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מכון ויצמן למדע
WEIZMANN INSTITUTE OF SCIENCE

September 9, 2024 Rehovot

RE: Evaluation of PhD thesis by Mgr. Daniela Knížková

Dear Committee members,
I have had the privilege of reviewing the PhD thesis entitled "***Molecular Mechanisms of Inflammation***" submitted by ***Mgr. Daniela Knížková***. I have examined the thesis in detail, along with the corresponding publications, and found both to be outstanding in multiple respects. Mgr. Knížková's work presents significant advancements in our understanding of the molecular mechanisms underlying inflammatory processes, with a particular emphasis on the regulation of cytokine signaling pathways, specifically those activated by IL-17 and TNF. The thesis is notable for its innovative approach and the high impact of its findings, which provide crucial insights into molecular mechanisms regulating immune activation by the above two pro-inflammatory cytokines. The thesis is well-written and presented, demonstrating a high level of scholarly rigor and clarity. Although I do not know Ms. Knížková personally, I had the opportunity to briefly encounter her at the BIOCEV 2023 conference, where she presented part of her PhD research on CMTM4. Her presentation was among the best and most compelling of the conference, showcasing her ability to convey complex scientific concepts effectively.

The thesis is structured into two primary sections. The first section provides a detailed molecular analysis of the IL-17 signaling pathway, while the second section explores the mechanisms involved in TNF signaling. In the first section, Mgr. Knížková builds upon two studies co-authored by her that were recently published in prestigious peer-reviewed journals.
The first study, co-authored by the student, employed an innovative mass spectrometry-based methodology to investigate the IL-17 receptor signaling complex (IL-17RSC). The research presents compelling evidence that IL-17 signaling is regulated negatively by the kinases TBK1 and IKKε through a robust feedback mechanism. This negative regulation is crucial for modulating the intensity of the IL-17 signaling cascade.
The second follow up study, which was spearheaded by the student, also exploited mass spec analysis to investigate proteins interacting with IL-17R signaling complexes, particularly those containing the IL-17RC subunit, which has a more restricted expression pattern compared to IL-17RA. Specifically, using mass spec analysis of mouse stromal ST2 cells treated with recombinant IL-17A, the student identified CMTM4—a multispaning transmembrane protein—as a component of the IL-17R signaling complex. CMTM4 was found to constitutively associate with IL-17RC, an interaction mediated by the transmembrane domains of both proteins. In CMTM4-deficient mouse and human cells, there was a noticeable reduction in the cell surface expression of IL-17RC compared to CMTM4-sufficient cells. This decrease was linked to inappropriate glycosylation of IL-17RC. Furthermore, IL-17A treatment of CMTM4-deficient cell lines led to impaired recruitment of ACT1 and TRAF6 to the IL-17R complex, resulting in reduced expression of IL-17-responsive genes that encode proinflammatory cytokines. Notably, these defects in IL-17 signaling were corrected by retroviral expression of CMTM4. Analysis of publicly available RNA sequencing databases revealed coexpression of CMTM4 and IL-17RC in human skin and small intestine tissues. In a psoriasis model driven by IL-17 signaling in keratinocytes, Cmtm4-deficient mice displayed milder inflammation and less severe skin pathology compared to wild-type controls. Collectively, these findings underscore CMTM4 as a critical component of the IL-17R signaling complex and suggest its potential as a

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therapeutic target for treating IL-17-mediated autoimmune diseases. The study was published in highly prestigious journal - *Nature Immunology* and received a major recognition in the field.

