

Thesis for
PhD Degree in 4.3 Ilka Tsvetkova

REVIEWER
Rossitza Konakchieva, Prof. Dr Habil

REVIEW

by Prof. Dr. Habil. Rositsa Konakchieva

Faculty of Biology – Sofia University "St. Kliment Ohridski"

on the Thesis work of **Ilka Tsvetanova Tsvetkova-Ivanova** for the awarding of the educational and scientific degree "**Doctor**" in professional direction **4.3. Biological sciences**, scientific specialty "Immunology", code 01.06.23, entitled: "**Investigations of the mechanisms of cell death and the role of the effector Gasdermin D, in the induction of the NLRP3 inflammasome. Relevance to Male Fertility Disturbances**"

Academic supervisor: Prof. Soren Hyrabedyan, Dr. Habil.

Current review evaluation of the dissertation work is based on the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its Application, Order No. 108/17.10.2023 of the Director of IBIR-BAN for the appointment of a Scientific Jury, and is in accordance with the recommended requirements adopted by the National Assembly of IBIR-BAS for acquiring scientific degrees and holding academic positions.

The PhD student Ilka Tsvetkova-Ivanova has submitted an original dissertation thesis and all necessary documents for the course of study. Attached is a Report from the IMI-BAN plagiarism system, the analysis of which shows the absence of significant similarities in the individual parameters above those recognized by the metadata platform. The attached Reference for the fulfillment of the minimum national requirements according to the PZASRB proves the presence of the necessary minimum of 30 points for a doctoral student in professional direction 4.3 Biological Sciences.

I declare that I have no conflict of interest, incl. co-authorship in the candidate's publications.

Scientific profile of the PhD student

Ilka Tsvetanova Tsvetkova-Ivanova's higher education includes a bachelor's degree in Molecular Biology in 2017, a master's degree in Developmental Biology in 2019, obtained at the Faculty of Biology of the University of St. Kliment Ohridski". Immediately after his graduation, he continues as a doctoral student (2019 — 2023) in the Laboratory of Reproductive OMIKs Technologies, Institute of Reproductive Biology and Immunology Acad. Kiril Bratanov, BAS. Since 2019, he has been an assistant in the Laboratory under the supervision of Prof. Soren Hayrabedyan and Prof. Krasimira Todorova. In her short scientific career, Ilka Tsvetkova was distinguished with prestigious scientific awards, some of which were received in teams of young scientists as a member of the Department of Genetics, Faculty of Biology, SU St. Kliment Ohridski", Sofia: 1/Second Prize for Oral Presentation, 2019 at the 6th Black Sea International Immunology School, Teteven, Bulgaria; 2/Team silver medal, 2017 International Genetically Engineered Machine (iGEM) competition, Boston, USA; 3/ Team bronze medal, 2016 International Genetically Engineered Machine (iGEM) competition, Boston, USA. In the period 2018-2023, she co-authored five publications in journals with an impact factor and participated in several scientific symposia.

State-of the art

The chosen topic is of high relevance to the field of reproductive medicine and reproductive immunology in particular. Through a fundamental scientific approach the study investigates the problem of immunologically determined damage to the spermatogenic function due to an experienced infection, or the so-called idiopathic disorders of unexplained nature. Manifestation of an autoimmune reaction to germ cell antigens is one of the possible causes of sterility. Under normal conditions, the blood-testicular barrier creates an immunologically tolerant environment suitable for maturing spermatids. Under various conditions such as trauma or chronic inflammatory damage to the endothelium, the integrity of this barrier may be compromised. As a result, cells of the immune system can reach and attack elements of the own reproductive system in the testicles, resulting in the formation of antibodies against its components. Sertoli cells are among the main participants, storing and protecting germ cells. The presence of caspases and the

possibility of the release of inflammatory cytokines, provoked by prolonged inflammatory reactions, could lead to a violation of their functions, affecting to a large extent the possibility of the proper course of spermatogenesis. The activation of apoptotic pathways is the more gentle pathway for cell death, leading to the protection of neighboring cells, but strong inflammatory processes and the cell's inability to cope with an infectious agent lead to the activation of pyroptotic pathways, provoking neighboring or immune cells. Gasdermin-mediated pyroptosis activates pathogen clearance and can help restore homeostasis, but dysregulated pyroptosis underlies many inflammation-mediated pathological conditions. Based on this hypothesis, the author focuses on the tracking and detailed overview of the specific type of cell death occurring as a result of activation of pro-apoptotic and pro-pyroptotic caspases, their interrelationship and activation pathways.

