

Prague 09.09.2021

Re: Examiner's Report on Bc. Liliana Tušková's Master thesis:

MHC II-EGFP knock-in mouse model as a suitable tool for quantitative gut immunology under conventional and germ-free conditions

MHC II-EGFP knock-in myší model jako vhodný nástroj pro kvantitativní střevní imunologii za běžných podmínek a podmínek bez bakterií

This study describes an investigation regarding the development and optimization for protocols designed for the efficient and comprehensive assessment of the frequencies and absolute cell number of various cell populations/subpopulations in secondary lymphoid organs (SLOs) using light sheet fluorescent microscopic (LSFM) and multiparametric flow cytometric (mFC) approaches. Despite many years of research, we still lack a complete picture of the number and total cellularity of various immune cell subsets which reside or transverse through these immune organs. Even more importantly, their classification and nomenclature, which has been based on the expression of surface as well as intracellular markers is sometimes fuzzy. Thus, although a few exceptions exist, such cell-type specific markers are not available. As a standard, most of these cell subsets are defined not only by a combination of different markers but also their level of expression. In addition, as we have learned from single cell RNA analysis of whole cell populations, practically every cell is unique in its expression profile, with many cells and a small number of cells that fall on the border between two or more well defined cell subsets.

The main premise of this pilot study was to show that the protocols and panels of antibodies used and optimized would be suitable for characterizing cell subsets present in the spleen, mesenteric lymph nodes (MLNs), and Payer's Patches (PPs). This has been tested in experiments where mouse MLNs and PPs isolated from conventional (SPF) and germ free (GF) housing were subjected to a comparative assessment by LSFM and mFC. It is important to emphasize that in this framework, the MHCII-EGFP mouse model which was co-developed by Prof. Černý nearly 20 years ago at MIT in USA, significantly simplified the preparation of MLNs and PPs and served as an internal control for staining MHCII molecules on various cell subsets. Data obtained was analysed and visually imaged using the unsupervised clustering (FlowSOM) which was complemented with a multidimensional reduction algorithm (t-SNE) to provide better visual resolution of cell subsets. These technically demanding approaches are a must for modern immunology. 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. It is clear that Liliana has excelled in completing these tasks to a desired pilot level, which is a relatively big achievement for a master student. Obviously, further improvement is needed to implement this protocol for the intended project investigating the entero-mammary pathway, which should be undertaken in the lab of Prof. Černý shortly. This work has nicely shown a basic layout for an effective and indispensable analytical tool of this projects.

The thesis has been written in a standard format, in English, in a very pleasant and readable form. It consists of 9 chapters, the Introduction, Theoretical background, Aims of study, Material and Methods, Results, Discussion, Conclusions, References, and Supplementary figures. Each of these chapters has been written to a high standard. I really enjoyed reading the Introductory chapter and the discussion where Liliana demonstrated her intellectual capability and sharpness in describing the current status of cell markers for different immune cell populations. In the discussion, she not only summarized her results but also provided comments to several important and unresolved issues.

The results were very interesting. Liliana has performed various experiments to establish two independent antibody panels, myeloid and lymphoid, each for 9-10 multicolor FC analysis, which identified major cell subsets in the immune-related organs analyzed. She discovered that several cell populations differed significantly between SPF and GF conditions in the MHCII-EGFP mouse model. Among many quantitatively interesting results, the best example, $\gamma\delta$ Tcells and Th and Tc cells which were determined to be significantly decreased in the GF condition, while B cells were increased. Some of this quantitative data seems to be unique such as the diminution of $\gamma\delta$ Tcells, since no other relevant data is available on this topic in the public domain. Thus, this work reports several interesting observations which open new avenues to study specific immune populations which are affected by the absence of microbiota. This is currently a very hot topic and in conjunction with the study of the entero-mammary pathway, which may lead to interesting discoveries.

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

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

1/ The Literature overview: in chapter 2.4, the author listed multiple cell types, hematopoietic and nonhematopoietic, which are capable of expressing MHCII molecules. However, one cell subset, in which the expression of MHCII is absolutely crucial to prevent autoimmunity has been omitted: mTECs.

2/ Methods, protocols, results:

-page 28, chapter 4.1.6: it does not state how GF mice were transferred to the lab in Prague and how much time, or how many days it took. It has been reported that the conventionalization of GF mice can occur very fast.

