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Review of doctoral dissertation by MSc. Ewa Marta Sroka entitled "The role of alternative sources of antigenic peptides for the major class I histocompatibility complex in the formation of immune responses and immune tolerance" realized at the International Center for Cancer Vaccine Research. The supervisor of this dissertation is Dr. Robin Fahreus

1. Formal assessment:

The basis for the implementation of this review is a letter from the Chairman of the Biological Sciences Discipline Council of the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk, prof. Mariusz Grinholc of July 1, 2022. The doctoral dissertation presented to me for review consists of 142 pages of typescript in the form of a computer printout in paperback, divided into the following parts: table of contents, abstract (pp. 9-11), abstract (pp. 2-3), list of abbreviations (pp. 4-7), introduction (pp. 8-43), aims of studies (pp. 43-46), materials and methods (pp. 47-62), results (pp. 63-100), discussion and perspectives (pp. 101-117), conclusions (pp. 118-120), biography (pp. 121-139), scientific achievements (pp. 140-142), acknowledgments (pp. 143). The layout of the dissertation, the proportions between the chapters, and their order are typical of a scientific monograph. The doctoral dissertation, apart from the summary in Polish, is written entirely in English. The author freely uses the English-language, and specialized scientific vocabulary. The text of the dissertation is carefully edited. It is a pity that there is no list of 28 figures and 8 tables in the text, which would be helpful in quickly finding interesting content. The four illustrations used in the introduction come from review publications. References to the sources are provided in the captions under these pictures. Descriptions of the figures presenting the experimental data are clear and unambiguous, and the attached statistical significance analysis facilitates their evaluation. The bibliography contains 240 references to the current professional literature, mostly published after the year 2000. The use of references to the cited literature is correct and does not raise any objections. I state that in formal terms, this dissertation meets all the criteria customarily adopted for this type of work and proves that the Author has mastered the art of writing scientific texts and presenting the results of research in English.

2. Substantive assessment:

The subject of this dissertation is the development and validation of new in vitro and in vivo research models that would be helpful in assessing the role of antigenic peptides encoded outside canonical exon sequences in maintaining immunological tolerance and generating an anti-tumor response. The above topic results directly from the new understanding of the

dominant sources of origin of antigenic peptides presented by the molecules of the major histocompatibility complex class I (MHC I). Based on the breakthrough discoveries of recent years, including those announced by the team of Professor Fahreus, it is recognized that the main source of antigenic peptides presented by MHC-I is not the products of proteolytic degradation of native proteins, but fragments of proteins resulting from pioneering or various defective RNA translation mechanisms by the ribosomes. Moreover, it is postulated that the pioneering round of translation of primary RNA transcripts (containing introns) takes place inside the cell nucleus and is carried out by a separate class of ribosomes (so-called immunorbosomes). This last aspect of research goes beyond the commonly accepted dogmas in molecular biology and is the subject of an ongoing academic debate. Undoubtedly, however, the technical progress in the field of visualization of the translation process using the ribopuromycylation method (RPM), or the demonstration of the importance of the pioneer round of translation process in the creation of antigen peptides made in recent years by teams of professors Shastri, Yewdell and Fahreus significantly strengthens the credibility of the postulated theses. However, there is still a need for experimental evidence allowing for a better assessment of the physiological significance of the above-described phenomena. Hence, in my opinion, the subject of the dissertation presented to me for evaluation is fully justified and is in the vanguard of concepts combining the fundamental processes governing the synthesis and degradation of proteins, and the maintenance of immune tolerance and stimulation of the immune response.

The seven sections of the introduction present an extensive literature review covering the most important issues related to the topic of the dissertation. The entire text is clear, and the argumentation in a very suggestive way focuses the reader's attention on the issues described in the resulting part of the work. The gift of a synthetic presentation by the author of many complex research concepts and detailed molecular mechanisms is noteworthy. It is also worth emphasizing the mature caution with which the author presents the research results, not fully accepted by the scientific community, eg on page 33. Reading the introduction gives the impression of the PhD student's commitment to in-depth study of the literature on the subject of the dissertation and presenting it to the reader in an interesting and accessible way.

The two main objectives of the doctoral dissertation, i.e. in vitro research on a gene construction encoding the immunogenic peptide SIINFEKL in intron 2 of the beta globin gene and in vivo research on the produced knock-in mice, in which the analogous gene structure as above was introduced into both alleles of the beta globin gene. There are also 6 specific tasks mentioned for in vitro testing and 8 specific tasks for in vivo testing on mice. All the research goals mentioned are clear and correctly formulated.

In the "materials and methods" chapter, the description of the research methods used in the work has been treated in detail and reliably. The variety of laboratory techniques used is worth emphasizing. The PhD student has mastered the methodology of lentiviral vector creation, directed mutagenesis, ribosome fractionation, PLA (proximity ligation assay) technique, nucleic acid isolation and analysis (RNA and gDNA), protein analysis by Western

blotting, and numerous cell techniques, including adoptive transfer of T lymphocytes and flow cytometry.