Compliance with PhD thesis requirements

The presented dissertation is structured and formatted according to the requirements. The dissertation is written on 136 pages, contains three schemes in the literature review, one diagram referring to the working hypothesis and 40 complex figures combining photographic microscopic material or histograms with graphic figures reflecting quantitative indicators after statistical processing. The introduction introduces the topic and contains the reasons for developing the dissertation. Followed by a Literature review chapter of 35 pages, ending with a summary, Aim and tasks - 2 pages, Materials and methods - 13 pages. The largest part of the work is occupied by the Results chapter - 50 pages, followed by a thorough Discussion on 11 pages. Conclusions – 1 p., Contributions – 1 p. 213 literary sources were used, including contemporary publications on the subject incl. latest - after 2015 This is testimony to the relevance of the presented work and the candidate's desire to supplement and improve her professional experience.

The aim of the dissertation is short and clear formulated: To investigate the mechanisms of cell death in Sertoli cells and the associated signaling pathways involving the main caspases and Gasdermin D.

Basic thematic tasks, exhausting the subject of the dissertation, are set. I find that task 3. is not set specifically enough and presents rather a methodical task subordinated to the others.

State-of-the-art methods from the arsenal of cellular and molecular biology and immunology have been used, which allow to analyze and derive original results with statistical reliability and fundamental contribution. Along with the routine methods of cryopreservation and cell culture, the use of highly informative analytical methods such as flow cytometry, quantitative DNA analysis (RT-qPCR), new generation nanopore sequencing of Sertoli cells 15P-1, ELISA methods, fluorescent microscopic analysis of caspase activity and cell death, and others. Experimental cell models have been used to activate macroautophagy and mitophagy in Sertoli cells. The statistical analysis was carried out and reflected competently, which makes it possible to derive original reliable dependencies reflected in ch. Conclusions.

The methods are described in good faith and sufficiently informative without unnecessary details. Descriptions of the apparatus, chemicals and kits are attached, which indicates employment of a high-class equipment and a modern professional level. The free and clear handling of the terminology and the adequate description of the methods speak of the rich experimental and methodical experience accumulated by the dissertation. The description of this section gives a brief idea of the extensive and labor-intensive work carried out, as a result of which convincing data were obtained allowing original conclusions to be formulated.

The results of the dissertation work are presented on 50 pages and follow the set tasks. The activation of TLR4 and the Nlrp3 inflammasome in the context of pyroptotic cell death was investigated; caspase-1 activity on the occurrence of pyroptotic cell death after stimulation of the TLR4 receptor with LPS and activation of Nlrp3 with ATP. The types of cell death that occurs after the activation of the inflammasome pathway were studied by monitoring the expression of the inflammasome Nlrp3 and the associated activation of caspase-1 and caspase-3; investigated the effect of the expression of the adapter protein ASC in Sertoli cells on the activation of caspase-1 and caspase-3 after stimulation of the TLR4 receptor with LPS and activation with ATP; the influence of Asc on the expression of Nlrp3 transcripts was investigated; the activation of caspases at different time points was dynamically followed in relation to the activation of apoptosis in Sertoli cells. Nanopore sequencing of Sertoli cells of line 15P-1 was performed, which contributed to an established connection of the receptor Cd300a with the inflammasome pathway of Nlrp3 as well as the pyroptotic cell death executor GSDMD with caspase-1 and caspase-3 directing the cell to a different type of cell death.

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The discussion contains a comprehensive analysis of the investigated signaling factors and cellular processes in Sertoli cells in various experimental models related to pro-inflammatory status in mammals and humans. The results are discussed in light of recent findings related to the expression of inflammasome-activating caspase-dependent signaling pathways and the targeting of Sertoli cells to a different cell fate.

On the basis of an analysis of the obtained data for the individual tasks, six conclusions have been formulated, which correctly reflect the research work and the results obtained, and I accept them in essence. The contributions reflect substantially the original findings of the dissertation and I accept them in full. Overall, the obtained results give reason to consider the dissertation work as a comprehensive fundamental work with valuable scientific results obtained in original experimental models and perspectives for application in reproductive immunology and medicine.

Conclusion

The current dissertation reflects a deep independent scientific development, realized with a wide arsenal of methods of modern immunology and molecular biology, which allowed the establishment of original scientific results by obtaining information at a modern scientific level. Bearing in mind the professional qualities of the doctoral student, whom I have had the pleasure of knowing since her student years at the Faculty of Biology of Sofia University "St. Kliment Ohridski", as well as the scientific achievements in the present work, I express my positive assessment of the presented dissertation work.

I strongly recommend to the Honorable Members of the Scientific Jury appointed by Order of the Director of IBIR-BAN to award Ilka Tsvetanova Tsvetkova-Ivanova the educational and scientific degree "Doctor" in professional direction 4.3. "Biological Sciences", scientific specialty Immunology (sh. 01.06.23).

02/03/2024

Reviewer:

/Prof. Dr. Habil. Rositsa Konakchieva/