RNDr. Dominik Filipp, Csc.
Laboratory of Immunobiology
Institute of Molecular Genetics AS CR
Videnska 1083
CZ-142 20 Prague 4
Czech Republic

Tel. (+420) 241.063.158
Mobil: (+420) 774.889.410
Email: dominik.filipp@img.cas.cz

Prague, May 27, 2025

Re: Examiner's Report on Bc. Nikola Malinska' Master thesis:

Study of Maternal Microchimerism Established during Breastfeeding and its Impact on the Offspring's Immune System

This comprehensive study investigates maternal-newborn microchimerism (MNM), focusing on the quantification and phenotyping of maternal cells transferred to selected offspring organs—including the spleen, thymus, mesenteric lymph nodes (MLNs), Peyer's patches (PPs), peripheral blood, and bone marrow, via suckling. The author also provides a detailed characterization of the cellular composition of mouse mother's milk from two heterologous sources: the mother's mammary gland and the pup's stomach. Furthermore, the study compares these findings between two closely related mouse strains. This work is particularly valuable, as challenges in isolating milk-derived cells have created a significant gap in knowledge regarding the cellular content of mouse mother's milk and its comparison to human milk.

The thesis is written in Czech and follows a standard academic structure, comprising nine chapters: Introduction, Literature Review, Aims, Materials and Methods, Results, Discussion, Conclusions, References, and Supplement. It represents a rigorous scientific effort, underpinned by high-quality data and employing a variety of genetic and transgenic mouse models in combination with advanced technologies. The findings lay a strong foundation for further mechanistic exploration of maternal-newborn microchimerism (MNM), a direction that is likely to be pursued in future research. Moreover, the dataset is robust and highly relevant, with the potential for immediate submission to a peer-reviewed journal. It is also worth noting that in 2024, Nikola Malinska published a first-author paper on maternal-fetal microchimerism in *Physiological Research*, further underscoring the quality and relevance of the work presented in this thesis.

The Literature Overview chapter *(*Teoretický úvod*)* is a particularly strong component of the thesis. The author presents a comprehensive and well-organized set of references to outline both the current understanding and the remaining gaps in knowledge surrounding maternal microchimerism. I found this chapter especially engaging, as it offers a critical and unbiased assessment of the existing literature, reflecting a deep familiarity with the field and a balanced perspective on ongoing debates.

A major strength of the overall work is its application of state-of-the-art methodologies to generate high-quality data. These include multicolor flow cytometry, single-cell RNA sequencing (scRNA-seq), and advanced imaging techniques such as confocal and light-sheet fluorescence microscopy. Notably, light-sheet microscopy was employed in combination with CUBIC tissue clearing to visualize and analyze specific organs, including Peyer's patches (PPs) and mesenteric lymph nodes (MLNs), further enhancing the spatial resolution and interpretability of the findings.

Although I regard the thesis as an excellent and original contribution to translational and clinical research, there are a few suggestions and questions that should be addressed:

1/ The methodology employed, particularly flow cytometry, scRNA-seq, and advanced microscopy, is technically demanding. For the sake of transparency and to aid both reviewers and readers, it would be helpful if the author clearly stated her individual contributions to these experimental procedures.

2/ The current structure includes two independent chapters*:* Chapter 2: Mateřské mléko (Mother's Milk) *and* Chapter 3: Maternální mikrochimerismus (Maternal Microchimerism). These chapters should be reformulated as subchapters within a broader *Introduction* or *Literature Review* section, to improve contextual flow. Also, it would be beneficial if the review papers listed in the section references are marked and distinguished from the primary literature sources.

3/ On page 17, the author cites evidence that maternal cells are detected in mouse embryos from embryonic day (E)13.5, with increasing frequencies from E15.5, and universally present by E18.5. However, previous studies have reported maternal $CD45^+$ macrophages in embryos even earlier, before E7.5 (e.g., Bertrand et al., *Blood* 106, 3004–3011, 2005; Balounová et al., *Nature Communications* 10, 5176, 2019). It would strengthen the thesis if these earlier findings were acknowledged and reconciled with the cited data.

