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**REPORT ON THE DOCTORAL THESIS OF M.SC. MARIA CAMILA TOVAR FERNANDEZ**

PhD thesis title: **Origin of antigenic peptides in MHC class I pathway**

PhD thesis supervisor: **Prof. Dr. Robin Fahraeus**

The PhD thesis presented by Maria C. Tovar Fernandez is devoted to a better understanding of the origin of antigenic peptides presented on the surface of the major histocompatibility complex (MHC) class I molecules. Doctoral studies were carried out under the supervision of Prof. Robin Fahraeus, who is a world leader in the field of immunology, especially in the aspect of regulation of the MHC class I pathway in immune response. Presentation of antigenic peptides on MHC-I molecules is a key component of the immune system that allows to distinguish between self and non-self peptides. Self peptides originating from mutated proteins and non-self peptides of viral origin are presented by MHC-I class molecules, and recognized by cytotoxic CD8-positive T cells. This process leads to the killing of the antigen presenting cells. Interestingly, previous studies carried out by Prof. Robin Fahraeus and his research group revealed that MHC-I molecules can also present peptides originating from the pre-spliced messenger RNAs (mRNAs); these peptides were called pioneer translation products, in short: PTPs. These interesting and novel findings have raised the question of the origin of antigenic peptides for the MHC-I pathway. To address this question, Maria C. Tovar Fernandez focused on two different sources of antigenic peptides: (i) autophagy - a protein degradative mechanism, and (ii) pioneer translation products derived from pre-spliced mRNAs. Thus, the PhD thesis of Maria C. Tovar Fernandez relates to important and still unsolved or poorly understood issues in the area of regulation of the MHC class I pathway in immune response.

The doctoral dissertation is composed of: Abstracts (Polish and English versions), General Introduction, PART 1 and PART 2 (each PART comprises separate chapters: Introduction, Aim, Material and Methods, Results, Discussion and Concluding Remarks), Acknowledgements, Bibliography and Annexes (including: supplementary figures, tables and a published paper). Presenting the results in two parts, comprising separate chapters: Introduction, Aim, Material and Methods, Results, Discussion and Concluding Remarks, does not follow a classical format of PhD theses. I am not sure whether it was the best idea to present the results of PhD studies in such a form. There is an impression that the manuscript comprises two separate PhD theses. I think that, at least, concluding remarks summarizing both parts of the PhD studies should be placed, to envision both parts as a whole. I am also wondering why the numbering of figures was not continuous throughout the dissertation, while the numbering of chapters, belonging to separate parts of the dissertation was? Moreover, in my opinion, adding a list of abbreviations would be beneficial and make the PhD thesis easier to follow. Additionally, I find inconsistency in the way of presenting results: “We” vs “I”. I have also a general comment regarding the nomenclature: gene names should be italicized (the HUGO nomenclature); e.g. page 2: “While genes coding for both chains of class II are HLA-DN, DM, and DO in

humans (…)", or page 84: "RPL0 is also interesting because is a gene associated with Lupus Erythematosus and inflammatory Bowel disease".

