Title: Giant viruses inhibit superinfection by downregulating phagocytosis in *Acanthamoeba*

Authors: Isabella L. M. Aquino[1], Erik Sousa Reis[2], Rafaella Oliveira Almeida Mattos Moreira[1], Nídia Esther Colquehuanca Arias[1], Matheus Gomes Barcelos[1], Victória Fulgêncio Queiroz[1], Raquel Duque do Nascimento Arifa[3], Larissa Mendes Barbosa Lucas[3], Juliana Miranda Tatara[4], Daniele G. Souza[3], Adriana Costa[5], Luiz Rosa[6], Gabriel M. F. Almeida[4], Erna Geessien Kroon[1], Jônatas S. Abrahão[1]

Affiliations

[1] Laboratório de Vírus, Instituto de Ciências Biológicas, Departamento de Microbiologia, Universidade Federal de Minas Gerais, Belo Horizonte city, Minas Gerais, Brazil

[2] Laboratório de Virologia Básica e Aplicada (LVBA), Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte city, MG, Brazil

[3] Laboratory of Microorganism-Host Interaction, Department of Microbiology, Universidade Federal de Minas Gerais, Belo Horizonte city, MG, Brazil.

[4] The Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, UiT - The Arctic University of Norway, Tromsø city, Norway.

[5] Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte city, MG, Brazil.

[6]Laboratório de Microbiologia Polar e Conexões Tropicais, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte city, Minas Gerais, Brazil.

Abstract

In the context of the virosphere, viral particles can compete for host cells. In this scenario, some viruses block the entry of exogenous virions upon infecting a cell, a phenomenon known as superinfection inhibition. The molecular mechanisms associated with superinfection inhibition vary depending on the viral species and the host, but generally, blocking superinfection ensures the genetic supremacy of the virus's progeny that first infects the cell. Giant amoeba-infecting viruses have attracted the scientific community's attention due to the complexity of their particles and genomes. However, there are no studies on the occurrence of superinfection and its inhibition induced by giant viruses. This study shows that mimivirus, moumouvirus, and megavirus, exhibit different strategies related to the infection of *Acanthamoeba*. For the first time, we have reported that mimivirus and moumouvirus induce superinfection inhibition in amoebas. Interestingly, megaviruses do not exhibit this ability, allowing continuous entry of exogenous virions into infected amoebas. Our investigation into the mechanisms behind superinfection blockage reveals that mimivirus and moumouvirus inhibit amoebic phagocytosis, leading to significant changes in the morphology and activity of the host cells. In contrast, megavirus-infected amoebas continue incorporating newly formed virions, negatively affecting the available viral progeny. This effect, however, is reversible with chemical inhibition of phagocytosis. This work contributes to the understanding of superinfection and its inhibition in mimivirus, moumouvirus, and megavirus, demonstrating that despite their evolutionary relatedness, these viruses exhibit profound differences in their interactions with their hosts.

Importance

Some viruses block the entry of new virions upon infecting a cell, a phenomenon known as superinfection inhibition. Superinfection inhibition in giant viruses has yet to be studied. This study reveals that even closely related viruses, such as mimivirus, moumouvirus, and megavirus, have different infection strategies for *Acanthamoeba*. For the first time, we have reported that mimivirus and moumouvirus induce superinfection inhibition in amoebas. In contrast, megaviruses do not exhibit this ability, allowing continuous entry of exogenous virions into infected amoebas. Our investigation shows that mimivirus and moumouvirus inhibit amoebic phagocytosis, causing significant changes in host cell morphology and activity. Megavirus-infected amoebas, however, continue incorporating newly formed viruses, affecting viral progeny. This research enhances our understanding of superinfection inhibition in these viruses, highlighting their differences in host interactions.

Keywords: superinfection; giant virus; phagocytosis; *Acanthamoeba*; virus-host relationship

Introduction

The virosphere is diverse, and different strategies for infecting host cells have evolved throughout evolution, [1]. Various studies have demonstrated that, during the early stages of infection, some viruses block the entry of other viral particles into cells through a mechanism known as superinfection inhibition [2-5]. This phenomenon can genetically favor the particles that first infect the cells in an inter- and intraspecific competition. Another hypothesis explaining the selection of superinfection inhibition mechanisms is based on the fact that an already infected cell would not naturally be a

fully productive host for progeny formation [31]. Thus, inhibition of penetration would allow such particles to remain in the extracellular environment and eventually find uninfected cells. A notable example of superinfection inhibition has been described for the vaccinia virus, a nucleocytovirus that, while infecting the host cell, not only blocks the infection by other particles but can also repel exogenous particles to neighboring uninfected cells via actin tail formation, accelerating the infection of the host cell population [3,4].

Other nucleocytoviruses, such as mimiviruses, have drawn the attention of the scientific community due to their gigantic particles (750 nm) and complex genome (1.2 Mb) [6]. Mimiviruses are giant viruses that, under laboratory conditions, infect *Acanthamoeba*. Recent studies have demonstrated significant genetic diversity within this viral group, supporting its division into different genera, including *Mimivirus*, *Moumouvirus*, and *Megavirus* [7]. Although most basic studies have been conducted using mimivirus, it is believed that moumouvirus and megavirus have virus-host interaction mechanisms similar to those described for mimiviruses. For instance, their viral particles enter the host cells after being phagocytosed [8,9]. The acidification of the phagosome induces the release of the genome and early-phase proteins into the host cell cytoplasm [8,10]. Within a few hours, viral factories, micrometric structures involved in the morphogenesis and maturation of viral progeny, are formed [8-11]. A few hours later, the host cell lysis releases viral progeny [9]. During the amoeba infection by mimivirus, more than one particle can be phagocytosed simultaneously and initiate the replication cycle [8,9]. However, it has not been experimentally shown whether infected amoebas block the penetration of exogenous virions, under what

circumstances and when this happens. No such data exists for moumouvirus and megavirus either.

