Prague 27.05.2021

Re: Examiner's Report on Bc. Anna Kratochvílová's Master thesis:

Vliv chladového stresu na imunitní systém za působení infekčních agens

This study describes the investigation into the impact of infection on the rat immune system during and/or after the organisms is exposed to cold stress or adaptation. Central to this topic are Toll-like receptors (TLRs) which sense the microbial metabolites of infectious agens. This topic draws the attention since there is only a limited knowledge about the interplay of these two stress factors which apparently were dominant forces that shaped our physiological responses to surrounding environment during our evolution. Since the adaptation to cold represents itself a complex phenomenon, a concommitant involvement of infection increases this complexity to a whole new level. To find the guiding principles which rule these processes and their potential intersection, is by the nature of these regulatory circuits a huge challenge. Thus, one must admire Anna's courage and enthusiasm to deliver substantial effort to unearth the basic principles how infection affects the immune system during the cold exposure and adaptation. On the other hand, one must realize that the stepping stones to carry out such tedious experiments are high standard protocols, tested and validated reagents, multiple repetitions, significant knowledge and keen analytical approach.

Thus, with this in mind, Anna divided the experiments into two parts. The main aim of the first set of experiments was to describe the effect of TLR stimulation on splenocytes and peritoneal cells *in vitro* after a short and long exposure of rats to cold. The author also measured the intracellular and/or serum levels of selected cytokines (IL-17, TNFα, IL-10 and IL-1β) and the surface level of TLR2 and TLR4 (receptor for Pam3CSK4 and LPS, respectively) by Western blot. The second aim was similar, however, on the last day of rat exposure to cold, irrespective of its duration, e.i. whether short or long, the animals were pretreated with the peritoneal administration of Pam3CSK4. The relative frequencies of various splenic immune subsets were characterized either immediately after their extraction from the experimental animals, or were further cultured in the presence or absence of LPS or Pam3CSK4. The intracellular levels of selected cytokines were also measured.

The thesis is written up in a standard format, in Czech language. It consists of 9 chapters, the Introduction, Literature overview, Aim of study, Material and Methods, Results, Discussion, Conclusions, References and Other sources of information.

The work reports several interesting observations which could lead to better understanding of how infection and exposure to cold affect each other and suggests which could be the principal immune cells through which the crosstalk between the adipose tissue and immune system during cold adaptation is mediated. I also assume, that due to COVID pandemic, not all planed experiments could be performed, which have made the situation a bit stressful.

While I feel that the thesis is of a standard quality, and the majority of data are original (data belonging to Anna's colleagues are correctly annotated), there are several important questions and suggestions that could be further discussed and clarify.

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

1/ The Literature overview, in general, is written well and cites not only past and recent papers, but also current literature from the last 2-3 years. I have several technical remarks:
-the arrangements of several chapters is a bit confussing since their information content is overlapping. For example, the chapter 2.4.1 (Imunitný systém a chlad) content-wise is overlapping with the chapter 2.4.2. (Opověd imunitního systému na chladový stres);
-the flow between some chapters is not always straightforward, one has to go back and forth to realize the context and content;
- some thoughts are not finished. For example, page 9, the second paragraph from top where the idea of the shift from proinflammatory to anti-inflammatory responses is mentioned, but not explained. On the same page, top paragraph, the explanation how increased Ig levels are linked to immune suppresion is ommitted;
-the chapter 2.5.2.1., which introduces Tregs and their role in BAT is extremely short and not adequate for this thesis;
-the Intro chapter contains only two figures, which is unfortunate, since the visualization of such complex proceses as the cold adaptation in conjunction with infection could be the right place for reader's engagement.
Overall, it seems that the author could put more effort to improve the quality of this chapter.

2/ Methods, protocols, results:
-even though this is not a concern, the work should indicate whether this project was approved by the Ethical Committee. Also, because the students are not allowed to work with live animals and to euthanize them, the name of responsible person who was assisting the author should be clearly indicated.
-the author refers to the fact, that in the experimental part 1, all together 22 rats were examined in each experimental group. In this regard, all data should be presented rather as a scattered plots/graphs to see each value obtained for each cohort;
-why the amount of Pam3CSK4 for intraperitoneal administration was chosen at 100μg/kg?
-the protocol described in the chapter "4.8. Flow cytometry", is disputable:

- First, it is not clear why the red blood cells (RBCs) were not removed from the spleen single-cell suspension before their culture in the 24-well plate and were removed only afterwards. Why splenocytes in this matter were treated differently than the cells from peritoneal lavage? Does the number 3.10^6 splenocytes seeded in each well include RBCs as well?

