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Opponent's evaluation of the PhD thesis

Title: Non-immunoglobulin binding proteins with immunomodulatory potential in autoimmune diseases
Study program: Molecular and Cellular Biology, Genetics and Virology
Author: Mgr. Yaroslava Groza
Supervisor: RNDr. Petr Malý, CSc.

The doctoral thesis by Yaroslava Groza focuses on an interesting, timely, and important topic-the development of specific protein binders with potential application in autoimmune diseases and cancer.
The author specifically focused on the receptors for Il-6 and Il-22, the PD1 immune checkpoint, and TREM2, which are relevant targets in biomedical research. The thesis is an annotated collection that includes five papers published in respected journals.

Original papers include articles in:
- Cell Communication and Signaling (IF 8.2, Q1), first author
- Cell Communication and Signaling (IF 8.2, Q1, in revision), fourth author
- Journal of Translational Medicine (IF 6.1, Q1), tenth author

Review articles include articles in:
- Cytokine and Growth Factor Reviews (IF 9.3, D1), first author
- International Immunopharmacology (IF 4.8, Q1), second author

A complex set of experimental approaches was employed to evaluate the newly developed binders. All papers were peer reviewed by experts in the field, supporting the validity of the findings.
The thesis is written in good English and spans 207 pages, including the abovementioned papers. Figures and tables are illustrative and adequately support the text. The bibliography includes 330 references that are relevant to the topic. The Abstract is well-structured and effectively summarizes the main focus and findings of the thesis. The largest part of the Introduction is dedicated to Il6, the main subject of the research, as evidenced by the two first-author publications. In addition to discussing individual molecular targets, the Introduction provides essential information on the diseases related to the mediators and receptors.
The Aims of the thesis are clearly stated and align well with the published papers.
The Results section comprise of a concise summary of the individual papers that follow. Contribution of the author to each of the papers is provided. The text is informative, and I find the inclusion of the Graphical Abstracts very useful.
The Discussion is organized into sections corresponding to the individual papers. For the IL-6 review article, it effectively emphasizes the knowledge gap that the review addresses. The

discussion of the original research is appropriate, although at times it delves too deeply into the results rather than placing them in a broader context. A brief section providing a more unified perspective on the collection of papers forming the basis of the thesis would be a valuable addition.
The Conclusions summarize the key findings of the individual manuscripts.

Critical comments:
While the Introduction is generally well-structured, there are some areas that could be improved. For instance, section 1.2 Il-6 role in cancer has subchapters 12.1 „Direct effect on cancer cells“ and 1.2.2 „Tumor microenvironment“ which would rather be Effect on the tumor microenvironment. Section 1.7.2 „ABD scaffold“ should have a more general title, as it also discusses the myomesin-1 derived scaffold. Additionally, 1.7.4 seems more appropriate as a subchapter of 1.7.3. Furthermore, 1.8. is placed after the methodological sections, which disconnects it from the other more biologically oriented chapters. Regarding Table 2, the information about IL-6/Il-6R inhibitors seems not to be completely up-to date especially in relation to „Completed clinical trials“- the references for monoclonal antibodies are not current and e.g. tocilizumab is now FDA-approved for several indications not mentioned in the table. Additionally, information on clinical trials is missing for recombinant proteins and small molecules, though I could find it for some of these. In my opinion, omitting this column would not detract from the table's overall value.
In the Results, including supplementary or unpublished data would, in my opinion, effectively complement the textual summaries of the articles. The thesis does not include supplementary figures from the papers and some, such as FigS2 for the Il22 study, are not accessible online as the manuscript is still under review. The TREM-2 review is a slightly self-standing topic. I believe its purpose was to summarize information about another potential target for non-immunoglobulin binding proteins, but this intent could be more clearly emphasized.
Overall, the text is clear and well-written; however, there are occasional sentences and formulations that could be refined (e.g. p5 “In contrast, soluble gp130 selectively inhibits the IL-6/sIL-6Rα complex-mediated [33].”; Table 1. “Oligodendro**glyo**cytes”; Figure 3 “Another kinase, PIP3,”- PIP3 is not a kinase; p26 “For the purpose of protein binder engineering, single-domain small protein is used as a scaffold for mutagenesis. Such a population of diversified DNA fragments is called a DNA library.”; Table 3 lacks references, Library size 10^{12} for Ribosome display conflicts the 10^{14} value listed in 1.7.4, etc.). Frequently, noun stacking makes the text a bit cumbersome, such as "IL-6 role in inflammatory bowel disease" instead of "The role of….". Additionally, some abbreviations are not explained and I could not find many of them in the abbreviation list either. After reading the Abstract, the Aims (“To develop a collection of IL-6Rα-binding NEF variants”), and the entire text I still remain curious why “NEF“ is used for the ABD-derived small binders.
On page 37, there appears to be an error in the graphical abstract; the graph on the right (PaTu) seems to display results of a proliferation assay (Fig. 6b), not a migration assay. I also noticed that two figures are labeled by the same number (six).

Reading of the thesis evoked the following questions:

1) In the Il6 study, the link between the biological effect of NEF binders and Il6R inhibition seem to be rather weak. The inhibitory effect on STAT3 phosphorylation in Fig. 4h is quite modest compared to both concentrations of the gold standard TCZ; NEF108 does not inhibit it at all. In contrast, TCZ has no effect on biological properties (growth, migration) in several in vitro assays, while e.g. NEF108 (but not NEF172, which inhibited

pSTAT3) strongly inhibits the growth of PaTu cells. How does this fit with the conclusion on p 172- “NEF variants can slow down cancer cell proliferation and this effect can be specifically attributed to IL-6 signaling inhibition by NEF variants.” Was Il6-mediated signaling differentially abrogated in the cells where you see (and not see) biological effects? Also, in Fig. 8a, addition of Il6 in combination with NEFs further **impairs** wound closure- how do you explain this? What is the effect of Il-6 itself?

2) NEF binding to PBMCs- a clearly positive population of cells is identified in Fig. 9a using the anti-Il6Rα antibody. The individual NEFs are not clearly indicated, but for some there seems to be a higher percentage of cells to which they bind. This would suggest some off-target binding. Is Il6Rα the only target?
3) In the PD1 study, a strong signal is observed in the liver. However, e.g. Hettich 2016 (DOI: 10.7150/thno.15253) reports only a weak signal in the spleen and lymph nodes using PD1 immunoPET in healthy mice. How could the specificity of your liver accumulation be verified?
4) Two different scaffolds were utilized for creating the binders. How was the choice for individual targets made? Are there some general principles that help select which scaffold will be more suitable?

Conclusion:

Despite the aforementioned critical comments, the submitted work clearly shows that Yaroslava Groza, M.Sc., has strong creative abilities and has mastered several advanced methods in biochemistry and cellular biology. Furthermore, she demonstrated the capability to design experiments, interpret data, and effectively summarize her findings.

I recommend the thesis for defense and acceptance as part of the requirements for awarding a Ph.D. degree.

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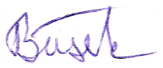

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