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Lack of evidence of mimivirus replication in human PBMCs

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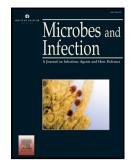
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1	Letter to editor
2 3	Lack of evidence of mimivirus replication in human PBMCs
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23	Abstract
24	The Acanthamoeba polyphaga mimivirus (APMV) was first isolated during a
25	pneumonia outbreak in Bradford, England, and since its discovery many research
26	groups devoted efforts to understand whether this virus could be associated to human
27	diseases, in particular clinical signs and symptoms of pneumonia. In 2013, we observed
28	cytopathic effect in amoebas (rounding and lysis) inoculated with APMV inoculated
29	PBMCs (peripheral blood mononuclear cell) extracts, and at that point we interpreted
30	those results as mimivirus replication in human PBMCs. Based on these results we
31	decided to further investigate APMV replication in human PBMCs, by transmission
32	electron microscopy (TEM) and qPCR. No viral factory was observed in APMV
33	inoculated PBMCs, at any analyzed time and M.O.I.s (multiplicity of infection), by
34	checking 550 cells per condition tested. We also measured the variation of viral DNA
35	by qPCR targeting helicase gene during the course of the TEM experiment in PBMCs,
36	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.

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Keywords: mimivirus; PBMCs; acanthamoeba

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### **Main Text**

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In 2003, the scientific community was surprised by the remarkable discovery of 44 Acanthamoeba polyphaga mimivirus (APMV), an Acanthamoeba-infecting virus with 45 46 outstanding features, including a giant virion (800nm) and a long and complex genome 47 (approximately 1,2Mb) [1]. APMV was first isolated during a pneumonia outbreak in 48 Bradford, England and since its discovery many advances have been done concerning the characterization of mimivirus evolution, life cycle in Acanthamoeba, diversity and 49 interactions with amoebas and other organisms [1-7]. Among mimivirus explored 50 topics, some research groups devoted efforts to understand whether this virus could be 51 52 associated to human diseases, in particular clinical sings symptoms of pneumonia [8,9]. 53 Different approaches have been explored to address, directly or indirectly, this issue: (i) 54 the search for antibodies against mimivirus and or viral DNA in pneumonia affected and 55 non-affected patients sera [9-13]; (ii) the search for APMV virions or DNA in 56 bronchoalveolar (BAL) samples, oral-nasal swabs and other samples (e.g. feces) [11-57 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, 58 including phagocytes (cells lines and PBMCs) [17,18]. Many of those studies 59 60 demonstrated the presence of pieces of mimivirus DNA in different human samples, including BAL, feces and serum; antibodies against mimiviruses were also detected in 61 62 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 63 mimivirus was proposed, by using very high M.O.I.s; and the replication of mimivirus 64 in mammalian phagocytes was proposed based on partial or indirect evidence, such as 65 qPCR and observation of cytopathic effects in amoebas inoculated with the extract of 66 67 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 68 69 is a member of the virome of humans and other vertebrates [19,23]. However, the role 70 of mimivirus as causative agent of diseases in humans is controversial and needs to be 71 substantially more investigated.

73 Inspired by a previous work that indicated the replication of mimivirus in mammalian 74 macrophage and monocyte derived cells by genome quantification, we investigated the 75 response of primary human peripheral blood mononuclear cells (PBMCs) against 76 mimiviruses at different M.O.I.s [17,18]. At different times post infection, the 77 mimivirus-inoculated PBMCs were collected and directly inoculated in Acanthamoeba 78 in order to detect and titer the virus by the endpoint/TCID<sub>50</sub> method. Interestingly, we 79 observed cytopathic effect in amoebas (rounding and lysis, after 4-6 days) inoculated 80 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. 81 82 used and conditions tested, resulting in growth curves. In the following months after the 83 publication, we decided to investigate the cycle of mimivirus in human PBMCs by 84 transmission electron microscopy. PBMCs were infected (2 pools of 5 healthy donors) 85 as described by Silva et al, 2013 and were collected at time 0, 2, 4, 6, 8, 12 and 24 hours 86 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 87 88 out according to UFMG Ethics Committee guidelines. We were not able to observe any 89 clear modification in the morphology of PBMCs after APMV infection, even at later 90 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 91 92 M.O.I.s. We were able to visualize APMV particles inside compartments, regardless the 93 time p.i.. At times 8 and 12 p.i. APMV particles seemed to be under degradation (fibrils 94 shorter, atypically interlaced), but not under morphogenesis (Figure 1). We also 95 measured the variation of viral DNA by qPCR targeting the helicase gene [24] during 96 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 97 98 extracts (supernatant and cells) in amoebas resulted in cytopathic effect (rounding), 99 although more discreet if compared to the previous experiments. The calculation of the 100 hypothetical title in this new set of experiments resulted in just 2 logs of difference 101 between time 0 and 24 p.i. (for both MOIs), while we observed about 4 logs in the first 102 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 103 104 those cells induces type I Interferons (IFNs), as described previously. Interestingly, we 105 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

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- 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
- 116 (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
- 121 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.
- 126 In addition, due the need of a large number of cells for experimentation, we prepare
- 127 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
- 130 response against mimivirus should also be performed in vertebrate's cell lines (e.g.
- 131 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.
- 135 Despite individual (immunological) and methodological bias related to PBMCs
- experimentation, we have no new evidence that support our previous conclusion that

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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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250	Figure 1 Legend
251	Transmission electron microscopy of human PBMCs inoculated with APMV, 12
252	hours post inoculation. A and B – Overview of inoculated cells. Arrows: APMV
253	particles inside endosomes. No viral factory can be visualized. C-E: APMV particles
254	fibers and capsid likely under degradation in PBMCs compartments.

