Author's response to reviews

Title: Excessive proinflammatory cytokine and chemokine responses of human monocyte-derived macrophages to enterovirus 71 infection

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Author's response to reviews: see over
Dr. Michael Baier
Editor

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Dear Dr. Michael Baier,

Re: [MS: 111533066619530]- Excessive proinflammatory cytokine and chemokine responses of human monocyte-derived macrophages to enterovirus 71 infection

Thank you for sending us the reviewers’ comments and we have revised our manuscript accordingly. And the point-to-point responses are listed below.

**REVIEWER 1:**

**Comment 1:** Did macrophages show cytopathic effect or not? Did they survive 24h post-infection? In Fig1C, not all the cells looked being infected by EV71 even though EV71 was infected at MOI of 5. Were all cells infected if the authors employed a higher MOI? In D, the authors showed kinetics of viral multiplication using realtime PCR. Did macrophages produce infectious progeny particles or not? The increase in the amount of viral genome was only three-fold. If the viral genome increased only such small amount, I wonder it can be detected by IFA. Is it possible to conclude “Effective infection…” written in the headline of the paragraph?

**Answer:**

To test the susceptibility of human monocyte-derived macrophages (MDMs) to EV71 virus, we initially tested the infection at different infectious doses, MOI of 0.1, 0.5, 1 and 5, by VP1
fluorescence staining. The infection showed a dose-dependent. And around 40% of MDMs were infected by EV71 at 24-h P.O.I. even though at a MOI of 5, the maxim one available for our study due to the limitation of the titer of virus stock prepared. These data have been already added in our revised manuscript (page 4, line 20-23; Figure 1).

Cytopathic effects of EV71 on MDMs, demonstrating as swelling cell and glassy appearance could be detected on 48-h POI. In addition, we also screened if there is the activation of apoptosis-related genes during EV71 infection in MDMs. Both FasL and TRAIL mRNA expressions were significantly increased in EV71-infected MDMs at both 24-h and 48-h POI. The data was not present in the study. Further investigations on apoptosis and autophagy of MDMs with EV71 infection will be performed.

To identify whether MDMs produce infectious progeny particles or not, virus titers of culture supernatants were obtained by measuring the 50% tissue culture infective dose (TCID50) on Vero cells and calculated by using the Reed and Muench formula. The results showed that virus yields increased gradually from 6-h to 48-h POI. during EV71 infection. These data have been already added in our revised manuscript (results Line 6-10, Page 5; figure 2B). As evidenced by the expression of viral VP1, viral replication and the production of infectious progeny particles in EV71-infected MDMs, effective infection in MDMs with EV71 was detected in our research.

**Comment 2:** In Fig. 2, the authors presented data for production of cytokines after EV71 infection. They claimed that induction of IL-1β, IL-6 and TNF-a was observed. However, this reviewer thinks that the levels of the cytokines were very small and it would be very difficult to discuss the difference between the cytokine levels of infected and mock-infected samples. Even if they were certainly different, this reviewer was not convinced that these small amounts of
cytokines are effective in vivo and that “excessive proinflammatory cytokine and ...” in the title of the manuscript is adequate.

**Answer:**

The data was based on the *in vitro* infection model with EV71 virus. As shown in figure 3, the increased levels of IL-6 and TNF-α was over ten-fold in EV71-infected MDMs than that in mock (IL-6: 40.48 pg/ml vs. 2.56 pg/ml, p=0.001, n=8, 24-h POI, TNF-α: 72.34 pg/ml vs. 6.68 pg/ml, p=0.001, n=8, 12-h POI.). Significance differences were observed on cytokine levels of infected samples compared to that of mock-infected ones. As discussed in our manuscript, strong responses of systemic or local TNF-α could be triggered by EV71 in human cases. IL-6, IL-1β and TNF-α are thought to be the potent pyrogens inducing fever. Furthermore, the subsequent responses of acute phase proteins and chemokine activations mediated by IL-6, IL-1β and TNF-α could exacerbate virus-induced inflammation and pathology. Therefore, our *in vitro* findings support the clinical findings and indicate that macrophages are an important target for EV71, and they can trigger pro-inflammatory response against viral infection.

**Comment 3:** *In page 9, lines 5-6, the authors discussed that viral proteins of EV71 may be recognized by TLR-2. This is not logically correct. Even though TLR2 expression is elevated after EV71 infection, this does not mean that the EV71 is recognized by TLR2.*

**Answer:**

We agree with you and we have clarified it in the discussion section in our revised manuscript (Line 9-12, Page 9). As reported by Triantafilou K, the reorganization and response to a particular motif or molecular pattern on the virus capsid was mediated by TLR2, one member on cell surface of TLRs family[1]. The up-regulations of IL-8 production and TLR2 mRNA
expression were found in both live virus- and UV-inactivated virus-infected MDMs as compared with mock. Therefore, we speculated the response of MDMs maybe induced via the interaction between TLR2 on cell surface and viral proteins.

REVIEWER 2:

Comment 1: As reported previously, children less that 5-year-old were the most susceptible groups as compared with older children. In this study, adult PBMCs were used instead of children PBMCs. In addition, as shown in this paper by "Seidler et al. 2010. Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. BMC Immunology, 11:30", adult derived monocyte-derived macrophages may possibly secrete different levels and/or cytokines/chemokines as compared with children monocyte-derived macrophages. Are the results demonstrated in this study reflect natural in vivo infections? Authors are required clarified and also discussed it in the discussion.

Answer:

We have addressed the points in the revised manuscript (line 19-25, page9, line 1-6, page10). Actually, the adults can also be infected with EV71. The underlying mechanism for the severity of EV71 in younger children (<5ys) remains unknown. And enhanced proinflammatory cytokines and chemokines were presented in severe cases of children patients with encephalitis or PE as previously reported. In the study, we focused on the responses of macrophages to EV71 virus. Moreover, the clinical severity in adult patients with acute encephalitis was similar to those of EV71 infection in children [2]. Our previous study on influenza virus suggested that a similar cytokines/chemokines profile was found in virus-infected adult and neonatal MDMs and the levels of most of the cytokines/chemokines were comparable [3]. The age-related severity in avian influenza virus infection was associated with differential expression levels of chemokine
receptors on MDMs. Our on-going study on the association of age-related severity in EV71 was being conducted.

**Comment 2:** Immunoflorescence and RT-PCR to confirm EV71 replication in monocyte-derived macrophages. Virus titration should also be performed to confirm the findings.

**Answer:**

We agree with you. Now we have tested virus titers of infectious progeny particles released by EV71-infected MDMs by measuring 50% tissue culture infective dose (TCID50) on Vero cells. Those data was added in the revised manuscript. Please refer to result section, Figure 2 B.

We are grateful to you and the Reviewers for your constructive suggestions in improving our manuscript. We checked our revised manuscript thoroughly including the text, figures, and references. We hope the revised manuscript can be accepted for publication. Please do not hesitate to contact us for further clarifications.

Your sincerely,

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**References**
