21.5.2021

RE: Evaluation report – MSc Thesis by Johan Šlemín

Dear Committee Members,

The presented thesis by Johan Šlemín deals with important topic of prevention of autoimmunity onset by the modulation of intestinal microbiota. The microbiota composition determines susceptibility to experimentally induced autoimmune uveitis and the unfavorable autoimmune outcome can be reduced by the colonization of experimental animals by commercially available *E. coli* strain (Heissigerova J. *el al* 2016, Dusek O. *et al.* 2020). This thesis is exploring the impact of *E. coli* colonization on the antigen presenting cells (APCs), hypothesizing that this type of stimulus is changing their status prior autoimmunity induction to more tolerogenic state.

The great plus is that the thesis is written in English with limited amount of spelling mistakes and it is based on classical structure and contains all required parts. However, during the review process several problematic aspects of the thesis were identified by the reviewer:

1) The results chapter starts with a brief introduction stating that for the purpose of the better text flow and better introduction of the scientific problem the author presents some experiments already published in Dusek O. *et al*. 2020 study (in this publication JŠ contributed to the Suppl. Table 3 only). In other words, the first and the largest part of the results section of diploma thesis was not done by JŠ and he was not contributing anyhow to presented data. In this light, putting this dataset in the results section is suboptimal and this data should be rather presented in the introductory part of the thesis. More importantly, the Dusek O. *et al*. 2020 paper is only cited once in the beginning of the Result chapter and there is not a single additional citation through the whole chapter, nor when figures directly copied from the publication or just slightly modified by changes in color code are presented (e.g. Fig 2 in methods section, or Fig. 3- 8 in results). Following the similar note, in conclusion section the citation referring to the Dusek O. *et al*. 2020 paper is missing, making the distinction between the impact of original paper and the submitted thesis rather blurry ("*Our study provides the first data documenting a reduction of EAU severity by EcN treatment. This thesis has identified several differences in the immune effects of EcO and EcN which could impact their effectiveness in treatment of EAU.*" – p.69). Lastly, there are parts of the text that were directly copied/just slightly modified from the above-mentioned publication. For instance, in the Methods section:

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Thesis:

> *EAU was induced by subcutaneous injection of interphotoreceptor retinoid-binding protein peptide 1-20 (IRBP; [Homo sapiens], H2N-GPTHLFQPSLVLDMAKVLLD-OH, New England Peptide, Gardner, MA, USA) 500 μg per mouse emulsified in CFA containing 3.3 mg/mL of heat-killed Mycobacterium tuberculosis H37Ra (Difco, Franklin Lakes, NJ, USA) immediately followed by intraperitoneal application of pertussis toxin (PTx; List Biological Laboratories, Inc., Campbell, CA, USA) 1.2 μg, as previously reported (Klimova et al., 2016).*

Dusek O et al. 2020:

> *EAU was induced by subcutaneous injection of interphotoreceptor retinoid-binding protein peptide 1-20 (IRBP; [Homo sapiens] H2N-GPTHLFQPSLVLDMAKVLLD-OH, New England Peptide, Gardner, MA, USA) 500 μg per mouse emulsified in CFA containing 3.3 mg/mL of heat-killed Mycobacterium tuberculosis H37Ra (Difco, Franklin Lakes, NJ, USA) immediately followed by intraperitoneal application of pertussis toxin (PTx; List Biological Laboratories, Inc., Campbell, CA, USA) 1.2 μg, as previously reported [36].*

Or the figure legend of Fig. 4 in the thesis is virtually similar to Fig,4 figure legend in the original publication:

> *EcN treatment is only effective when given before or at the time of EAU induction. (A) EAU severity was analysed by in vivo fundus biomicroscopy on the 21st and 27th day postinduction and (B) by histological analysis at day 28 postinduction. Differences were quantified by unpaired Mann–Whitney test; * $p < 0.05$ ** $p < 0.01$; n = 8 (placebo prevention), 13 (EcN prevention), 19 (placebo early + late treatment), 22 (EcN early + late treatment), 10 (placebo late treatment) and 20 (EcN late treatment) in total, graphs show individual values and red lines represent mean. Black squares represent individual inflammation severity scores from the placebo group and open circles from the EcN group.*

Dusek O et al. 2020:

> *Live EcN decreases severity of EAU if administered at time of disease induction. The EAU severity was analyzed by in vivo fundus biomicroscopy on the 21st and 27th day post-induction (A) and by histological analysis at day 28 postinduction (B) and the differences were quantified by unpaired Mann–Whitney test; * $p < 0.05$ ** $p < 0.01$; n = 8 (placebo prevention), 13 (EcN prevention), 19 (placebo early + late treatment), 22 (EcN early + late treatment), 10 (placebo late treatment) and 20 (EcN late treatment) in total, graphs show individual values and red lines represent mean. Black squares represent individual inflammation severity scores from the placebo group and open circles from the EcN group.*

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2) The introductory section about Anatomy and Function of Intestinal Immune system (p. 15-19) is heavily based on three reviews/textbooks (Hořejší et al., 2017; Mowat, 2003; Tokuhara et al., 2019). There is a minimal amount of other citations and this part of the text is just reprinting information from textbooks showing minimal synthesis of information from distinct and updated resources. Moreover, this part of the text is completely ignoring Th17 response, one would argue the most important type of response in the context of epithelial immune reaction with a clear pathogenic role in autoinflammatory diseases.

