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Title: ToxoRO Y6Ⅰ/Ⅲ induced M2 phenotype of macrophages protects Caco-2 cells via inhibition of M1 associated inflammation

Comments #1:

Summary This in vitro study aims to verify the potential role of parasite-derived effector Toxoplasma ROP16Ⅰ/Ⅲ in ameliorating inflammatory bowel disease (IBD) pathology, through promotes the polarization of M2 macrophages and downregulate the M1 associated inflammatory response. The RAW264.7/Caco-2 co-culture system was established as an inflammatory model of IBD in vitro. the results presented in the manuscript demonstrated that, the RAW264.7 macrophages stimulated by LPS (M1 cells) showed increased production of iNOS, NO, TNF-α, IL-1β, and IL-6 and facilitated Caco-2 cells apoptosis in the co-culture system, while ToxoRop16Ⅰ/Ⅲ transfected RAW264.7 macrophages bias to M2 cells, enhanced the synthesis of Arg-1, IL-10, TGF-β1, and IL-13. M2 mixed with M1, exhibited downregulation of the pro-inflammatory factors and alleviated Caco-2 cells apoptosis in the co-culture system. According to their experimental results, the authors conclude that the ToxoRop16Ⅰ/Ⅲ exhibited a protective role on Caco-2 intestinal epithelial cells by promoting the polarization of M2 cells and dampen the M1-mediated inflammatory response. Strength: - The manuscript addressed a promising strategy for IBD immunotherapy with parasite-derived effector ToxoROP16Ⅰ/Ⅲ. In vitro, the ToxoROP16Ⅰ/Ⅲ showed a potential to promote M2 cells polarization and ameliorate the M1 mediated inflammatory response. Which is considered a major approach in fighting the IBD. Suggest that the ToxoRop16Ⅰ/Ⅲ might have potential in ameliorating bowel inflammation by driving intestinal epithelial macrophages to M2 cells phenotype and maintaining the equilibrium of the gut macrophage subsets. - The experiments are well

designed in term of sampling, control and data validation.

Weakness:

- To verify the role of ToxoRop16$_{I/III}$ on intestinal homeostasis, the authors only examined the Caco-2 cells apoptosis, which not necessarily reflect the functionality of the Caco-2 cells. It would be worth to examine the effect of ToxoRop16$_{I/III}$ on Caco-2 monolayer integrity and permeability.

Response: We appreciate the reviewer's suggestion. We understand that apoptosis, and integrity and permeability of the Caco-2 cells as well, are the key indicators of cell damage. The present study is only a primary approach for the potential role of parasite-derived molecule, ToxoROP16$_{I/III}$ which has been shown to be able to drive macrophages to M2 polarization, in immune therapy of IBDs that have been recognized as a type I dominant inflammatory autoimmune disease. Additional research is going on for deep insight into the effect of ROP16$_{I/III}$-induced M2 macrophages on protection of intestinal epithelial cells both *in vitro* and *in vivo,* including the integrity and permeability of the cells to be measured.

The authors used a co-culture system of the intestinal epithelial Caco-2 cells (human cell line) and macrophage RAW264.7 cells (mice cell line), which raise a concern about the applicability of this model. Besides, the authors didn't mention the origin of the cell lines used in the manuscript.

Response: Thank you for the inquiry. The Caco-2 (human cell line) used in the experiments was purchased from MOL Biotechnology

co., Ltd (HeFei, Anhui), whereas RAW264.7 (mouse cell line) , from the lab (ATCC® SC-6003). We understand that Caco-2 and RAW264.7 cell lines originate from different species and incompatibility might exist. We used this co-culture system just based on the previous report which indicated that Małgorzata Kujawska et al, Shao-Jung Wu et al. and Shashank Singh et al. and more experimental studies have used RAW264.7/Caco-2 to establish in vitro intestinal inflammatory.

