Prague 06.09.2019

Re: Examiner's Report on Daniel Vašek's Master thesis:

Vliv chladové adaptace na imunitní systém

This study, describes the investigation of the impact of cold stress and cold adaptation on the rat immune system. This is very interesting topic since it involves an interplay between adipose tissue, neuroendocrine and immune system, which from the point of view of adaptation to cold has not been studied extensively. While this still provides enough room for new discoveries, such adaptation is indeed a very intricate and complex physiological process, the study of which requires fine-tuned and unified protocols, reagents, many internal control samples, meticulous and tedious work which together can provide reproducible results. Thus, while the scope of the work is obviously complex and methodologically challenging, Daniel decided to take on this project and divided experiments into two parts. The first set of experiments specifically addresses the question of changes of immune cell populations in the spleen during the exposure to cold as well as changes in immune responses of non-stimulated or mitogen-stimulated splenocytes and peritoneal macrophages (pMFs) measured by their proliferation and the secretion of selected set of cytokines, such as IL-4, IL-6, IL-10 and NO. The author also measured the serum level of epinephrine (NE), and changes in spleen RNA levels of its neagtive regulator, the enzyme MAOA during short and long term adaptation. The second part of experiments is dealing with the assessment of impact of adrenergic signaling on these changes utilizing adrenergic inhibitors β2AR and β3AR.

The thesis is written up in a standard format, in Czech language. It consists of 8 standard chapters, the Introduction, Literature overview, Aim of study, Material and Methods, Results, Discussion, Conclusions and References.

In general, the work brings about several interesting observations concerning the adaptation of rat immune system to cold, which, once confirmed and shown with all necessary controls, is publishable in a relevant scientific journal. Definitively, overall, the idea of the project, collaborative effort with the group of bioenergetics and muscle physiology from the Faculty of Sciences, as well as results which show the kinetics of rat adaptation to cold, changes in the cellularity of splenic immune subsets and the contribution of adrenergic signaling to these changes is very encouraging and sound. These results also suggest that it would be worth to continue in this effort and now, after the initial assessment of this process, the authors can ask other, even more complex questions within the frame of this topic.

While I feel that the thesis is of good quality, described data are original and valuable for a broad research community, there are several suggestions and questions that could be further discussed.

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

1/ The abstract and a very short Intro chapter are not very informative, do not allow the reader to grasp immediately what is the premise of the study. In the abstract, there is not even one word about obtained results. The working hypothesis is completely missing.

2/ The Literature overview contains a lot of information, but it doesn't select or accentuate which of this information is more important for this specific study then the other. Sometimes, the very important information is ommitted. For example, on page 7, the author describes three types of βARs as receptors for NE, but only in the Discussion he reveals that the affinity of these receptors to NE differs and uses this argument to explain his data. Similarly ignored is also description of how does the used inhibitor of β2AR, or β3AR, work, or a previously observed compensatory effect if only one of these inhibitors is used. This info should be included before the Result chapter, so the reader can directly relate to this info when reading and thinking about the results described. Overall, it seems that the literature overview contains many facts which are listed without apparent effort to put them in some better interconnected and more logical context, which in return, would better navigate the reader toward understanding of why this project is important, what is known and what is stil unknown. The author reiterates in several places that practically nothing is known about responses of immune system to a long-term adaptation to cold, which itself is arguable. There are several papers on this topic which he cites and discusses in the Discussion section. However, specific results of these studies, in the context of Daniel's work, are not described with depth needed to understand, what exactly is the novelty of Daniel's results and what is just the confirmation of the facts reported previously. In addition, the text contains several misspelled words, even in some headings (for examples Fig.5, "Makorfagy" instead of Makrofagy"), which could be easily removed if the thesis is read carefully.

3/ Fig.3 and even more blatantly, Fig.4, taken from the literature, seems to be there just for decoration. They are not explained properly, many abbreviations as well as the color code of arrows is not explained. Fig.4 is unreadable and uncomprehensible. This is unfortunate, because right here, Daniel could present his deep interest in the topic by preparing his own figures, which would much better align with the text of his thesis.

