INSTITUTE OF BIOORGANIC CHEMISTRY
Polish Academy of Sciences

Noskowskiego 12/14, 61-704 Poznań
tel.: +48 61 852 85 03, secretariat +48 61 852 89 19
fax: +48 61 852 05 32, e-mail: ibch@ibch.poznan.pl
REGON 000849327 VAT no. PL 7770002062
http://www.ibch.poznan.pl

Prof. dr hab. Jerzy Ciesiołka

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Review of the PhD thesis of mgr inż. Ewa Maria Sroka
presented to the Scientific Council of Biological Sciences of the University of Gdańsk with a view to obtaining a PhD degree in the field of Natural Sciences in the discipline of Biological Sciences

Thesis title: The role of alternative sources of antigenic peptides for the major histocompatibility complex class I in the formation of immune responses and immune tolerance

The research presented in the thesis was conducted at The Intercollegiate Faculty of Biotechnology of the University of Gdansk and the Medical University of Gdansk, under the supervision of Dr Robin Fahraeus.

The thesis is organized in a standard way and its main sections are as follows: Introduction (36 pages), Aims of the study (3 pages), Materials and Methods (16 pages), Results (38 pages), Discussion and Perspectives (17 pages), Conclusions (3 pages), and the Bibliography list containing 240 items. The thesis also contains the Abstract, List of Abbreviations and the list of Scientific Achievements of the Author.

The work described in the thesis aims at a better understanding of the physiological role of intron-derived antigenic peptides for the major histocompatibility complex class I (MHC I) pathway. In brief, a model mRNA was created which encodes the 8-aminoacid-long SL8 epitope in intron 2 of β-globin mRNA. Studies performed in vitro showed that the SL8 sequence can be translated from pre-spliced mRNA, without affecting the main open reading frame and the rate of translation of full-length proteins. Isolation of ribosome footprints confirmed translation initiation within a 27-nucleotide-long stretch upstream of the SL8 insert. Subsequently, a novel mouse model encoding SL8 sequence was developed and the role of intron-derived SL8 oligopeptide for the MHC I pathway was investigated in vivo.

In the first chapter of the Introduction, the major discoveries regarding the knowledge how the immune system perceives self- versus non-self-recognition is reviewed from a historical perspective. Special emphasis is put on the achievements of Nobel prize winners who paved the path for the development of knowledge in the field of immunology and cellular biology. In the following chapters of the Introduction section, the Author briefly describes the main features of the MHC class I and II

presentation pathways, and further focuses in detail on the MHC class I pathway for direct antigen presentation. In the next, shorter chapters, the MHC II pathway of antigen processing and presentation is described, as well as the MHC I cross-presentation by Dendritic cells, and the self- and non-self-recognition is characterized. In the final chapter, the Author outlines the role of peptides in MHC I and II complexes in central and peripheral immune tolerance. Overall, the Introduction chapter is very informative, clearly focusing on the major aspects of studies relevant to those described in the thesis. From the Reviewer's point of view, I have to note the lack of numbering of chapters in the Introduction section. The note regarding the formal arrangement of the dissertation content also applies to the Materials and Methods section, which is divided into chapters in the text, which are not, however, listed in the Table of Contents.

The Results section is divided into two parts based on the experimental models used for the evaluation of the functional role of intron derived antigenic peptides - in vitro and in vivo. In the first part of the in vitro studies, encompassing chapters I to V, the Author describes the development of model β-globin-SL8 mRNA encoding the 8-aminoacid-long SL8 insert in intron 2. Evidence is provided that the insert does not affect splicing of that mRNA when an appropriately designed RT-qPCR assay is used. Subsequently, an in vitro antigen presentation assay was applied to confirm that the intron-derived-SL8 sequence can generate antigenic peptides and be presented on the Kb MHC class I molecules on the cell surface. Further experiments were designed to establish the RNA source from which the intron-derived SL8 peptide was translated. Detailed analysis of the polysome profile from the transiently transfected cells with the model mRNA construct, performed also in the presence of splicing inhibitor Isoginkgetin, showed that pre-spliced mRNA is actively translated from an alternative open reading frame. Another assay, the proximity ligation assay, successfully detected the presence of intron-derived peptide products. Subsequently, the Author put a lot of effort in identifying the translation initiation site of the β-globin-SL8 mRNA construct. To this end, several constructs were synthesized with mutations in presumable translation START and STOP codons. Analysis of RNA expression and antigen presentation using the mRNAs with substitutions of potential START codons did not show substantial changes compared to the results observed with the initial mRNA. An analysis of antigen presentation from the constructs in which potential STOP codons were mutated appeared to be more successful. The results strongly suggest that the synthesis of the SL8-carrying peptides is initiated within 27-nucleotide-long stretch of the model mRNA. This observation was verified by performing a modified ribosome profiling experiment with the use of inhibitors maintaining the ribosome at the position of translation initiation. After RNase treatment, the expected 27-nucleotide-long oligonucleotide was identified among RNA fragments protected by the ribosome using reverse transcription with a specifically designed stem-loop primer followed by qPCR. The Author hypothesized that two adjacent leucine codons CUG present in-frame

with SL8 encoding sequence may be implicated in translation initiation but their mutation did not affect expression of the SL8-carring peptide substrate.