Irrespective of the comments above, I would like to emphasize that the scientific background, scientific aims of the project, materials and methodology section, research results and discussion are presented in a clear and legible manner. The General Introduction sets up the topic of the research and provides basic information on the major histocompatibility complex molecules. Then, follows the first part of the dissertation. In the first part, Maria C. Tovar Fernandez focused on autophagy and its role in the generation of peptide substrates for the MHC class I pathway. In the Introduction section related to this part of the dissertation, the PhD candidate nicely presented the scientific background and the respective literature concerning the autophagy pathway, the possible links between autophagy and antigenic peptides generation for the MHC class I pathway, and substrates for the autophagy pathway. Autophagy is a major, and highly regulated intracellular degradation process responsible for the clearance of cytoplasmic proteins, certain pathogens and dysfunctional organelles via the lysosomal pathway. Autophagy has also been implicated in removing from the cell harmful protein aggregates. Protein aggregates can cause several neurodegenerative disorders, for example polyglutamine (polyQ) diseases. PolyQ diseases result from a continued expansion of polyglutamine, that triggers misleading interactions between proteins and results in pathological effects such as aggregation. Currently, little is known about the different functionalities of autophagy in the antigen presentation. It is also not clear what is the link between autophagy and antigenic peptides generation for the MHC class I pathway. Based on the literature data, it is suggested that this association is substrate-dependent. Immune response towards cells carrying protein aggregates is also relatively unknown. To address all these issues, Maria C. Tovar Fernandez evaluated MHC class I antigenic peptides production from autophagy processing using two different types of substrates: (i) EBNA1 viral protein (of Epstein-Barr virus), which is characterized by the glycine-alanine repeat (GAr) domain conferring an aggregate-prone protein conformation, and to be autophagy processed via the Atg5/12 pathway, to produce antigenic peptides for the MHC class II pathway, and (ii) ovalbumin protein fused to aggregate-prone glutamine repetitions (OVA-PolyQ).

Experiments carried out with the use of H1299 cells (Human non-small cell lung carcinoma) transfected with specific constructs encoding EBNA1 protein or OVA protein (and their derivatives), revealed: (i) no difference in presentation of peptides derived from the viral EBNA1 protein following suppression of autophagy by knocking down Atg5 and Atg12 by siRNA, (ii) downregulation of OVA-derived peptides in antigen presentation upon knockdown of Atg5 and Atg12 by siRNA, (iii) that fusing the aggregate-prone PolyQ to the ovalbumin had no effect on antigen presentation via autophagy, (iv) that fusing the EBNA1-derived gly-ala repeat (GAr) sequence to ovalbumin prevented the presentation of peptides from OVA to the MHC class I pathway via autophagy. In conclusion, the collected results suggested a substrate-dependent presentation of antigenic peptides for the MHC class I pathway via autophagy, and that protein aggregation is not a key feature to provide antigenic peptides for the MHC class I pathway via Atg5/12-dependent autophagy. Moreover, collected data indicated that the GAr domain of the EBNA1 may serve as a novel virus-mediated mechanism for immune evasion of autophagy-dependent antigen presentation. I have no doubt that these new data will lead to a better comprehension of viral immune evasion.

I would like to emphasize here that this part of PhD studies has already been published in Cellular Immunology (Elsevier), $IF_{2021}$ 4.178: *Substrate-specific presentation of MHC class I-restricted antigens via autophagy pathway*. **Tovar Fernandez MC**, Sroka EM, Lavigne M, Thermou A, Daskalogianni C, Manoury B, Prado Martins R, Fahraeus R., Cell Immunol. 2022 Apr;374:104484, and Maria C. Tovar Fernandez is the first author on this publication.

I have a question to this part of the PhD thesis: do other viruses have the glycine-alanine repeat (GAr) domains, or similar domains, that let them to evade autophagy-mediated MHC class I-restricted antigen presentation?

In the second part of the PhD thesis, Maria C. Tovar Fernandez followed the previous studies conducted by Prof. Robin Fahraeus and his research group. The results of those studies have revealed that some MHC class I antigenic peptides can originate from pre-spliced mRNAs. In the Introduction section related to the second part of the dissertation, the PhD candidate provided data on a possible, alternative source of MHC class I antigenic peptides, i.e. alternative mRNA translation, including information on: antigenic peptide synthesis by alternative translation initiation, antigenic peptides derived from intron sequences and antigenic peptides derived from the pioneer round of translation. The PhD candidate focused on antigenic peptides originating from pre-spliced mRNA, i.e. PTPs. PTPs are defined as antigenic peptides products derived from pre-spliced mRNA synthesized through pioneer round of translation in the nucleus. Once synthesized, PTPs reach the proteasome to be further processed. Maria C. Tovar Fernandez applied different approaches to support the existence of PTPs and identify the ribosomal proteome (riboproteome) responsible for PTPs synthesis.