3) The presented thesis builds upon data presented in the Dusek O. *et al*. 2020 paper. The authors showed that protective effect of probiotic treatment on the development of autoimmune uveitis is dependent on the live probiotic bacteria and that the treatment with heat-killed probiotics has minimal impact ("*Next, we assessed whether viable EcN was required for a beneficial effect on EAU severity. Treatment of mice with autoclaved EcN (aEcN) had a small but non-significant effect on EAU severity, suggesting that viable organisms are required to protect against EAU (Figure 3). This suggests that an EAU suppressive effect is dependent on live probiotic bacteria.*" Dusek O. *et al*. 2020). In clear contradiction with above mention results the data presented in diploma thesis uses stimulation of bone-marrow derived antigen presenting cells by lyophilized probiotics.

4) There is a claim in methods section that the FBS used for media supplementation was produced by the Institute of Molecular Genetics in Prague (and the same is claimed about EDTA). I guess this claim is not correct.

5) The representative FACS plots and gating strategies are missing (Fig 11 and 14).

6) The number of replicates measured in particular experiment is missing (n=?, Fig. 10-14)

7) There are some irregularities associated with citation formatting, e.g. the same paper is cited in the text as *Hajjar, Ernst, Tsai, Wilson, & Miller, 2002* (p. 18) or *Hajjar et al., 2002* (p. 20). Several citations are listed in following format in the list of references: *Agus, A., Denizot, J., Thévenot, J., Martinez-Medina, M., Massier, S., Sauvanet, P., … Barnich, N. (2016). Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive E. coli infection and intestinal inflammation. Scientific Reports, 6(1), 19032.* Replacing some of the authors with dots is not acceptable. Lastly, the *Sokol H et al. 2008* citation is listed twice in reference list using slightly different formatting.

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I would like to take this opportunity and ask several questions, some of them related to above mentioned points:

Ad 3) Can you please justify why you were measuring impact of stimulation by the lyophilized probiotics on bone-marrow derived antigen presenting cells? Your colleagues were already able to show, that the impact of heat-killed bacteria administration on development of induced uveitis autoimmunity is minimal. What was the rationale behind these experiments? Moreover, to what extend actually the bone-marrow derived antigen presenting cells resemble the antigen presenting cells in the small intestine?

Ad 5) I would have some concerns about viability of the cells when 1000 µg/ml concentration of lyophilized bacteria was used (Fig. 10-14). There would be probably very high level of cellular death resulting into observed increased variability of results in this particular datapoint. Moreover, I guess the representative FACS data related to these figures are going to be presented during the thesis defense.

In the chapter "*Extra-Intestinal Inflammatory Diseases*" you are mentioning how the (induced) autoimmunity can be modulated by various (largely probiotic) microorganism. Interestingly, you are not mentioning one (patho)biont with the ability to modulate Th17 response. This organism was shown to modulate the outcome of several autoimmune diseases, including EAE, autoimmune arthritis or diabetes. Do you know, what is the status of your experimental animal colony (and the First Medical Faculty of Medicine colony) regarding this patho(biont)?

Lastly, imagine you have an almost unlimited budget, what would be your experimental approach to deal with the scientific question of your thesis? What experimental design and methods you would use for determining the impact of probiotics treatment on intestinal antigen presenting cells. How you would measure the change in antigen presenting capacity of antigen presenting cells in the antigen (IGRP) specific manner? How you would distinguish between antigen presenting cells originating from the intestine and subsequently migrating to the inductory place of IGRP-based autoimmunity and other locally presented antigen presenting cells?

To summarize, the thesis is focusing on fascinating and potentially both medically and commercially interesting aspect of host – probiotics interaction. However, there are, softly said, some suboptimal parts of the thesis mentioned above. Clearly, direct copying of whole text parts is not acceptable. Some parts of the introductory and discussion text are lacking necessary depth and synthesis of information from different sources. And the reasons why some experiments were performed using particular experimental setup are not really clear. Ignoring above mentioned aspects, the vast majority of introductory, results and discussion sections are clearly written and the

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flow of the text is smooth. I really appreciate for instance mentioning the work about not so clear effect of probiotics in various pathologies and mentioning that there exist conflicting data regarding this aspect. However, I would actually expect more complex discussion on this issue in the thesis. I am leaving the overall classification of the thesis on committee.

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