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Review of the Doctoral Dissertation by Konkonika Roy,
entitled *"The Dark Side of Endotoxin Tolerance: Increased Susceptibility to Cancer"*

The doctoral dissertation of Konkonika Roy was carried out at the Department of Immunology under the supervision of dr hab. Sylwia Wrotek, Professor at NCU, with dr Tomasz Jędrzejewski serving as the assistant supervisor.

The dissertation has been prepared in the form of a collection of three original, multi-author publications. The manuscript of one of these works has been submitted to the journal. The articles published to date appeared between 2023 and 2025 in reputable journals indexed in the *Journal Citation Reports* (JCR) database. The first article was published in *Cancers* (IF 4.5; 200 points according to the Ministry of Science and Higher Education journal ranking), and the second in *Cellular Immunology* (IF 4.1; 100 points). The third work is a completed manuscript that has been submitted for peer review to an immunology journal.

In all the aforementioned publications and in the submitted manuscript, the Doctoral Candidate is the first author and, moreover, serves as the corresponding author. Based on the attached information, it is evident that the doctoral student made a significant contribution to all the indicated studies, which included, among other tasks: designing and conducting experiments, analyzing and interpreting experimental data, collecting materials for analyses, preparing manuscripts and figures, as well as proofreading the manuscripts after peer review.

This PhD dissertation builds on an idea proposed by William Coley, a pioneer known as the "father of immunotherapy," who emphasized the role of fever in his treatment approach. Notably, many cancer patients display a reduced ability to develop fever during infections. The dissertation specifically hypothesizes that this impairment may result from endotoxin tolerance (ET), which could weaken the anticancer immune response. The PhD Candidate rigorously tests this hypothesis. To date, ET has remained an underexplored area within cancer immunology,

and this dissertation directly addresses that gap. The research concept originated from the supervisor, who successfully attracted the interest of NCN experts, resulting in the award of a *Preludium bis* grant to support these studies. Part of the results—particularly those obtained using the SCENITH technique—were generated by the PhD student during a research internship at the Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg University (Mainz, Germany).

The doctoral dissertation submitted for review consists of 114 pages and, in addition to the cited publications, includes all necessary components such as: a list of abbreviations, a detailed description of the Doctoral Candidate's contribution to the development of the publications, abstracts in both English and Polish, and a comprehensive introduction to the research topics addressed in the thesis. The dissertation also clearly states its objectives, provides a detailed description of the materials and methods used for both *in vitro* and *in vivo* studies, and contains a discussion, summary, conclusions, and a list of cited literature. The structure of the dissertation is correct and typical for this type of doctoral work, in which a significant portion of the results has already been published in the form of scientific articles.

In the introduction, the Doctoral Candidate presents issues closely related to the subject of the dissertation, which focuses on the molecular mechanisms involved in the immune response to endotoxin (LPS) and the role of macrophages in infections and tumorigenesis. The work provides a detailed analysis of LPS recognition within the TLR4 signaling cascade and discusses downstream pathways, including both MyD88-dependent and TRIF-dependent signaling. It also addresses the activation of canonical and non-canonical inflammasomes, the significance and molecular basis of fever as part of the inflammatory response, and the phenomenon of endotoxin tolerance, characterized by a diminished immune reaction following repeated exposure to LPS. Furthermore, the dissertation examines the role of macrophages in regulating immune responses during infections and their involvement in cancer development. Overall, it serves as an excellent introduction to the topics addressed in the thesis. In presenting the individual topics, the Doctoral Candidate provides a well-argued rationale for the subjects addressed in her doctoral dissertation, which concern the role of endotoxin tolerance (ET) in cancer development. The research aims to fill a gap in knowledge regarding how ET modifies immune responses, particularly in macrophages, and how these alterations affect tumor progression.

The scientific objectives are clearly formulated and, together with the scope of the experiments conducted, are presented in detail in well-defined points.

In the experimental study published in *Cancers* (Roy, K.; Kozłowski, H.M.; Jędrzejewski, T.; Sobocińska, J.; Maciejewski, B.; Dzialuk, A.; Wrotek, S. *Endotoxin Tolerance Creates Favourable Conditions for Cancer Development*. *Cancers* 15(20), 5113, 2023), the Doctoral Candidate

focused on investigating the impact of ET on cancer progression through its effects on the tumour microenvironment. The primary objective was to determine whether macrophages rendered endotoxin-tolerant (MoET) contribute to the creation of a pro-tumourigenic environment. To this end, MoET were generated and assessed for their effects on breast and colon cancer cells using two experimental approaches: direct co-culture and stimulation with MoET-conditioned media. The behaviour of cancer cells under these conditions was evaluated through various functional assays, including viability, clonogenicity, motility, and scratch assays, as well as 3D spheroid formation. The results demonstrated that factors secreted by MoET significantly enhanced cancer cell viability, motility, and clonogenic potential, indicating that ET fosters the development of a tumour-supportive microenvironment. Notably, despite the reduced expression of classical pro-inflammatory cytokines such as TNF-α and IL-6, MoET still displayed certain features of M1 polarization. This suggests a distinct reprogramming of macrophages under ET conditions, which may contribute to tumour progression across multiple cancer types.

