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Wrocław, 14.12.2023

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Review of the doctoral dissertation by Mikołaj Kocikowski entitled

OF DOGS AND MEN. TRACING IMMUNE CHECKPOINT SIGNATURES ACROSS CANCERS AND UNLEASHING THE POTENTIAL OF CANINE PD-1 ANTIBODIES

Supervisor: dr Theodore Hupp, prof. UG

Second supervisor: dr Javier Antonio Alfaro

Formal and legal basis for the review

The review was prepared based on the Resolution of the Scientific Council of Discipline of Biotechnology of the Intercollegiate Faculty of Biotechnology UG&MUG, according to which I was designated as the reviewer of the doctoral dissertation of Mikołaj Kocikowski, entitled „Of dogs and men. Tracing immune checkpoint signatures across cancers and unleashing the potential of canine pd-1 antibodies". The legal basis of this review is the Act of 20 July 2018 – The Low of Higher Education and Science (Journal of Laws of 2023, item 742).

The research problem and the scope of the thesis

Dogs have accompanied humankind for thousands of years sharing the same environment, and thus being exposed to the same as humans environmental factors such as air pollution, tobacco smoke, and various

chemicals. Recent developments in veterinary care have led to a significant extension of dogs' lifespan and allowed the diagnosis and treatment of a growing number of different diseases in this species. Among all diseases in dogs, cancer is considered the main cause of mortality. Until very recently, studies on the canine neoplasia were limited by the lack of well-characterized and widely available tools for *in vitro* studies. The latest advances in the development of genome-integrated molecular reagents and commercially available high-throughput methodologies specific for dogs have enhanced our ability to more thoroughly interrogate canine cancers and characterize shared and novel targets for therapeutic intervention. Importantly, specific biochemical pathways known to be drivers in human cancers are frequently observed in canines, offering the opportunity to target those mechanisms in dogs and allow accurate preclinical assessment of novel therapeutics. In this context, the study of naturally occurring cancers in dogs provides a valuable perspective; dogs develop a broad spectrum of naturally occurring cancers sharing many characteristics with those found in human counterparts. Interestingly, canine cancers often recapitulate the biology and heterogeneity of human disease, including complex interactions between the immune system and tumor cells. This provides a unique opportunity - the possibility of developing new immunotherapies, both for canine patients as well as for humans - using the dog as a model.

One of the current directions of research in cancer immunotherapy is blocking immune checkpoints (ICs) - signaling pathways that play a key role in regulating immune responses by modulating the activity of immune cells. IC receptors are present on the surface of the immune cells, like cytotoxic lymphocytes. which allows for control of the course of the immune reaction. Unfortunately, cancer cells may express inhibitory IC ligands on their surface, thanks to which they silence the cytotoxic effects of lymphocytes. Therefore, the topic undertaken by the doctoral student and the research goals are important and up-to-date. Disclosure of immune checkpoints in canine cancers and their relation to human cancer, development of antibodies targeting canine PD-1, and use of them in anticancer therapy constitute excellent research targets. These endeavors could not only advance our understanding of tumor immunology in dogs but also potentially offer valuable insights applicable to human cancer therapy, thereby bridging a significant gap in comparative oncology.

General description of the doctoral dissertation and the doctoral candidate's contribution to the research.

The doctoral dissertation submitted for review is written in English, has 233 pages (plus appendices), and contains 405 bibliography items. It contains also information about Funding and Support, the Preface, Lay

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Summary, Abstracts in English and Polish, Acknowledgements, Index of Contents, Appendices, and a list of candidate's publications and presentations. The description of the dissertation is divided into 3 parts corresponding to the relevant issues: 1) immune checkpoint landscapes of human and canine cancers; 2) comparative characterization of two novel antibodies against canine PD-1; 3) caninized antibodies: strategies for protein deimmunization. Each section contains an introduction, results, discussion, and methods. Additionally, the dissertation also includes a general introduction, conclusions and implications, perspectives, and bibliography. The structure of the doctoral thesis is logical and transparent and does not raise any objections. However, the dissertation lacked a list of abbreviations.

At the end of the dissertation, a list of publications and conference reports co-authored by the candidate is attached. Unfortunately, no statements confirming the doctoral student's participation in individual papers were included, nor is it known whether his participation was leading. Only in one of the presented publications doctoral student is the first author. Thus, please clarify which stages of the research were carried out by the doctoral student.

Another discrepancy concerns the list of supervisors - in the documentation there are two supervisors - in the dissertation appear also the third supervisor – Dr. Maciej Parys.

