Prague, 24th May 2023

RE: Evaluation Report – Diploma Thesis by Veronika Cimermanová

Dear Committee Members,

The main aim of the diploma thesis authored by Veronika Cimermanová was to elucidate the origin, phenotype and potential functional role of a newly discovered subset of $CD8^+$ T-cells with polyamine metabolism signature (T_{pam} cells).

The thesis is written in English and has a standard type of chapter composition. The introductory part nicely demonstrates the heterogeneity of $CD4^+$ helper T cells and the current lack of information concerning such a functional heterogeneity in the cytotoxic T cell subset. In general, this part is really well written, it is brief, focused, citing recent relevant literature and really easy to follow. Only a limited number of issues was identified (e.g. "*the expression of surface molecules CD127*-", *pg12*). Sometimes a rather generic type of sentence with low informational value is used (e.g. *"…specific markers depending on their specialized effector functions within the exact tissue"*, pg 12). In a similar fashion, on pg. 16 the potential caveats of stimulation experiments of the $ISAG^{hi}$ cells is described as *"…the marker used for the isolation of $ISAG^{hi}$ cells was not $ISAG^{hi}$ exclusive, since it was expressed by other T-cell subsets in their t-SNE projection*.". From my point of view, it would be important to stress what was the marker used and directly refer to the figure in the paper or if this was reanalyzed by the author or someone else in the laboratory, this should be directly shown in the thesis. This is a strong statement and should be sufficiently backed.

Lastly, the introduction of the newly described T_{pam} population should be more detailed. I noticed that this is an emerging population described recently by the laboratory, however as currently described in the thesis, the use of CD137 as the marker of T_{pam} is not really convincing. It seems that max. 25% of the T_{pam} cluster express gene encoding CD137 (*Tnfrsf9)* on the scRNAseq analysis provided on Fig. 3. Moreover, this seems to be expressed on a rather low level. I guess the laboratory has more evidence for claiming that CD137 is the unique marker of T_{pam} population. As the CD137 is used throughout the thesis to isolate or detect the T_{pam} cells, I would appreciate some validation to be present. Nevertheless, I consider these as minor issues, that do not disparage the overall high quality of the introductory text and I guess that all of these can be easily commented by the author during the thesis defense.

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The Materials and Methods section representatively describes the methods used and clearly enables the repeating of the experiments. I highly appreciate posting the code used for RNA-seq data analysis and re-analysis of single-cell RNA-seq data on GitHub.

In the results section, FACS analyses are used to determine the presence of T_{pam} cells in C57Bl/6 and Balb/c animals and in the animals bearing monoclonal TCR repertoire (on Rag WT and deficient background). These experiments clearly point to the overall presence of T_{pam} cells in different strains of mice with polyclonal repertoire. Next, the expression of CD137 was determined on thymic T-cells, suggesting that $CD137^+$ T-cells have an overall higher expression of CD5, pointing to higher signaling levels received during their thymic selection. Subsequently, the adoptive transfer of T_{pam} cells to T-cell deficient CD3 knockout animals was conducted, pointing to the reversibility of T_{pam} state, as they reverted to naïve phenotype after the transfer. The last set of experiments revealed that T_{pam} signature is acquired after the activation of conventional $CD8^+$ T cells, then they have faster activation than conventional $CD8^+$ cells and T cells with a T_{pam} signature can be found during the early phases of infection. Overall, the experiments are well-reported and documented. I have just a few questions driven mainly by curiosity:

1. In Fig 4b the frequency and count of T_{pam} cells in various strains of mice and their secondary lymphoid organs were determined. Were these animals littermates, were these animals of the same sex and age? I am wondering if you observed any changes in the count of T_{pam} cells dependent of the sex or age of the experimental animals.

2. Just a minor issue – I guess you are using the composite image in the case of the left panels of Figure 6a (TCR-b /CD137). It would be beneficial to indicate this in the figure legend. It took me a while to get this.

3. Related to Figure 6: Is there a possibility that some of the $CD8^+$ T cells with T_{pam} signature would re-migrate back to the thymus (this was previously shown for instance for Tregs)? Please suggest an experiment that would allow you to discriminate between cells that underwent recently thymic selection and those migrating from the periphery. Can you also somehow distinguish between cells localized intrathymically and those in the vasculature/bloodstream of the thymus?

4. Is there another option how to compare the signature of some clusters between two independently run single cell sequencing experiments? Why was the variant of comparing the limited signature based only on 12 chosen genes?

5. And lastly, a more general question. Can you think about an experimental approach that would allow you to determine *in vivo* if the T_{pam} cells are descendants of some population or if they give rise to some other?

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The discussion part is to the point and really well-built, pointing to possible caveats and experiments that need to be done to clearly point out the role of T_{pam} cells in the $CD8^+$ T-cell pool.

To summarize, I feel privileged to read this well-written thesis and I fully recommend it for the defense in the Immunology program.

With best regards

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