-page 28, chapter 4.2.1: mice are not executed but euthanized.

-page 31: it is quite unusual to first show Figure 4 and then Figure 3;

-page 33, chapter 5.2: the first sentence doesn't make a sense;

-In some figure legends, such as Figure 13 and 30, there are spelling mistakes;

-on several occasions in the text the author has described the acquisition of immune organ 3D structure, but none of them has been shown. Even though you have informed the reader that the data are not shown, this information should be rather completely omitted, or mentioned just once;

-page 46, Figure 14: it seems that the number of metaclusters from 25 to 26 according to this figure was increased in B – Spleen, lymphoid panel, and not in C- PP, as alluded in the text, page 44.

- Figure 14: it would be much easier, if the author navigates the reader by labelling the defined population according the type of cell subset they represent, as per text on pages 44-45, and not just by numbering as Pop1, Pop2, etc.
- Figure 10, 31, and some other: the stars which indicate the degree of statistical significance are displaced;
- starting from figure 15, up to figure 42, these figures are not referenced in the text, making the reading quite uncomfortable;
- Fig 15-23, the color code for the level of gene expression is missing;
- Fig. 40, the lower part of the bottom graph is cut off;
- page 102, Figure legend for S Fig.4 is misplaced and bottom panels are switched.

Questions for discussion:

1/ In Figure 5C you have shown the presence in the intestine of MHCII-EGFP mice of small shining dots, which you called the putative micropatches. What is the exact nature, cellular content and function of these structures and their relationship to the cryptopatches and ILFs? Does their localization near intestinal blood vessels suggest their potential role in intestinal immune responses? Can these structures be visualized in WT mice?

2/ The frequencies of MHCII-EGFP cells in PPs differ significantly when assessed by LSM (Fig. 8) versus mFC analysis. (Fig. 23, bottom). How these results can be reconciled given very different sensitivity of these two methods?

3/ Even after collagenase treatment of PPs (Fig. 10), there seems to be quite significant irreproducibility in the number of obtained cells? What factor does play a role here, and can it be eliminated?

4/ Comparative assessment of the frequencies of $\gamma\delta$ Tcells, Th and Tc cells and B cells between SPF and GF animals across several tissue samples showed significant differences. Unfortunately, these results are not discussed in the context of the effect of germ-free environment on the development of these populations. Can you at least speculate how microbiota could affect the frequencies of indicated cell subsets? Do you know whether a dramatically decreased numbers of $\gamma\delta$ Tcells in GF housing are irreversible after conventionalization of these mice to SPF housing? Lastly, it has been shown that $\gamma\delta$ Tcells play a critical role in thermogenesis. Given your discovery that these cells are underrepresented in GF conditions, mice living in GF conditions would have to experience difficulties to survive when exposed to cold conditions. By the generalization of this notion, one can suggest that it was the intestinal microbiota who endowed mammals with capacity to survive cold conditions via supporting development of $\gamma\delta$ Tcells which support thermogenesis. Can you please comment on this? Do you plan to extend this important observation in your future studies?

5/ The author suggests that no reliable F4/80 signal is obtained when cell suspension from MLN is stained for macrophages by a mixture of CD11b and F4/80 Abs. However, Fig. 11 shows that CD11b signal is also absent. On the other hand, CD11b provides a reliable signal using the splenic and MLN

suspension to detect cDC2 cell subset. This is a very strange result: while macrophages cannot be stained, cDC2 from the same tissue can be stained. Could it be that macrophages are just not present in sufficient numbers?

6/ The author suggests that developing the algorithm using Artificial intelligence will solve the problem with counting of DRAQ5 stained cells in a whole tissue. Can you briefly explain how this can be achieved?

Conclusions and recommendations

Liliana Tušková's diploma thesis represents a high-quality work which is worth continuing in the future. I want to emphasize that the listed concerns in no way diminish the quality of work. She has analyzed and presented her data clearly and openly discussed the pitfalls of her tedious experimental work. Based on this, I recommend this thesis to be accepted as the fulfilment of the requirement for awarding the Master degree to the candidate.

A handwritten signature in black ink that reads 'Dominik Filipp'. The signature is written in a cursive, flowing style.

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