[Kujawska M et al. Spray-Dried Potato Juice as a Potential Functional Food Component with Gastrointestinal Protective Effects. Nutrients 2018; 10(2) [PMID: 29495317 DOI: 10.3390/nu10020259]

[Wu SJ et al. Delivery of berberine using chitosan/fucoidan-taurine conjugate nanoparticles for treatment of defective intestinal epithelial tight junction barrier. Marine drugs 2014; 12(11): 5677-5697 [PMID: 25421323 DOI: 10.3390/md12115677]

[Singh S et al. Probiotic attributes and prevention of LPS-induced pro-inflammatory stress in RAW264.7 macrophages and human intestinal epithelial cell line (Caco-2) by newly isolated Weissella cibaria strains. Food & function 2018; 9(2): 1254-1264 [PMID: 29393319 DOI: 10.1039/c7fo00469a]

In the co-culture system, the RAW264.7 cells were seeded at a density of 2×106 cells. Whereas, the Caco-2 cells were seeded at a density of 5×105 cells, 4:1 ratio, which could increase the Caco-2 burden in this model.

Response: This ratio of Caco-2/RAW264.7 was used just following the previous reports [ref. 32-33, RAW264.7 cells (4×10^5 cells) and Caco-2 cells (4.4×10^5 cells)], with a slight modification.

The manuscript included an insufficient description of the Caco-2 differentiation status which could remarkably affect its response.

Response: Thanks for the suggestion. We just showed interest in apoptosis of Caco-2 cells, indicating the inflammation-induced damage to the intestine epithelial cells. Other exploration would be needed to reveal the Caco-2 differentiation status and its response.

The Arg-1 relative mRNA and protein expression results are not consistent. In figure (5, C) the relative mRNA expression of Arg-1 was markedly higher in LV-rop16Ⅰ/Ⅲ-Mφ group (8-fold) compared with M1+M2 group. Whereas, in figure (7.C) the Arg-1 protein expression was higher (2-fold) in M1+M2 group compared with LV-rop16Ⅰ/Ⅲ-Mφ group. Furthermore, in figure (4, G) the Arg-1 protein expression was 2-fold higher in LV-rop16Ⅰ/Ⅲ-Mφ group relative to Mφ, compared with 1-fold higher expression in figure (7.C).

Response: Thank you very much for the inquiry. In figure (5, C) the relative mRNA expression of Arg-1 was markedly higher in LV-rop16Ⅰ/Ⅲ-MΦ group compared with M1+M2 group was only in the genetic level, it could be that the encoding of genes and genetic modification was the first， and the formation of proteins may lag behind. and not in protein expression, so the high expression, but in figure (7.C) the Arg-1 was protein expression higher (2-fold) in M1+M2 group compared with LV-rop16Ⅰ/Ⅲ-MΦ group, At the same time, I also checked and calculated over and over again, and the results were the same.

In figure (4, G) the Arg-1 protein expression was 2-fold higher in LV-rop16Ⅰ/Ⅲ-MΦ group relative to MΦ, this was detected after virus transfer, so the Arg-1 protein expression higher, but in figure (7.C) the Arg-1 protein expression after the mixture mixed with M1 and M2, and the β-actin they use are also different.

The authors should correct the figures numbering, for example, the protein expression of Arg-1 and PD-L2 were presented in figure 7 not figure 6 as it was mentioned in the manuscript.

Response: It has been corrected.

- In the methods; the NO assay section, the sampling and the density of the cells were ambiguous and unclear.

Response: They have been revised.

Comments #2:

In this manuscript, the authors verified previously reported finding by other researchers that ToxoROP16 I/III induced M2 polarization in RAW264.7 macrophage cell line. They further showed that ToxoROP16 I/III induced M2 phenotype of RAW264.7 cells reduced the apoptosis in intestinal epithelial cell line Caco2 cells induced by M1 phenotype of RAW 264.7, the M1 type RAW264.7 cells were pre-induced using LPS. The data reported in this manuscript were generated using a cell culture model, which are difficult to directly related to IBD. However, the observation that a component of Toxoplasma gondii polarized M2, which in turn inhibited M1 induced epithelial damage is still interesting. Please see my comments below:

1. The manuscript has no page numbers and no line numbers, which makes it very difficult to communicate between the reviewers and the authors.

