Author’s response to reviews

Title: The importance of the PD-1/PD-L1 pathway at the maternal-fetal interface

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Prof. Dr. Kedra Wallace
Associate Editor for “BMC Pregnancy and Childbirth”

Dear Professor Dr. Kedra Wallace,

We were grateful for the comments of the reviewers on our manuscript PRCH-D-18-01367 “The importance of the PD-1/PD-L1 pathway at the maternal-fetal interface”.

We made a revision of our article considering all the suggestions of the reviewers.

The following correction was made:

Reviewer #1

Comment 1. “Manuscript should be further revised by a native English speaker in order to correct several typos.”
As the reviewer suggested the manuscript was revised and the typos were corrected.

Comment 2. “Inclusion/exclusion criteria should be better clarified (any other demographic or clinical criteria?).”

Thank you for this excellent comment, the following part was inserted into the Participants section.

“All subjects affected by any kind of pregnancy-related complication and/or infection, alcohol abuse, pre-pregnancy disease, AIDS, in vitro fertilization pregnancies, diabetes mellitus, renal diseases, immune-associated disease and deep vein thrombosis were excluded.”

Comment 3. “Was this study registered? I could not find any information about this point.”

According to the suggestion of the reviewer, the following section was inserted into the Declarations.

“Ethics approval and consent to participate

The collection of the samples and experimental procedures were approved by the Regional Ethics Committee at the Medical School, University of Pécs (approved protocol registration number: 6149) and written informed consent was obtained from all participant. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.”

Comment 4. “It is essential that the Authors describe limitations of their study. In this regard, the really small size of the sample is certainly one of the main limitations of the study. I recommend better clarifying this point.”

As suggested we inserted a limitation section into the Discussion:

“There were some limitations in the present study. Firstly, due to technical difficulties, we were not able to separate appropriate amount of cells from decidual tissues to perform all experiments on all samples. Second, functional assays of the investigated cells should be performed in the future to investigate the relationship between PD-1 positive immune cells and PD-L1 molecules.”

Comment 5 and 6. “Recent and novel evidence suggested that epigenetic changes, in particular altered expression of selective miRNA, may play a key role in both placental-induced diseases
such pre-eclampsia and intrauterine growth restriction. It would be mandatory to discuss (at least briefly) this topic, referring to: PMID: 28466013; PMID: 28282763”

“The real challenge in the era of molecular medicine is to find a biomarker (or even better a panel of biomarkers) for early diagnosis of pre-eclampsia, a disease for which the altered immune tolerance at the maternal-fetal interface may play a significant role for its initiation and progression. I would stress this point, referring to: PMID: 28243732; PMID: 26512423; PMID: 29923045.”

According to the reviewer’s suggestion the following part was added to the introduction.

“Investigation of the immune-checkpoint molecules at the maternal-fetal interface is crucial in order to clarify the exact immunological relationship between the mother and the fetus. This knowledge accompanied by epigenetic examinations (33) could help us to understand the background of pathological pregnancy conditions such as preeclampsia and intrauterine growth restriction. One of the biggest challenges in reproductive immunology is to find a proper biomarker or biomarker panel for early diagnosis of preeclampsia. We hope that our results supplemented with previous findings (34, 35) will contribute to understanding the background of this pathological condition.”

Comment 7. “At the end of the manuscript, reference list is not formatted according to the Journal’s guidelines. I recommend the Authors to follow this example: Smith JJ. The world of science. Am J Sci. 1999;36:234-5.”

The reference list was formatted as a reviewer suggested.

Reviewer #2

Comment 1. “The abstract states that 20 healthy pregnant women were involved in the project. However, the methods and results only indicate that a maximum of 13 healthy pregnant women were recruited. Please clarify”

As noticed by the reviewer, we corrected the abstract, since 13 pregnant women were included in the study. The number 20 was corrected to 13 in the abstract.

Comment 2. “7 samples of DIC and 10 of PMBCs from pregnant women. Were any samples lost of were cell irretrievable from tissue? Were the PBMCs and DICs isolated from different patients? Were DICs and PBMCs from pregnant women matched?”

The reason for the different number of the DICs and PBMCs was that we were not able to separate appropriate amount of cells from decidual tissues to perform all experiments on all samples. The PBMCs and DICs were isolated from the same patients and were matched.
Comment 3. “Line 60: The authors state that the innate rather than the adaptive immune system is dominant at the maternal fetal interface. However, the study seems to focus more on CD8+ T cells (adaptive immunity). Perhaps should revise sentence to "at the maternal-fetal interface suggests an important role of both the innate and adaptive adaptive immune systems".”

According to the reviewer’s suggestion we revised the incriminating sentence to „Furthermore, the elevated ratio of natural killer (NK) and NKT-like cells at the maternal-fetal interface suggests an important role of both the innate and the adaptive immune system.”

Comment 4. “The introduction is too long. Lines 85-107 should be condensed into 1 paragraph.”

According to the reviewer’s suggestion the introduction section was shortened.

Comment 5. “Authors need to introduce/discuss NKG2D, its function and how it relates to PD-L1/PD-L1 expression and function. The section in the results on NKG2D is the first you hear of it and readers may not be aware of this receptor and its function.”

As a reviewer suggested, the following part was inserted into the Introduction.

