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REVIEW of the doctoral dissertation by Raghunandan Mahadeva entitled "Iron Deficiency Uniquely Reprograms the Amino Acid and Lipid Metabolism of Red Pulp Macrophages to Support Enhanced Iron Recycling Functions".

The doctoral dissertation by Raghunandan Mahadeva describes the impact of iron-deficient conditions on the function and metabolic programs of splenic red pulp macrophages. These processes are very poorly understood, particularly the knowledge linking metabolic pathway engagement and the capacity of red pulp macrophages to maintain iron-recycling activity under conditions of restricted iron availability. For this reason, the topic undertaken by the doctoral candidate is extremely interesting; the work is innovative, and its results may contribute to a better understanding of the functional crosstalk between the liver and spleen in iron recycling from erythrocytes.

Formal assessment

The presented doctoral dissertation takes a form of a collection of two original articles – one in the form of a preprint available on the bioRxiv platform, and the second published in EMBO Reports. In the article available on bioRxiv, the doctoral candidate is the equal first author (out of four), while in the second publication he is the fourth co-author. Based on the information provided in the publications, it can be inferred that the doctoral candidate played an important role in the conducted research and simultaneously met the criteria for first and fourth authorship of the attached publications.

In "EMBO Reports" publication PhD candidate optimized several methods assessing the uptake of red blood cells, red blood cells ghosts and free hemoglobin, assisted in the execution of the experiments, participated in the interpretation of the obtained data. In manuscript available on bioRxiv, PhD candidate was responsible for the conceptual, experimental and analytical work defining how iron deficiency alters metabolic circuitries in red pulp macrophages. The input of the PhD candidate into this manuscript is very well defined and meticulously described.

The mandatory declarations of the Candidate's contributions to the articles are included after each manuscript. The dissertation is written in English and uses an appropriate and clear language.

In addition to the attached publications, the dissertation comprises an abbreviated form of a written thesis – an introduction, briefly stated four research objectives, a short results section

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summarizing the results included in both manuscripts, concluding discussion and future directions sections, and a bibliography. An introduction that shortly describes iron balance, role of red pulp macrophages as iron-recycling cells provides a brief overview and constitutes a sufficient introduction to the topic, though perhaps schematic figures explaining aminoacid metabolism and metabolic organization of efferocytic macrophages and red pulp macrophages would have made the content even clearer. The "Discussion" and "Future perspectives" sections are very well revealing the Candidate's potential for scientific reasoning and demonstrates his scientific maturity. The Candidate clearly identifies the significance of his work for the field and also critically interprets the obtained data. Importantly, PhD candidate very proficiently discusses the future directions, open questions and issues that remain unresolved and require further investigation.

Merits assessment

As part of the project, the PhD student characterized the metabolic adaptations of red pulp macrophages under iron-deficient conditions, with a focus on defining changes in mitochondrial respiration, substrate utilization, and identifying key metabolic pathways associated with enhanced erythrophagocytic activity. More specifically, he defined the role of branched-chain amino acid catabolism in supporting mitochondrial function and sustained erythrophagocytosis. The PhD candidate was also involved in investigating the contribution of lipid metabolism to the bioenergetic adaptation of RPMs and determination how metabolic pathways are functionally integrated with upstream iron-dependent signaling. By conducting such extensive research, PhD candidate has demonstrated an impressive knowledge of various experimental methods. The analysis of the attached publications indicates that the doctoral candidate skillfully employs and has a deep understanding of multiple techniques in biology and molecular genetics (metabolic analyses, flow cytometry, genetic modifications, protein and metabolite analyses) and animal work, tissue collection, and downstream sample preparation. PhD candidate is knowledgeable in his research field and conducts studies that go beyond the state of the art and significantly enhance current knowledge regarding the metabolic rewiring of red pulp macrophages under conditions of restricted iron availability.

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I have some questions and comments to which the PhD candidate may respond during the defense:

1. In iron-deficient mice, the erythrophagocytosis capacity of red pulp macrophages is enhanced – is it selectively observed for red blood cells, or in general phagocytosis of various particles is affected? How do these processes potentially affect the immune response to parasites and blood-borne infections? Given that Fc receptor CD16 in red pulp macrophages of iron-deficient mice was upregulated – could immunophagocytosis mediated by antibodies be increased in iron-deficient mice? Could the increased rate of phagocytosis be accompanied by potentiation of other functions of red pulp macrophages, such as antigen presentation, immunomodulation? Also, are other functions of red pulp macrophages (like cytokines secretion) affected under iron deficient conditions?
2. Could expanded mitochondrial functions and lysosomal biogenesis and function in red pulp macrophages be rather a result of enhanced erythrophagocytosis than the cause? Similarly, could increase in intracellular Ca2+ levels and activation of SYK in red pulp macrophages of iron-deficient mice compared to iron-balanced mice be again a consequence, rather than contributing to enhanced phagocytic capacity of red pulp macrophages? How can this cause-and-effect relationship be addressed?
3. How anaemia of chronic disease affects the erythrophagocytosis capacity of red pulp macrophages? Could anaemia of chronic disease be treated with hepcidin inhibitors? How would this treatment affect the erythrophagocytosis capacity of red pulp macrophages and what could be the clinical consequences in reference to the observation made by PhD candidate?
4. Does ferroportin overexpression induce red blood cell clearance in iron-balanced conditions?
5. Are branched-chain amino acid catabolism and β-oxidation processes known to be involved in phagocytosis and metabolic activities of red pulp macrophages in iron-balanced conditions? Or rather they are selectively involved in phagocytosis regulation upon iron deficiency?

Summary

My comments neither decrease nor question the scientific value of the presented work. The PhD candidate has conducted innovative research, the results of which are important for understanding the consequences and physiological significance of the enhanced erythrophagocytosis in iron deficiency conditions, as well as mechanisms governing functional and metabolic adaptation in red pulp macrophages upon iron deprivation. The PhD candidate

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possesses extensive knowledge, as well as the ability for critical analysis and interpretation of the obtained data.

Considering this, I, the undersigned, hereby state that the doctoral dissertation of Raghunandan Mahadeva meets the requirements specified in Article 187 of the Act of July 20, 2018 – Law on Higher Education and Science (c.t., Journal of Laws of 2024, item 1571, as amended). I hereby recommend to the Doctoral Committee of the International Institute of Molecular and Cell Biology in Warsaw to admit Raghunandan Mahadeva to the subsequent stages of the procedure for the conferment of the doctoral degree in the field of natural sciences, in the discipline of biological sciences.

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