-The usage of a scraper to remove cells from the surface of plastic well is very often deleterious for cells as they often die due to the mechanical stress and damage. Moreover, such removal is never complete. This can significantly alter the total cell numbers obtained and thus skews the enumeration. How did you control for such negative effect?

-Is not clear how the splenocytes were divided for staining given the four staining panels?

-The FMO controls for CD45RA and γδTCR are missing in Figures 3 and 4, respectively.

-$CD25^+$ cells from CD3 subsets are considered as activated T cells, but they definitively include also Tregs. Tregs are not analyzed at all.

-the protocol for intracellular cytokine staining does not indicate that brefeldin was used to halt the secretion of the measured cytokines into media. This is a standard procedure for cytokine staining. Why it was ommitted?

-Wouldn't be informative to perform also ELISA for indicated cytokines on serum samples derived from experimental rats?

-The gating strategy for assessing the the intracellular staining (Fig. 4) seems to be detecting only a few positive cells (≤5) which suggests that it could be the game of small numbers which is always prone to inaccuracies;

-the author always refers to CD80 as a marker of activated cells (B cells, myeloid). However, it is well known that CD86 is much better marker. B cells, activated or not, express only limited amount of CD80. Moreover, low levels of CD80 are expressed also by non-activated myeloid cells. What makes you argue that these are indeed activated cells?

-Western blot, Figure 12: can the author present all three blots with TLR2 and β-actin detection) from which the intensity of signals were measured? How long exposure for measurement of the signal was chosen?

-it is not clear how the absolute number of cells was accounted (Figure 6B and 6D)? What does this number refer to? The number of cells per whole spleen?

Questions for discussion:

1/ In the second set of experiments, there is an obvious disonnect between the site of administration of PamCSK4, i.e. peritoneal cavity versus the site of analysis, i.e. spleen. Wouldn't be more informative to focused on analysis of cells from the peritoneal cavity? By the same token, it is indeed a question in which niche (i.e. which of the secondary lymphoid oragans, or thymus, or perhaps intestine immune system), should one look for exposure-to-cold-related changes in immune system? Would there be a difference between an airborne, blood-born or skin-born infection? From the evolutionary point of view, which are more important for our physiology: responses to cold or responses to infection? In this context, what is the phenotype of β2-AR KO on all immune cells?

2/ In the context of the question above, and the fact that γδ T-cells are enriched in BAT, it is the BAT issue which should be intensively investigated in regards of the crosstalk between Immune system and adaptation to cold. What are your thoughts on this issue?

3/ Because several steps in the protocols used for the analysis of splenocytes in described experimental settings are problematic (see the *"formal concerns and technical questions",* above), some of the results should be considered with precaution. Specifically, in the Figure 27, the author suggests a model in which γδ T-cells play a dominant role on thermogenesis. Could the author clearly point out the main attributes of suggested model which are directly derived from her results?

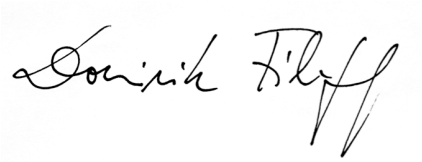
4/ it is not clear why the author suggests that exposure to cold significantly improved the survival of splenocytes (Fig.6). The author herself confirmed that the % of survival in all sample was the same

(Fig.6). Can the author lay out the argument for her conclusion in respect of better survival of spleenocytes in 14D sample?

5/ I think that a very interesting question is whether a systematic and perphaps prolonged exposure to cold can have a protective effect from infection. Is there any murine model to test this prediction?

Conclusions and recommendations

Anna Kratochvilova's thesis represents a standard quality of work which is worth to continue in the future. While I pointed out strong attributes of her work, I also have to emphasize, from my point of view, the weaker sides, as is required from this report. I'm sure that Anna is fully capable to improve her writting and presentation skills in the near future. Every student must go through this process and learn how to succeed. This work is just the first major milestone on her path. Based on the above analysis of the presented work, I recommend this thesis to be accepted as the fulfilment of the requirement for awarding Master degree to the candidate.



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