FACULTY OF SCIENCE
Charles University

Review of PhD thesis by Martin Selinger

entitled

Molecular Factors of Cell Antiviral Immunity

PhD thesis by Martin Selinger was prepared in the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences & Faculty of Science, University of South Bohemia in České Budějovice, under supervision of prof. RNDr. Libor Grubhoffer, CSc. The thesis focuses on the description of flavivirus-host interactions in case of tick-borne encephalitis virus (TBEV) and Zika virus (ZIKV).

The thesis contributes to our knowledge of (i) the TBEV-induced host responses in human neural cells and IFN-mediated protection, (ii) the identification of a new phenomenon of TBEV-induced host transcriptional and translational shut-off, and (iii) characterization of TBEV-derived molecules with hypothetical immunomodulatory characteristics, TuORF and ZIKV sfRNA.

Martin Selinger performed analysis, and is the first author, of the study of TBEV-induced host responses in human cells of neuronal origin and IFN-mediated protection (published in the *Journal of General Virology*). This paper was the first report to show that type I IFN pre-treatment of human medulloblastoma DAOY cells *in vitro* resulted in a decreased production of TBEV. Further examination of the effect of IFN-β and IFN-λ1 showed that only IFN-β pretreatment inhibited TBEV production. In this study in DAOY cells infected by TBEV, Martin Selinger showed presence of a wide panel of ISGs and proinflammatory cytokines, including type III but surprisingly not type I IFNs. He showed also a number of non-coding RNAs, including long non-coding RNAs. Upregulation of pro-inflammatory cytokines was observed following TBEV infection, while a high rate of down-regulation was observed for a wide panel of pro-inflammatory cytokines upon IFN-β treatment. These data can define a target of potent antiviral effects in cases of TBEV infection in human neuronal cells.

In another paper with the name of Martin Selinger as a first author, TBEV inhibits rRNA synthesis and host protein production in human DAOY cells (published in *PLoS Neglected Tropical Diseases*). In order to determine the mechanism how TBEV interacts with the host defence, he

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analysed *de novo* protein synthesis in DAOY cells. He observed a significant decrease in the rate of host protein synthesis, and in addition, TBEV infection resulted in a specific decrease of RNA polymerase I transcripts, but had no effect on the POLR3 activity. This is the first report of flavivirus-induced decrease of specifically POLR1 rRNA transcripts accompanied by host translational shut-off.

In addition, Martin Selinger participated in preparation and imaging of TBEV-infected cells for the study of expression of a second open reading frame (TuORF) present in the genome of TBEV (published in *Virus Genes*). However, the putative peptide TuORF was not expressed on detectable levels in the TBEV-infected human or tick cell lines, thus its role during TBEV infection is not clear. He was also responsible for the cloning of ZIKV sfRNA and sfRNA/IFN assays for the study of the full genome sequence and sfRNA interferon antagonist activity of ZIKA virus from Recife, Brazil (published in *PLoS Neglected Tropical Diseases*). In the latter study the collective of authors identified a sfRNA in ZIKV-infected cells that has antagonist activity against RIG-I-induced IFN-I. Very helpful for construction of his thesis was participation in the writing of review article entitled A bite so sweet – the glycobiology interface of tick-host-pathogen interactions (published in *Parasites and Vectors*).

The thesis is organized to 9 parts – Preface, Introduction, Aims and objectives, Research papers, Discussion, Conclusion and future perspectives, References, List of abbreviations and Curicilum vitae. The thesis successfully synthesizes heterogeneous subjects to unique study on the innate immunity to arboviruses. It contains well written literature overview and it is finished by an excellent discussion

Martin Selinger could clarify or discuss during defense of his thesis the following points.

1. Many PRR signalig pathways result in a parallel production of IFNs and proinflammatory cytokines. What is the relative importance of these cytokines in anti-TBEV response and pathogenesis? In the presented work mostly IFN but not proinflammatory cytokine responses were studied. In Introduction, the part concerning IFNs is clearly described. However, a part concerning proinflammatory cytokines is missing.

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2. Do you have some preliminary results with culture of primary human neurons or astrocytes?
3. What was a positive control to test IFN-λ1 activity? Did you test IFN-λ3? Is IFNLR expressed on the surface of DAOY cells? What are the major differences in the outcome of type I and type III IFN signaling?
4. What is known on comparison of TBEV and ZIKA virus receptors and target cells in humans, bats and arthropods?
5. What is the mechanism of sfRNA formation?
6. What is the quantity of virus in infected tick? Explain the mechanism of co-feeding from the point of view of quantity of transmitted virus. Why the blood borne infections like HBV, HCV, HIV are not transmissed by arthropods?
7. Why arboviruses seem to be resistant to tetherin/BstII?
8. Is there any effect of CCR5-delta32 mutation in pathogenesis of arbovirus infection?