The results described in this part of the thesis are very meaningful for the hypothesis assuming that the translation process, but not the degradation of full-length proteins, is the source of antigenic peptides for the MHC complex class I pathway. The interpretation of experimental results is clear and convincing. However, in my opinion, some additional information on possible translation initiation mechanisms could be drawn from an analysis on the mRNA level. Non-AUG codons have been implicated in translation initiation quite often and the role of the mRNA structure in this process has been acknowledged (for example, reviewed in: D. E. Andreev et al., 2022, Genome Biology 23:111). Has the Author checked how the nucleotide sequences around the analysed codons agree with the preferable context of non-standard translation initiation codons? Additional information could also come from computer prediction of secondary structure elements present in this mRNA region. I would appreciate if the Author could comment on that issue during the public defense of her thesis.

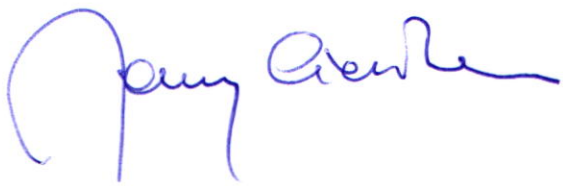
Mgr Ewa Sroka begins the second part of the Results section with describing the establishment of a mouse model encoding SL8 peptide in the intron 2 of β-globin gene. Transgenic mice were identified using the PCR analysis of DNA and separation of PCR amplicons by agarose gel electrophoresis. The expression level of SL8-specific pre-mRNA was the highest in spleen, bone marrow and blood while no detectable expression was observed in thymus, lymph nodes and liver. Western blot analysis of protein lysates from splenic and bone marrow cells confirmed that the SL8 insertion did not affect β-globin expression in mutant mice. Since β-globin is a subunit of hemoglobin it was also important to show no influence of modification on the morphology of red blood cells. Subsequently performed antigen presentation assay confirmed that pre-mRNA with SL8 insertion can be translated and present the antigen in vivo. Further experiments showed that adoptive transfer of specific and activated CD8+T cells causes immunological reactions in spleen of mutated mice. Intron-derived antigenic peptides expressed by tumors from the known to host genomic context reduce the overall capacity for anti-cancer response. Finally, the Author established double and triple transgenic mice models carrying β-globin-SL8 knock-in and SL8-specific transgenic CD8+T cells which can be attractive models for future studies of the physiological mechanisms induced by intron derived SL8 antigen.

In the Discussion section the obtained experimental results are widely discussed and they are juxtaposed with relevant literature data. A special emphasis is placed on those results which confirm the hypothesis of translation from mRNA non-coding regions as the source of peptides presented by the MHC type I complex. In this discussion, the Author does not hesitate to consider the latest attractive hypotheses concerning the process of translation, including those that have not been fully

confirmed yet. Among those are: translation initiation from non-AUG codons occurring also from different reading frames and involving various mechanisms, translation of spliced and pre-spliced mRNAs by different kinds of ribosomes or ribosomes with different translation initiator factors, and translation occurring in nuclear compartments. Overall, the Discussion section proves that mgr Ewa Sroka has an excellent understanding of the subject of her dissertation. Moreover, she presents the obtained results and literature data in a mature way, emphasizing doubts and alternative interpretations. This makes the Discussion read with great interest and pleasure. The only remark: the 17-pages-long text would be easier to read if it was divided into chapters.

To conclude, I rate the doctoral dissertation of mgr Ewa Sroka very highly. The obtained results are novel and make a significant contribution to the knowledge on the possible origins of antigenic peptides for the major histocompatibility complex class I. The results were obtained with the use of the latest techniques and highly advanced methods, applied by the best laboratories working in the field. The high importance of the obtained data is confirmed by the fact that some of them have been published in the prestigious journal Cell Immunology (IF 4.669), and the next part of the results is prepared in the form of a manuscript sent for publication. These remarkable achievements and very high competence of mgr Ewa Sroka in the subject of her dissertation prompted me to ask for considering bestowing an appropriate scientific award to her.

Taking all the above into account, I recommend that the Scientific Council of Biological Sciences of the University of Gdańsk would proceed with further procedural steps to confer the PhD degree on mgr inż. Ewa Maria Sroka.



Grounds for the application for the distinction of the doctoral dissertation of mgr inż. Ewa Maria Sroka

I express my high opinion regarding the doctoral dissertation of mgr Ewa Maria Sroka. The topic discussed in her dissertation is very important and valid, and the research was conducted at a very high professional level, with the use of a number of advanced techniques and research methods, applied by the best laboratories working in the field. The obtained results were discussed in a mature, definitely distinctive way, demonstrating a very high knowledge of the research issues. The author presented a number of research hypotheses, the verification of which may be an attractive option for further experimental research.

The results obtained by mgr Ewa Maria Sroka have been described so far in two experimental articles - one published in Cell Immunology (IF 4.669), of which she is the second author, and the other, of which she is the first author, sent for publication and currently being reviewed. This confirms the high scientific value of the results obtained by mgr Ewa Sroka in the course of preparing her doctoral dissertation. Moreover, mgr Ewa Sroka is the co-author of three review articles thematically related to her doctoral dissertation, published in the following journals: Molecular Immunology, Open Biology and Infectious Diseases and Therapeutics. She is also the first author of four conference reports, the topics of which are related to that of her doctoral dissertation.