclear cells are directly isolated from patient blood without any activations.

2) Previous work [2] and current data indicate that CB-SC-derived nitric oxide (NO) plays a key role in the immune modulation. When mononuclear cells pass through the Educator device, NO as a free radical released by CB-SCs can quickly transmit into their cellular membrane, without the aid of dedicated transporters.

3) To determine how soon for CB-SCs to begin their immune modulations, we explored different time points (including 1, 2, 4, 6, 12 hrs) by using human PBMC (peripheral blood mononuclear cells) co-cultured with CB-SCs, in the presence of phytohaemagglutinin (PHA) stimulation. Real time PCR array revealed that some
of early-response genes started to show marked changes one hour later after co-cultures, in comparison with CB-SC-untreated control. It suggests the action of CB-SCs is very quick. For practical applications in clinic, we designed and performed the clinical treatment protocol for Stem Cell Educator therapy in 8-9 hours [1,6]. During this period, patients are easily co-operated.

4. Discussion:

a) The description of the pathogenesis of diabetes is more about type 1 than type 2 regarding the different T cell subtypes. This issue has to be clarified.

   We appreciate your clarification. We have clarified this issue in the text.

b) It is a low inflammation in the pancreas with mostly macrophages and adipokines in the circulation. This issue has to be clarified.

   We appreciate your suggestion. We have clarified this issue and quoted literatures related to adipokines in T2D patients.

c) Macrophages are antigen-presenting cells, but with much lower capacity than dendritic cells. The co-stimulatory molecules are only important to identify the subpopulation of the educated immune cell type.

   We agree with the reviewer that dendritic cells (DCs) are professional antigen-presenting cells with more capacity than macrophages. Besides macrophages, dendritic cells and other immune cells including neutrophils and eosinophils, mast cells, natural killer cells, have been recently implicated in the pathogenesis of obesity and insulin
resistance [17-21,38]. We plan to explore the changes of DCs and other immune cells in T2D patients after receiving Stem Cell Educator therapy.

Additionally, we will work on more detailed mechanisms underlying the modulation of co-stimulating molecules and their functional relationships with T cells and Tregs in T2D patients.

Quality of written English: Needs some language corrections before being published

Our manuscript has been improved by a professional medical science writer.

Once again, we thank the reviewers for their constructive comments and useful guidance in revising our paper. We would appreciate favorable consideration of our revised manuscript.
Reference List


37. Mishra M, Kumar H, Bajpai S, Singh RK, Tripathi K: Level of serum IL-12 and its correlation with endothelial dysfunction, insulin resistance,