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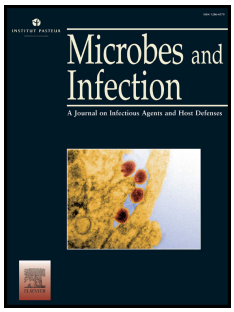

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Letter to editor

Lack of evidence of mimivirus replication in human PBMCs

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Abstract

The Acanthamoeba polyphaga mimivirus (APMV) was first isolated during a pneumonia outbreak in Bradford, England, and since its discovery many research groups devoted efforts to understand whether this virus could be associated to human diseases, in particular clinical signs and symptoms of pneumonia. In 2013, we observed cytopathic effect in amoebas (rounding and lysis) inoculated with APMV inoculated PBMCs (peripheral blood mononuclear cell) extracts, and at that point we interpreted those results as mimivirus replication in human PBMCs. Based on these results we decided to further investigate APMV replication in human PBMCs, by transmission electron microscopy (TEM) and qPCR. No viral factory was observed in APMV inoculated PBMCs, at any analyzed time and M.O.I.s (multiplicity of infection), by checking 550 cells per condition tested. We also measured the variation of viral DNA by qPCR targeting helicase gene during the course of the TEM experiment in PBMCs, but the DNA levels stayed the same as the first time-point post infection. In summary,

our newest qPCR and TEM results do not support previous statements (including ours) that mimivirus is able to replicate in humans PBMCs.

Keywords: mimivirus; PBMCs; acanthamoeba

Main Text

In 2003, the scientific community was surprised by the remarkable discovery of Acanthamoeba polyphaga mimivirus (APMV), an *Acanthamoeba*-infecting virus with outstanding features, including a giant virion (800nm) and a long and complex genome (approximately 1,2Mb) [1]. APMV was first isolated during a pneumonia outbreak in Bradford, England and since its discovery many advances have been done concerning the characterization of mimivirus evolution, life cycle in *Acanthamoeba*, diversity and interactions with amoebas and other organisms [1-7]. Among mimivirus explored topics, some research groups devoted efforts to understand whether this virus could be associated to human diseases, in particular clinical sings symptoms of pneumonia [8,9]. Different approaches have been explored to address, directly or indirectly, this issue: (i) the search for antibodies against mimivirus and or viral DNA in pneumonia affected and non-affected patients sera [9-13]; (ii) the search for APMV virions or DNA in bronchoalveolar (BAL) samples, oral-nasal swabs and other samples (e.g. feces) [11-15]; (iii) mice infection by using distinct doses of purified virus to check virus replication and pneumonia signs [16]; (iv) inoculation of mimivirus in mammalian cells, including phagocytes (cells lines and PBMCs) [17,18]. Many of those studies demonstrated the presence of pieces of mimivirus DNA in different human samples, including BAL, feces and serum; antibodies against mimiviruses were also detected in humans and others animals [10,11,19]; mimivirus has been isolated from human samples, including BAL and feces; an animal model to study pneumonia caused by mimivirus was proposed, by using very high M.O.I.s; and the replication of mimivirus in mammalian phagocytes was proposed based on partial or indirect evidence, such as qPCR and observation of cytopathic effects in amoebas inoculated with the extract of APMV-inoculated PBMCs [18,20,21,22,23]. Although a number of studies were not able to detect mimivirus in human samples, there is increasing evidence that mimivirus is a member of the virome of humans and other vertebrates [19,23]. However, the role of mimivirus as causative agent of diseases in humans is controversial and needs to be substantially more investigated.

Inspired by a previous work that indicated the replication of mimivirus in mammalian macrophage and monocyte derived cells by genome quantification, we investigated the response of primary human peripheral blood mononuclear cells (PBMCs) against mimiviruses at different M.O.I.s [17,18]. At different times post infection, the mimivirus-inoculated PBMCs were collected and directly inoculated in *Acanthamoeba* in order to detect and titer the virus by the endpoint/$TCID_{50}$ method. Interestingly, we observed cytopathic effect in amoebas (rounding and lysis, after 4-6 days) inoculated with APMV inoculated PBMCs extracts. At this point we interpreted those results as mimivirus replication in human PBMCs, and the obtained titers correlated to the M.O.I. used and conditions tested, resulting in growth curves. In the following months after the publication, we decided to investigate the cycle of mimivirus in human PBMCs by transmission electron microscopy. PBMCs were infected (2 pools of 5 healthy donors) as described by Silva et al, 2013 and were collected at time 0, 2, 4, 6, 8, 12 and 24 hours post inoculation at M.O.I.s of 1 and 10. The cells were then prepared to transmission electron microscopy (TEM) as previously described [5]. All experiments were carried out according to UFMG Ethics Committee guidelines. We were not able to observe any clear modification in the morphology of PBMCs after APMV infection, even at later time points. After the analyses of more than 550 PBMCs per condition by TEM, no viral factory was observed in APMV inoculated PBMCs, at any analyzed time and M.O.I.s. We were able to visualize APMV particles inside compartments, regardless the time p.i.. At times 8 and 12 p.i. APMV particles seemed to be under degradation (fibrils shorter, atypically interlaced), but not under morphogenesis (Figure 1). We also measured the variation of viral DNA by qPCR targeting the helicase gene [24] during the course of the TEM experiment in PBMCs, but the DNA levels stayed the same as the first time-point post inoculation (Ct 26). Titration of this set of infected PBMC extracts (supernatant and cells) in amoebas resulted in cytopathic effect (rounding), although more discreet if compared to the previous experiments. The calculation of the hypothetical title in this new set of experiments resulted in just 2 logs of difference between time 0 and 24 p.i. (for both MOIs), while we observed about 4 logs in the first set [18]. Although we could not observe APMV replication in human PBMCs as previously interpreted by our team, we could confirm that mimivirus inoculation in those cells induces type I Interferons (IFNs), as described previously. Interestingly, we also could confirm that U.V. inactivated APMV, but not infectious APMV, is able to

