JULY 24th, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 12262-FINAL REVISION - RESUBMITION.doc).

Title: SUBVERSION OF CELLULAR STRESS RESPONSES BY POXVIRUSES

Author: Thiago Lima Leão and Flávio Guimarães da Fonseca

Name of Journal: *World Journal of Clinical Infectious Diseases*

ESPS Manuscript NO: 12262

The manuscript has been improved according to the suggestions of reviewers:

OBSERVATIONS BY REVIEWER 00503124

1. The authors state, The inhibition of HSP90 function by novobiocin during infection impairs intermediate and late viral gene expression and, therefore, reduces viral DNA replication[115; 116' This is a confusing statement because intermediate and late viral gene expression occur after DNA replication, not before DNA replication. In addition, what the authors state here is completely contradicted by the abstract from the article they reference: Novobiocin inhibits vaccinia virus replication by blocking virus assembly. Novobiocin inhibits the replication of vaccinia virus in cultured BSC40 cells. All classes of viral proteins were synthesized during synchronous infection in the presence of drug. The onset of DNA replication was delayed slightly, yet the extent of DNA replication in the presence of novobiocin was comparable to that of a control infection. A delay in the temporal transition to late viral protein synthesis was in keeping with the effects on DNA replication. Although the precursor forms of the major viral structural proteins were synthesized normally at late times, the proteolytic processing of these polypeptides was inhibited, which suggested an impediment to virus assembly. Electron microscopy revealed that novobiocin blocked virus morphogenesis at an early stage. Conversion of the concatemeric DNA replication intermediates into hairpin telomeres occurred in the presence of novobiocin, confirming that telomere resolution was not coupled to virus assembly. Novobiocin is the latest addition to a class of antipoxviral agents, which includes rifampin and IMCBH, that arrest morphogenesis.

Response: *The reviewer is absolutely accurate in his observations. During writing of the manuscript we mixed up information relative to novobiocin with that of geldanamycin. In this part of the text we wished to discuss about the later, thus, all information on novobiocin was removed and replaced by information relative to the impact of geldanamycin on the poxvirus life cycle. This drug, differently from novobiocin, delays DNA replication and reduces viral late expression. To support our claims we introduced the reference* Hung JJ, Chung CS, Chang W. J. Virol. 2002; 76, 1379–1390 *(ref.# 36). The chronological inversion of intermediate/late gene expression and DNAS replication was also corrected.*

This particular paragraph now reads: "The inhibition of HSP90 function during infection by the use of geldanamycin - a drug that blocks the ATPase activity of that chaperone - impairs viral multiplication by delaying viral DNA replication and intermediate transcription, and also by reducing expression of late genes [36]."

2. How does the author know that C16 orthologs are non essential for virus replication? Have any been tested? Or is the author just assuming this? Authors should be careful in generalizing too much about poxviruses.

Response: *After reading again this part of the text we agreed with the reviewer criticism. We had made that assumption from studies that have been originally published about* Vaccinia virus *only. Thus, we toned down the statements by removing generalizations about C16 orthologs, as follows:*

*Page 12: "*The *Vaccinia virus* C16 protein is non-essential for virus replication but play important roles in the manipulation of the host cell biochemistry[97]"

Page 15: "Counteracting this immune signaling, the Vaccinia virus produces, early in infection, the C16 protein - which can bind to Ku70 blocking DNA-PK recruitment to DNA - and the N2 protein - a virulence factor that presents the ability to inhibit IRF3-dependent innate immune responses[126; 127]."

3. On XBP1, authors could mention TLR regulation of XBP1 activation. (Martinon, Nat. Imm. 2010). Vaccinia has a number of proteins that interfere with TLR signal transduction.

Response: *In order to mention the suggested aspect we included the following paragraph in the main text (page 9): "It is known that XBP1 can be activated by TLR-2 and TL4-4 stimulation in an IRE1 dependent manner; also known is the fact that Vaccinia virus and other cordhopoxviruses are able to interfere with TLR signaling. Therefore, this seems to be a virus-driven indirect strategy to down-modulate XBP1 activation. Because XBP1 has been shown to be important for sustained production of cytokines by macrophages, it seems logical that poxvirus may interfere with XBP1 activation as a way to cope both with the host innate responses as well as with the ER stress."*

4. On the discussion of CHOP, GADD34, ATF3, authors could include that E3L knockout significantly increases ATF3 expression. (Ludwig, JVI 2005)

