CHARLES UNIVERSITY
Faculty of science

Prague, 9th January 2023

RE: Evaluation Report – Doctoral Thesis by Lenka Vaneková

Dear Committee Members,

The doctoral thesis authored by Lenka Vaneková main aim was to develop a suitable mouse model reflecting the chronic type of hepatitis B (HBV) infection that can be utilized for preclinical testing of the novel therapeutics. One of the pathways utilized in general by the immune system to fight against infection is the cGAS-STING signaling pathway. Thus, a large library of STING-agonists was prepared and one compound was tested as the potential cure for the chronic type of hepatitis B infection utilizing above mentioned newly developed mouse model. In addition, a protocol for the detection of immune populations in the liver utilizing flow cytometry was developed.

In total, Lenka Vaneková is the (co)-author of five publications. This s a major achievement for a PhD student. Three of them were devoted to the design and synthesis of STING receptor agonists and were published in prominent journals such as the Journal of Medicinal Chemistry (2) and Structure (1). The next two, where Lenka Vaneková is the first author, are devoted to the development of a mouse model of chronic HBV infection and flow cytometry analysis of liver infiltrating immune cells were published in the local Physiological Research journal and in Methods and Protocols, respectively.

The thesis itself has a standard type of chapter composition and is written in English. The Introductory part is largely devoted to the cGAS and STING itself and their signaling pathway. Much less space is devoted to HBV and the immune response to it and the involvement of cGAS-STING pathway in this process. The Introductory part is rather short, yet contains all the important details necessary for the evaluation of the applicant's contribution to the field, and it is nicely supplemented with decent schematics. By saying that, it should be also noted that sometimes the text contains unfinished sentences or unclear statements (e.g. *"TLRs, for instance, can be divided into several 12 subfamilies based on their primary sequences (S. Pandey et al., 2015)." pg16*; *"Another important STING-dependent downstream signalling separate from IFNproduction is an autonomous cell defence mechanism, autophagy." pg 29; "Mice with deletion of lupus susceptibility gene Fcgr2b and without deletion of Sting1 gene showed improved survival and disease symptoms when (A.Thim-uam et al., 2020)."pg38.).* Based on this, I would like to ask:

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1. What do you mean by 12 TLR subfamilies?

Thus, the Introductory part would clearly benefit from additional proofreading.

In the results section, I will mainly focus on the papers, where Lenka Vanekova is the first author.

In the Physiological research study, they report the development of a novel mouse model of chronic HBV based on the delivery of expression plasmid with replication-crippled HBV. This leads to stable construct expression in the liver without the development of infectious particles, permitting the work with the newly established mouse model outside the BSL3 barrier. Beyond this study, the doctoral thesis reports yet unpublished data utilizing this newly established model for pre-clinical compound testing. I have several questions regarding these results:

2. Did you check the expression of the plasmid in other tissues or organs than the liver? Why it might be for instance important to check the expression of the construct in the thymus?

3. You are showing the cytokine production and activation of T-cells after the injection of the STING-agonist compound in Fig 15 (pg78). The FACS plots for data presented in Fig15C are not shown. Strikingly, strong and rapid T-cell activation is observed (up to 30-40% of all T-cells are activated when animals were treated with a high dose of the agonist for both – 4 hours or overnight). Did you observe any increased mortality or autoimmunity of experimental animals after this treatment or their premature death during the experiment?

4. Did you measure what happens with T-cells (or the immune system in general) concerning their activation in the course of the experiment, e.g. after several weeks?

5. Please specify how the percentage of virus-positive cells was measured and calculated in Figure 18 (text on pg.82). No statistics for the virus positivity is shown, it is not clear how many replicates were used…

6. How do you think the observed decrease in chronic HBV is achieved in your model? Can you at least hypothesize what cells might be mechanistically involved in this process? How you would test this? Can you please suggest some options?

7. You are mentioning the advantages of your new mouse model for chronic HBV. Are there any disadvantages of it in comparison to the established ones?

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The second study in Methods and Protocols is describing the isolation of immune populations from the liver. Combining perfusion (to eliminate the blood-borne immune cells from the organ when focusing on the organ infiltrating ones), commercial kit permitting enzymatic digestion of the organ, Percoll-based separation of stromal/epithelial cells from immune cells and flow cytometry analysis. All really standard steps when analyzing organ-infiltrating immune cells. Personally, I feel this is a quiet standard protocol supplemented with the representative flow cytometry analysis. Once again, here I have several specific questions:

8. Was the Fc-block used when staining the immune populations? Why is this important when dealing for instance with macrophages or DCs?

9. In your representative gating strategy you are showing the Cd49b/Cd19 double negative gate. Four children gates originate from this parent gate. It seems to me, that these children gates contain different numbers of cells (two in the center vs two on the side)? Is this correct, how this happened?

10. Kupffer cells were from macrophages separated solely based on the $F4/80^{+}$ and $CD11b^{low}$ expression. I do not think this is sufficient. Can you think of an additional marker that can help you to distinguish macrophages from Kupffer cells? Actually, you are using this marker in your gating strategy. It would be beneficial to see how this marker works together with CD11b and F4/80 (you can utilize back-gating for instance).

11. Is the CD8 sufficient marker to claim that you are discriminating cDC1 from cDC2 population? Can you, please, suggest better markers to discriminate these populations?

To summarize, the parts devoted to the design, synthesis and *in vitro* testing of the STING-agonists represents decent advancement within the field and the development of such agonist have clearly great value both clinically and commercially. The generation of a novel mouse model of chronic HBV is a nice achievement and represents an interesting tool for the potential mechanical testing of novel compounds. The publication of protocol is in my eyes rather problematic.

With best regards

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