In the present work, we describe for the first time, that mimivirus and moumouvirus induce the process of superinfection inhibition in amoebas. Interestingly, despite being genetically related, megaviruses do not block superinfection, allowing long-lasting entry of exogenous virions into infected amoebas. Investigation of the superinfection blocking mechanisms suggests that mimi- and moumouvirus inhibit the amoebic phagocytosis process, causing significant changes in the morphology and activity of host cells. The constant incorporation of newly formed megavirus virions by infected amoebas impacts the viral progeny released into the supernatant, but the phenomenon is reversible when phagocytosis is chemically inhibited. This work provides information on superinfection and its inhibition in mimi-, moumou-, and megaviruses, highlighting that despite being evolutionarily related, these viruses exhibit profound differences in their relationship with their hosts.

Results

Megavirus particles can be found within vesicles at 6 hours post-infection

During our ongoing efforts to discover new amoeba viruses, we recently isolated megavirus caiporensis from an urban lagoon in Belo Horizonte, Brazil. This isolate prompted a comparative study of the surface fibrils of mimi-, moumou-, and megavirus particles [12]. Despite the differences in the organization of these fibrils, the viral factories of these three viral groups are similar, being about 3-4 micrometers in size, electron-dense, and with particles sprouting on their surfaces during morphogenesis (Figure 1). However, transmission electron microscopy (TEM) analysis of cells infected

by megavirus caiporensis revealed the presence of some viral particles enclosed by membranes after the formation of viral factories, 6 hours post-infection (h.p.i.), suggesting these particles might be released by exocytosis (Figure 1 and 2). Although such a release mechanism has been described for other giant viruses like Pandoravirus, cedratvirus, Marseillevirus, and Orpheovirus, there is no evidences that the progeny of mimi-, moumou-, and megavirus are exclusively released by cell lysis [13,14,19,28].

To verify if this phenomenon is exclusive to the megavirus caiporensis isolate, we analyzed five other megavirus isolates obtained by our group and one isolate from the University of Tromso, Norway, by TEM. The analyses revealed that of the seven isolates analyzed, all showed viral particles enclosed by membranes in the stages of infection when the viral factory was fully formed and producing new particles. This result suggested that considering the analyzed megavirus isolates, all appear to release part of their progeny by exocytosis. Analysis of our image library of five mimivirus isolates and five moumouvirus isolates did not reveal the presence of viral particles enclosed by vesicles in the late stages of infection (after the formation of the viral factory). This result suggests the presence of viral particles enclosed by membranes from 6 h.p.i. seems to be an exclusive characteristic of megaviruses compared to the other two viral groups.

Megavirus progeny is not released by exocytosis

A viral release experiment was conducted to evaluate whether megavirus caiporensis particles can be released by exocytosis, [13,14]. *Acanthamoeba castellanii* trophozoites were infected with mimi-, moumou-, or megavirus at a multiplicity of

infection (M.O.I.) of 10. At 0, 2, 4, 8, 12, and 24 hours post-infection, viable amoebas were quantified, and viral particles in the supernatant were titrated. The rationale of this experiment was to verify if there is an increase in viral particles in the supernatant without a reduction in the total number of viable cells, indicating the exocytosis of particles into the supernatant before cell lysis. Although TEM images suggested the possible release of megavirus particles by exocytosis, the release experiment demonstrated that the increase in mimi-, moumou-, and megavirus titers in the supernatant of infected cultures was accompanied by a reduction in the number of viable amoeba cells (Figure 3A-C). As an experimental control, amoebas were infected with cedratvirus, known to be released by exocytosis. As shown in the graph, an increase in cedratvirus particles in the supernatant between 4 and 12 hours was observed, not associated with a decrease in the number of viable amoebas, indicating release by exocytosis (Figure 3D). TEM analysis showed cedratvirus particles inside exosomes at 8 hours post-infection (Figure 3E-G). These results, therefore, do not support our initial hypothesis of megavirus release by exocytosis.

Mimivirus and moumouvirus, but not megavirus, inhibit superinfection

Considering that the hypothesis of megavirus particle exocytosis was not confirmed, the investigation of megavirus particles enclosed in membranes in the late stages of the cycle continued. Since the particles in vesicles were not being released, we hypothesized that they might be entering cells already infected by megavirus. To verify this hypothesis, we designed an experiment where amoebas infected with mimi-, moumou-, or megavirus were exposed, two hours post-infection, to a "bait" to check for phagocytic activity. After four hours of exposure to the bait, six hours post-infection, the samples were analyzed by confocal immunofluorescence (IF) microscopy. The bait

used in this experiment was Orpheovirus particles, at a ratio of 10 particles per amoeba. Orpheovirus was chosen as bait because it cannot replicate in *Acanthamoeba* and thus would not cause major interference with the analyzed cells, despite being internalized. The host of Orpheovirus under laboratory conditions is *Vermamoeba vermiformis* [28].

IF analyses revealed the presence of green-marked Orpheovirus particles primarily attached outside the cells or at intercellular space among cells infected by mimi- or moumouvirus (Figure 4A). However, in about 27% of megavirus-infected cells, Orpheovirus particles were visible inside cellular vacuoles (darker regions in the amoeba cytoplasm) (Figure 4A-B and 5A). Most megavirus-infected cells contained one to two Orpheovirus particles, but some cells had up to six incorporated Orpheovirus particles (Figure 4 B and 5B). Besides the observation and quantification of phagocytosed baits, the supernatant of cells infected by mimi-, moumou-, or megavirus and subsequently exposed to Orpheovirus was titered in *Vermamoeba vermiformis* six hours post-infection. The results show that almost all the Orpheovirus input used as bait for mimi- or moumouvirus-infected cells was recovered from the supernatant. In contrast, there was a significative reduction in the input of Orpheovirus particles exposed to megavirus-infected cells, indicating that the cells partially incorporated the baits (Figure 5C). This result was confirmed by TEM images, where cells containing formed megavirus viral factories alongside vesicles containing Orpheovirus particles were observed (Figure 5D).

The above results indicate that mimi- and moumouvirus, but not megavirus, block the incorporation of new viral particles into infected cells. To evaluate from what time

superinfection is inhibited by mimi- and moumouvirus, amoebas were infected by these viruses or megavirus and exposed to Orpheovirus as bait at different times: 0, 2, 4, 8, and 12 hours. After 4 hours, the supernatant was collected and titered in *Vermamoeba vermiformis*. The results indicate that from 2 hours post-infection, mimi- or moumouvirus-infected cells reduce bait incorporation. In contrast, bait incorporation by megavirus-infected cells was observed even at 12 hours, suggesting that superinfection is not entirely blocked by this virus, even in the late stages of infection (Figure 5E).