Response: *We acknowledge that the host stress responses, innate immune responses and apoptosis/necrosis pathways are intrinsically connected and have many signaling cascades that are intertwined throughout these processes. However, keeping in mind our limitations concerning the manuscript length and complexity, we decided to dissect some processes and focus in their participation during the development of stress responses, exclusively. Thus, some aspects were purposely left aside, which is the case here, and we would rather keep it like that. However, if the reviewer feels that this is absolutely essential to the paper, we will be more than happy to include such discussions.*

5. On IRE1/ATF6 dependent stress pathways, TRAF 2 is a binding partner for IRE1, and vaccinia interferes with TRAF2 (Ember, JGV 2011; Haga, JVI 2014)

Response: *As mentioned above, we had to hold back on certain discussions in order to maintain our proposed focus and paper length. In the case of the paper by Ember et al., JGV 2012, the authors have shown that the C4 Vaccinia virus protein inhibits "NF-κB activation at, or downstream of, the inhibitor of kappa kinase (IKK) complex". TRAF2 and 6 are upstream of the IKK complex and, therefore, are not targets of the C4 protein. In the case of Haga et al., JVI 2014, authors demonstrated that TRAF2 is indeed important for Vaccinia virus replication, however, they conclude that "TRAF2 is a proviral factor for VACV that plays a role in promoting efficient viral entry, most likely via the plasma membrane". At this early step of the infection, it is not clear whether the observed phenomena are related to the role of TRAF2 in the stress response (we think not!). Thus, we opted not to discuss this particular aspect (if the reviewer agrees, of course).*

6. There are several problems with references. Authors should go through the entire list to check for accuracy.

Response: *As suggested by the reviewer we revised all references throughout the paper in order to keep their accuracy.*

Authors state that C16 play an important role in cell biochemistry, but the reference does not support this statement. There are no data on cellular biochemistry in that manuscript.

Response: *We modified that statement in order to assure support from the used reference. Now it reads: "The Vaccinia virus C16 protein is non-essential for virus replication but seems to play an important role in the down-modulation of the host immune responses[97]. Further studies showed that this protein can inhibit HIF1α hydroxylation through direct interaction with the PHD2 enzyme even when ectopically expressed[98"]".*

Reference 40 that is supposed to be on ROS accumulation is on urbanization of house finches with no data on ROS.

Response: *That is embarrassing, and we will not even try to explain it. The strange reference was removed and adequate references (ref. #69 and #105) were kept on the paragraph.*

Reference 23 does not appear to be related to the sentence on "poxviruses that … encode their own redox machinery in order to mediate disulfide bond formation in newly made viral proteins."

Response: *It seems to be another case of reference misplacement. It was removed and the adequate references were maintained (ref. #107 to 109).*

Ref 139, is this correct on IRE dependent stress pathways?

Response*: Yes it is. In the mentioned reference authors used a T7 RNA polymerase-expressing recombinant Vaccinia virus (vTF7.3) to ectopically express several MHV proteins. In all but one construction there was no activation of ER stress, showing that the Vaccinia virus infection itself was not causing stress. Only when the Spike (S) protein was expressed by the Vaccinia vector stress responses were mounted, indicating that the coronavirus S protein was the stress inducer.*

7. Authors state "Poxviruses increase intracellular ROS accumulation[40] to promote growth of infected cells and immune evasion." Do the authors mean all poxviruses do this? Or only myxoma virus? Teoh ML, Turner PV, Evans DH. JV 2005.

***Response**: Indeed, the mentioned references report such aspect for the myxoma and shope fibroma viruses only; we have extrapolated to other poxviruses because many of them encode genes that are similar (if not identical) to the ones described for the leporipoxviruses (as is the case of the SOD-1-like A45R gene from Vaccinia virus). The sentence was modified as follows to clarify this point:*

"It has been shown that Myxoma virus and Shope fibroma virus increase intracellular ROS accumulation to promote growth of infected cells and immune evasion. This is achieved via inhibition of Cu/Zn-SOD1 activity through the expression of catalytically inactive homologs of cellular SOD1 that cannot bind Cu - which is essential for dismutase activity - but retains the Zn-binding properties and, similarly to their cellular homologs, form stable heterodimeric complexes with cellular Cu-dependent chaperones that are essential for SOD1 function[105; 69]. It is likely that other poxviruses cause a similar effect during their multiplication cycle as some encode SOD-1 like genes; one such example is the A45R SOD-1-like gene from Vaccinia virus." (page 13).

