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Examiner's Report on Mgr. Anetta Härtlová's PhD Thesis:

Molecular mechanisms of the interaction of intracellular pathogen *Francisella tularensis* and antigen presenting cells.

This study describes an investigation into the identification of novel lipid raft-associated molecular structures supporting interaction between the intracellular pathogen F. tularensis and antigen presenting cells functioning as a host. It details a top of the art mass spectrometry technology and related protocols build and designed for isolation, processing and proteome wide identification and quantification of membrane proteins regulating essential biological processes. Presented thesis reports on results from two seemingly independent studies that overlap in terms of their unified effort to identify the key membrane-associated elements controlling the entry of F. tularensis into the host cells. Specifically, it is focused on (i) establishment of F.tularensis infection model *in vitro*; (ii) identification and functional characterization of sequestosome-1/p62 protein which links the process of bacterial entry with intracellular degradation processes; and (iii) identification of Bst-2 protein as IFN-γ upregulated lipid-rafts associated protein with possible involvement in the process of F. tularensis infection.

The thesis is well written up. It consists of 9 chapters and supplementary figures on 144 pages with 27 figures, 7 tables and 294 references. The Introduction, Background, Aim of study 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, and in my view especially that of the first subproject, is the most interesting one: it clearly demonstrates complexity of host-pathogen interaction in terms of molecular processes underpinning the destruction of this pathogen. Experimental results are unambiguous as they position p62 protein as the essential linker functionally connecting internalization, ubiquitination and autophagy-mediated degradation of F. tularensis. This finding could be of significant importance for understanding the process of F. tularensis infection and for finding a means of possible protection against infection. From scientific point of view, it represents an important step towards characterizing the precise steps underpinning molecular mechanism of F. tularensis infection.

The second part of experimental section is also very interesting to read. It characterizes IFNγ-induced upregulation of Bst-2 protein on antigen-presenting cells. Interestingly, this upregulation is completely blocked upon F. tularensis FSC200 infection. While the mechanism linking infection process with inhibition of Bst-2 expression is not provided, it certainly attests to existing crosstalk between signaling pathways regulating early phases of infection and so far uncharacterized process regulating Bst-2 expression, the protein previously implicated in the antiviral immune responses. It seems that this is the very first report on the involvement of this protein in the process of bacterial infection. Thus, Bst-2 could represent a global regulator of signalling pathway limiting the rate of infection, whether of viral or bacterial origin.

Anetta Härtlová in this section demonstrates her skilfulness and intellectual ability to undertake and solve the difficult task of elucidating the mechanism underlying host-pathogen interaction, what is always one of the most difficult aspects of biological research. Apart from these excellent results, she used very demanding methodological approaches usually applied to identification of unknown protein speciments in a high-tech research. The fact that Anetta Härtlová was able to apply these advanced approaches to her research strategies is a great achievement for PhD student. The Discussion and Conclusion sections summarize these successful attempts and put them into the context of current knowledge surrounding the host-pathogen interactions.

The obvious strength of the study is the use of technologically very advanced, and so far rarely used approaches for characterizing narrowly defined subpopulation of molecules from a sub-cellular compartment which plays a major role in the pathogen entry into the cell. In this case, Anetta Härtlová applied this approach to lipid rafts/DRM fractions associated proteins and cell surface proteins in IFN-γ activated macrophages. From the results presented herein it is clear that all major objectives have been largely achieved and are suitable for publication(s) in relatively high profile international journals.

However, while I feel that Chapter 5 (Results) is very strong in its characterization of proteins critically involved in the host-pathogen interactions, there are few major and several minor concerns that need to be clarified.

Major concerns:

1/ Efficient concentration of IFN-γ used for treatment of macrophages. All results of the second subproject are derived from experiments where the macrophages were treated with defined concentration of IFN-γ. Two specific questions concern this experimental design:

-Fig. 5.14 and the text on the page 83, chapter 5.2.3., states that the time of pre-treatment and concentration of 1000IU of IFN-γ was chosen based on “efficient restriction of F. tularensis FSC200 proliferation inside macrophages”. However, no IFN-γ dose-dependent proliferation assay at indicated time points nor reference for such experiment is provided. Thus, it is not clear whether using lower or higher concentration of IFN-γ would be even more efficient. Has such titration experiment been performed?

-how does 1000 IU of IFN-γ relate to physiological concentration of this cytokine present in normal physiological conditions and during bacterial infection? Is such a high concentration ever achieved during infection?

2/ Figure 5.18.: Figure legend reads that the proliferation of FSC200 in stimulated and un-stimulated macrophages are shown. However, all six types of samples shown in this bar graph are

treated with IFN-γ: three of them before and three of them after FSC200 infection. It would be reasonable to show the proliferation of FSC200 in IFN-γ untreated samples, so a clear conclusion about the effect of IFNγ could be derived. Also, the statistical analysis confirming the significance of described differences in bacterial proliferation between samples should be provided.

3/ Figure 5.19.B. This figure demonstrates that only 24-hour treatment of macrophages with IFN-γ exhibits statistically significant (approx.10-fold) surface upregulation of Bst-2 protein, while 8 and 12 hour treatments result only in a marginal upregulation (2- and 3-fold, respectively). Yet, only 8 hour-lasting pre- or post-treatment of macrophages with IFN-γ were used in combination with Francisella infection (fig 5.19.B). For this reason it is impossible to distinguish whether Francisella infection, initiated 8-hours after the treatment of cells with IFN-γ, blocks Bst-2 upregulation or rather is able to downregulate it. Experiments including 24-hour pre-treatment of macrophages with IFNγ followed by infection for 1, 4, 8, 12 and 24 hours should be performed in the future to complement this set of data.

