TP TRANSLATIONAL PEDIATRICS
AN OPEN ACCESS JOURNAL COVERING ALL ASPECTS OF PEDIATRICS RESEARCH
IMPACT FACTOR 4.047

Peer Review File

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Reviewer Comments

In this manuscript, Li et al. demonstrate the effects of TNF-alpha on c-Jun and P-gp expression. These findings are very interesting, but a major question is the high concentration of TNF-alpha required to demonstrate such effects (much higher than physiological concentrations), leading to the question of clinical relevance of these findings. Major revisions would be necessary before this paper could be considered acceptable for publication.

Major comments

1. 20 ng/mL of TNF-alpha was cytotoxic, so subsequent experiments focused on the concentration of 10 ng/mL. However, maternal plasma or serum levels of TNF-alpha during pregnancy are nearly 1000-fold lower than this (please see PMID 17047289 and PMID 11591402). In non-pregnant patients with septic shock, the median serum level of TNF-alpha was still below 10 ng/mL (mean 0.7 ng/mL, median 0.25 ng/mL, see PMID 2791581). This brings to question the clinical relevance of 10 ng/mL of TNF-alpha affecting placental cell transporters.

Reply: Thanks for the precious suggestion. In obesity patient, TNF-a is frequently upregulated and resulted in inflammatory condition. This makes us to hypothesize that TNF-a may result P-gp and its molecule-transporting activity. In this study, we used Human placental choriocarcinoma cell lines, Bewo, JEG-3 and JAR. This may result in a relatively low sensitivity to TNF-a. In further investigation, it is worth confirming whether physiological concentration of TNF-a could exert these regulatory roles.

2. The methods section describing the digoxin experiments is missing.

Reply : This section was added as follows:

Detection of efflux ratio of digoxin

Cells were seeded onto 24-well plates (BD-Corning, Corning, NY, USA) at a density of 1× 105 cells/well and allowed to be cultured overnight. After three washes using PBS, 0.5 ml of HBSS supplemented with 0.1 μM of [3H]digoxin and P85 (0.1% and 1%), tween 80 (0.1% and 1%) was added into seeded cells to initiate digoxin accumulation. Cells were then washed using ice-cold HBSS for three times and supernatant was removed. Then, cells were lyzed using 0.1 mL of 10% SDS solution, mixed with 0.5 mL Optiphase cocktail solution (Perkin Elmer Inc.; Boston, MA, USA), and stabilized for overnight incubation. The radioactivity of [3H]digoxin in the cell lysate was measured using a Microbeta 2 liquid scintillation counter (Perkin Elmer Inc.; Boston, MA, USA).

Changes in the text: We added the related content (see Page 7, line 188 to 196).

3. Some data appear to be missing. There is a statement that in Figure 6C, the efflux ratio was

significantly decreased by the addition of SP600125, SR11302 or vinblastine. However, Figure 6C only shows vinblastine. The data for SP600125 and SR11302 are missing.

Reply: Sorry for the mistake. In digoxin efflux experiment, we only used vincristine instead of SP600125 and SR11302. We have made changes in the manuscript.

Changes in the text: We removed the description of SP600125, SR11302 from the manuscript.(See Page 10, line 294-296)

4. In the first sentence of the discussion section, the authors state that P-gp is the most abundant transporter in the placenta. What about OAT4 or BCRP? (Please see PMID 32591415.)

Reply: Sorry for the misleading description, this sentence has been modified as below:

P-glycoprotein (P-gp) is an abundant transporter in the placenta and exerts protective effects against cytotoxic agents during pregnancy.

Changes in the text: see Page 10, line 298 to Page 299.

5. The authors state that they failed to analyze c-Jun mRNA. However, Figure 3C appears to show data for c-Jun mRNA.

Reply: It has been modified as below:

In this study, we analyzed c-Jun mRNA and protein levels in response to TNF- α treatment and found that TNF- α induced c-Jun expression and phosphorylation in a dose- and time-dependent manner in Bewo cells.

Changes in the text: see Page 11, line 312 to Page 315.