Mimivirus and moumouvirus cause cell size reduction and inhibit the formation of vacuoles and pseudopodia in amoebas

During the previously described experiments, we observed that amoebas infected with mimivirus and moumouvirus exhibited distinct morphological characteristics compared to amoebas infected with megavirus and the control group (uninfected cells). To better understand this process, amoebas infected with mimi-, moumou-, or megavirus were observed by immunofluorescence 6 hours post-infection, using Evans blue again as a cytoplasmic marker. The cell size and the number of cytoplasmic vacuoles present in the cells were measured. These vacuoles may be related to the natural phagocytic activity of amoebas, as this pathway represents their main form of feeding/nutrition. Even under axenic conditions, such as laboratory culture, amoebas maintain constant phagocytic activity, forming vacuoles or phagosomes.

Measurement of the cells, considering the largest observed axis, revealed that cells infected with mimi- or moumouvirus showed a significant size reduction, with an

average size of 13 and 14.5 micrometers, respectively. In contrast, cells infected with megavirus had dimensions similar to uninfected cells, with an average size above 20 micrometers (Figure 6A-B). This result suggests that mimi- and moumouvirus cause compaction of the host cells. The count of cytoplasmic vacuoles revealed that cells infected with mimi- or moumouvirus showed a significant reduction compared to cells infected with megavirus or uninfected cells. On average, about four vacuoles were observed in megavirus-infected cells, while one or no vacuoles were observed in cells infected with mimi- or moumouvirus (Figure 6C). Considering possible cellular alterations caused by preparation for IF, we also evaluated amoebas infected by mimi- , moumou-, or megavirus using scanning electron microscopy. Besides the cellular compaction observed by IF, we also observed that cells infected with mimi- or moumouvirus reduced the number and size of pseudopodia at six hours post-infection (Figure 6D). It is important to note that pseudopodia are essential structures for initiating the phagocytosis process in amoebas. Megavirus-infected and control cells exhibited typical trophozoite size and appearance, around 20 micrometers. Therefore, taken together, our results indicate that mimi- and moumouvirus cause host cell compaction, in addition to reducing the number of pseudopodia and intracellular vacuoles.

The consequences of megavirus superinfection in amoebas

As previously described, the measurement of viral particles in the supernatant of infected cells, revealed a distinct profile for megavirus compared to other viruses (Figure 3A-C). Although it was clear that the release of megavirus particles is dependent on cell lysis, we observed that the titers of megavirus particles released into the supernatant are lower than that observed for mimi- and moumouvirus, from the

beginning to the end of the productive phase of the cycle. It is important to emphasize that the entry process of giant virus particles into host cells is promoted by phagocytosis. As far as we know, it is independent of cellular receptors. Thus, for a mimi-, moumou-, or megavirus to initiate infection the host must actively phagocytize the viral particle. This naturally causes methodological difficulties in synchronizing the cycle in a population of amoebas, even at high MOIs. Therefore, if they are active, newly formed and released viral particles can be phagocytized by neighboring cells. Considering that, we hypothesized that part of the viral progeny released by lysis from megavirus-infected cells could be incorporated by neighboring cells that are also infected but not yet lysed.

To better understand this process, cells infected by megavirus, 12 hours post-infection, were analyzed by TEM. Similar to what we observed in microscopy of cells infected by megavirus six hours post-infection (Figure 1), we also visualized megavirus particles being massively incorporated by already infected amoebas. However, interestingly, at 12 hours post-infection, most of the phagocytized particles were in the process of uncoating, releasing their genome and viral proteins into a cytoplasm already in an advanced state of degradation due to the primary infection (Figure 7A-E). Thus, numerous empty megavirus capsids or their inner membrane fused with the phagosome membrane were observed (Figure 7A-E). In some images, up to three megavirus capsids were seen in a single phagosome, all in the process of uncoating (Figure 7A). This result suggests that part of the megavirus progeny formed during the cycle can be lost when incorporated by already infected cells, resulting in the superinfection of a cell in an advanced state of degradation. The consequence is a

smaller number of megavirus particles in the supernatant of infected cultures compared to cultures infected by mimi- or moumouvirus.

The inhibition of phagocytosis reverses the superinfection by megavirus and increases the number of viral particles in the system

As presented, previous results showed that mimivirus and moumouvirus cause a reduction in the formation of pseudopods, compaction of the cellular cytoplasm, reduction in the formation of intracellular vesicles, and lower incorporation of baits. In contrast, cells infected by megavirus exhibit a phenotype similar to non-infected cells in terms of their morphology and phagocytic activity. Considering the hypothesis that the inability to inhibit superinfection causes a reduction in the total megavirus titers in the supernatant, we decided to investigate the impact of phagocytosis on this process.

For this purpose, amoebas were infected with mimivirus, moumouvirus, or megavirus, and the cells were washed thirty minutes after infection to remove remaining particles. Two hours post infection the amoebas were treated with different endocytic and phagocytic pathways inhibitors. Chloroquine was used as an inhibitor of endocytosis, EIPA as an inhibitor of macropinocytosis and cytochalasin as an inhibitor of phagocytosis. After 24 hours of infection (22 hours of treatment), the viral particles in the supernatant were quantified. As a control group, amoebas were infected by the viruses and treated two hours later with the vehicle of the inhibitors. Therefore, in this experiment, we verified the impact of inhibitors on viral progeny incorporation and its effect on the final titer in the culture supernatant.

No changes in the titers of mimivirus and moumouvirus were observed. In both cases, amoebas treated with the inhibitors showed titers similar to those observed in the control (Figure 8). However, a significant increase in megavirus titers in the supernatant was observed in the group treated with cytochalasin (Figure 8C). Together, this result indicates that inhibiting phagocytosis reduces the phagocytic action on exogenous particles by amoebas previously infected by megavirus, mitigating the process and consequences of superinfection.

