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Oborová komise pro doktorský studijní obor **Molekulární a buněčná biologie**
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Examiner's Report on Mgr. Zuzana Mikulková's PhD Thesis:

**Study of cell immunity of patients with type 1 diabetes mellitus.
(Studium buněčné imunity u pacientů s diabetes mellitus typ 1.)**

This study describes an investigation into differences of the critical components of immune system between the patients with the type 1 diabetes (T1D) and multiple sclerosis (MS), their close relatives and healthy control individuals. Specifically, it is focused on the cellular frequency and function of naïve, activated and memory T lymphocyte populations such as CD4⁺ and CD8⁺ as well as the T-regulatory (Treg) and T suppressor (Ts) cells. It highlights the utility of modern immunological techniques like fluorescence-activated cell sorting (FACS) analysis, magnetic separation of cells, multicolour cytometric bead array assay for clinical studies.

Presented thesis reports on results from three seemingly independent studies that overlap in terms of their unified effort to identify the cellular mechanism underlying an/or accompanying the development of autoimmune disease, T1D in particular. These three parts are focused on (i) the cellularity of Treg and Ts cells and the correlation of their suppressive function with the type of cytokine expression; (ii) the cellularity of naïve, activated and memory CD4⁺ and CD8⁺ cells and the correlations of their frequencies with TH1, TH2, Th3 and Th17 cytokine profiles in T1D and MS patients compared to controls; and (iii) the reactivity of T lymphocyte population derived from patients with T1D and the controls to diabetogenic peptides presented or not by activated dendritic cells (DCs).

The thesis is well written up. It consists of 8 chapters and the supplement containing copies of four publications related to presented thesis. It contains 118 pages with 20 figures, 13 tables and 257 references. The Introduction, Background, Aim of the study and Material and Methods sections help the reader to follow the logic of the candidate's argument as she constructs the rationale for the study, describes its design, procedures and the methods required for its analysis. The result section is very interesting to read. It clearly demonstrates the complexity of immune cellular network and a frequent disbalance often seen in the number and function of its components that accompanies patients with T1D or could predispose their relatives to develop this disease. Interestingly, the chapter 4.1, graph 1, 2 and 4, point to the fact that healthy relatives of T1D, e.g. those at certain risk of developing this disease, already differ in immunological parameters from healthy controls. Even though the immune cells of this group of individuals were not subjected to other immunological tests described in this study, these results suggest that healthy relatives of patients with T1D could be the appropriate target group to identify genes, mechanisms and cells whose altered function could initiate and contribute to pathogenesis of this disease. This could be a paradigm shifting finding for which the author of this thesis deserves a full credit.

In the result section, Zuzana Mikulková demonstrates her skilfulness and intellectual ability to undertake the first steps on the way to elucidate the mechanism underlying the misregulation of immune homeostasis leading to the onset of autoimmunity. Apart from very interesting results she used quite demanding methodological approaches applied usually in a high-tech research. The fact that Zuzana Mikulková was able to apply these advanced approaches to her research strategies is a great achievement for a PhD student. The Discussion and Conclusion sections summarize these successful attempts and put them appropriately into the context of current knowledge surrounding the dysregulation of immune regulatory circuit in diabetes and multiple sclerosis.

The obvious strength of the study is the connection between clinical and basic immunological research, a necessary requirement for translational medicine. From the results presented herein it is clear that all major objectives have been largely achieved and results have been published in well-recognized immunological and neurological journals.

While I feel that the Result section is very strong in providing a fresh insight into the complex regulation of cellular network in T1D, there are several concerns that the author needs to clarify:

1/ based on results described in the chapter 4.1. and as commented above, it seems that the group of healthy T1D relatives with dysregulated frequencies of regulatory T cell population would be worth of testing in activation assays using DCs and DP mix. Why this group was not used in these experiments? Do you plan to do so later? What reactivity of this group to DP would you expect?

2/ it seems that the distribution of y-axis values related to %CD3⁺CD8⁺CD28⁺ for healthy kids (ZD deti) and ZD-adults (ZD dospěli) presented in the graph 7 are very similar and their interquartile ranges (green boxes) are nearly completely overlapping. However, the author shows that statistical analysis detected significant difference ($p < 0.05$) between these two groups using Mann-Whitney U-test. As the raw data are not included in the thesis, an independent analysis is not possible. Would you explain how exactly this statistic analysis was performed and whether the raw data are publically available? Similar feeling surrounds statistical results presented in the graph 11d (ZD vs T1D, $p = 0.0042$) and 14 (right panel, ZD vys riz vs. ZD niz.riz, $p < 0.05$).

3/ From the graph 4 it is clear that Foxp3 marker is present only in approx. 60-80% of CD4⁺CD25⁺CD127⁻ regulatory cells. What experimental evidence do you have to prove that CD4⁺CD25⁺Foxp3⁺CD127⁻ cells also function as Treg cells?


4/ on the page 12 and 25 the author states that under certain conditions pancreatic β -cells induce the expression of MHCII molecules and this, in turn, initiates the inflammatory reaction against them. Wouldn't MHCII expression and presentation of self-antigens on these cells lead rather to peripheral tolerance (induction of anergy and/or deletion) than inflammation and immunity as the co-stimulatory receptor of B7 family might not be co-expressed? And wouldn't be more efficient to present self-antigens in the pancreatic draining lymph nodes rather than on β -cells? Explain your view.

Conclusions and recommendation

I have identified both the strengths and weaknesses of the thesis, although I have concentrated mainly upon the latter as is expected in such report. I want to emphasize however, that the above listed concerns in no way diminish the high quality of work presented in this thesis.

Zuzana Mikulková's thesis represents a first class work presented in a well-written standard format which brought a significant advancement in the field of T1D pathogenesis. Multiple experimental approaches, many advanced procedures and techniques described, open presentation and discussion about successful but also less successful experiments, decent analysis of obtained results as well as the discussion demonstrate that the author is fully prepared for the scientific carrier she has chosen and is able to work independently. Zuzana Mikulková has already published five papers in well recognized international journals specialized in T1D- and regulatory cell-related topics, on two of them as the first author.

Given the quality and importance of experimental results of Zuzana Mikulková's work, I fully recommend this thesis to be accepted as the fulfilment of the requirement for awarding PhD degree to the candidate according to the law §47 section 4.



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