6. This statement is confusing: SP600125 failed to affect the fold enrichment of c-Jun in the promoter region of the ABCB1 gene indicates that endogenous phosphorylated c-Jun has no obvious function in BeWo cells. What is the connection between SP600125 and phosphorylated c-Jun?

Reply: This statement has been modified as below:

a 4-hour pretreatment with 1.75 μM SP600125, a inhibitor of c-Jun phosphorylation, failed to affect the fold enrichment of c-Jun in the promoter region of the *ABCB1* gene, indicating that endogenous phosphorylated c-Jun has no obvious function in Bewo cells.

SP600125 is a specific inhibitor of c-Jun phosphorylation. Aim to evaluate the effects of phosphorylation of c-jun on binding to ABCB1 promoter region.

Changes in the text: see Page 11, line 332 to Page 335.

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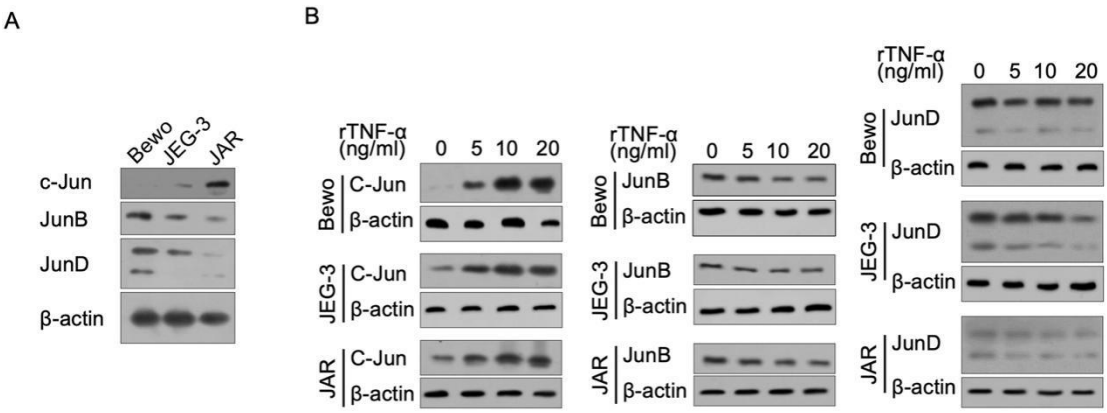
7. In Figure 1B, from which cell line was the beta-actin? Shouldn't there be multiple rows of beta-actin for each cell line?

Reply: Sorry for the mistake. In figure 1B, for each cell line, beta-actin was measured respectively. The figure has been re-arranged and attached as below:



Changes in the text: see Page 16

8. Does Figure 6A show c-Jun expression in the BeWo cell line? For the first column (Mock), there now appears to be much more c-Jun than had been observed in Figure 1A. Is this a different cell line, or did the conditions change?

Reply: The endogenous level of c-Jun in Bewo is quite low. In figure 6A, we extend exposure time to make sure clear band of c-Jun could be observed.

9. It would be helpful to draft a scheme or figure showing the overall mechanism of TNF-alpha having effects on P-gp expression or function. As written now, it is very confusing because the text states that SR11302 is a P-gp inhibitor, but the text also states that SR11302 is an AP-1 inhibitor. Likewise, the text states that SP600125 is a P-gp inhibitor, but the text also states that SP600125 is a c-Jun inhibitor. These statements make it very difficult to follow the intent of some of the experiments and makes it difficult to understand the overall mechanism.

Reply : Sorry for the misleading statement. SR11302 is an AP transcriptional factor inhibitor. SP600125, an inhibitor of c-jun N-terminal kinase. The manuscript has been clearly modified to abolish the misleading statement.