To prove that antigenic peptides for MHC class I are produced from pre-spliced mRNA, Maria C. Tovar Fernandez used H1299 cells and reporter constructs encoding the β-globin carrying the highly immunogenic SIINFEKL (SL8) peptide in either the intron or the exon region. PTPs were visualized directly by polyclonal antibodies specific against SL8 peptide and the flanking sequences from the specific β-Globin intron. Because immune peptides are rare and rapidly degraded, the proximity ligation assay (PLA) was applied (this assay allows to detect two antibodies in close proximity). To study the translation machinery involved in PTPs synthesis, the polysome fractionation approach was applied. This technique allowed to determine the localization of mRNAs in the different ribosomal fractions. In this experiment, the PhD candidate used as a model system H1299 cells transfected with β-Globin carrying the SL8 peptide sequence located in the intron. Collected results revealed that the immune peptide sequence in the pre-mRNA was found in the light polysomes (pre-spliced mRNAs could be find in the light polysomes fraction, in contrast to the spliced mRNAs, that are present in the heavy polysomes fractions). In summary, in the PART 2 of the dissertation, the PhD candidate provided additional support for intron translation theory by: (i) presenting that MHC class I antigenic peptides can be derived from intron sequences, (ii) visualization of intron-derived peptides and (iii) identification of pre-spliced mRNA antigenic peptide sequence in the light polysome fraction. These findings suggest that there is a specialized ribosomal machinery (different from the machinery used in the canonical translation) synthesizing antigenic peptides for the MHC class I pathway.

I have some minor comments to this part: (i) page 79; Figure 6C, description: „The white scale bars are equivalent to 10." - the unit of measure should be specified, (ii) page 80; it would be beneficial, for the legibility of Figure 7, to name polysome fractions presented in the graph, in Figure 7A.

I have also a general question to the PhD candidate: whether (and how) the knowledge gained during her PhD studies can be used in applied immunology, for example in the designing of anti-cancer drugs or vaccines protecting from viruses?

To summarize, I would like to underline that the results presented by Maria C. Tovar Fernandez in the PhD thesis might be of general interest for the researchers working in the field of molecular biology and immunology. The PhD candidate used a variety of advanced techniques from the area of molecular biology, immunology and immunechistochemistry. The experiments were adequately designed, and clearly presented, important and necessary controls were included. The interpretation and discussion of the results were adequate and proved the expertise of the PhD candidate in the field of antigen presentation through the MHC class I molecules. I appreciate the novelty of the presented research, especially concerning the area of MHC class I antigenic peptides originating from alternative mRNA translation and specialized ribosomal machinery involved in the synthesis of antigenic peptides for the MHC class I pathway. I also would like to emphasize that Maria C. Tovar Fernandez is the first

author on the manuscript *Substrate-specific presentation of MHC class I-restricted antigens via autophagy pathway* (data presented in this manuscript make up a large part of the PhD thesis).

Taking all the above into account, I state that the PhD thesis presented by Maria Camila Tovar Fernandez certainly meets the requirements laid down for the degree of PhD in biology by the status in the Journal of Laws of the Republic of Poland (Dz. U. 2018 poz. 1668. Z późń. zm.). 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 in biology on mgr Maria Camila Tovar Fernandez.

Z pełnym przekonaniem stwierdzam, że przedstawiona mi do oceny rozprawa doktorska spełnia warunki określone w ustawie Dz. U. 2018 poz. 1668. USTAWA z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce z późniejszymi zmianami i wnioskuję do Rady Dyscypliny Nauki Biologiczne Uniwersytetu Gdańskiego o dopuszczenie mgr Marii Camili Tovar Fernandez do dalszych etapów przewodu doktorskiego.

Dr hab. Anna Kurzyńska-Kokorniak, prof. ICHB PAN