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Examiner's Report on Mgr. Václav Janovec's PhD Thesis:

Vliv malých DNA virů na funkci plasmacytoidních dendritických buněk

Effect of small DNA viruses on function of plasmacytoid dendritic cells

This study describes an investigation regarding the interplay between Toll-like receptor 7/9 (TLR7/9) and Regulatory Receptors (RRs) which dictates the functional outcome in the production of type I interferons (IFN-I and-III) from plamacytoid dendritic cells (pDCs) upon their infection by small enveloped DNA viruses, hepatitis B virus (HBV) and human immunodeficiency virus (HIV). Usually, upon the recognition of DNA of these viruses in early endosomal compartment, pDCs are capable of secreting an enormous amount of IFN-I, which serves together with other cytokines, to induce antiviral responses in surrounding cells with the aim of inhibiting or slowing down viral replication and spreading. The premise of the study was to explore the previous observations which suggested that viruses could target the RR signaling pathway which in pDCs acts as a negative feedback loop to inhibit TLR7/9 signaling which diminishes the production of IFN-I and effective antiviral responses. It is important to emphasize that the understanding of this inhibitory mechanism on the molecular level is lacking. The work of Mgr. Janovec represents several important steps towards the resolution of these uncertainities as they provide fresh insight into a deeper understanding of the molecular processes underpinning this phenomenon. Importantly, the author has also shown that blocking the RR-mediated inhibition of TLR7/9 signaling pharmacologically can indeed be a very effective way of restoring the immunogenic activity of pDCs which can be potentially used for the treatment of chronically infected patients with HBV. His work also contains many other interesting results which contribute to a better understanding of the effect of HBV and HIV on the biology of pDCs.

It is necessary to emphasize that the four main papers which are directly linked to Mgr. Janovec' PhD thesis have been published in international and impacted journals, such as *Frontiers in Immunology* (IF_{2020} = 7,5), *Scientific Reports* (IF_{2020} = 4), and *Viruses* (IF_{2020} =3,816). These papers have been well received by the scientific community. As an example, the review by *Hirsch, Janovec, Stranska and Bendriss-Vermare* in *Front. Immunol (2017)* has already received more than 9,000 views and 28 citations. In addition, the primary paper published by *Janovec et al* also in *Front. Immunol (2018) has already received nearly 5,400 views and 15 citations.* Moreover, there are three other publications which are unrelated to the presented PhD thesis, two of them were co-authored by Mgr. Janovec (*FEBS* (IF_{2020} = 4,392), *Cells* (IF_{2020} = 4,366)), and one published in *Cancers* (IF_{2020} = 6,126), with Mgr.

Janovec as the first author. In this regard, the work represents an indispensable addition to scientific literature publically available on this topic.

The results from these studies are indeed interesting. From my point of view, among the most impressive results generated by Mgr. Janovec is the elucidation of the role of MEK1/2-ERK signalling pathway in dampening IFN-I production in pDCs. In this scenario, the high-avidity engagement of BCDA2 RR by mAbs on pDCs effectively inhibits IFN-I and IL-6 production via the activation of ERK and c-FOS. This could be potentially a very important insight which warrants further investigation. Also, very promising is data from the study using TLR dual-acting agonists to produce PMBC-derived cytokines which are capable of inhibiting HBV in human hepatocytes *in vitro*. Together, these results could be potentially far reaching for clinical practise since a step-by-step improvement in this proces can potentially improve patient lives.

The thesis is well written. It is presented in a shorten version with 267 references. It contains the abstract, list of abbreviations used throughout the text, introductory chapter which highlights the main characteristics, activation process, signaling of pDCs, mode of viral recognition, and the effect of viral infection on pDCs. Chapter #3, "Aims of the work", introduces the main goals of this study followed by a short chapter #4 which lists of methods used. Then, chapter #5, Results, lists 7 papers which were published during the PhD studies, four of which are directly linked to the presented PhD thesis and their main conclusions are concisely summarized. This chapter highlights the significant achievements of Mgr. Janovec in this relatively competitive field of research.

While I feel that the conclusions of this study are very important and strong, there are several suggestions and questions that could be further discussed. First, I have several formal concerns:

1/ I feel, unfortunately, that the title of the thesis "Effect of small DNA viruses on function of plasmacytoid dendritic cells", seems not to be reflective of the actual science presented in the thesis. The generalization of the results obtained from experiments using only two small DNA viruses, HBV and HIV, could be misleading. Second, the function of pDCs has been assessed only in the case of the former (*Janovec et al, Front in Immunol, 2018*), but not the HIV story, which examined instead the phenotype of pDCs (*Font-Haro, Janovec et al, Viruses, 2018*).