The thesis represents a valuable contribution to our knowledge of molecular factors of cell antiviral immunity. It is well written and easy to read. The informational content is appropriate according to the topic of the thesis, and contains proper and essential references. The thesis is written in English with minimal number of mistakes and typing errors. I read the whole thesis and the attached papers with a great interest.

In conclusion, the thesis of Mgr. Martin Selinger perfectly fulfills requirements demanded for the level of dissertation work. The thesis would be highly ranked at the Board of the doctoral study program Molecular and cell biology, genetics and virology at the Faculty of Science, Charles University. Martin Selinger is the first author of two excellent papers published in impacted journals. I heartily recommend submitted work for defense, and depending on the outcome of defense procedure for approval of doctor degree.

Prague, March 3, 2020

Prof. RNDr. Ivan Hirsch, CSc

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Review of PhD thesis by

Mgr. Martin Selinger

Molecular Factors of Cell Antiviral Immunity

PhD thesis by Martin Selinger, which was done in the laboratory of prof. Libor Grubhoffer is focused on factors of innate immunity induced by the infection with tick-borne encephalitis virus. The thesis is written in English and consists of five publications and several accompanying chapters. All author's publications were published in high quality international journals. In two of these papers, Martin Selinger is the first and in one case the second author indicating his substantial share in these studies.

The thesis is written in good English with only minor errors or only petty language imperfections. The thesis is introduced by very nicely and synoptically arranged Introduction, which provides a comprehensive summary of the field addressed during the PhD study. Results are presented in the form of five publications where Martin Selinger participated and it is apparent that he contributed substantially or led the study in three of them. The publications present a great set of very interesting and novel data representing very significant contribution to the understanding of the innate immunity response of the cell infected with tick-borne encephalitis virus and the modulation of this response by the virus itself. The PhD thesis is finished by Discussion where the author clearly summarizes the achieved results and discusses them in the context of already known data. The PhD thesis makes apparent that the author learned a range of very advanced methods, familiarized himself with the field and significantly contributed to the field.

I have a few objections concerning the formal aspects of the thesis. Some abbreviations were not explained in the text and even though they were intuitive, it was necessary to look them up in the list of abbreviations at the end of the thesis. Since I was very interested by the author's publications I would appreciate inclusion of at least the most important supplementary figures into the thesis so I don't need to search and download them from the journal web page. Here, I have objections to some discrepancies between legends and figures in the publications. I think that there is an incorrect legend in the chart in Figure 6b in Selinger et al., 2017, where I would expect term IFN-λ instead of TBEV. One more weighty discrepancy jumped out in Selinger et al., 2019 in supplementary Figure S3, which is almost correctly described in the RESULTS chapter, although it is mentioned that samples were analyzed 12h post transfection in the text, but 24h in the figure. More distinct discrepancy is in

the figure legend where the times after transfection disagree, probably a control different from the control, which was really used in the experiment, is indicated and description of the figure does not match the arrangement of the figure. I guess that this originated from additional edits of the figure where the appropriate editing of the legend was forgotten. It is a pity since it partially degrades otherwise very nice and interesting work.

In spite of the aforementioned objections, I have to say that I enjoyed reading the thesis and I studied it with a real interest.

The look into the authors CV reveals that Martin Selinger participated in six publications, experienced a short-term stay in Glasgow and gained a grant support for his project during his study. Again, it indicates his great qualification for further successful scientific work.

With regard to the fact that we can afford a sort of speculative discussion at the defense I have a few questions to the author:

1. With regard to the maturation, flaviviruses can be divided into so called *trans*-maturing flaviviruses causing a hypertrophy and dilatation of endoplasmic reticulum and more rare *cis*-maturing lacking such outcomes. Is it known whether these two groups of flaviviruses differ in the innate immunity response induced in the infected cells or in the extent of the response?
2. Is it possible that TBEV RNA polymerase shares some cofactor specifically with RNA polymerase I and so these RNA polymerases could compete for the cofactor thus only RNA polymerase I would be affected in the cell? Alternatively, is it possible or probable that viral infection induces a pathway resulting in suppression of viral RNA polymerase or degradation of viral RNA and due to some similarity also the cellular RNA polymerase I would be suppressed or its products degraded? Is it possible to find some similarities of these RNA polymerases or their products?