Discussion

The mechanisms involved in superinfection inhibition are varied and widespread throughout the virosphere. As mentioned, the phenomenon has been extensively studied in the nucleocytoplasmic vaccinia virus. It has been demonstrated that the early expression of the A33 and A36 proteins by the vaccinia virus is necessary to repel exogenous particles by forming actin tails on the surface of infected cells [3]. Interestingly, four hours after infection, the vaccinia virus induces a 90% block of superinfection [4]. Superinfection inhibition is also known for bacteriophages. The expression of the lipoprotein gene ltp (TP-J34) of the temperate phage Streptococcus thermophilus phage TP-J34 interferes with the infection of exogenous phages by blocking the injection of viral DNA into the cell [5]. Although the mechanism has not been fully elucidated, it has been shown that the p33 protein of the citrus tristeza virus is essential for inhibiting intraspecific superinfection [15]. The HIV virus blocks superinfection by reducing the expression of the viral coreceptor CCR5, making the penetration of exogenous particles unfeasible [2]. As far as we know, the entry of giant viruses into amoebas occurs by phagocytosis and does not require a cellular receptor,

another point that differentiates these viruses from most of the canonical virosphere. Thus, since amoeba trophozoites constantly perform phagocytosis, it would not be intuitive to predict that giant viruses would cause superinfection inhibition.

Free-living amoebas, such as *Acanthamoeba*, must constantly activate the phagocytosis process to obtain nutrients. Weisman and Korn experimentally demonstrated that the phagocytosis process in amoebas is stimulated by particles larger than 500 nanometers [16]. Therefore, most environmental bacteria stimulate phagocytosis in *Acanthamoeba*, their primary source of nutrients. However, the discovery of giant viruses, such as mimi-, moumou-, and megaviruses, revealed that this feeding strategy of amoebas can trigger infection and death of this organism, as these viral entities have particles larger than 500 nanometers. Some theories hypothesize that the gigantism of giant viruses may have been selected to exploit the niche of entry by phagocytosis [17]. Besides gigantism, a series of structural and enzymatic adaptations in viral particles were required to exploit the phagocytic pathway, such as enzymes to control oxidative stress in the phagosomal environment, new uncoating portals like the stargate axis, and the configuration of an inner membrane within the capsid, essential for the genome delivery process in the cytoplasm [18]. Therefore, phagocytosis seems to be a crucial phenomenon for the relationship between giant viruses and amoebas.

In the present study, our results indicate that mimi- and moumouviruses induce superinfection inhibition in amoebas approximately from 2 hours post-infection. These viruses also induce profound changes in amoeba morphology and physiology, such as cytoplasm condensation, reduction in the size and number of pseudopods, diminution

in the phagocytosis rate, and a decrease in the number of cytoplasmic vacuoles. In contrast, cells infected by megavirus retain the fundamental property of amoeba trophozoites: phagocytosis. One consequence of this is the constant incorporation of exogenous viral particles until the late stages of the cycle. However, this property is reversed when a pharmacological phagocytosis inhibitor is employed. To our knowledge, the present study is the first to demonstrate the existence of the superinfection process and its inhibition by giant viruses. It is also the first time that inhibition of the phagocytosis process is associated with the inhibition of viral superinfection in amoebas.

Together, our data indicate that the absence of the ability to inhibit superinfection causes a reduction in megavirus titers, as part of the newly formed progeny is promptly phagocytosed and uncoated in already infected cells. During our microscopy analyses, none of the cells infected by megavirus presented more than one viral factory. This indicates that the content uncoated by exogenous megavirus particles does not result in progeny formation. This conclusion is corroborated by the lower titer observed for megavirus compared to mimi- and moumouviruses, regardless of the productive phase of the cycle. From the amoeba population perspective, the phagocytosis of newly formed megavirus particles could be considered an antiviral process, as it reduces the number of viral particles in the extracellular medium, decreasing the chance of neighboring healthy cells' infection. Possibly, mimi- and moumouviruses have overcome this disadvantage by developing mechanisms to control the phagocytic activity of their hosts. The viral proteins involved in this process remain to be investigated, with molecular tools available to delete viral genes involved in cytoskeleton control. In addition, an interesting perspective to investigate is the

consequence of superinfection inhibition during virophage co-infections. Although the virophage entry mechanisms are unknown, it has been hypothesized that these satellite viruses might enter amoebas associated with mimivirus fibrils. In this case, the blocking of superinfection could affect virophage infection.

Although the discovery of the first giant amoeba virus occurred just over 20 years ago, numerous unprecedented processes in the relationship between these viruses and their hosts have been described [6, 19-23]. The constant investigation of this relationship is crucial, as amoebas and their ancestors are some of the oldest known eukaryotic organisms. The hypothesized scenario for the origin of giant viruses also dates back to ancient times in the context of proto-eukaryotic cells [24]. Therefore, although studies involving giant viruses and their hosts represent great novelties in modern virology, such relationships are possibly among the oldest in the history of life on Earth. Understanding these relationships can contribute to a better understanding of the virosphere as a whole.

Methods

Virus multiplication, purification, and titration

The megavirus caiporensis was isolated 2017 from the Pampulha Lagoon, Belo Horizonte, Brazil [12]. The moumouvirus B60 was isolated in 2018 from a river in a savanna biome in Brazil [25]. The Brazilian cedratvirus was isolated in 2017 from a fishbowl, in Belo Horizonte, Brazil [26]. The mimivirus (APMV) and Orpheovirus were kindly provided by Dr. Bernard La Scola (Aix Marseille University). *Acanthamoeba castellanii* ATCC 30010 cells were kindly provided by Dr. Adriana Oliveira Costa (UFMG). *Vermoameba vermiformis* cells were also kindly provided by Dr. Bernard La

Scola (Aix Marseille University). The study was registered at the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen). For production and purification, each virus was inoculated at a M.O.I. of 0.01 in cell culture T175 flasks containing 1.4×10^7 *Acanthamoeba castellanii* trophozoites and 25 mL of peptone-yeast extract-glucose (PYG) medium supplemented with penicillin (100 U/mL; Cellofarm, Brazil), streptomycin (100 µg/mL; Sigma-Aldrich, Burlington, MA, USA), and amphotericin B (0.25 µg/mL; Cultilab, Brazil). The cells were incubated at 30°C. After observation of the cytopathic effect caused by viral infection (i.e., rounding cells and cellular lysis), the flask contents were collected and ultracentrifuged (36,000 × g) in a 22% sucrose cushion for 50 min. The pellet containing purified viral particles was suspended in phosphate-buffered saline (PBS). All titration assays were performed using the endpoint method [25].