The importance and urgency of the undertaken scientific problem and its formulation

The topic that the doctoral student discusses in his dissertation is current, innovative, and of great scientific value. The first issue researched by the PhD student is the Immune checkpoint landscapes of human and canine cancers. In this study PhD candidate pursued three objectives: a) to assess the abundance of immune checkpoints across the canine cancer types; b) to compare these IC profiles with those found in human cancers; c), to analyze the IC patterns of human cancer subtypes and individual patients.

PhD candidate decided to assess the abundance of immune checkpoints across the canine cancer types, to compare their IC profiles with those found in human cancers, and to analyze the IC patterns of human cancer subtypes and individual patients. The research provided a thorough analysis of IC expression patterns in both human and canine cancers. This revealed potential canine models for human cancer immunotherapy and identified prospective IC targets common to both species. By comparing the 'IC signatures encompassing 44 IC genes in 27 human and 14 canine cancer types, the study uncovered distinct and shared patterns across species, cancer types, and individual patients. In my opinion, these findings are

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of great importance in comparative oncology and have the potential to significantly advance our understanding of cancer treatment strategies across different species

In the second part of the research, the doctoral student performed a comparative characterization of two novel antibodies against canine PD-1: PD1-1.1 and PD1-2.1. The antibodies were characterized by their performance in key molecular assays and their therapeutically crucial checkpoint-blocking ability. The third goal was to develop strategies for protein deimmunization. Antibody caninization was performed using various methods that have been tested in detail.

The mentioned research goals are ambitious and innovative and have high scientific value. Subsequent issues have been clearly defined, described, and explained. It is visible that the Ph.D. candidate is able to formulate research issues and conduct experiments to obtain answers to scientific questions.

The methodological correctness of the research

The assessed dissertation represents a well-planned research project that uses modern research methods and tools including advanced bioinformatics techniques. The presented approach allows for obtaining original and reliable results facilitating the comparison of data obtained for dogs with those from human medicine. I am convinced that the doctoral student correctly selected the methods and tools to check his hypotheses. The level of methodological advancement of the presented dissertation is admirable.

At one of the stages of obtained antibodies characterization, difficulties occurred in performing the challenges of the PBMC-based IFN-γ secretion assay. Why hasn't an alternative method for stimulating and/or detecting PBMC activation been explored?

The level of knowledge on the topic of the work and its presentation. The skills in analysis and interpretation of research findings, driving conclusions and presentations of the results.

The dissertation submitted for evaluation is a very well-prepared monography. It contains extensive and detailed descriptions introducing the topic and at the same time showing the doctoral student's extensive knowledge. Each section includes a comprehensive methodology, enabling the replication of the experiment. The Discussion, Summary, and Future Perspective parts are perfectly prepared and fully exhaust the discussed topic. Everything is provided with high-quality figures, diagrams, and graphics enables a thorough analysis of the results. The conclusions drawn by the doctoral student come from the analysis of the described results, which means that the candidate is able to analyze data and draw proper

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scientific conclusions. Notably, the doctoral candidate can discern and articulate the limitations of the conducted research while also considering possible implications effectively suggesting avenues for future studies in the field. Such a critical approach from a young scientist is very valuable. The quality of the presented dissertation proves the Ph.D. candidate's scientific maturity, but also his diligence - which is an important feature of a scientist.

Originality of the results and their contribution to knowledge enhancement

The presented results for the first time described a landscape of immune checkpoint (IC) expression in canine cancers. Ph.D. candidate characterized 44 ICs in 14 canine cancer types, significantly enriching the body of knowledge in canine oncology. Moreover, the studies provided new, validated reagents to accelerate research in canine immunology - two antibodies against the canine PD-1 immune checkpoint. Finally, validation of a novel, streamlined antibody production method was provided, and a functional chimeric antibody was generated (caninized antibodies). Overall, the research presented in the dissertation is pioneering in nature and requires the doctoral student to work creatively in the context of developing methods and searching for appropriate tools. The results of the work are excellent - undoubtedly making a significant contribution to contemporary comparative oncology.

Final conclusion

Summarizing the entire review it could be concluded that: a) the doctoral dissertation is a well-prepared monography with a coherent theme; b) the doctoral dissertation demonstrates the candidate's general knowledge in the discipline of Biotechnology and the ability to conduct scientific research independently; c) the topic of the doctoral dissertation is an original solution to a scientific problem. Taking into consideration the above, I can state that the doctoral dissertation by Mikołaj Kocikowski, entitled: „Of dogs and men. Tracing immune checkpoint signatures across cancers and unleashing the potential of canine PD-1 antibodies" meets all the requirements set out in Article 187 of the Act of 20 July 2018 – The Low of Higher Education and Science (Journal of Laws of 2023, item 742). Therefore, I hereby apply to the Scientific Council of Discipline of Biotechnology to admit Mr. Mikołaj Kocikowski to further stages of the doctoral procedure.

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