4/ Material and Methods chapter, doesn't specify how the peritoneal macrophages (pMFs) were isolated. It seems that all cells obtained by peritoneal lavage are considered as pMFs. This is incorrect and can significantly distort results shown in Fig.5 and 11. What was the purity of pMFs? Did the total number of cells from peritoneal lavage change during the adaptation to cold? Similar problem could be envisioned with BAT tissue. It is hard to understand why this tissue was not used for experiments in accurate weight aliquots. How hard it is to prepare a cell suspension from this tissue and why you have not opted for organizing your experiments with BAT this way?

5/ The thesis contains two figures with the label "Obrazek 5".

6/ Why the changes accompanying the cold adaptation are not also expressed in total numbers of cells in measured subsets? This can be even more important then their frequencies. The notion in the discussion that the author is aware of this fact is even more disturbing, because it could be relatively easily fixed before submitting his thesis.

7/ CD11b/c^{hi} cell subset can't be labeled Macrophages, rather myeloid cells.

8/ Is CD45RA in rats a specific marker of B cells? Wouldn't be better to use CD19 or other B cell-specific marker?

9/ Fig.5B, p. 34, 10-hr time point is missing without any explanation.

10/ Fig.6C a 6E and the statement that the proliferation data after stimulation with LPS correlates with cytometric analysis is likely not entirely correct. While the B cells in 10D and 5T are increased by approx. 25% compared to the control sample, proliferation rate nearly doubled and tripled, respectively. By the same token, it is not clear, why the proliferation of T cells in 10D and CLR, but not 5T, is decreased (Fig.6A, B versus 6D). In this figure, the responses to ConA do not correlate with the celularity of T cells in the samples tested. How do you explain this? My concern is also, that the standard deviation in the proliferation assay are too narrow. They are usually a bit broader because of technical issues with measurement of radioactivity and radioscintilation protocol. Who did perform these experiments? How many samples were used to evaluate their average values?

11/ It is not clear, nor it is explained in the text, why the inhibitors were administered between day 3 and 9 of the 10-day experiment. What was the reason?

12/ What is the target specificity of β2/3RA inhibitors? Can they overlap?

12/ Fig.10A, was the difference between K and K-B2, and CH and CH-B3 significant?

13/ Discussion, p.49, top, the author states that Th2 CD4+ cells do not express β2AR. Did you check if it is not gradually upregulated on these cells after few days in cold conditions? That could explain the polarization to Th2 durind the adaptation period.

14/ I'm profoundly missing a final cartoon or figure where the obtained data is included in the revised and or suggested model of cold adaptation. Given the richness of the Daniel's study, the conclusion should also clearly state, which are the brand-new results, what is the novelty, and which data represent the confirmation of previous reports.

Questions for discussion:

1/ Why did you decide to use rat instead of mouse model where you can much better proceed with various mutant, transgenic or KO strains?

2/ From the Fig.10A, it seems that the activation of β2RA on immune cells play a negative role in the production of BAT. What would be the evolutionary advantages of such physiology? What could be the mechanism?

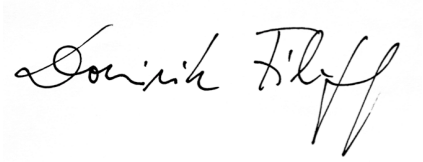
3/ β2RA are expressed on many other organs in the body, not only on cells of immune system. How you can be sure that the data obtained by using β2RA inhibitor and observed changes are indeed related to blocking this signaling pathway in immune cells? Other possible scenarios should be also discussed.

4/ Wouldn't be more obvious to look into changes associated with the immune system directly in the BAT tissue since this tissue is central to the process of cold adaptation?

5/ How do you envision to utilize cold adaptation process for treatment and/or preventing obesity, which you mention in the abstract, but then this sound idea is not discussed in a broader context in your thesis?

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. I want to emphasize however, that the Daniel Vašek's thesis represents a good quality of work which has a potential for future scientific endevours. Some insufficiencies can be found in the way how the author works with the text, i.e. how his thesis is written and results presented. However, this is a process which every student must learn with time and I'm sure that Daniel's scientific career is on the right path to be succesful. Based on this, I recommend this thesis to be accepted as the fulfilment of the requirement for awarding Magister degree to the candidate.



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