Electron microscopy

A. castellanii cultures infected with different viruses were analyzed by SEM and TEM. Experiments and analyses were performed in the Center of Microscopy at the Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil (http://www.microscopia.ufmg.br). For SEM assays, samples were fixed by immersion in a solution containing glutaraldehyde (2.5%) in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. Postfixation was followed for each sample with 2% osmium tetroxide (OsO4) for 2 h at room temperature. Fixed samples were dehydrated using a growing series of ethanol dilutions (30%, 50%, 70%, 95%, and 100%) for 10 min in each step. Then, samples were dried with CO2 at a critical point using CPD 030 equipment (Bal-Tec, Liechtenstein). Next, samples were supported in aluminum stubs and metalized with a thin layer (5 nm) of gold particles using MED 020 equipment (Bal-Tec,

Liechtenstein). Samples were observed in a FEG-Quanta 200 FEI microscope (FEI Co., Eindhoven, Netherlands) at 15 to 20 kV.

For TEM assays, samples were fixed by immersion in a solution containing glutaraldehyde (2.5%) in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h. After fixation, postfixation was performed with a solution of 1% osmium tetroxide in sodium cacodylate buffer (0.1 M, pH 7.2) for 1 h followed by en bloc counterstaining with uranyl acetate (2% uranyl acetate in deionized water). Samples were gradually dehydrated by immersion in 70%, 80%, and 90% ethanol once for 15 min each and twice in 100% ethanol for 15 min. Next, samples were embedded in Epon resin. Ultrathin sections were obtained using an ultramicrotome with diamond knives (Leica Microsystems), and these sections had a thickness of 70 nm and were placed on a 200-mesh copper screen. The screens were counterstained with Reynold's lead citrate solution for 10 min. Images were obtained using a Tecnai G2-12-SpiritBiotwin FEI electron microscope (FEI Co., Eindhoven, Netherlands) at an acceleration voltage of 120 kV using a charge-coupled-device camera.

Immunofluorescence microscopy

For immunofluorescence, *A. castellanii* trophozoites were infected with viruses at an M.O.I 10. and, approximately 2×10^5 cells were collected and centrifuged at 800 × g for 10 min. The pellet was resuspended in 50 µL of Page’s amoebae saline (PAS), and the cells were attached to a slide via cytospin and fixed in methanol for 15 min. After fixation, cells were incubated with 3% bovine serum albumin (BSA)-PAS for 30 min, followed by three rinses with 0.1% PAS-Tween. Cells were stained with polyclonal anti-Orpheovirus whole particle antibody produced in mice – CEUA 235/2023 - (1:400

diluted in 3% BSA-PAS) for 1 h at 37 °C, followed by three rinses with PAS-Tween 0.1%. After a 1 h incubation with an anti-mouse secondary antibody (1:400 diluted in 3% BSA-PAS), one drop of 0.01% Evans Blue (Sigma), which was sufficient to cover the cells, was added and incubated for 15 min at 37 °C, followed by three rinses with PAS-Tween 0.1%. Uninfected cells (control) were also fixed and prepared as described. Fluorescently labeled cells were observed using a confocal Axio Imager Z2-Apotome 2 microscope (Zeiss). The Zen Lite software from Zeiss microscopy was used for image processing. The ImageJ software (version v1.53k, National Institutes of Health) was used to measure 50 different cells during the acquisition of images. The measures were used to calculate the medium sizes and deviate.

One-step-growth curves and cell counting

A. castellanii trophozoites were infected by mimivirus, moumouvirus, megavirus or cedratvirus, at M.O.I. of 10, in T25 culture flasks. Thirty minutes after infection, the supernatants were removed, and fresh medium was added. Then, the supernatants of infected cells were collected at different time points and titrated in 96-well plates containing 40,000 *A. castellanii* cells, by the endpoint method [25]. In parallel, the remaining cells of each time and experimental group were quantified using a Neubauer chamber. Viable cells were identified by using trypan blue (Sigma).

Phagocytic activity and superinfection assays

One million of *A. castellanii* trophozoites were infected by mimivirus, moumouvirus or megavirus at M.O.I. of 10, in T25 culture flasks. Thirty minutes after infection, the supernatants were removed, and fresh medium was added. Two hours post-infection, purified Orpheovirus particles were inoculated into the amoeba’s monolayers at the

M.O.I. of 10. At six hours post-infections (four hours post-Orpheovirus inoculation), cells were collected and analyzed by IF, as previously described. Only particles colocalizing with vacuoles were considered phagocytized. The same experimental groups were analyzed by TEM. The supernantants of each flask were collected and titrated in *Vermoameba vermiformis*, using the endpoint method. To verify the superinfection and its inhibition, $1x10^6$ of *A. castellanii* cells were infected by mimivirus, moumouvirus, or megavirus at M.O.I. of 10, in T25 culture flasks. At different time points, Orpheovirus was inoculated in a M.O.I. of 10. Four hours after this inoculation, the supernatants were collected and titrated in *Vermoameba vermiformis*, using the endpoint method [25]. IF images obtained from this experiment are presented in both Figures 4 and 6.

Chemical inhibitors and the reversion of superinfection

Different chemical inhibitors were used to investigate the reversion of superinfection in megavirus-infected cells, such as cytochalasin D – a phagocytosis inhibitor, chloroquine – clathrin and caveolin -dependent of acidification pathways inhibitors, and 5-(N-ethyl-N-isopropyl) amiloride (EIPA) – a specific macropinocytosis inhibitor. Cytochalasin D and chloroquine had already been confirmed as inhibitors of endocytic pathways in *Acanthamoeba* [29,30]. A total of 5×10^5 *A. castellanii* trophozoites were infected with mimivirus, moumouvirus, or megavirus, and thirty minutes after infection the cells were washed with PBS to remove remained particles. Two hours post-infection, the cells were treated with different inhibitors of endocytic and phagocytic pathways: with 2 µM of cytochalasin (Sigma-Aldrich, United States), 100 µM of chloroquine (Sigma-Aldrich, United States) or 1 µM of EIPA (Sigma-Aldrich, United States). The cytotoxicity of the inhibitors was tested in *Acanthamoeba,* and the choice

of inhibitor concentrations was based on previous studies [8,13]. After 24 hours of infection (22 hours of treatment), the viral particles in the supernatant were quantified by the endpoint method. in this experiment, we verified the impact of inhibitors on viral progeny incorporation and its effect on the final titer in the culture supernatant.