Response: Thank you for your advice. In the revised article, I will correct it.I'm very sorry to read the difficulties in the article.

2. Conclusion in the abstract: "These findings may be helpful for gaining a better understanding of the underlying mechanism". What mechanism do the authors refer to?

Response: The aetiologies of this disease, IBD, remain unknown, a variety of factors cause IBD, including environmental, genetic and immune factors and the gut microbiota. The mechanism that causes IBD is still unclear, so mang factors induces IBD. In the study of this article, the main mechanism we mentioned is the role of immunization in IBD. ToxoROP16$_{I/III}$ induced RAW264.7 polarization to M2 macrophage, down-regulation the M1 associated inflammation response and playing a protective role on Caco-2 intestinal

epithelial cells. This articl ideal come from helminths (parasitic worms) can induce type 2 immune intestinal inflammatory responses by promoting the expansion of protective bacterial communities that inhibit proinflammatory bacterial taxa. (Ramanan et al. Helminth infection promotes colonization resistance via type 2 immunity. Science 2016; 352(6285): 608-612).So, these findings may be helpful for gaining a better understanding of the underlying immunization mechanism.

3. Introduction: "with changes to living conditions, the incidence of IBD is increasing". Do the authors mean the incidence of IBD in China?

Response: The incidence of IBD is increasing not only in China also in the world. Established high-prevalence populations of IBD in North America and Europe experienced the steepest increase in incidence towards the second half of the twentieth century. Potentially relevant environmental influences span the spectrum of life from mode of childbirth and early-life exposures (including breastfeeding and antibiotic exposure in infancy) to exposures later on in adulthood (including smoking, major life stressors, diet and lifestyle). [Ananthakrishnan AN. Epidemiology and risk factors for IBD. Nature reviews Gastroenterology & hepatology 2015; 12(4): 205-217].

4. "During the process of inflammation, macrophages play a central part in cell polarization". Please specify which cell polarization.

Response: In this article, Macrophages cell normally resides in a spherical, detached and undifferentiated state, known as the initial cell state, so wei called M0 phenotype of macrophages (RAW264.7 macrophages). In article "During the process of inflammation, macrophages play a central part in cell polarization", mean that MI phenotype of macrophages stimulated by LPS (M1 cells), so during the process of inflammation, M1 phenotype of macrophages play a central part in cell polarization.

5. “Jensen previously demonstrated (28)”. The first author of reference 28 is Melo MB, why was the second author mentioned?

Response: I'm so sorry. In the process of writing the article, I made a mistake. So sorry. “Melo MB previously demonstrated (28)”. in this article, I have made a change.

6. Table 1: references should be included about who have designed these primers.

Response: Thank you very much for finding this mstake. I have a change in the article. Primer synthesis was completed by Sangon Biotech (Shanghai, China).

7. Figure 5. Why TNFa was not there?

Response: In this article, LPS polarized macrophages to the M1-like phenotype, the mRNA and ELISA expression levels of IL-6, IL-1β and TNF-α were significantly increased in M1 cells so TNF-a in Figure 4. The IL-10, TGF-β1 and Arg-1 in M2 cells was markedly upregulated in Lv-*rop16* $_{I/III}$-Mφ, so TNF-a not in Figure 5. I separate M 1 and M 2.

8. Discussion: ROP16 is a kinase that can directly phosphorylates the Stat3/Stat6. DoesToxoROP16 I/III contain the full gene encoding ROP16 or only a fragment?

Response: In this article, ToxoROP16 I/III contain only a fragment gene encoding ROP16 I/III. An amino acid substitution of ROP16I/III phosphorylates Stat3/6, the 503 of type I and III (such as RH, CTG) ROP16 is

leucine (ROP L503 (i.e., ROP16 I/ III), induced macrophage to M2 polarization.

So, in article, ToxoROP16 I/III contain only a fragment gene encoding ROP16.