

Dear Lian-Sheng Ma, Science Editor, Company Editor-in-Chief, Editorial Office:
Thank you very much for your letter, and the peer-review report. Based on your comment and request, we have made minor modification on the original manuscript. Here, we attach revised manuscript for your approval. A document answering every question from the reviewer is also summarized and enclosed.
Here below is our description on revision according to the reviewer's comments.

Part A (Reviewer 1)

Specific comments: Please add a section in discussion regarding other triggering factors, except mutations, that may be linked with a late onset type 2 familial hemophagocytic lymphohistiocytosis.

By searching the relevant literatures about FHL, I have found several additional factors associated with the late-onset of the disease. A detailed description is given below.

I Gene dosage effect and triggering factors

FHL is generally considered as an autosomal recessive disease, Cetica et al.^[1] analyzed 500 patients with HLH and found 43 (18%) of the 240 patients with sporadic HLH had monoallelic mutations in one FHL-related gene. On testing 28 of the 43 patients, 11 had a partial degranulation defect, indicating that these mutations have a relation to the degranulation pathway. They put forward that there is a gene dosage effect whereby FHL can no longer be regarded as a straightforward recessive disease. Patients with HLH are highly enriched for monoallelic mutations in those same genes that produce FHL when both alleles are mutated. The authors speculated that some patients may have missed a second FHL-related mutation due to the defects of gene sequencing technology, whole-exome sequencing could help solve the problem. Some patients with monoallelic mutations do not develop FHL, which may as a result of insufficient gene dose effect. They also concluded that there is always a triggering factor in the onset of HLH, while in FHL, the most prevalent trigger is the viral infection that can not be eliminated by genetically defective CTLs. In terms of triggering factors, EBV or other herpesviruses are the most frequently reported, there are also some findings about arthritis driven inflammation, macrophage activation syndrome (MAS) and leishmaniasis. They suggest that the clinical characteristics of HLH are usually the result of a combination of an exogenous trigger and genetic predisposition, and the different weights of the two factors leads to a wide disease spectrum that ranges from HLH secondary to severe infection to FHL. Therefore, we speculate that people with FHL-related genetic defects are susceptible to the above triggering factors. Meanwhile, when the influence of the triggering factors is powerful enough, the disease can be induced to develop.

Based on the above discussion, in the case of known gene mutations, the occurrence time and severity of exogenous triggers may explain the difference in the onset time of the disease.

A recent report including 137 adult HLH cases in Germany published in 2020 put forward the spectrum of triggers of the disease^[2]. Although only 20 patients performed perforin sequencing, 3 of them were positive and were diagnosed as FHL2. It can be inferred that the spectrum has certain reference value for FHL. They

concluded that the most common triggering factors were infections (44.5%) and malignancies (35%). In infection-associated HLH, viral infections especially EBV and CMV were most common, bacterial, fungi and parasite infection accounted for a very small proportion. In malignancy-associated HLH, hematologic neoplasia, in particular lymphomas of B-lymphoid origin (21.9%) and T-lymphoid origin (7.3%) were much more prevalent. Another 9.5% of patients were diagnosed to be triggered by autoimmune or inflammatory diseases.

II Correlated effect of FHL-related mutations involved in the cytotoxic pathway

In a study of adult-onset FHL, Zhang et al.^[3] mentioned two double heterozygous patients with the A91V mutation in *PRF1* and a second mutation in either *STXBP2* or *MUNC13-4* developed FHL in adulthood.

In another article they wrote published in *Blood* in 2014^[4], from 2701 patients with clinically suspected FHL, there were 28 patients with single heterozygous mutations in 2 FHL-related genes. The onset age of heterozygotes with variants in *PRF1* and one gene involved in cytotoxic lymphocyte degranulation pathway (*UNC13D* (*MUNC13-4*), *STX11*, *STXBP2*, and *RAB27A*) (*PRF1*/Deg) was later than that of heterozygotes with variants in two degranulation genes (Deg/Deg), *PRF1* single heterozygotes, and *PRF1* homozygotes or compound heterozygotes. Heterozygous mutations of degranulation genes seemed to partially offset the adverse effects of *PRF1* mutations and delay the onset time. The correlated effects of these gene mutations on the related proteins needs further study. Interestingly, 72.7% (8 of 11) of *PRF1*/Deg patients had low perforin expression, but only 20% (1 of 5) of Deg/Deg patients showed decreased perforin expression. This phenomenon also indicated that low perforin expression has no direct correlation with early onset.

Although only compound heterozygous mutations of *PRF1* were detected in our patients, the above hypothesis can be used as another explanation for the delayed onset of FHL.

The above points have been summarized and inserted into the manuscript, as shown in the red font.

References

- 1 **Cetica V**, Sieni E, Pende D, Danesino C, Fusco CD, Locatelli F, Micalizzi C, Putti MC, Biondi A, Fagioli F, Moretta L, Griffiths GM, Luzzatto L, Aricò M. Genetic predisposition to hemophagocytic lymphohistiocytosis: Report on 500 patients from the Italian registry. *J Allergy Clin Immunol* 2016; 137: 188-196 [PMID: 26342526 DOI: 10.1016/j.jaci.2015.06.048]
- 2 **Birndt S**, Schenk T, Heinevetter B, Brunkhorst FM, Maschmeyer G, Rothmann F, Weber T, Müller M, Panse J, Penack O, Schroers R, Braess J, Frickhofen N, Ehl S, Janka G, Lehmborg K, Pletz MW, Hochhaus A, Ernst T, Rosée PL. Hemophagocytic lymphohistiocytosis in adults: collaborative analysis of 137 cases of a nationwide German registry. *J Cancer Res Clin Oncol* 2020; 146: 1065-1077 [PMID: 32076823 DOI: 10.1007/s00432-020-03139-4]
- 3 **Zhang K**, Jordan MB, Marsh RA, Johnson JA, Kissell D, Meller J, Villanueva J, Risma KA, Wei Q, Klein PS, Filipovich AH. Hypomorphic mutations in *PRF1*, *MUNC13-4*, and *STXBP2* are associated with adult-onset familial HLH. *Blood* 2011;

118: 5794-8 [PMID: 21881043 DOI: 10.1182/blood-2011-07-370148]

4 **Zhang K**, Chandrakasan S, Chapman H, Valencia CA, Husami A, Kissell D, Johnson JA, Filipovich AH. Synergistic defects of different molecules in the cytotoxic pathway lead to clinical familial hemophagocytic lymphohistiocytosis. *Blood* 2014; 124: 1331-4 [PMID: 24916509 DOI: 10.1182/blood-2014-05-573105]

Part B (Science editor)

Specific comments:

(1) The “Author Contributions” section is missing. Please provide the author contributions.

The author contributions have been added into the manuscript.

(2) The authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s).

A funding agency copy of approval document has been uploaded. The information of the corresponding author of is highlighted on page 7 of the document.

(3) The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

The original figures has been rearranged by PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Thank you and all the reviewers for the kind advice. We hope that the revision is acceptable and look forward to hearing from you soon.

Sincerely yours,

Feng-Ling Min