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Acknowledgements

We would like to thank all colleagues from the Giant Viruses Study Group (www.giantviruses.com) and from Laboratório de Vírus of Universidade Federal de Minas Gerais (UFMG). Also, we thank Centro de Microscopia of UFMG for the contribution of microscopy images. We thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), FAPEMIG (Fundação de Amparo à Pesquisa do estado de Minas Gerais), Pro-reitorias de Pós-graduação e Pesquisa da UFMG and the Centre for New Antibacterial Strategies (CANS) of the Arctic University of Norway (project ID #2520855). The manuscript text was corrected by artificial intelligence (ChatGPT). This research is registered at SISGEN. J.S.A., E.G.K, L.H.R., and D.G.S. are CNPq researchers.

Figures captions

Figure 1: Particle, viral factory, and progeny features of mimi-, moumou-, and megavirus observed by transmission electron microscopy. This panel shows that mimi-, moumou-, and megavirus particles exhibit differences in the organization and

abundance of surface fibrils, while their viral factories appear very similar. Notably, at 6 hours post-infection, some megavirus particles are observed inside vesicles (black arrowheads).

Figure 2: The occurrence of megavirus particles within cytoplasmic vesicles. The replication cycles of megavirus caiporensis and other isolates were analyzed using transmission electron microscopy. All images were captured after the mature viral factory appearance, approximately 4-6 hours post-infection. Vesicles contain from one to several megavirus particles.

Figure 3: Mimi-, moumou-, and megaviruses are not released via exocytosis in *Acanthamoeba*-infected cells. One-step growth curves of the supernatant from *Acanthamoeba* infected with (A) mimivirus, (B) moumouvirus, (C) megavirus, and (D) cedratvirus. The left Y-axis plots viral titers at different times post-infection. The right Y-axis plots data on viable cells at different times post-infection. The blue box in (D) highlights the period when cedratvirus is primarily released in the supernatant via exocytosis (the number of viable, non-lysed cells remains stable). Solid lines with circles represent virus titers, while dashed lines with squares represent viable cell counts. Panels (E-G) depict cedratvirus particles being released inside exosomes, 8 hours post-infection.

Figure 4: Megavirus-infected cells phagocytose exogenous particles. Amoebas were infected by mimi-, moumou-, and megavirus, and two hours later were exposed to a 'bait,' the Orpheovirus particles. At hour six post-infection, cells and particles were analyzed by immunofluorescence microscopy. Orpheovirus particles, probed with a

mouse primary antibody, appear green, while the amoeba cytoskeleton stained by blue Evans appears red. (a) Cells infected by mimi-, moumou-, or megavirus and exposed to Orpheovirus (bait). Only Orpheovirus particles colocalizing with amoeba vacuoles (black cytoplasm regions) were considered phagocytized (highlighted with dotted lines inside cells). (b) This panel shows several individual *Acanthamoeba* cells with phagocytized Orpheovirus particles.

Figure 5: The consequences of superinfection and its inhibition in mimi-, moumou-, and megavirus-infected cells. (A) Megavirus-infected cells phagocytized significantly more exogenous particles than mimi- and moumouvirus-infected cells. This graph was obtained after observing 50 cells infected by mimi-, moumou-, or megavirus and analyzed by immunofluorescence microscopy. The average of three independent replicates is presented. (B) Number of cells vs. Orpheovirus-incorporated baits. The graph depicts all analyzed cells belonging to three independent replicates. (C) Titration of the residual Orpheovirus input in *Vermoameba vermiformis* cells. Mimi-, moumou-, and megavirus-infected cells were inoculated with Orpheovirus baits to verify phagocytosis activity. Four hours later (six hours post-infection), the supernatants were collected and titered in *Vermoameba vermiformis*, the laboratory host of Orpheovirus. This graph shows that almost the complete input of Orpheovirus baits was recovered from the supernatant of mimi- and moumouvirus-infected cells, suggesting a significant reduction in phagocytosis activity compared to megavirus-infected cells. (D) Transmission electron microscopy of megavirus-infected cells exposed to Orpheovirus baits. Asterisks denote megavirus particles; VF represents megavirus virus factory; red dashed circles indicate phagosomes containing Orpheovirus particles. (E) Evaluation of superinfection inhibition by mimi-, moumou-,

573 and megavirus. *Acanthamoeba* cells were infected by mimi-, moumou-, or megavirus
574 at an M.O.I. of 10. At times 30 min, 1h, 2h, 4h, 6h, and 12h, cells were exposed to
575 Orpheovirus baits. Four hours after this exposure, culture supernatants were collected
576 and titered in *Vermoameba vermiformis*. The graph shows the almost complete
577 recovery of Orpheovirus inputs from mimi- and moumouvirus-infected cultures from 2
578 hours post-infection. This result indicates that mimi- and moumouvirus start to block
579 phagocytosis of exogenous particles at early times post-infection. ***: $p < 0.001$, ****:
580 $p < 0.0001$ (ANOVA, one-way).
581
582 **Figure 6: Mimi- and moumouvirus cause compaction of the cellular cytoplasm**
583 **and reduction in the formation of pseudopods and intracellular vacuoles.**
584 *Acanthamoeba* cells were infected with mimi-, moumou-, or megavirus at an M.O.I. of
585 10. At 6 hours post-infection, cells were analyzed by immunofluorescence microscopy.
586 The amoeba cytoskeleton stained with blue Evans is represented in red. (A) Cells
587 infected with mimi- and moumouvirus appear rounded and compacted compared to
588 megavirus-infected and uninfected cells. Cell dimensions were measured (B), and
589 vacuole counting (C) was performed by analyzing 50 cells randomly in three
590 independent replicates. (D) Scanning electron microscopy of amoebas infected with
591 mimi-, moumou-, or megavirus, 6 hours post-infection. This panel shows that cells
592 infected with mimi- and moumouvirus are smaller and exhibit fewer pseudopods
593 compared to cells infected with megavirus or uninfected cells. ****: $p < 0.0001$
594 (ANOVA, one-way). Some of the images presented here (panel A) were also shown in
595 Figure 4A-B, as they were obtained from the same experiment.
596