The results are presented in 8 subsections. Figure 5 (page 64) shows the gene construction named by the Author β-globin-SL8. It has been shown that introns 1 and 2 are effectively spliced after transient transfection into target cells. It is not clear to me, however, whether the splitting efficiency of intron 2 modified by sequences encoding the neomycin resistance cassette (NeoR) and the SL8 peptide, in this case, is greater, less than or equal to the unmodified intron 2? In my opinion, this is important because the reduction of splicing efficiency may have a direct effect on the creation of a pool of RNA transcripts retaining intron 2, which is indirectly confirmed by the results from Figure 6, where the Ex2 / I2SL8 and NeoR / NeoR primers show only a slight decrease in expression compared to Ex2 / Ex2 amplicon. In relation to this experiment, as well as to the experiment in Fig. 9, 14, and in figure 21 I also have a technical reservation. The materials and methods section on page 57 describes the methodology for RNA isolation using the RNeasy Plus Mini Kit. It uses the genomic DNA degradation procedure. Has the effectiveness of this procedure been determined, for example by amplifying a non-reverse transcribed (-RT) RNA sample? Such a procedure would undoubtedly exclude the presence of a plasmid or contamination of genomic DNA in the samples, which could have a negative effect on the obtained results. Figure 17 shows adapted ribosome profiling to establish an alternate translation initiation site for the SL8 peptide. It would be worth presenting in panel B the negative result obtained with the use of the Fw2 primer. How far from the predicted alternate translation start site does this primer connect to the template relative to the Fw1 primer? Figure 21 describes the expression of the transgene in various organs. How to explain the expression level obtained in the spleen (about 10^3) with the expression level in splenocytes (<4)? Figure 22 suggests that HBB expression is higher in the bone marrow than in WT mice. Would the l densitometric evaluation of the signal strength show statistical significance in this case? Figures 25 C and D show the histopathological analysis of the spleen after intravenous infusion of activated CD8 + OT-1 T cells into HBB mice. Due to the high expression of HBB mRNA in the bone marrow previously shown in Figure 21, in the case of SL8 peptide presentation, one would expect a loss of erythropoietic precursors in the bone marrow and spleen, which would consequently lead to progressive symptoms of anemia in the blood of HBB mice. Has the blood count in this regard been analyzed?

The above questions and comments, resulting from the reviewer's duty, are of an organizational nature and are the starting point for discussions. They in no way question whether they detract from the positive results obtained by the PhD student as part of the doctoral project. In my opinion, it is particularly significant in the conducted research to show that the peptide encoded by the intron sequence is expressed in vivo, which has been documented in Figure 24 C, where adoptive transfer of OT-1 CD8 + lymphocytes to HBB mice caused their proliferation almost three times higher than for WT mice. Additionally, using the ribosome footprinting technique, a potential translation initiation site of this peptide was identified, suggesting a mechanism of its formation (Figures 16 and 17). Equally significant is the observation presented in Figures 24 and 28, where the transfer of dendritic or tumor cells presenting the SL8 peptide by MHC-I to HBB mice resulted in a significantly lower proliferation

of specific CD8 + T cells in these mice compared to control mice suggesting that the SL8 peptide encoded in the intron is able to stimulate the mechanisms of immune tolerance. The expected analysis of the maturation of T lymphocytes in the thymus in the currently created OT-1 / RAG KO / HBB triple-transgenic mouse model will allow determining, among other things, to what extent the SL8 peptide is able to cause the deletion of CD4 + 8 + thymocytes, which would indicate the stimulation of the central immune tolerance mechanism.

In the discussion, the author confronted the obtained results with the literature on the subject. She discussed the cognitive significance of the experiments in a balanced and critical way. In this context, the critical evaluation of the innovative PLA technique used in the work to determine the subcellular localization of the SL8 peptide precursors deserves special mention (page 107). This chapter also presents a very interesting perspective of using the obtained results to enhance the presentation processes of neoplastic neoantigens for the purposes of immunotherapy (page 119).

3. Summary:

To sum up, the doctoral dissertation presented to me for review is an original solution to a significant scientific problem, demonstrates the general knowledge of the doctoral student and the ability to independently conduct scientific research, and present and critically discuss their results. The scientific value of the presented research is significant considering that original research models were created and characterized, the analysis of which allowed for drawing valuable conclusions about alternative sources of antigenic peptides, important for the processes of immunological tolerance and that may be used in cancer immunotherapy. The results of these studies contribute to a significant progress in the scientific discipline represented by the PhD student.

4. Final conclusion:

In my opinion, the doctoral dissertation submitted to me for review meets the conditions set out in Art. 13 sec. 1 of the Act of March 14, 2003 on academic degrees and academic title as well as degrees and title in the field of art (Journal of Laws No. 65, item 595, as amended).

That is why I submit an application for admission to the High Scientific Discipline Council of Biological Sciences of the University of Gdańsk and the Medical University of Gdańsk, MSc. Ewa Maria Sroka to the next stages of the doctoral dissertation. Taking into account the scientific value of the obtained results and the original solution of the research problem expanding the knowledge of the fundamental mechanisms involved in the production of antigenic peptides for the major histocompatibility system, I apply for this doctoral dissertation to be distinguished.

Arkadiusz Miazek

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