-also, it is not clear to which experimental condition quantified for Bst-2 protein in the figure 5.19B (last six bars) is the FACS analysis data, shown in the figure 5.20A (upper left histogram) related.

Minor points:

-page 23, chapter 2.1.7.4.3., titled Inflammasome – a link between pyroptosis and autophagy is a bit misleading as no link between pyroptosis an autophagy is provided or explained, and autophagy *per se* is not even mentioned in the text of this section. Also, it would be reasonable to clearly define the process of pyroptosis in the context of this chapter.

-figure 5.1. demonstrates that the internalization of F.tularensis into J774.2 cells reaches saturated level 10 minutes after incubation. However, the paper published by Tamilselvam and Daefler (JI, 2008, 180: 8262; figure 4.B) shows saturated levels only after 50-60 minutes of exposure to pathogen. How do you reconcile these two sets of experimental data?

-figure 5.3B, page 63: part B of this figure is projected as the magnification of the figure A at the position where pathogen interacts with the macrophage membrane. However, it seems that B is not derived from A, as a small, finger-like membrane protrusion is not visible in A. Thus, the author should clarify the origin of B.

-the text on page 88 describes observed downregulation of Icosl (CD275) surface protein measured by FACS analysis after IFN-γ treatment. However, figure 5.16 shows its higher level of expression in IFN-γ treated samples (red line) compared to untreated one (light grey line) (see also the insert with the quantification of this value in bottom right position of this figure. How do you explain this discrepancy?

-the title of 5.2.6. section “The protein expression of the CSC proteins, Bst-2 and CD54, on J774.2 in response to Francisella tularensis infection” does not correlate well with its content. It rather shows the rate of proliferation of FSC200 in IFNγ treated macrophages prior or after the infection. This title would serve its purpose much better in the following section 5.2.7.

Editing points

-page 5: abbreviation SOSC3 should read SOCS3.

-page 16, 3rd row from the top: "the choice of receptors used by *pathogens* to internalize *macrophages*……"……..should read "the choice of receptors used by *macrophages* to internalize *pathogens*……."

-page 23, 2nd row from the top: PRRS should read PRRs

-page 25, 13th row from the top: …."glycophingolip"…. should read …."glycophingolipid"……

-page 29, Figure legend: "Evolution of the membrane raft concept from.", from should be deleted.

-page 30, 20th row from the top:…..(Figure 1.4) should read (Figure 2.4.)

-page 37, 16th row from the top: 2DE abbreviation is not listed in the list of abbreviations used. Instead 2D-PAGE is listed. 2DE abbreviation is used several times in this thesis. Similarly, on the same page, the expression "**2DE gel electrophoresis"** is used. What is the meaning of **E** in its context?

-page 37, 24th row from the top: text "**differential in-gel electroporesis (DIGE)"** does not correlate with the explanation of **DIGE** in the list of abbreviations, where it is defined as **2-D Fluorescence Difference Gel Electrophoresis**. Only one of these two can be correct.

-page 70, figure 5.7A: the resolution is too small. It is hard to read the text. I'd suggest that this figure is projected in a landscape format using the entire surface of this page.

-page 88, 11th row from the top: text….."(Figure 5b)"….this figure doesn't exist in this thesis.

-page 89, **figure 5.16**: this figure is not labelled properly. According to the figure legend there should be part A and B, which are missing.

-page 90, 7th row from the top: text "…increase already in fours upon IFN-γ stimulation". Should read …."increase already in four hours upon IFN-γ stimulation".

-page 101, 5th row from the top: the author posits that the function of Bst-2 is still unknown. This contradicts the information in the last paragraph on the same page where its involvement in antiviral immune responses is described. References used in this context originate from very prestigious journals such as Nature and PlosPathogens. Thus author could provide more information on the structure of this molecule and suggest at least a putative model of its involvement in the observed phenomenon.

Curiosity-driven questions:

Based on presented data, there are several curiosity-driven questions related to this study:

- The formation of macrophage-derived pseudopod-like structures upon contact with Francisella begs the question about its relation to lipid raft membrane structures, as this work confirms that

this is indeed the entry point for pathogen. If you treat macrophages with MBCD, would you see the formation of this structure upon infection?

- Do you think that heat-killed F. tularensis would get internalized by the same mechanism as if alive? Would it require functional lipid rafts?

- The author posits that in the future is planning to define the origin of ubiquitinated proteins surrounding the pathogen immediately after internalization. How such experiment will be performed?

- Bst-2 protein upregulation is efficiently shut down upon infection. Do you think it is a general phenomenon or rather specific for F. tularensis infection. Would an infection with other type of intracellular pathogens cause the same effect? Also, the author suggest that infection alone is not sufficient to block Bst-2 upregulation. What other factors are involved?

- The author suggests that another lipid raft-associated protein called NBR1, known autophagic receptor, does not change its quantity during early phases of infection and hence its involvement in the process of autophagy of F. tularensis should be precluded (page 97). Does the author suggest that quantitative changes of a given protein is a solely predictor of its involvement in host-pathogen interaction in rafts?

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 a report. I want to emphasize however, that the above listed concerns in no way diminish the high quality of work presented in this thesis.

Anetta Härtlová thesis represents a first class work presented in a well-written standard format which brought significant advancement in the field of host-pathogen interactions. 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. The author has already published three papers in well recognized international journals specialized in this topic in biological research (Microbial pathogenesis and Microbiology&Immunology). Moreover, two additional manuscripts highlighting current results described in her PhD thesis are under preparation (prof. J.Stulik, personal communication).

Given the quality and the experimental richness of Anetta Härtlová'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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