induces the expression of Mx1 and IFI6, two interferon stimulated genes (ISG), repeating the same observation from our 2013 paper (data not shown).

Discussion

When comparing results from both approaches (2013 paper and new presented data), we came to conflicting conclusions. While the first has been interpreted as virus growth and correlation of that to the IFN system antiviral activity and inhibition by the virus, the second dataset (based on complementary methodological approaches – MET and qPCR) does not reveals any signs of mimivirus replication in PBMCs, but confirmed the interaction with the human IFN system. The activation of IFN system by mimivirus is not a surprise, considering that the virion is large and potentially presents many PAMPs (pathogens associated molecular patterns).

PBMCs are a mixture of heterogeneous primary cell populations. That means that each time a PBMC purification is made, different ratios of cell types can be obtained. And since these cells are collected from the blood of donors, the immune status and other parameters unique to that specific donor at the time of collection can affect how the cell preparation will be. Although blood donors are only accepted when healthy (no reportable disease nor disease symptoms), subclinical infections by other pathogens, exposure to any particular substance or even previous exposure to mimivirus (which would result in circulating antibodies) are not taken into account. Also, the interaction between different types of cells makes the system more complex and prone to variation. In addition, due the need of a large number of cells for experimentation, we prepare PBMCs by making pools of 5 to 10 blood donors, which support an even more complex scenario. Although we could observe a similar immunological response to that observed in our first study [18], we believe that future studies concerning immunological response against mimivirus should also be performed in vertebrate's cell lines (e.g. RAW, THP-1), aiming to reduce such variables associated to PBMCs by experimenting on less variable systems. We believe that variations on amoeba-toxicity induced by extracts of APMV-inoculated PBMCs could be explained by the heterogeneity inherent to PBMC system.

Despite individual (immunological) and methodological bias related to PBMCs experimentation, we have no new evidence that support our previous conclusion that

mimivirus is able to replicate in human PBMCs. The reason why extracts of PBMCs exposed to mimivirus induce cytopathic effects in *Acanthamoeba*, in a given circumstance, remains to be investigated. In the absence of replication, we hypothesize that it can be related to immune response factors produced after mimivirus inoculation in PBMCs, which increases in concentration during the experiment time-course and ends up affecting the amoeba used for titrating the samples. It is important to note that adding extract from PBMCs which were not exposed to mimivirus to amoebas does not lead to cytophatic effect, suggesting that the activation of PBMCs response against this large set of PAMPs (mimivirus virion) lead to the production of such compound(s) that causes amoebas rounding. The nature of this putative toxic inhibitor of amoebas produced by PBMCs when exposed to mimivirus is not known and its production seems to be inhibited by PBMCs pre-treatment with IFN-beta 1, but not by IFN-alfa 2. The fact that mimivirus inhibits Mx1 and IFI6 genes even in the absence of apparent replication while inactivated mimivirus does not is especially intriguing and one possible explanation could be that mimivirus virions contains U.V. labile inhibitory factors; or that mimivirus would be able to express genes which blocks Mx1 and IFI6, regardless of replication and morphogenesis in human PBMCs. In summary, our newest qPCR and TEM results do not support previous statements (including ours) that mimivirus is able to replicate in humans PBMCs. Despite the absence of replication, mimivirus virions seem to be able to interfere with the expression of Mx1 and IFI6 in human PBMCs, two ISGs. It is important to note that regardless of the scenario, the interaction between the human IFN system and mimivirus (or its impact on human cells) is interesting and deserves more investigation. The relevance of the recently proposed IFN-beta antiviral mechanism mediated by IRG1 and itaconic acid is still valid and may be expanded to the immunological response to other viruses [25].

Finally, we would like to declare that our group has no direct evidences that establish a clear causality relationship between mimivirus and any disease in humans or other animals. However, we do support and encourage other research groups to investigate this relevant topic, respecting a rigorous experimental design, with adequate controls. We also support the use of isogenic animals and cell lines (instead primary cultures) to reduce variability and lack of reproducibility. Lastly, we would like to highlight the importance of using multiple methodological approaches to address a given question, to avoid misinterpretations – as we experienced.

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Figure 1 Legend

Transmission electron microscopy of human PBMCs inoculated with APMV, 12 hours post inoculation. A and B – Overview of inoculated cells. Arrows: APMV particles inside endosomes. No viral factory can be visualized. C-E: APMV particles fibers and capsid likely under degradation in PBMCs compartments.