Peer Review File

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Reviewer Comments

Comment 1: The authors investigated the role of SOCS3 in primary human cytotrophoblast apoptosis. After an overexpression and knockdown of SOCS3 in cell culture via lentivirus as a vector, both groups and one without infection as control were exposed to 100 ng/ml IL-6 for 24 hours. The authors were able to prove that overexpression of SOCS3 attenuates the phosphorylation of STAT3 and reduces the IL-6-dependent apoptosis while its knockdown leads to increase of cell death. Although the paper shows valuable and significant data, some major and minor issues clearly need to be addressed.

Reply 1: Thanks for your recognition of our work and helpful suggestions. The responses to specific questions are as follows.

Major issues

Comment 2: The authors do not mention which phosphorylation site of STAT3 the investigated. Although the Tyrosin 705 phosphorylation is expected in an inflammatory model, one of the main causes of placenta insufficiency is the insufficient blood flow that can lead to phosphorylation of Serin 727 due to oxidative stress of the mitochondria. (Jeremy A Meier , Moonjung Hyun , Marc Cantwell , Ali Raza, Claudia Mertens , Vidisha Raje, Jennifer Sisler, Erin Tracy, Sylvia Torres-Odio, Suzana Gispert, Peter E Shaw, Heinz Baumann, Dipankar Bandyopadhyay, Kazuaki Takabe, Andrew C Larner, Stress-induced dynamic regulation of mitochondrial STAT3 and its association with cyclophilin D reduce mitochondrial ROS production, Sci Signal. 2017 Mar 28;10(472):eaag2588.doi:10.1126/scisignal.aag2588.)

Reply 2: We apologize for the missing information of phosphorylation site of STAT3. The phosphorylation site of STAT3 we analyzed in this study was Serin 727 and we have added the information in the revision (see Page 11, Line 226-227).

Changes in the text:

1) **Page 11, Line 226-227:** p-STAT3 (Ser727) (Cell Signaling Technology, Cat No. 9136S, 1:1000).

Comment 3: The authors do not compare their groups to a cytotrophoblast from a carried to term pregnancy.

Reply 3: In previous study, we have determined the expression of IL-6, SOCS3 and p-STAT3 in placental tissues (Zhou et al, 2015). The expression of IL-6 is

significantly higher in the placental tissues from the pre-term labor group (50.6 ± 9.4 ng/mL) when compared with those from both term labor group (15.2 ± 4.1 ng/mL) and term not in labor group (7.1 ± 2.7 ng/mL). This result was in accordance with that the infection in preterm placentas was more obvious than in term placentas. In addition, the placental tissues from term labor group and term not in labor group showed similar expression level of SOCS3 protein, while that in the pre-term labor group was significantly increased. Besides, although the expression of p-STAT3(Ser727) in placental tissues increased significantly in term labor groups, no significant difference was observed between the preterm labor and term not in labor groups. Considering the feedback inhibitory role of SOCS3 in JAK/STAT3 signaling pathway, the relatively low level of p-STAT3(Ser727) in preterm group might result from enhanced SOCS3 expression. These results suggested that SOCS3 might be engaged in pre-term birth. However, the exact role of SOCS3 was not investigated. Therefore, we focused on the role of SOCS3 in preterm labor in this study and demonstrated that SOCS3 could protect preterm placental tissue-derived cytotrophoblasts from IL-6 induced apoptosis by inhibiting JAK2/STAT3 signaling pathway, which was in accordance with high expression of SOCS3 and low level of p-STAT3(Ser727) modification in preterm placental tissues in our previous study. Although p-STAT3(Ser727) modification was inhibited by SOCS3 in preterm placental tissues, the outcome of preterm birth implied that there were still other mechanisms remained to be illustrated, which would be in the scope of our further studies.

Reference:

1) Zhou, X., Jiang, Z., Zou, Y., Yin, Y., Zuo, Q., & Sun, L. (2015). Role of SOCS3 in the Jak/stat3 pathway in the human placenta: Different mechanisms for preterm and term labor. Acta Obstetricia et Gynecologica Scandinavica, 94 (10), 1112–1117.

Comment 4: The authors do not address limitations of their investigation such as the possibility of upregulation of other JAK/STAT pathways after knockdown of SOCS3 (Peter J. Murray, The JAK-STAT Signaling Pathway: Input and Output Integration, J Immunol March 1, 2007, 178 (5) 2623-2629; DOI: https://doi.org/10.4049/jimmunol.178.5.2623) as well as role of the increased sensitivity and response to inflammatory cytokines of the fetal monocytes (Elisabeth R. Krow-Lucal, Charles C. Kim, Trevor D. Burt, Joseph M. McCune, Distinct functional programming of human fetal and adult monocytes, Blood (2014) 123 (12): 1897–1904. https://doi.org/10.1182/blood-2013-11-536094)

Reply 4: Thank you for your valuable advice and we have addressed the

limitations of our investigation in the section of Discussion in our revised manuscript (see Page 16-17, Line 333-358; Page 22-23, Line 472-491).

Changes in the text:

1) Page 16-17, Line 333-358:

IL-6 is a crucial modulator of mammalian responses to tissue damage caused by infection and inflammation, which are among the major causes of human preterm birth. Consistent with this, our previous study showed that the expression of IL-6 was notably increased in preterm placental tissues (20). As SOCS3 played a negative feedback role in IL-6-induced activation of JAK/STAT3 signaling (20, 29), the overexpression of SOCS3 in preterm placental tissue-derived cytotrophoblasts also inhibited IL-6-induced p-STAT3, while SOCS3-knockdown had the opposite effect.