8. Language: Under HOST TRANSLATIONAL SHUTOFF, it should say Most Viruses, as obligate intracellular parasites,…

***Response**: Corrected as suggested.*

Under Oxidative stress, I do not know what "Poxviruses explore…" means

***Response**: We meant "exploit", not "explore". It was corrected accordingly.*

In the miscellaneous cell signaling section, the meaning of this sentence is unclear, "promoting Akt phosphorylation and downstream events leading to the suppression of apoptosis and cell growth, survival, and proliferation[146; 129]. Is promoting AKT phosphorylation leading to the suppression of apoptosis, cell growth, survival and proliferation, or is it leading to the suppression of apoptosis and cell growth?

***Response**: The phrase was modified. Now it reads: "Class IA PI3K proteins were shown to play an important role in poxviruses infection, promoting Akt phosphorylation and downstream events leading to the suppression of apoptosis, cell growth, survival, and proliferation[140; 141]."*

9. On the second page of the introduction authors claim that poxviruses dedicate more than 50% of their genome to immune evasion. This figure is higher than I believe and the review from G smith says 1/3 to ½ of the genome.

***Response**: The sentence was modified as follows: "Indeed, most poxviruses (especially chordopoxviruses) spare up to 50% of their genomes to code for immune evasion-related and host-interaction genes[7]".*

OBSERVATIONS BY REVIEWER 02521807

This is an interesting review. They describe diverse strategies that poxviruses use to subvert host cell stress responses. The manuscript needs a thorough re-editing job so that scientists both in and outside the immediate filed can better follow it. In this sense, they should offer a brief introduction of the cellular stress responses and their several cascades of events.

***Response**: We agree that the host stress responses form a quite complex set of cell functions and some introduction to basic and more frequent pathways would be beneficial to readers. However, we have to keep in mind our limitations concerning the manuscript length and complexity; thus, we decided not to review the cellular stress pathways themselves, as this could substantially increase the length of the paper and also shift the main focus of the review. Rather, we dedicated to focus the article on stress responses that are actively modulated by poxviruses. Nonetheless, in order to minimally present some background on cell stress responses we added the following paragraph to the introduction section (page):*

"All the above mentioned virus-driven interferences within the cell may lead to the transduction of cell stress signals and consequent cell stress responses. The cell may respond to stress in a variety of ways, including the activation of pathways that promote survival or the elimination of damaged cells through programmed cell death (apoptosis, necrosis and/or autophagy). There is a multitude of pathways that may be elicited upon different types of stress, and the resulting signal transduction cascades are often shared by other cell processes such as the activation of innate immunity, cell cycling and so on. Nevertheless, the most common stress responses include those elicited against heat shock, ER stress (the unfolded protein response – UPR), DNA damage, and oxidative stress."

Additionally, some background on each type of stress response is given at each specific subsection (HEAT SHOCK RESPONSE, UNFOLDED PROTEIN RESPONSE and so on). The article was also thoroughly edited for clarity.

The authors only represent in a Figure those events ascribed to unfolded protein response. It is a useful tool to follow the description on text. They should incorporate additional figures – besides Figure 1- representing other events (such as heat shock response; host translational shutoff; hypoxic response; oxidative stress response; DNA damage response) related with poxviruses immune escape. By this strategy, some descriptions could be shortened thus appearing more clear. Most of the mechanisms described are related to "poxviruses". It should be specified whether are related to vaccinia (most of the references are related with), or another well-defined poxvirus. Minor comments Golgi is a last name, so it must be written with Upper case.

***Response**: A new set of figures were created and included to illustrate most subsections of the paper, as suggested.*

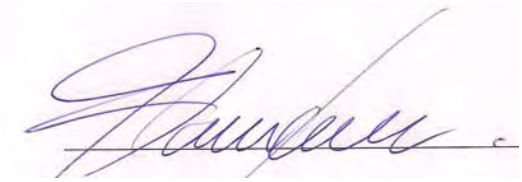
Most of the mechanisms described are related to "poxviruses". It should be specified whether are related to vaccinia (most of the references are related with), or another well-defined poxvirus. Minor comments Golgi is a last name, so it must be written with Upper case.

***Response**: The text was modified to clarify whether a given information is common to all poxviruses or it is specific to a virus or group of viruses. Please see examples on pages . The name "Golgi" was corrected to the proper letter case thoroughly.*

We sincerely hope that our manuscript has met the reviewers' expectations.

Please observe that my e-mail address has changed to fdafonseca@icb.ufmg.br

Sincerely yours,



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