Review 1: "Cytotoxic lymphocytes are dysregulated in multisystem inflammatory syndrome in children"

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RR:C19 Evidence Scale rating by reviewer:

- **Potentially informative.** The main claims made are not strongly justified by the methods and data, but may yield some insight. The results and conclusions of the study may resemble those from the hypothetical ideal study, but there is substantial room for doubt. Decision-makers should consider this evidence only with a thorough understanding of its weaknesses, alongside other evidence and theory. Decision-makers should not consider this actionable, unless the weaknesses are clearly understood and there is other theory and evidence to further support it.

Review:

SARS-CoV-2 coronavirus disease 2019 (COVID-19) is associated with the emergence of a novel multisystem inflammatory syndrome in children (MIS-C), which appears 4 to 6 weeks after the SARS-CoV-2 infection peak in areas with wide-spread infection. Some clinical features of MIS-C, such as fever, rash and conjunctivitis, overlap with Kawasaki Disease (KD). MIS-C can lead to systemic inflammation and multiorgan failure, however the immunological events involved in its development remain unknown, and a better understanding of its pathogenesis is needed.

In this preprint, the authors present transcriptome analysis of whole blood samples collected from patients with MIS-C, pediatric COVID-19, and healthy controls (HCs) from the New York area. Although the authors could only identify a few genes that were significantly differentially expressed between MIS-C and HCs, by cell deconvolution they found reduced proportions of circulating CD8⁺ T cells in MIS-C patients. Co-expression network analysis led to the identification of 11 gene modules. Comparing these results to a publicly available dataset of KD patient transcriptomes, the authors identified few shared modules between MIS-C and KD. Compared with HCs, the "Skyblue module", which contains genes related to cytotoxic and exhausted CD8⁺ T cells, was critically downregulated in both MIS-C and KD patients. Furthermore, *TBX21*, a transcription factor considered a biological switch for exhausted T cells and a key driver of the Skyblue module, was also downregulated in MIS-C patients. Based on this analysis, the authors misleadingly conclude that MIS-C and KD may share a similar etiology, and both diseases are associated with the downregulation of cytotoxic and exhausted CD8⁺ T cells responses.

However, this study suffers from important limitations in the study cohort design, such as the small size of the experimental groups, the repeated sampling from same individuals and the lack of an adequate control group. Indeed, 8 children with MIS-C and 7 children with pediatric COVID-19 were analyzed and compared with just 4 HCs, and this small number of individuals per groups severely underpowers the analysis. Furthermore, the reason repeated samples were collected from the pediatric COVID-19 group, and the time course of their collection remain unclear. Moreover, the pediatric COVID-19 group used may not immunologically be representative of pediatric COVID-19 patients, as 6 out the 7 enrolled children were chronically immunocompromised and treated with either chemotherapy or immunosuppressive drugs. Therefore, gene expression data obtained from this group must be considered with extreme cautions. Another limitation is the lack of adequate age-matched control group, as the HC volunteers were adults. Although the authors "artificially" corrected the gene expression variation due to age differences in their analysis, there is no information provided regarding the COVID-19 status of the adult HCs used in this study. Ideally, the control group should comprise age-matched individuals selected based on a negative SARS-CoV-2 RT-PCR test or serologic test.

This study also furthers the misconception that MIS-C and KD are similar diseases with the same etiology. Although MIS-C and KD appear to share some clinical features, it is evident today that MIS-C and KD are two different entities, as MIS-C patients also suffer from gastrointestinal, neurological and hematological symptoms which are rarely observed during KD, and are more reminiscent of toxic shock syndrome (1). The authors' conclusion that KD and MIS-C share a molecular signature is misleading, as only 4 of the 12 gene modules presented in Figure 4 are enriched in the same direction in MIS-C and KD. Since most of the identified modules are not shared between the 2 conditions, this analysis indicates at best some partial overlap. In addition, the gene modules identified as overlapping between MIS-C and KD may just be reflective of a shared intense acute inflammatory response, with two different etiologies.

The conclusion that both MIS-C and KD exhibit a dysregulated cytotoxic T cell response associated with the downregulation of genes related to CD8⁺ T cell exhaustion needs to be considered with extreme caution, as this study relies entirely on transcriptome analysis and inferred gene expression. In contrast, using high dimensional flow cytometry and cellular immunophenotyping, Vella *et al.* demonstrated in a recent preprint that frequencies of circulating exhausted or chronically stimulated CD8⁺ T cells, identified by the co-expression of both PD-1 and CD39, are substantially increased in MIS-C patients compared to pediatric COVID-19 patients (2).

In summary, although the topic investigated is of high interest and the evidence provided in this preprint might potentially be informative, the data must be considered with caution due to an imperfect experimental design, highly descriptive results and some misleading conclusions.

REFERENCES

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2. Vella L, Giles JR, Baxter AE, Oldridge DA, Diorio C, Alanio C, et al. Deep Immune Profiling of MIS-C demonstrates marked but transient immune activation compared to adult and pediatric COVID-19. *Medrxiv*. 2020.