The second study presented by the Doctoral Candidate (Roy, K.; Jędrzejewski, T.; Sobocińska, J.; Spisz, P.; Maciejewski, B.; Hövelmeyer, N.; Passeri, B.; Wrotek, S. *Divergent Impact of Endotoxin Priming and Endotoxin Tolerance on Macrophage Responses to Cancer Cells. Cellular Immunology*, 411–412, 104934, 2025) focused on examining the impact of ET on the interaction between immune cells—particularly macrophages—and the tumour microenvironment. The research investigated how repeated exposure to endotoxins, such as lipopolysaccharide (LPS), induces an immunosuppressive state that may facilitate cancer development and progression. Using a combination of *in vitro* and *in vivo* approaches, the study demonstrated that endotoxin-tolerant macrophages undergo phenotypic reprogramming in the presence of cancer cells, adopting a pro-tumorigenic M2-like state. Aditionally, endotoxin tolerance enhances the metabolic flexibility of macrophages within the tumor microenvironment, enabling them to utilize alternative metabolic pathways beyond classical glycolysis and oxidative phosphorylation. Furthermore, *in vivo* experiments confirmed macrophage infiltration into tumours under both ET and non-ET conditions, with a markedly suppressed immune landscape observed in the presence of ET.

The manuscript submitted for publication (Roy, K.; Maciejewski, B.; Jędrzejewski, T.; Spisz, P.; Sobocińska, J.; Di Pentima, M.; Passeri, B.; Wrotek, S. *Endotoxin Tolerance Enhances Breast Cancer Aggressiveness and Alters Inflammatory Marker Expression in Tumor and Spleen of Mice*. is based on a study investigating the impact of ET on breast cancer progression in a mouse model. In this study, tumor-bearing mice with induced ET (ETBC) were compared to non-tolerant control mice (BC). The ETBC group exhibited significantly faster tumor growth, reduced leukocyte counts, and decreased spleen weight, all indicative of systemic immunosuppression. Gene expression analysis of spleen and tumor tissues revealed downregulation of pro-inflammatory cytokines such as IL-6 and IFN-γ, alongside upregulation of immune-modulating and pro-

tumorigenic factors including IL-1β, NOS2, COX-2, VEGF, and CSF-1. These results suggest that ET impairs immune surveillance and promotes a tumor-supportive microenvironment. Overall, the findings highlight the potential of ET as a relevant target for therapeutic intervention in cancer.

The conclusions presented in the *Summary and Conclusion* chapter are fully consistent with the results obtained during the course of the research. In her work, the Doctoral Candidate demonstrated that ET profoundly affects both systemic and local immune responses by inhibiting pro-inflammatory mediators, altering leukocyte populations, and modifying the expression of immune-related genes in the spleen and tumour tissues. ET reprograms macrophages toward an M2-like, immunosuppressive phenotype, accompanied by metabolic changes that facilitate tumour growth. Tumour cells exposed to ET exhibit increased survival, migration, clonogenicity, and spheroid formation, while *in vivo* experiments confirm that ET accelerates tumour progression and fosters the development of an immune environment conducive to cancer growth. Overall, the study's findings identify ET as a potential therapeutic target, underscoring the need for further research into strategies for its detection and reversal in cancer prevention and treatment.

All the objectives set out in the doctoral dissertation have been achieved. In the reviewed dissertation, the Author achieved several notable research accomplishments that significantly enhance the scientific value of the work:

1. Application of diverse and advanced methodologies – the study integrates molecular, cellular, immunological, surgical, and metabolic approaches. Particularly noteworthy are the surgical procedures enabling stress-free temperature monitoring via biotelemetry, as well as the use of advanced metabolic analysis methods such as SCENITH, which exceed current gold standards in the field.

2. Use of multiple experimental models – the research combines *in vitro* cell line studies with *in vivo* animal experiments, enabling the assessment of both systemic immune responses in mice and tissue-specific immune mechanisms, thus ensuring a comprehensive and multidimensional approach to the research problem.

Despite the very promising results obtained by the Doctoral Candidate, it should be noted that the overall applicability of the findings remains uncertain, as it is unclear whether they can be broadly extrapolated to different cancer models. Please clarify whether there are any types of cancer in which endotoxin tolerance plays a minimal or negligible role, and, if so, explain why.

In summary, I conclude that all the presented works form a logical whole, culminating in a coherent and well-substantiated conclusion that links immune system dysfunction to tumour progression.

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The doctoral dissertation by Ms. Konkonika Roy fully meets all the formal requirements set out in the Polish legal act *Ustawa – Prawo o szkolnictwie wyższym i nauce* (Dz. U. z 2020 r. poz. 85 z późn. zm.). The work represents an original and significant contribution to solving a scientific problem in the field of biology, offering a unique analysis not only of the mechanisms by which endotoxin-tolerant macrophages promote tumor development, but also of the reciprocal influence exerted by cancer cells on these macrophages. This dynamic, bidirectional interaction is explored in depth, providing valuable insights into the complex immunodynamic processes that shape tumor biology.

The Doctoral Candidate's scientific achievements extend beyond the scope of the presented publications and include two additional articles in prestigious international journals, presentations at scientific conferences, the award of a research grant in a competitive call funded by Nicolaus Copernicus University in Toruń, participation in research internships, as well as active involvement in the academic development of junior researchers. Collectively, these accomplishments reflect the Candidate's extensive expertise and scientific maturity.

In summary, I assess Ms. Konkonika Roy's doctoral dissertation very highly. I therefore recommend to the Council of the Discipline of Biological Sciences at Nicolaus Copernicus University in Toruń that Ms. Konkonika Roy be admitted to the subsequent stages of the doctoral procedure. Furthermore, given the high scientific merit of the dissertation, the practical significance of the findings, and the overall excellence of the Candidate's academic achievements, I also propose that the doctoral dissertation be distinguished with an appropriate award.