ÚOCHB AV ČR
IOCB PRAGUE
Ústav organické chemie a biochemie
Akademie věd České republiky, v. v. i.
Institute of Organic Chemistry and Biochemistry
of the Czech Academy of Sciences

Opponent's review of the PhD thesis
Cancer Immunotherapy exploiting engineered antibody fragments against prostate-specific membrane antigen

Author: Msc. Gargi Das
Supervisor: Dr. Cyril Bařinka, PhD

This doctoral thesis focuses on the development of various antibody fragments from the antibody 5D3, targeting the crucial therapeutic marker PSMA in prostate cancer. The development of innovative targeting strategies against PSMA holds significant importance. The thesis successfully establishes that antibody fragments derived from 5D3 represent a promising tool (published in *International Journal of Molecular Sciences*, the candidate is a co-author). Building on this knowledge, a novel 5D3-based BiTE (published in *ACS Pharmacology and Translational Science*, the candidate is the primary author) was created to guide T cells towards PSMA-expressing cells. The yet-unpublished segment of the thesis introduces the development of 5D3/anti-CD64 (FcγRI) engagers, demonstrating their potential in triggering immune responses in monocytes when in the presence of PSMA-expressing cells. The presented data are not only relevant and highly important but also establish a robust foundation for further investigations.

The thesis follows a conventional structure encompassing introduction, aims, methodology, results, discussion, and conclusions supplemented by a list of literature and two appendices (containing the publications). Unfortunately, the individual chapters and subchapters lack numbering, impeding text navigation and a considerable number of typos, grammatical errors, and unclear language hinder overall readability.

I won't analyze the published data as they have already undergone review and been accepted. Instead, my focus will be on the unpublished portion, where I have identified the following shortcomings:

1) Statistical significance: There is no mention of whether the presented data were repeated, and the explanation of what the error bars represent is lacking. This makes it difficult to assess the reproducibility of the submitted data.
2) The flow cytometry experiments lack an explanation of the gating strategy. This is particularly crucial in Figure 11 (more below), where it is unclear how and why the quadrants were chosen the way they are displayed.

3) Fig 9 C, D, E, F: It is not clear which cells were used for the experiments. Additionally, it appears that the functional assays (ROS, phagocytosis) were conducted in different cell lines, posing challenges in correlating Kd values from the binding assay with results from functional studies. Could you provide clarification on this?
4) Fig 11 A: This is the most problematic part! Nowhere is it explained what each dot blot is or why the quadrants were set in this specific way!

Furthermore, I have the following questions to the candidate:

1) Previously, it has been shown that CD64 triggering with a monomeric ligand leads to rapid internalization of the receptor to the endosomal compartment, followed by quick recycling to the cell surface. To initiate CD64 signaling, achieving ligand multivalency is crucial. On page 38, the author notes: "5D3-CP33 was designed to directly connect PSMA-positive cells to FCγRI-positive cells and trigger clustering of FCγRI at the cell surface, resulting in the signaling for ROS production". How does the clustering of CD64 occur when the receptor is stimulated through monomeric antibody-peptide fragments?
2) In Fig 9D, it is shown that the binding affinity of the 5D3-CP33 fragments to cell-bound CD64 is around 140 nM. This value is 10-100 times lower than the published affinity of IgG1 binding to CD64, shown to be 1-10 nM. Considering that CD64 in the blood is thought to be saturated with plasma IgG1, do you believe that your antibody-peptide fragments can displace IgG1? How do you envision your fragments working *in vivo*?
3) Apart from creating the 5D3-derived cell engagers, are there other therapeutic applications for the antibody itself or its fragments? Could you please discuss the potential options, advantages, and disadvantages?

Considering all aspects, although the candidate should enhance her attention to detail, I believe she has acquired a diverse range of experimental techniques and has authored two nice publications. This experience will undoubtedly benefit her in her future endeavors. **Therefore, I recommend the thesis for defense and propose awarding Gargi Das the Ph.D. title**.

In Prague, January 22nd, 2024 Tereza Ormsby, Ph.D.

Flemingovo nám. 2
166 10 Praha 6
Czech Republic
+420 220 183 333
uochb@uochb.cas.cz
www.uochb.cz
IČ: 61388963
DIČ: CZ61388963