Qeios

Peer Review

Review of: "Phosphatidylserine (PS)-Targeting Chimeric Interferon (IFN) Fusion Proteins for Anti-Tumour Applications"

Jonas Wolf[1]

1. Universidade Luterana do Brasil, Brazil

The manuscript titled "Phosphatidylserine (PS)-Targeting Chimeric Interferon (IFN) Fusion Proteins for Anti-Tumour Applications" presents a highly innovative and compelling approach to cancer immunotherapy. The authors describe the development of a first-in-class fusion protein combining Type I and Type III interferons linked to the phosphatidylserine-targeting domains of Gas6. The rationale of exploiting the chronic externalization of phosphatidylserine in the tumor microenvironment to deliver a localized immunostimulatory payload is scientifically sound and addresses a critical need in treating cold tumors. The study is comprehensive, spanning molecular design, in vitro biochemical validation, and in vivo syngeneic tumor models. However, to maximize the clinical and translational impact of this work, several methodological clarifications, experimental controls, and textual revisions are required. The following critical review outlines broad and specific opportunities for improvement to strengthen the manuscript for publication.

Regarding broad opportunities for improvement, the most significant limitation of the current study design is the methodology used for the in vivo tumor models. The authors demonstrate anti-tumor efficacy by injecting E0771 and B16-F10 cells that have been stably transfected to constitutively secrete the fusion proteins. While this elegantly proves that the presence of the cytokine in the tumor microenvironment alters tumor growth, it completely bypasses the pharmacokinetic and pharmacodynamic hurdles of systemic drug delivery. The ability of the Gas6 domain to successfully home to the tumor site from systemic circulation is a cornerstone of the therapeutic premise, yet it remains untested in vivo. The authors must explicitly state this limitation in the abstract and discussion,

 doi.org/10.32388/7OSSRU

acknowledging that future studies using intravenous administration of the purified recombinant biologic are essential. Additionally, the manuscript suffers from widespread typographical errors related to the Greek letter lambda, which is frequently and erroneously replaced by numbers such as 12 or 22 throughout the text. A thorough proofreading is necessary to correct these nomenclature errors.

Specifically, the following modifications are required across the manuscript pages. On Page 1 within the Abstract, the text should be amended to clarify that the in vivo efficacy was demonstrated using ectopic expression models rather than systemic delivery of a purified biologic. On Pages 5 and 6 in the Materials and Methods section, the authors describe the purification of recombinant proteins using Expi293T cells and TALON resin. The manuscript would benefit from the inclusion of specific quantification regarding the final protein yield and purity percentages, perhaps in a supplementary table, to confirm the scalability of this manufacturing process.

On Page 12, the authors note a crucial observation regarding off-target toxicity. They mention that mice bearing tumors expressing a combination of single interferon molecules developed ascites and anaemia, whereas those with the fusion protein did not. Because reducing systemic toxicity is a major advantage of targeted therapies, this observation should be supported by quantitative data, such as complete blood counts or histopathological scoring of the liver, rather than relying solely on gross anatomical photographs.

On Page 14, the authors briefly mention that the fusion proteins significantly reduce the efferocytosis of apoptotic cells by macrophages, referencing Supplementary Figure 3g. Given that the blockade of efferocytosis fundamentally alters the immunological landscape of the tumor and could potentially induce secondary necrosis, this finding is too important to be relegated to the supplement. This data should be moved to a main figure, and the text on Page 14 should be expanded to discuss the dual impact of efferocytosis blockade and interferon signalling on macrophage polarisation.

On Page 24, the bulk RNA sequencing experiment utilizes murine lung epithelial cells treated with the fusion protein. The authors must add a justification on this page explaining why a benign lung epithelial cell line was chosen for transcriptomic profiling instead of the E0771 breast cancer or B16-F10 melanoma cell lines that were actually used for the in vivo efficacy models.

To further assist the authors in refining their study, I present the following ten questions for consideration and response.

Question One: Why was ectopic expression within the tumour cells chosen over systemic intravenous administration of the purified Gas6 fusion protein for the in vivo efficacy models?

Question Two: Can the authors provide quantitative pharmacokinetic and half-life data for the purified fusion protein to determine its stability in systemic circulation?

Question Three: What is the proposed mechanistic explanation for why the physical fusion of interferon-beta and interferon-lambda reduces the systemic toxicity, such as ascites and anaemia, that was observed when the two cytokines were co-expressed as separate molecules?

Question Four: Why were murine lung epithelial cells specifically selected for the RNA sequencing transcriptomic profiling instead of the established E0771 or B16-F10 tumour cell lines?

Question Five: In the efferocytosis assay, does the blockade of macrophage clearance by the fusion protein carry a theoretical risk of inducing secondary necrosis and unintended autoimmune responses in healthy tissues?

Question Six: How does the binding affinity and dissociation constant of the Gas6 Gla-EGF domains compare to standard Annexin V when evaluated quantitatively via surface plasmon resonance?

Question Seven: Given that the RNA sequencing and flow cytometry data demonstrate that the fusion protein upregulates PD-L1 expression, are there ongoing or planned in vivo experiments to combine this biologic with anti-PD-1 or anti-PD-L1 checkpoint inhibitors?

Question Eight: What specific statistical normality tests were utilised to ensure that the data met the assumptions of normal distribution and equal variance prior to performing the reported ANOVA analyses?

Question Nine: How might the presence of the 6X His tag affect the immunogenicity or clearance rate of the fusion protein during prolonged exposure in the murine models?

Question Ten: Could you clarify the exact biochemical composition of the flexible linker used between the domains and discuss whether its specific length was optimised to prevent steric hindrance between the two distinct interferon receptor binding sites?

Declarations

Potential competing interests: No potential competing interests to declare.