In conclusion, I would like to say that PhD student Martin Selinger gained very valuable and original data and proved his ability of its elaboration, interpretation and publication. He learned a wide range of methods and skills. I found his PhD thesis very well done and I decidedly recommend his thesis for defense and for approval of doctor degree.

V Praze 3.3.2020

RNDr. Filip Šenigl, Ph.D.
ÚMG Praha

PhD thesis review for the Department of Molecular and Cell Biology and Genetics, Faculty of Science, University of South Bohemia (USB) submitted by Professor Pat Nuttall, 28th February 2020.

Candidate: Martin Selinger

Thesis title: Molecular factors of cell antiviral immunity

Review comments for the candidate:

1) The thesis Preface provides a rationale for the thesis project and places the research in the context of the research strengths of the laboratory in TBEV-host interactions. However, the final sentence of the Preface states that studies were made of "the modulatory effects of particular viral components from TBEV and ZIKV." What is the rationale and context for studying ZIKV?

2) Introduction:
How could the Introduction be re-structured to reflect better the research described in the publications?
p. 2. What key factor is missing in the description of dengue virus?
p. 8. How strong is the evidence KFDV is transmitted by soft tick species?
p. 8. "TBEV is expanding" – where has it most recently been newly reported?
p. 9. What is "virus transmission between ticks"?
p. 9. What is trans-stadial transmission?
p. 11. What are "subjective" and "objective" sequelae?
p. 11. "The TBEV E protein is responsible for yet unknown receptor binding and membrane fusion" – what does this mean?
p. 15. "Immune system and its response to ~~the~~ viral infections" What responses of the human immune system are particularly relevant to flaviviral infections?
p. 16. "However, Langerhans cells are susceptible to TBEV infection and may contribute to the spread of TBEV to uninfected cells at the same time [47]." Explain what is meant by this sentence.
p. 16. "The humoral immune response is crucial in controlling flaviviral infection.." Explain the situation in humans for TBEV and ZIKV infections.
p. 16. "On the other hand, the phenomenon of antibody-dependent enhancement (ADE) of infection was described for DENV [89, 90], thus the humoral immune response can also favour arboviral infection as a side effect." What is ADE and how can it favour arboviral infection?
p. 17. "The complexity of flavivirus-adaptive immunity interactions is demonstrated by in vivo studies with TBEV, where activation of the cytotoxic T lymphocytes (CTLs) contributes to the disease pathogenesis [55, 98]." What is the situation for ZIKV?
p. 17. "It includes the rapid recognition of pathogen-associated molecular patterns (PAMPs) in immune and non-immune cells by the pattern-recognition receptors (PRRs) and subsequent response including..." Explain this recognition system.
p.24. The paragraph describing the effects of TRIM proteins on TBEV and ZIKV refers to differences in inhibitory mechanisms. Why might this be?
p. 25. "Interestingly, IFITM5 was not shown to be IFN-inducible and is expressed only in osteoblasts." In what way is this interesting in the context of the thesis?
p. 26. "Since flaviviruses belong to the group of enveloped viruses, it is not surprising that IFITMs were proved to inhibit some of them." Why is TBEV an exception?
p. 28. "Surprisingly, OASL did not restrict DENV replication in A549 and HEK293T cells [185]." Why is this surprising?
p. 28-33. Viral RNA – why is there no mention of TBEV and ZIKV in this section?
p. 34. What is known about TBEV NS1, NS2A, NS4A/B in the context of host inhibition?

3) Aims and objectives
p. 35. Why is there no mention of ZIKV?

4) Publications
Five publications comprise the body of the thesis research of which the candidate is first author of two. All the publications are in internationally recognised journals, and they include two publications in PLoS Neglected Tropical Diseases, a journal which has a relatively high impact factor (>4).

PhD thesis review for the Department of Molecular and Cell Biology and Genetics, Faculty of Science, University of South Bohemia (USB) submitted by Professor Pat Nuttall, 28th February 2020.

Candidate: Martin Selinger

Thesis title: Molecular factors of cell antiviral immunity

Recommendation:

The thesis is of a standard consistent with that expected of a PhD and is appropriate for a defence.

