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Peer Review File

Gasdermin-E-mediated pyroptosis drives immune checkpoint inhibitor-associated myocarditis via cGAS-STING activation

Editorial Note: This manuscript has been previously reviewed at another journal. This document only contains reviewer comments and rebuttal letters for versions considered at Nature Communications.

REVIEWER COMMENTS

Reviewer #5 (Remarks to the Author):

In this manuscript by Sun et al. the authors explore the role of gasdermin E in immune checkpoint inhibitor (ICI)-associated myocarditis. Hereto, two models of ICI-associated myocarditis were used. I have the following comments.

1) Authors should explain the rationale why using the two specific ICI-myocarditis models also in view of the clinical scenario. This should be implemented in the results and discussion.
2) In the methods section, info about the sex of the mice is missing, which is not according to the ARRIVE guidelines. This should be updated particularly considering the impact of sex on the immune system and its relevance in myocarditis.

Point-to-point response to Reviewer #5

Reviewer #5

In this manuscript by Sun et al. the authors explore the role of gasdermin E in immune checkpoint inhibitor (ICI)-associated myocarditis. Hereto, two models of ICI-associated myocarditis were used. I have the following comments.

Reply: We would like to extend our heartfelt gratitude to you for prompt and professional review. The suggestion regarding two models of ICI-associated myocarditis is indeed helpful in enhancing the quality of our manuscript. In accordance with your guidance, we have revised our manuscript. Thank you again for your positive comments on our work.

Q1. Authors should explain the rationale why using the two specific ICI-myocarditis models also in view of the clinical scenario. This should be implemented in the results and discussion.

Reply: We appreciate this important comment. According to your comment, we added the rationale of using the two specific ICI-myocarditis in both “Results” and “Discussion” sections (**Line 105-108, 287-294** and **506-514**). Thank you immensely for this suggestion, as it greatly enhances the clinical relevance of our manuscript.

overdose anti-mouse PD-1 for one month (**Figure 1A**). This approach aimed to mimic the clinical scenario where cancer patients are treated solely with anti-PD-1 antibody, as approximately 70% of total cardiac irAEs are attributed to the administration of anti-PD-1 antibody alone.[21] Treatment of aPD-1 successfully inhibited tumor growth (**Supplemental Figure 1A**) and inhibited tumor-induced death (**Supplemental Figure 1B**). Flow cytometry

Line 105-108

Deficiency of Gsdme ameliorates myocarditis in Pdcd1$^{-/-}$ receiving anti-Ctla4 treatment

In clinical practice, it has been observed that combined therapy with anti-PD1 and anti-CTLA4 in advanced cancer patients is associated with a higher incidence of ICI-induced myocarditis.[32,33] Additionally, studies in mice have shown that combined inhibition or simultaneous deletion of PD-1 and CTLA4 replicates pronounced ICI-induced myocarditis without additional interventions such as tumor.[7,8,34,35] Taking these findings into consideration, we employed the second animal model of ICI-induced myocarditis involving combined inhibition of PD-1 and CTLA-4 without tumor to validate our aforementioned observations in tumor-bearing mice treated with aPD-1 alone. We generated PD-1/GSDME double knockout

Line 287-294

504 In our study, we employed two mouse models with ICI-associated myocarditis. One model
505 was induced by injecting antibodies against PD-1 into tumor-bearing mice, while the other was
506 induced by injecting antibodies against CTLA4 into PD-1 knockout mice. The former model
507 was designed to replicate the treatment of new-onset malignant patients typically with only
508 anti-PD-1 antibody. Notably, approximately 70% of total cardiac irAEs are associated with the
509 administration of anti-PD-1 antibody alone.[21] The latter model was utilized to simulate the
510 treatment of advanced cancer patients undergoing combined therapy with both anti-PD-1
511 antibody and anti-CTLA4 antibody for improved survival[32,33]. As a result, our experimental
512 setup encompassed both clinical scenarios. Remarkably, consistent results were observed in
513 both models of ICI-induced myocarditis: the deletion of GSDME inhibited the development of
514 ICI-induced myocarditis. Regarding the model induced by injecting antibodies against PD-1
515 into tumor-bearing mice, it should be noted that anti-PD-1 treatment alone in normal C57BL/6

Line 506-514

Q2. In the methods section, info about the sex of the mice is missing, which is not according to the ARRIVE guidelines. This should be updated particularly considering the impact of sex on the immune system and its relevance in myocarditis.

Response: We are sorry for this neglect and deeply appreciate your valuable comment. Typically, our cardiac research only employed male mice due to the widely recognized differences in cardiac function between male and female mice. Taking into account that female tend to exhibit greater resistance to myocarditis compared to males[1-3], we opted to exclusively employ male mice in our study. This experimental approach aims to minimize variability factors in our results to the greatest extent possible. The information of sex of mice was added into the revised manuscript in “Method” section (**Line 644-646**) and “Abstract” section (**Line 45**).

643 mice. The monoclonal anti-mouse Ctla4 antibody (BioXcell, IgG2b, clone: 9D9, #BE0164) at
644 a normal dose (200 μg/dose, i.p., 4 injections in 20 days) was administrated. In this study, only
645 male mice were utilized to induce ICI-myocarditis, aiming to minimize variability factors, as
646 females tend to display greater resistance to myocarditis compared to males[67].

Line 644-646

43 myocarditis (anti-PD-1 treatment in tumour-bearing mice and anti-Ctla4 treatment in PD-1$^{-/-}$
44 mice) and in cancer patients diagnosed with ICI-induced myocarditis. Deficiency of GSDME
45 in male mice alleviates ICI-induced infiltration of T cells, macrophages, and monocytes, as
46 well as cardiac mitochondrial damage and inflammation. GSDME is prominently expressed in
47 cardiomyocytes within the heart. Notably, restoration of GSDME expression specifically in

Line 45

Reference

1. Gerdts, E. & Regitz-Zagrosek, V. Sex differences in cardiometabolic disorders. *Nat Med* **25**, 1657-1666 (2019).
2. Fairweather, D. *et al.* Sex and gender differences in myocarditis and dilated cardiomyopathy: An update. *Front Cardiovasc Med* **10**, 1129348 (2023).
3. Kyto, V., Sipila, J. & Rautava, P. Gender differences in myocarditis: A nationwide study in Finland. *European Heart Journal* **34**, 3505 (2013).