Changes in the text: see Page 2 line 42, and Page 11,line 332

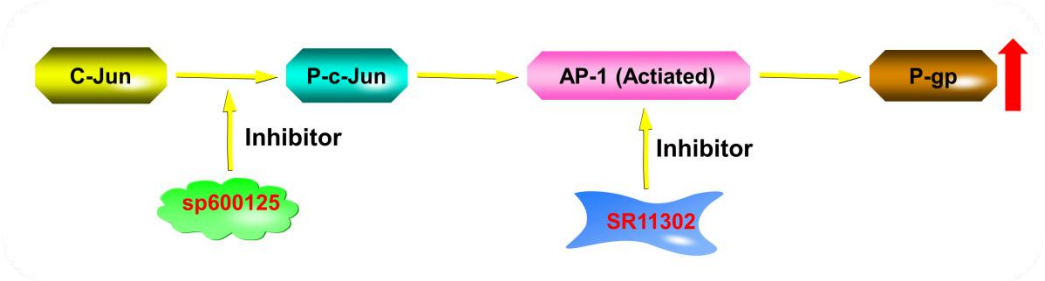


Figure A scheme of the overall mechanism of TNF-alpha having effects on P-gp expression

10. If SR11302 and SP600125 are P-gp inhibitors (or even if they are not), wouldn't it be valuable to show the effects of these inhibitors on DiOC2(3), Rh123, and digoxin uptake or efflux?
Reply: Sorry for the misleading statement. SR11302 is an AP transcriptional factor inhibitor. SP600125 is an inhibitor of c-jun N-terminal kinase.

11. In Figure 6B, the P-gp inhibitor vinblastine did not show any increase in fluorescence for either DiOC2(3) or Rh123. Likewise, in Figure 6C, DMSO+vinblastine did not show any decrease in the digoxin efflux ratio (in fact, it appears to show a slight increase). These findings bring into question the effectiveness of vinblastine as a P-gp inhibitor to explore the mechanisms pertinent to this investigation.
Reply: In figure 6B and 6C, addition of vinblastine in DMSO group did not show any decrease in the digoxin efflux ratio is due to the relative low level of efflux. After TNF-a treatment, efflux ratio increased, and thus, the effects of vinblastine were observed. In this case, it is a efficient inhibitor to explore the mechanism.

Minor comments
1. Grammatical corrections are needed throughout the manuscript, including figure legends.
Reply: Thanks for the suggestion. The manuscript has been modified commercially.See the attachment.

2. Is Na3VO4 correct?
Reply: It has been corrected into Na_3VO_4.
Changes in the text: see Page 3, line 78.

3. The phrase "Reverse Transcriptional quantitative PCR" should probably be put in bold heading font.
Reply: It has been modified.
Changes in the text: see Page 5, line 132.

4. The statement "All Jun proteins were obviously regulated by the additional of rTNF-alpha in a dose-dependent manner" does not seem accurate. It seems to apply to c-Jun only, not JunB or JunD.
Reply: Thanks for the remainder. It has been modified properly.
Changes in the text: see Page 8, line 212.

5. The statement "As expected, treatment with 20 μM rifampin…also significantly increased the P-gp protein (Figure 3D" does not seem accurate. Figure 3D does not indicate any significant difference for rifampicin.

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Reply: Sorry for the mistake. Rifampin was used as a control in this study. However, it is not presented in this section. Thus, we deleted this sentence to make it accurate.

Changes in the text: see Page 9, line 247-249.

6. The statement "SR11302 negligibly affected c-Jun protein levels" does not seem accurate. What is the P-value for comparison of TNF-alpha + SR11302 vs. TNF-alpha in the second panel of Figure 6A?
Reply: Sorry for the mistake. We have modified this sentence in the manuscript.

Changes in the text: see Page 10, line 289.

7. The legend for Figure 1 defines P-values for * or **, but no asterisks appear in the figure.
Reply: It has been modified in figure 1 legend.

Changes in the text: see Page 16, line 473.

8. Most of the figure legends do not specify which cell line was investigated.
Reply: The cells used was described in results section.(three placental cell lines, Bewo, JEG-3 and JAR cells)

Changes in the text: see Page 7, line 205-206.

9. In Figure 3D, last panel, it is surprising that there is a significant difference for 20 ng/mL TNF-alpha but not for 5 ng/mL (which appears to have a narrower error bar and a slightly higher value).
Reply: It may due to the high background.

10. Was vinblastine dissolved in DMSO when introduced to the cells? I am trying to understand why a DMSO control would otherwise be inserted with the figures.
Reply: Yes, vinblastine was dissolved in DMSO. And same volume of DMSO was used as negative control.