Figure 7: Exogenous particles incorporated by 12-hour megavirus-infected cells appear to be undergoing the uncoating process or are already empty. Amoebas were infected with megavirus at an M.O.I. of 10. Twelve hours post-infection, infected cells were analyzed by transmission electron microscopy. (A-C) Megavirus exogenous particles (inside vesicles) undergo the uncoating process. In the images, it is possible to visualize the particle's inner membrane fused with the phagosome membrane. (D-E) Empty exogenous particles inside vesicles (indicated by black arrowheads).

Figure 8: Cytochalasin D reverses the superinfection promoted by megavirus and increases the number of viral particles in the system. *Acanthamoeba* cells were infected with mimi-, moumou-, or megavirus at an M.O.I. of 10. Two hours post-infection, the amoebas were treated with different inhibitors of endocytic and phagocytic pathways: 2 µM of cytochalasin, 100 µM of chloroquine, or 1 µM of EIPA. Twenty-four hours post-infection, the culture supernatants were titrated. No significant changes in viral titers were observed in cells infected by mimi- (A) or moumouvirus (B) and treated with any of the mentioned inhibitors. However, cells infected with megavirus (C) and treated with cytochalasin produced significantly increased titers. ****: $p < 0.0001$ (ANOVA, one way).

References

1. Roossinck, M. The good viruses: viral mutualistic symbioses. Nat Rev Microbiol **9**, 99–108 (2011). https://doi.org/10.1038/nrmicro2491

2. Schwartz E.J., Biggs K.R.H., Bailes C., Ferolito K., Vaidya N.K. HIV dynamics with immune responses: Perspectives from mathematical modeling. Curr. Clin. Microbiol. Rep. 2016;**3**:216–224. doi: 10.1007/s40588-016-0049-z.

3. Doceul V, Hollinshead M, van der Linden L, Smith GL. Repulsion of superinfecting virions: a mechanism for rapid virus spread. Science. 2010 Feb

12;327(5967):873-876. doi: 10.1126/science.1183173. Epub 2010 Jan 21. PMID: 20093437; PMCID: PMC4202693.

4. Christen L, Seto J, Niles EG. Superinfection exclusion of vaccinia virus in virus-infected cell cultures. Virology. 1990 Jan;174(1):35-42. doi: 10.1016/0042-6822(90)90051-r. PMID: 2294646; PMCID: PMC7130625.

5. Hutchison C.I., Sinsheimer R. Requirement of protein synthesis for bacteriophage X174 superinfection exclusion. J. Virol. 1971;**8**:121–124. doi: 10.1128/jvi.8.1.121-124.1971.

6. La Scola B, Audic S, Robert C, Jungang L, de Lamballerie X, Drancourt M, Birtles R, Claverie JM, Raoult D. A giant virus in amoebae. Science. 2003 Mar 28;299(5615):2033. doi: 10.1126/science.1081867. PMID: 12663918.

7. Aylward FO, Abrahão JS, Brussaard CPD, Fischer MG, Moniruzzaman M, Ogata H, Suttle CA. Taxonomic update for giant viruses in the order Imitervirales (phylum Nucleocytoviricota). Arch Virol. 2023 Oct 31;168(11):283. doi: 10.1007/s00705-023-05906-3. PMID: 37904060.

8. Andrade ACDSP, Rodrigues RAL, Oliveira GP, Andrade KR, Bonjardim CA, La Scola B, Kroon EG, Abrahão JS. Filling Knowledge Gaps for Mimivirus Entry, Uncoating, and Morphogenesis. J Virol. 2017 Oct 27;91(22):e01335-17. doi: 10.1128/JVI.01335-17. PMID: 28878069; PMCID: PMC5660479.

9. Suzan-Monti M, La Scola B, Barrassi L, Espinosa L, Raoult D. Ultrastructural characterization of the giant volcano-like virus factory of Acanthamoeba polyphaga Mimivirus. PLoS One. 2007 Mar 28;2(3):e328. doi: 10.1371/journal.pone.0000328. PMID: 17389919; PMCID: PMC1828621.

10. Schrad JR, Abrahão JS, Cortines JR, Parent KN. Structural and Proteomic Characterization of the Initiation of Giant Virus Infection. Cell. 2020 May 28;181(5):1046-1061.e6. doi: 10.1016/j.cell.2020.04.032. Epub 2020 May 8. PMID: 32392465; PMCID: PMC8054779.

11. Mutsafi Y, Zauberman N, Sabanay I, Minsky A. Vaccinia-like cytoplasmic replication of the giant Mimivirus. Proc Natl Acad Sci U S A. 2010 Mar 30;107(13):5978-82. doi: 10.1073/pnas.0912737107. Epub 2010 Mar 15. PMID: 20231474; PMCID: PMC2851855.

12. de Aquino ILM, Serafim MSM, Machado TB, Azevedo BL, Cunha DES, Ullmann LS, Araújo JP Jr, Abrahão JS. Diversity of Surface Fibril Patterns in Mimivirus Isolates. J Virol. 2023 Feb 28;97(2):e0182422. doi: 10.1128/jvi.01824-22. Epub 2023 Feb 2. PMID: 36728417; PMCID: PMC9972986.

13. Silva LKDS, Andrade ACDSP, Dornas FP, Rodrigues RAL, Arantes T, Kroon EG, Bonjardim CA, Abrahão JS. Cedratvirus getuliensis replication cycle: an in-

depth morphological analysis. Sci Rep. 2018 Mar 5;8(1):4000. doi: 10.1038/s41598-018-22398-3. PMID: 29507337; PMCID: PMC5838162.