Questions for discussion:

1/ It is evident, that maternal microchimerism (MM) is not universally present across all vertebrate species. For example, in egg-laying species, such as fish, reptiles, and birds, there is currently no evidence for the existence of MM. In your opinion, what is likely a major evolutionary driver behind the development and retention of maternal microchimerisms in mammals? In respect to MM, is anything known about this phenomenon in non-placental mammals, marsupialis and monotremes? Do we have evidence that milk in marsupials or monotremes contains viable immune or other somatic cells, and if so, is there any indication of postnatal, milk-mediated microchimerism in these lineages?

2/ The current model proposes that microbiota specific RORgt+ Tregs suppress IgA production in the gut thereby limiting IgA-mediated opsonization of commensal bacteria. This promotes microbial persistence and contributes to immune tolerance and homeostasis. However, in your study (page 6), you suggest that the amount of maternal IgA in milk can shift this balance: (i) high levels of IgA in breast milk are associated with reduced $ROR\gamma t^+$ Tregs and increased IgA-secreting plasma cells**,** leading to greater resistance to intestinal infection, (ii) in contrast, low maternal IgA favors higher $ROR\gamma t^+$ Treg

frequencies and fewer IgA-producing plasma cells, which correlates with increased resistance to colitis, cancer, and allergies**.** How do you reconcile these apparently divergent outcomes? Could these findings suggests that maternal IgA modulates not only early pathogen defense but also long-term immune tolerance?

3/ Published data suggests that maternal cells can survive and persist in offsprings for extended periods, i.e. months, years, or even decades. This raises a fundamental immunological question concerning how are these allogeneic cells tolerated. Can you suggest an immune mechanism explaining the long-term persistence of maternal microchimeric cells despite their expression of non-inherited maternal antigens (NIMAs)?

4/ The timing and mechanism of gut closure are critical factors influencing the window of opportunity for maternal cell transfer via milk. Available data points to striking interspecies differences between human, mouse and pigs, with the pigs undergoing gut closure within 24–48 hours postpartum, mice at 10-14 days and in humans this process is more gradual and variable. Would you consider the rapid gut closure in pigs as evidence that maternal microchimerism is not a universal or essential survival strategy for all mammals? In your view, what is the critical postnatal window for the establishment of maternal microchimerism and is the first 48 hours sufficient to enable lasting engraftment of maternal cells?

5. There appears to be a discrepancy in the interpretation of Figure 9**,** which presents anti-CD45.2 staining of maternal cells transferred to Ly5.1 offspring in Peyer's patches (PPs) and mesenteric lymph nodes (MLNs). However, Figure 6 indicates that a substantial portion of this population co-expresses CD45.1, and Figure S6 confirms the presence of $CD45.2^{+}CD45.1^{+}$ double-positive cells in Ly5.1 pups nursed by $CD45.2^{+}$ mothers. Given the lack of CD45.1-negative controls in Figure 9, what supports the conclusion that these cells are truly of maternal origin and not due to staining artifacts? It would be important to clarify how you differentiated maternal cells from recipient-derived populations that might upregulate or acquire CD45.2.

6/ There is some confusion surrounding the data presented in Figure 16 (Chapter 6.3, pages 63–64), which analyzes immune cell content in milk collected either directly from the mammary gland or the pup's stomach. According to the methods, there is no mention of cross-fostering between Ly5.1 and C57BL/6 mouse colonies during this analysis. However, the results appear contradictory. Notably, Ly5.1 mothers show significantly lower milk volume and $CD45^{+}$ cell numbers than C57BL/6 mothers. Yet, Ly5.1 pups have more $CD45^{+}$ cells in their stomach milk content than C57BL/6 pups, the opposite trend. In the discussion (Chapter 7.3, page 75), it seems implied, though not explicitly stated, that pups may have been fostered between strains. Could you clarify whether mother-pup swapping was performed in this experiment? If so, a clear description of the experimental setup, including foster pairing and timing, would be essential for correct interpretation.

7/ Several atypical or rare cell types present in the mother's milk have been revealed by scRNAseq. Could you ellaborate more on their uniqueness and potentially specific role in the developing newborn immune system?

Conclusions and recommendations

I have identified both the strengths and weaknesses of this thesis, although I have concentrated mainly upon the latter as expected from critical evaluation. However, I want to emphasize, that the concerns raised in no way diminish the overall high quality of the work. The thesis presents a well-executed study with clear scientific merit and promising implications for translational medicine. Based on these strengths, I recommend that this thesis be accepted as fulfilling the requirements for the award of the Master's degree to the candidate.