Given the high expression of SOCS3 and low expression of p-STAT3 in preterm placental tissues (20), it remains to be determined whether cytotrophoblast apoptosis is inhibited *in vivo*. Furthermore, it has been reported that SOCS3 can regulate the quantity and type of STAT signal generated from the IL-6 receptor (IL-6R), and the absence of SOCS3 significantly induces the phosphorylation of both STAT3 and STAT1 (29, 30). Thus, the phosphorylation status and other STAT proteins' exact roles in preterm placental tissues and cultured cytotrophoblasts remains to be determined.

On the other hand, both the fetal and the maternal immune systems were actively involved in maintaining normal pregnancy (31–33). It has been reported that IL-6 stimulation promotes STAT3 phosphorylation in both fetal and adult monocytes (34). Although fetal and adult monocytes express similar levels of SOCS3, the expression of IL-6R in adult monocytes was significantly lower than that in fetal monocytes, which resulted in markedly higher STAT3 phosphorylation in fetal monocytes exposed to IL-6. Illustration of the mechanism underlying IL-6/STAT3/SOCS3 signaling regulation in both fetal and adult monocytes would provide new insights into inflammation-associated preterm birth.

2) Page 22-23, Line 472-491:

(28) Takahashi Y, Carpino N, Cross J C, et al. SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. EMBO J 2003, 22(3): 372-384.

(29) Croker B A, Krebs D L, Zhang J G, et al. SOCS3 negatively regulates IL-6 signaling in vivo. Nature Immunol 2003, 4(6), 540–545.

(30) Murray P J. The JAK-STAT signaling pathway: input and output integration. J Immunol 2007, 178(5), 2623–2629.

(31) Erlebacher A. Immunology of the maternal-fetal interface. Ann Rev

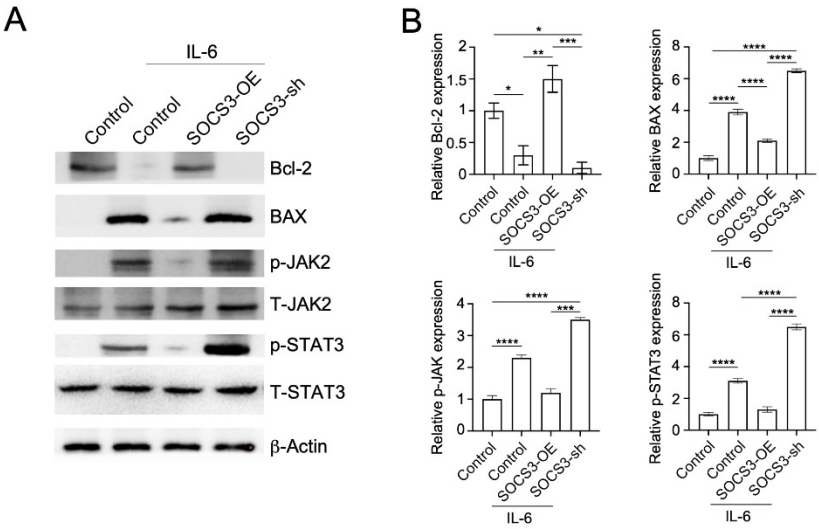
Immunol 2013, 31, 387–411.
(32) Burt T D. Fetal regulatory T cells and peripheral immune tolerance in utero: implications for development and disease. Am J Reprod Immunol 2013, 69(4), 346–358.
(33) Miller D, Gershater M, Slutsky R, et al. Maternal and fetal T cells in term pregnancy and preterm labor. Cell Mol Immunol 2020, 17(7), 693–704.
(34) Krow-Lucal, E R, Kim, C C, Burt, T D, et al. Distinct functional programming of human fetal and adult monocytes. Blood 2014, 123(12), 1897–1904.
(35) Ding Y B, Fu L J. Apoptosis Related Gene Expression Profile of Trophoblasts Derived from Recurrent Spontaneous Abortion. Chin J Cell Biol 2009, 31(2): 250-256.

Comment 5: Figure 4 is incomplete and does not fully convey the data set from the Western Blot analysis. A graphical presentation with statistical analysis is definitely required.

Reply 5: Thank you for your valuable advice and we have added the data of statistical analysis as Figure 4B (see Page 25, Line 527-531; Page 27, Line 558).

Changes in the text:

1) **Page 25, Line 527-531:** B: The levels of Bcl-2, BAX, p-JAK2, and p-STAT3 were measured by ImageJ software and determined by taking the ratio of the target protein band intensity against that of β-actin. Data are expressed as the mean ± SEM of the mean of three independent measurements. *P < 0.05, **P < 0.01, ***P<0.001 and ****P<0.0001.
2) **Page 27, Line 558:**



Minor Issues

Comment 6: I suggest a clearer definition of pregnancy (amenorrhea is defined as “absence of menstruation for 3 months”) and a differentiation between the stages of preterm birth.

Reply 6: We apologized for the inappropriate statement of preterm birth. The statement “Placentas were collected from non-smoking, healthy women with pregnancies delivered by Caesarean sections less than 37 weeks of amenorrhea” was amended as “Placentas were collected from cesarean sections of non-smoking, healthy women with a pregnancy less than 37 weeks' gestation (hereafter called "preterm labor")” in the revised manuscript (see Page 7, Line 131-133).

Changes in the text:

Page 7, Line 131-133: Placentas were collected from cesarean sections of non-smoking, healthy women with a pregnancy less than 37 weeks' gestation (hereafter called "preterm labor").

Comment 7: I recommend to the authors to edit the paper lexically and grammatically.

Reply 7: Thanks for your advice. The gramma of the manuscript has been edited by the editors from AME Editing Service and the changes have been highlighted in blue in the revised manuscript.