Signed: P. A. Nuttall

Professor Patricia A. Nuttall, external examiner

Date: 28th February, 2020

I. In this paper the candidate is the second author. He contributed Fig. 3, showing the unsuccessful detection of TBEV TuORF peptide expression in cell cultures, and helped revise the manuscript.
I.1. How does this work fit within the aims of the thesis?
I. 2. What revisions did the candidate make and why?

II. This publication describes work on of Zika virus; the candidate was the 14th author. The candidate undertook the cloning of ZIKV subgenomic flavivirus RNA and sfRNA/IFN assays, and participated in revision of the manuscript.
II.1. How does this work fit within the aims of the thesis?
II. 2. What revisions did the candidate make and why?

III. The candidate was first author of this publication in and was responsible for designing the study, undertaking experiments, data processing and analysis, and writing the manuscript. This manuscript is clearly a significant part of the thesis.
III. 1. The manuscript was received in March 2017 and accepted in June 2017. What comments were made by the reviewers and how did the candidate respond?
III. 2. The results confirmed that DAOY (human medulloblastoma) cells are of neuronal origin and in a dedifferentiated state typical of cancer cells. How might TBEV infection of dedifferentiated neuronal cells differ from infection of differentiated neuronal cells?
III. 3. A Corrigendum was published in June 2017 because of a mistake in the data. How was this mistake detected and what was the process to correct the mistake?

IV. This paper reviews the glycobiology of the tick-host-pathogen interface. The candidate is the 6th author and was responsible for the section on "N-linked glycans of flaviviruses" and participated in the manuscript's revision.
IV.1. How does this work fit within the aims of the thesis?
IV. 2. What revisions did the candidate make and why?

V. This is perhaps the most significant publication for the thesis. The candidate was first author of this publication, designing the study, undertaking experiments, data processing and analysis, and writing the manuscript.
V.1. The manuscript was received in March 2019 and accepted in September 2019. What comments were made by the reviewers and how did the candidate respond?
V. 2. The publication claims to be the first report of a specific flavivirus-induced decrease of POLR1 rRNA transcripts accompanied by host translational shut-off. Have there been any subsequent reports of a similar nature?
V. 3. What is the relevance of this publication in the hunt for antivirals to treat flavivirus infections?

5) Discussion
p. 141. Besides DAOY cells, two other human cell lines were employed: bone marrow metastasis of Evans stage 4 neuroblastoma (UKF-NB4) and glioblastoma astrocytoma (U373 MG Uppsala). Why were these 3 lines chosen and what knowledge was gained when results from the 3 different cell lines are compared?
p. 142. "Surprisingly, no type I IFNs were expressed in response to TBEV infection." Was this the case for all 3 cell lines?
p. 142. "On the other hand, pre-treatment with either IFN-β or IFN-λ1 resulted in an increased protection of A549 cells (human lung adenocarcinoma) against TBEV." Why was this particular cell line chosen?
p. 142. "This finding represents one of the main outcomes from this study – an evidence how important is the context of infection in terms of tissue response specificity to various IFNs. In accordance to our findings, the tissue and cell type specificity in terms of IFN signalling was described for both, IFN-β or IFN-λ [124, 200]." What does this mean? If this is considered one of the major outcomes of the thesis it needs to be explained more fully.
p. 142. "The panel of up-regulated cytokines/chemokines as described here would mostly result in the activation/stimulation and chemotaxis of effector immune cells. This finding correlates with TBEV-associated immunopathogenesis in the brain [54, 55]." Explain how the upregulation of these cytokines correlates with immunopathogenesis.

p. 143. "It is not surprising that the panel of genes activated upon IFN-β treatment differs strongly from the panel of genes, whose expression is activated in response to TBEV infection naturally." Explain why it is not surprising.
p. 143. "An elegant study by Richardson et al. revealed that IFI6 prophylactically protects uninfected cells against DENV, WNV, YFW, and ZIKV by preventing the formation of virus-induced ER membrane invaginations [247]." Which cell type(s) was used? How can the results of Richardson et al. be extrapolated to TBEV?
p. 144. "Unfortunately, we are currently not able to distinguish the exact rate of host and virus contribution to the observed translational shut-off." What does this mean (rate of...contribution)?
p. 144-145. "To our knowledge, the virus-driven reduction in host rRNA levels has not been described before for any flavivirus." What is the most likely explanation why flavivirus-driven reduction in host rRNA levels has not been reported previously?

6) Conclusions and future prospects
6.1. What is the candidate's greatest achievement in this thesis?
6.2. Is there anything in the thesis the candidate would do differently if he was starting again with the benefit of hindsight?