2/ The very first paragraph in the Chapter 2.3, page 18, is seemigly missing several primary references. Specifically, the author has failed, or for some reason did not want to use the primary references for description of PRRs, PAMPs and DAMPs. Charlie Janeway, the father of modern Innate Immunity, was a central figure who not only coined this nomeclature and predicted the existence of PRRs and PAMPs

in 1989 but also experimentally demonstrated their presence in the mammalian system in 1997. Similarly, Polly Matzinger also predicted the existence of DAMPs. I guess that their names and seminal papers should be referrenced in the PhD thesis.

3/ The presented PhD thesis is very well written, however, there is a slight biased towards a "virological central view of TLR9 function". Clearly, TLR9, -7 and -8, are indispensable in the role of the detection of viral nucleic acids (NAs). However, there is plenty of evidence that TLR9 can also efficiently sense the presence of endogenous DNA from apoptotic/necrotic cells and such engagement triggers immune responses representing important "DAMP" or "danger" signals. Thus, I would recommend, to tone down statements such as "pDC are cells specialized to recognize viral infections". Instead, in the Introductory chapter, I would recommend to characterize the function of TLR9 in a broader sense, i.e. recognition of exogenous as well as endogenous NAs and only then elaborate on its more specific role in the recognition of viral NAs.

Questions for discussion:

1/ It is not clear how BDCA-2 via activation of ERK-1 and c-Fos inhibits TLR9-mediated production of IFN-I and III, chemokines and cytokines. Is the action of c-FOS direct or indirect? Also, in your paper (*Front in Immunol, 2018*) you stated that c-FOS expression induced by BDCA-2 mAb was only partially inhibited, pointing to the existence of MEK1/2-independent regulation of this expression. Are there other signaling pathways which bifurcate following BDCA-2 triggering? You have also stated that this inhibitory capability depends on a high-avidity interaction of RRs with their natural ligands, but the outcome of such interaction is not always the same for all RRs (for example BCDA-2 and ILT7) in terms of molecules other than IFN-I, specifically co-stimulatory molecules CD80 and CD86. Furthermore, one of your observations presented in *Figure 10D* of the same paper shows that CpG-mediated TLR9 signaling also induces ERK phosphorylation but without activation of c-FOS. What is the current mechanistical view on underlying causes of these different signaling conditions?

2/ Please explain how both the ITAM and ITIM motifs of adaptor proteins through which RRs signal are equally efficient in abrogating TLR9-mediated production of IFN-I and III?

3/ Your work suggests that pharmacological targeting of MEK1/2-ERK signaling could be a strategy to re-establish the immunogenic activity of pDCs. This could be is clinically highly relevant. However, given that MEK1/2-ERK signaling is used ubiquitously, how does the targeting of this signaling axis, specifically to pDCs and perhaps to hepatocytes, could be achieved? Can you please provide any information regarding a suitable and/or available strategy?

4. In your paper published in *Sci. Report, 2020*, you have shown that conditioned media from PBMCs stimulated with dual-acting TLR agonists inhibited the production of HBV in freshly PHH but failed to reduced cccDNA levels. Does this mean that HBV can persist in a latent state for an unlimited time? Then, when the humoral inhibition is removed, do the viral titres return to infectious levels? In general, what is the relationship between production versus persistence of HBV? Are those 8% of HBV patients who are efficiently cured with a IFN-α monotherapy completely clean of cccDNA?

Conclusions and recommendation

I have identified both the strengths and weaknesses of the thesis. I want to emphasize, however, that the above concerns in no way diminish the high quality of work presented in this thesis and author's publications.

The thesis of Mgr. Janovec contains important work that has been presented in a well-written and shortened format that has brought further advancement in the understanding of HBV- and HIV-modulated immune mechanisms regulating IFN-I responses in pDCs. This contribution in the long run can improve the management of HBV patients with respect to their potential cure. In addition, modern experimental approaches, an open presentation, discussion and analysis of the obtained results demonstrate that the author is fully prepared for his professional scientific carrier. His papers which have been published in well-recognized international journals lend further support to this statatement. Given the quality of Mgr. Janovec work, I fully recommend this thesis to be accepted as the fulfilment of the requirement for awarding the degree to the candidate according to the law §47 section 4.

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