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Reviewer's report on the dissertation thesis of

MSc. Gargi Das

"Cancer immunotherapy exploiting engineered antibody fragments against prostate-specific membrane antigen"

The present dissertation thesis was prepared at the Faculty of Science of Charles University as part of the Immunology Ph.D. study program under the supervision of RNDr. Cyril Bařinka, Ph.D. at the Laboratory of Structural Biology at the Institute of Biotechnology, Czech Academy of Sciences (BIOCEV). It is written in English on 71 pages of text and 46 pages of appendices, which are the attached two publications forming the professional basis of the thesis. Both publications were already published by peer-reviewed journals; the Ph.D. candidate is the first author of one of the two articles, and on the other, she is a co-author. Apart from these publications, the thesis also contains separate materials and methods, results, and discussion sections related to the yet unpublished experimental work of the candidate. This last piece of research work, together with the first-author manuscript, forms the essential part of the thesis, with respect to the amount of experimental work performed by the candidate and concerning the space given to this topic within the thesis.

The thesis is structured in a classical subdivision, with the introduction section covering the topics of prostate cancer and its possible immunotherapies, protein and antibody engineering, with the focus on anti-PSMA recombinant protein engagers, followed by the thesis's objectives, materials, and methods, results (also including commentary on the published scientific articles), discussion, conclusion, and references sections. While I appreciate the concise introduction, the commentary on the published works is rather succinct and limited to describing their main findings; however, the specific candidate's contribution toward them could also be explained.

This minor criticism, however, does not detract from the professional standard of the work, which is very high (both formally and graphically – although the thesis's chapters could have been numbered and more visibly shown in the text). I am confident to say that the results of the present work have advanced this research by a great deal. The candidate mastered a wide range of molecular biological and biophysical techniques, from heterologous expression of proteins to their functional and structural characterization. This is especially evident from the included (yet unpublished) results on the production and biological characterization of the monocyte-activating bispecific engagers, which I consider an innovative and promising piece of science. An overall evaluation of the results obtained is made in the final discussion, as well as their thorough comparison with the present state of knowledge. Comparing the set goals and the achieved results, I cannot but conclude that the candidate has achieved everything she set out to do at the beginning of the thesis.

In conclusion, I am pleased to say that the submitted dissertation thesis is, in my opinion, a very successful work. With its elaboration, MSc. Gargi Das showed distinct creative skills and knowledge and the ability to work independently as a scientist. The thesis fulfills the requirements of the relevant legal provisions, and I therefore fully recommend it for defense.

<u>Questions for discussion</u>:

1) What is the species of origin of the used 5D3 monoclonal antibody? Is it a mouse IgG? Would it pose an obstacle for the intended therapeutic use of your bispecific engagers? If yes, what would be your approach to tackle it?

2) In your discussion on page 46, you explain that to ensure optimal BiTE functionality, the depletion of Treg cells is essential. At the same time, stimulation or expansion of the cytotoxic effector T cells would be desirable. Have you considered a combination therapy with some of the IL-2-based immunostimulatory therapeutics, such as recombinant immunocytokines (e.g., those described in DOI: 10.4049/jimmunol.2300467) capable of biasing the immune response in this desired direction?

3) Do you have any plans for commercialization / technology transfer / clinical trials of your engagers? Are there any similar products already on the market or under development?

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