The second section of the thesis addresses the TNF signaling pathway, which, despite extensive research, still contains unresolved elements. This part of the research, currently under revision for publication in *Nature Communication*, examines the role of adaptor proteins TANK and AZI2 in protecting against TNF-induced cell death. The results reveal that while TANK and AZI2 exhibit redundant functions within TNF signaling, their presence is essential for preventing TNF-induced cell death, highlighting their crucial role in maintaining cellular integrity

The application of a novel mass spectrometry-based approach to address key immunology-related questions is very original and innovative, and has helped bring new insights into the molecular composition and regulation of cytokine signaling, including the negative regulation of IL-17 signaling by TBK1 and IKKε, as well as the crucial role of CMTM4 in autoimmune diseases. The investigation into TANK and AZI2's roles in TNF signaling adds valuable knowledge to the field of inflammatory disorders, with potential therapeutic relevance for conditions involving these cytokines.

Although the thesis effectively compiles individual studies, it would benefit from a more explicit discussion and integration of the findings, including their limitations. An expanded analysis of how these insights contribute to the broader context of cytokine signaling would enhance the overall narrative. Additionally, a thorough exploration of potential future research directions and the implications of these findings for therapeutic strategies would greatly strengthen the thesis and provide valuable guidance for subsequent research efforts.

In conclusion, the student has delivered an impressive, comprehensive, and insightful thesis that significantly advances our understanding of cytokine signaling pathways. This work has resulted in three seminal publications, including a notable first-author paper by the student that has garnered substantial international recognition. The thesis is methodologically rigorous, well-structured, and makes substantial contributions to the field of immunology. It reflects a high level of scholarly excellence and provides valuable insights that will impact future research in the field.

Overall, I commend Mgr. Knížková for her excellent work and highly recommend her thesis for acceptance

Sincerely,

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Specific follow up questions:

1) The thesis provides a thorough review of the major sources of IL-17 proteins. However, the expression patterns of the various IL-17 receptor subunits are only briefly addressed in the background section. It would be beneficial if the student could discuss the expression patterns of the individual IL-17 receptor subunits across different tissues, highlighting any notable overlaps and differences of the individual subunits. Presenting this information in a graphical format would enhance clarity and provide a more comprehensive understanding of the receptor distribution.
2) The study mainly relies on in vitro approaches and models to assess CMTM4's role. Although it includes some in vivo validations, the scope of in vivo experiments is relatively limited. Could the student think of additional in vivo studies using mouse models, which would provide a better understanding of CMTM4's role in different physiological and pathological contexts? Please give some examples of projects based on such models you would give to your starting PhD students if you were a PI.
3) Please describe a dream experiment which you would like to do to in a follow-up project, given you would be given unlimited resources.
4) Could the student provide hypotheses regarding the potential phenotypes in human patients with either loss-of-function (LOF) or gain-of-function (GOF) mutations in the CMTM4 gene? Additionally, what therapeutic approaches might be suggested to address these conditions?
5) Please discuss the common phenotypes associated with deficiencies in either IL-17RC or IL-17RA in humans. How do these phenotypes compare, and are there any notable differences between the two types of deficiencies?
6) Is there any evidence indicating that CMTM4 is associated with receptors other than IL-17R? Are there other receptors that you hypothesize CMTM4 might modulate?
7) Do you anticipate that CMTM4 might also play a role in modulating IL-17RE-dependent signaling?
8) The co-expression of CMTM4 with IL-17RC in stromal tissues is intriguing. This observation suggests that CMTM4 might not influence IL-17 signaling in hematopoietic cells as they appear to lack CMTM4 expression. Do you agree with this interpretation? In your opinion, which is more crucial for maintaining immune homeostasis and preventing fungal overgrowth: IL-17 signaling in hematopoietic cells or in stromal cells?
9) Loss of function mutation of either IL17A or IL17F results in clear immune phenotype, characterized by chronic candida infection. Why in your opinion there is no compensation/redundancy?
10) Please compare the roles of IL17C vs IL17A/F in autoimmunity and anti-candida response. How do they differ? Does any of these two types more dominant in autoimmune disorders vs anti-candida response.
11) Please discuss how your discovery of CMTM4 as a regulator of IL17 signaling could be exploited for therapeutic purposes.

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