“NKG2D is an activating receptor expressed by NK and NKT-like cells, but it is also present on CD8+ T cells. After binding to its ligands such as UL16 binding protein 1 (ULBP1), ULBP2, ULBP3, major histocompatibility complex class related molecules A and B (MICA, MICB) it has a costimulatory function and contribute to the cytotoxic activity of these effector cells [31]. Although MICA expression restricted to gastric and glandular epithelial cells, the expression pattern of ULBP appears to be extensive in healthy adult tissues since ULBP transcripts were observed in kidney, prostate, uterus, tonsil, lymph node tissues [32] and trophoblast cells [33]. Currently one of the most efficient immunotherapy is to block the PD/PD-L1 checkpoint inhibitor pathway [34, 35]. A recent study reported a connection between a higher PD-L1 and lower NKG2D ligand expression in a radioresistant cell [36]. Therefore it might be interesting to investigate the co-expression of PD-1 and NKG2D receptors by CD8+ T and NKT-like cells since NK cells do not express PD-1 receptor.”

Comment 6. “Are data normally distributed or did authors test for normality? If so, please state this in the statistics. With such small Ns this is a concern.”

Normality test was performed during the statistical analyses and in every case normal distribution was established. According to the reviewer’s suggestion the following section was inserted into the statistical analysis section.

“Normality of our data was tested by Shapiro-Wilk test. A normal distribution was confirmed.”

Comment 7. “Was power analysis performed for this study?”
Unfortunately, the power analysis was not performed before the initiation of our study. As we mentioned in the discussion, small sample size is a limitation of our manuscript.

Comment 8. “Was the T-test paired or unpaired?”

According to the question of the reviewer the statistical analysis section was clarified. Unpaired and paired sample t-test were used based on the investigated study groups.

Comment 9. “According to sample flow cytometry gating strategy, Tregs were identified from the same sample of cells stained for NK cells. In the methods, both CD56 and Foxp3 are on APC, how could the authors distinguish these cells types with two markers in the same panel on the same Fluor? Wouldn't the Tregs appear as CD56+ and be identified as NKT cells?”

Thank you for your valuable remark, we corrected the misleading figure showing our gating strategy. The FoxP3 intracellular antigen staining was performed after the CD3 and CD4 surface antigen staining. The CD56 surface antigen was stained accompanied only with CD3, CD4 and CD8 surface antigens. The gating strategy was modified according to the reviewers comment.

Comment 10. “Line 299: How do CD8+ T cells give a Th1 immune effect? Do the authors mean type 1 instead of Th1? Similar question for Line 329.”

Thank you for the excellent comment, in line 299 the “Th1 effects” and in line 329 the “Th1 immune responses” phrases were changed to “inflammatory effects” and “inflammatory immune response” respectively.

Comment 11. “Lines 351-354: If soluble ligands for NKG2D bind to the receptor how does this reduce cytotoxicity? This seems contradictory to the authors previous description of NKG2D as an activating receptor. Does binding to NKG2D trigger cytotoxic function or inhibit it?”

The engagement of NKG2D by its ligands initiate a rapid pro-inflammatory mechanism and contribute to the expansion of specific T cells (31, 32, 33). However many studies reported that NKG2D ligands shed by tumor cells could connect to NKG2D receptor on effector lymphocytes and interfere with the recognition of these tumor cells (50, 51, 52). One possible explanation is that the soluble or surface form of NKG2D ligands have a different effect on the receptor-expressing cells.

According to the reviewer’s question, the following sentence was inserted into the discussion section. “It seems that soluble or surface form of NKG2D ligands have a different effect on the receptor-expressing cells.”
Minor Concerns:

Minor comment 1. “English type editing required”

As the reviewer suggested the manuscript was revised and the typos were corrected.

Minor comment 2. “Line 110-111: What did the study say about how PD-L1 influences Treg cells? Did the influence promote survival or demise of the fetus?”

Habicht et al. investigated the interaction between PD-L1 and Treg at the feto-maternal interface in a mouse model (38). They revealed a quantitative expansion of alloreactive T cells in parallel with reduced Treg function by blocking the surface PD-L1 which resulted in decreased fetal survival. It seems that the PD-L1 promote fetal survival by maintaining adequate Treg function.

The following sentence was inserted into the introduction.

“An interesting study has been reported that in allogeneic pregnancy a quantitative expansion of alloreactive T cells in parallel with reduced Treg function by blocking the surface PD-L1 resulted in decreased fetal survival. It seems that the PD-L1 promote fetal survival by maintaining adequate Treg function.”

Minor comment 3. “Ethical information should be included in participants section of Methods”

As a reviewer suggested ethical information was added into the Declarations.

Minor comment 4. “Intracellular staining method should be before flow cytometry method”

According to the reviewer’s suggestion the intracellular staining method section was relocated before the flow cytometry method section.

Minor comment 5. “Authors should identify NK cells as CD56+NK, CD56Dim NK and CD56brightNK rather than just NK, NKdim and NKBright”

According to the reviewer’s suggestion NK, NKdim and NKBright appellations were changed to CD56+NK, CD56Dim NK and CD56bright NK.

Minor comment 6. “What type of controls were used for the flow cytometry? FMOs, isotype controls?”

For our flow cytometric measurements, isotype controls were used.
We hope that the revised manuscript has now been sufficiently improved to merit publication.

Sincerely,

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