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Re: Examiner's Report on Bc. Alžběta Synáčková Master thesis:

Search for new regulators of MyD88-dependent signaling

This study, describes the investigation into the identification of gene products which are involved in the regulation of MyD88 signaling by forming a multimeric complex. It relies on the immunoprecitation of strep-flag-tagged MyD88 protein which serves as a bait to immunoprecipitate the MyD88 complex-interacting components upon induced IL-1α signaling. The author succesfully isolated and determine several new components of activated MyD88 signaling complex, specifically, TBK1 kinase with its two adaptor proteins, TANK and AZI2. Using a standard biochemical approaches, the author provides evidence that TANK and AZI2 recruit TBK1 to MyD88 complex and, importantly, that this recruitment negatively regulates the outcome of IL-1α-induced MyD88 signaling. The author also demonstrated that Tank/Azi2 double KO (dKO) display a partial embryonical lethality and born animals die prematurely between week 3 and 40 due to inflammatory phenotype and dermatitis resulting in the multiorgan failure. This immunopathological phenotype which is caused by overactivated MyD88 signaling can be partially rescued by the ablation of MyD88 expression. The author also tested the prediction that IRAK4, a kinase which is downstream of MyD88, could be a potential therapeutic target to obviate this inflammatory phenotype.

The thesis is written up in a standard format, in English. It consists of seven standard chapters, the Literature overview, Aim of the study, Material and Methods, Results, Discussion, Conclusions and References. This is a high quality work, serious science with a solid data employing new transgenic mouse models. Upon its completion the projects has a potential to be published in a high profile immunological or signaling journal.

While I feel that the thesis is of excellent quality, described data are original and valuable for a broad research and potentially also for clinical community, there are several suggestions and questions that should be addressed and discussed.

First, I have several **formal concerns and technical questions**:

1/ the methodology used in this study includes biochemical, genetic and microscopic approches. The author should be more explicit in providing information which experiments and analyses were performed by the author herself and which by her colleagues.

2/ The abstract and text contains abbreviation IL-1R-SC or IL-1R-RSC which are not explained in the text nor indicated in the abbreviation list.

3/ Figure illustrating the TNF signaling pathway as described on pages 17-19 would be of benefit to the readers of this thesis. Figure 2 on page 22 does not schematically correspond to the description of the TNFR1 signaling with two modules: complex I and II, described on page 17.

4/ Even though the thesis is written with a very good command of english, very occasionally, there are several imperfections which make it hard to understand the meaning of given sentence. For example, on page 38: "To investigate the impact of MyD88 signaling on the phenotype of Tank/Azi^{DKO} mice".

5/ Material and Methodology section does not provide a necessary description of approches and methods used in this study. For example, on page 38, it is not mentioned how was the Azi2/MyD88^{DKO} allele prepared and this information in also omitted in the Method section. Did you check for the presence of potential LncRNAs in the deleted region? In addition, it is not appropriate to write that, quote: „Azi2 and Myd88 are located close to each other on the genome". It would be more accurate to describe it in some acceptable measures, for example in kbp, Mbp or cM (centimorgans). The author also refers to the Area under Curve statistical method, with no explanation and description how it was technically performed.

6/ Fig. 4C. Perhaps, it would be biologically more relevant to measure the levels of TNFα and IL-6 in the supernatants by ELISA then to quantify their transcript levels by qRTPCR?

Questions for discussion:

1/ Fig. 3B. In the discussion the author mentioned that TBK1 and AZI2 were detected as components of MyD88 complex but due to inconsistencies in detection these constituents were left out from the table. How many other potential components of MyD88 complex behaved in a similar manner and were thus not shown in Fig. 3B? TBK1 is labelled in red but it is not explained why. The meaning of iBAQ values are also not explained. Did you test by IP whether IKKe is in the MyD88 signaling complex as well?

2/ Have you tried to visualized by the microscopy the recruitment of TANK-AZI2-TBK1 complex to Myd88. Since IL-1R is on the cell surface, perhaps, in stimulated cells these complexes should be detectable right underneath the membrane.

3/ Could you elaborate on the mechanism how p-TBK1 can deliver a negative regulatory function to MyD88 complex?

4/ Fig. 6B. From the levels p-TBK1 versus p-p105, pIKKα/β, p-JNK and p-p38 at 15 mins (as used in Fig. 3B), it seems that only the hyperphosphorylated level of p-p105 correlates with the overstimulation of MyD88 signaling. How do you explain this set of data?

5. It seems that the Tbk1$^{fl/fl}$ mice do exist. If you got hands on them, have you tried to look which innate immune cells are responsible for an overt inflammatory phenotype due to excessive MyD88 signaling? In this context, would it be possible, even theoretically, to detect which cells in the most inflammed tissues of the Tank/Azi2 dKO mice exhibit the highest MyD88 signaling?

6/ Tank/Azi2/Myd88 TKO, compared to Tank/Azi2 DKO mice start to die approx. 20 weeks later. However, once this process starts, the steepness of the dying curve is the same. Thus, one can argue that dying 40-week-old Tank/Azi2/Myd88 TKO mice could suffer from the same level of multiorgan inflammatory autoimmunity as 20-week-old Tank/Azi2 DKO mice. Did you perform H-E staining on the same tissues as in Fig. 7C-F in 40-week-old Tank/Azi2/Myd88 TKO? If not, what would you expect to see? Did you measure the serum levels of TNFα in 20-week-old Tank/Azi2 DKO versus 40-week-old Tank/Azi2/Myd88 TKO? In other words, do they die because of the same pathology?

7/ why it is difficult to pharmaceutically target MyD88? Is there any potential for small therapeutical molecules to efficiently block either MyD88-IL-1R or Myd88-IRAK4 interaction?

6. **Conclusions and recommendations**

I have identified both the strengths and weaknesses of the thesis, although I have concentrated mainly upon the latter as it is expected from such report. However, I want to emphasize, that the above listed concerns in no way diminish the high quality of work presented in this thesis with significant potential to translational medicine. Based on this, I recommend this thesis to be accepted as the fulfilment of the requirement for awarding the Master degree to the candidate.

Best regards,