14. Pereira Andrade ACDS, Victor de Miranda Boratto P, Rodrigues RAL, Bastos TM, Azevedo BL, Dornas FP, Oliveira DB, Drumond BP, Kroon EG, Abrahão JS. New Isolates of Pandoraviruses: Contribution to the Study of Replication Cycle Steps. J Virol. 2019 Feb 19;93(5):e01942-18. doi: 10.1128/JVI.01942-18. PMID: 30541841; PMCID: PMC6384056.

15. Folimonova SY. Superinfection exclusion is an active virus-controlled function that requires a specific viral protein. J Virol. 2012 May;86(10):5554-61. doi: 10.1128/JVI.00310-12. Epub 2012 Mar 7. PMID: 22398285; PMCID: PMC3347309.

16. Weisman RA, Korn ED. Phagocytosis of latex beads by Acanthamoeba. I. Biochemical properties. Biochemistry. 1967 Feb;6(2):485-97. doi: 10.1021/bi00854a017. PMID: 4860149.

17. Rodrigues RAL, Abrahão JS, Drumond BP, Kroon EG. Giants among larges: how gigantism impacts giant virus entry into amoebae. Curr Opin Microbiol. 2016 Jun;31:88-93. doi: 10.1016/j.mib.2016.03.009. Epub 2016 Apr 1. PMID: 27039270.

18. Colson P, La Scola B, Levasseur A, Caetano-Anollés G, Raoult D. Mimivirus: leading the way in the discovery of giant viruses of amoebae. Nat Rev Microbiol. 2017 Apr;15(4):243-254. doi: 10.1038/nrmicro.2016.197. Epub 2017 Feb 27. PMID: 28239153; PMCID: PMC7096837.

19. Arantes TS, Rodrigues RA, Dos Santos Silva LK, Oliveira GP, de Souza HL, Khalil JY, de Oliveira DB, Torres AA, da Silva LL, Colson P, Kroon EG, da Fonseca FG, Bonjardim CA, La Scola B, Abrahão JS. The Large Marseillevirus Explores Different Entry Pathways by Forming Giant Infectious Vesicles. J Virol. 2016 May 12;90(11):5246-55. doi: 10.1128/JVI.00177-16. Erratum in: J Virol. 2021 Aug 25;95(18):e0107121. PMID: 26984730; PMCID: PMC4934737.

20. Oliveira G, Silva L, Leão T, Mougari S, da Fonseca FG, Kroon EG, La Scola B, Abrahão JS. Tupanvirus-infected amoebas are induced to aggregate with uninfected cells promoting viral dissemination. Sci Rep. 2019 Jan 17;9(1):183. doi: 10.1038/s41598-018-36552-4. PMID: 30655573; PMCID: PMC6336878.

21. Yoshikawa G, Blanc-Mathieu R, Song C, Kayama Y, Mochizuki T, Murata K, Ogata H, Takemura M. Medusavirus, a Novel Large DNA Virus Discovered from Hot Spring Water. J Virol. 2019 Apr 3;93(8):e02130-18. doi: 10.1128/JVI.02130-18. PMID: 30728258; PMCID: PMC6450098.

22. Boratto P, Albarnaz JD, Almeida GM, Botelho L, Fontes AC, Costa AO, Santos Dde A, Bonjardim CA, La Scola B, Kroon EG, Abrahão JS. Acanthamoeba polyphaga mimivirus prevents amoebal encystment-mediating serine proteinase expression and circumvents cell encystment. J Virol. 2015 Mar;89(5):2962-5. doi: 10.1128/JVI.03177-14. Epub 2014 Dec 17. PMID: 25520511; PMCID: PMC4325711.

23. Queiroz, V.F., Tatara, J.M., Botelho, B.B. et al. The consequences of viral infection on protists. Commun Biol **7**, 306 (2024). https://doi.org/10.1038/s42003-024-06001-2

24. Krupovic M, Dolja VV, Koonin EV. The virome of the last eukaryotic common ancestor and eukaryogenesis. Nat Microbiol. 2023 Jun;8(6):1008-1017. doi: 10.1038/s41564-023-01378-y. Epub 2023 May 1. PMID: 37127702;

25. Dos Santos Silva LK, Rodrigues RAL, Dos Santos Pereira Andrade AC, Hikida H, Andreani J, Levasseur A, La Scola B, Abrahão JS. Isolation and genomic characterization of a new mimivirus of lineage B from a Brazilian river. Arch Virol. 2020 Apr;165(4):853-863. doi: 10.1007/s00705-020-04542-5. Epub 2020 Feb 12. PMID: 32052196.

26. Rodrigues RAL, Andreani J, Andrade ACDSP, Machado TB, Abdi S, Levasseur A, Abrahão JS, La Scola B. Morphologic and Genomic Analyses of New Isolates Reveal a Second Lineage of Cedratviruses. J Virol. 2018 Jun 13;92(13):e00372-18. doi: 10.1128/JVI.00372-18. PMID: 29695424; PMCID: PMC6002711.

27. Reed, L.J.; Muench, H. (1938). "A simple method of estimating fifty percent endpoints". The American Journal of Hygiene. **27**: 493–497.

28. Souza F, Rodrigues R, Reis E, Lima M, La Scola B, Abrahão J. In-depth analysis of the replication cycle of Orpheovirus. Virol J. 2019 Dec 16;16(1):158. doi: 10.1186/s12985-019-1268-8.

29. Alsam S, Sissons J, Dudley R, Khan NA. Mechanisms associated with Acanthamoeba castellanii (T4) phagocytosis. Parasitol Res. 2005 Aug;96(6):402-9. doi: 10.1007/s00436-005-1401-z

30. Moon EK, Kim SH, Hong Y, Chung DI, Goo YK, Kong HH. Autophagy inhibitors as a potential antiamoebic treatment for Acanthamoeba keratitis. Antimicrob Agents Chemother. 2015 Jul;59(7):4020-5. doi: 10.1128/AAC.05165-14.

31. Biggs KRH, Bailes CL, Scott L, Wichman HA, Schwartz EJ. Ecological Approach to Understanding Superinfection Inhibition in Bacteriophage. Viruses. 2021 Jul 17;13(7):1389. doi: 10.3390/v13071389.