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1 **Ultrastructural modifications induced by SARS-CoV-2 in Vero cells : a**
2 **kinetic analysis of viral factory formation, viral particle morphogenesis and**
3 **virion release**

4

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21

22 Abstract

23

24 Many studies on SARS-CoV-2 have been performed over short time scale, but few have
25 focused on the ultrastructural characteristics of infected cells. We used TEM to perform kinetic
26 analysis of the ultrastructure of SARS-CoV-2-infected cells. Early infection events were
27 characterized by the presence of clusters of single- membrane vesicles and stacks of membrane-
28 containing nuclear pores called annulate lamellae (AL). A large network of host cell-derived
29 organelles transformed into virus factories was subsequently observed in the cells. As
30 previously described for other RNA viruses, these replication factories consisted of double-
31 membrane vesicles (DMVs) located close to the nucleus. Viruses released at the cell surface by
32 exocytosis harbored the typical crown of spike proteins, but viral particles without spikes were
33 also observed in intracellular compartments, possibly reflecting incorrect assembly or a cell
34 degradation process.

35

36 **Keywords:** Covid-19; SARS-CoV-2; virus/cell interactions; electron microscopy.

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42 Introduction

43 Coronaviruses are infectious agents that infect many species of mammals and birds, and have
44 high zoonotic potential. Some, such as HKU1, OC43, 229E, and NL63, circulate seasonally
45 and cause benign respiratory illnesses in children or adults [1-3]. However, others, such as the
46 severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 [4] and Middle East
47 respiratory syndrome coronavirus (MERS-CoV) in 2012 [5] have emerged more recently,
48 through cross-species transmission between one or more mammals and humans. These
49 emerging viruses can cause severe respiratory disease in adults. At the end of 2019, the
50 identification, in China, of SARS-CoV-2 as the causal agent of atypical pneumopathies,
51 provided the latest example of these emerging coronaviruses [6, 7]. SARS-CoV-2 is less
52 virulent than SARS-CoV [8], and infected patients seem to be contagious before the onset of
53 symptoms and even asymptomatic individuals appear to be able to transmit the disease [9]. This
54 renders the identification of infected patients and chains of transmission much more complex
55 and has resulted in the massive spread of this new virus worldwide, and the declaration of a
56 pandemic by the WHO on March 11, 2020. The associated disease was named COVID-19
57 (coronavirus disease 2019). The pathophysiology of SARS-CoV-2 seems to differ in several
58 ways from those of SARS-CoV and the benign human coronaviruses that generally circulate.
59 However, it remains difficult to determine the exact contributions of viral and host factors to
60 these differences.

61 Coronaviruses, with a single-stranded positive RNA genome of 26-32 kb, have the
62 largest genomes of RNA viruses. The first two-thirds of the genome encodes two polyproteins

63 that are subsequently cleaved by proteases to give rise to non-structural proteins involved in
64 viral replication [10]. The last third of the genome encodes four structural proteins: spike (S),
65 envelope (E), membrane (M), and nucleocapsid (N). Some coronavirus virions contain an
66 additional membrane protein, a hemagglutinin esterase (HE). These proteins assemble to form
67 enveloped virions of about 120 nm in diameter. The first stages of the viral cycle occur on virus-
68 induced double-membrane structures derived from the endoplasmic reticulum (ER), commonly
69 referred to as "double-membrane vesicles" (DMVs) [11]. These structures are mostly located
70 in the perinuclear zone, and are also generated by other RNA viruses, such as arteriviruses,
71 picornaviruses, and the flaviviruses of genus *Hepacivirus* [12, 13]. In coronaviruses, the nsp3
72 and nsp4 proteins appear to be sufficient to induce the formation of these structures. The viral
73 genome replication complexes are anchored in the DMV membranes, and their fibrous content
74 consists mostly of *de novo* synthesized double-stranded viral RNA. In SARS-CoV, these DMVs
75 are not isolated, but integrated into an interconnected reticulovesicular network derived from
76 the ER [14]. Later in the viral life cycle, DMVs fuse into larger cytoplasmic vacuoles containing
77 numerous assembled virions that have budded, this budding probably occurring in the ERGIC
78 (ER-Golgi intermediate compartment) [15].

79 There are many examples of the usefulness of electron microscopy for improving our
80 understanding of the infectious cycle of viruses [16]. The Vero cell line has been shown to be
81 a relevant model for studies of SARS-CoV-2/cell interactions [17]. We therefore used these
82 cells for a kinetic study of virus-induced DMVs, viral morphogenesis and release for this new

83 pathogen, based on immunofluorescence on confocal microscopy and transmission electron
84 microscopy.

85

86 **Materials and Methods**

87 **Viral strain and cells**

88 The SARS-CoV-2 strain used in this study was isolated with a nasal swab sample collected
89 from one of the first COVID-19 cases confirmed in France: a 47 y-o female patient hospitalized
90 in January 2020 in the Department of Infectious and Tropical Diseases, Bichat Claude Bernard
91 Hospital, Paris [18]. This sample was collected according to the declaration of Helsinki and
92 received approval from local ethics commission. The complete viral genome sequence obtained
93 using Illumina MiSeq sequencing technology was then deposited after assembly on the GISAID
94 EpiCoV platform (Accession ID EPI_ISL_411218) under the name
95 BetaCoV/France/IDF0571/2020. Vero-81 cells (ATCC, CCL-81) were maintained in DMEM
96 medium supplemented with 10% FBS at 37°C, under an atmosphere containing 5% CO₂. To
97 generate virus stocks, we facilitated SARS-CoV-2 infection by transducing these cells with a
98 lentiviral vector expressing transmembrane protease serine 2 (TMPRSS2). TMPRSS2 was
99 inserted into a pTRIP vector, and lentiviral vectors were produced by the transfection of
100 HEK293T cells with pTRIP-TMPRSS2, pHCMV-VSVG and HIV gag-pol in the presence of
101 Turbofect (Life Technologies) according to the manufacturer's instruction. Supernatants
102 containing lentiviral vectors were used to transduce cells twice, 48 h apart. SARS-CoV-2 was

103 then propagated in these Vero-81 cells expressing TMPRSS2. The kinetic study following
104 infection with this strain was performed on regular Vero-81 cells.

105 **Kinetics of SARS-CoV-2 infection**

106 Cells were plated in 24 wells plates (with or without glass coverslips) and infected the next day
107 at a MOI of 0.25 for 1h. Then the cells were rinsed twice with PBS and incubated at 37°C. At
108 various time point post-infection cell supernatants were collected to quantify the secretion of
109 virus and the cells were processed for different analyses (immunostaining, immunoblot and
110 qRT-PCR).

111 **Immunofluorescence**

112 Infected cells were fixed by incubation in 3% paraformaldehyde for 20 minutes, and stored in
113 PBS at 4°C until required for immunolabeling. Cells were permeabilized by incubation with
114 0.1% Triton X-100 for 5 minutes, and were then blocked by incubation for 30 minutes with 5%
115 goat serum (GS) in PBS. Infected cells were labeled with a mixture of the mouse J2 monoclonal
116 antibody against dsRNA (Scicons, diluted 1:1000) and a human monoclonal antibody directed
117 against the spike protein (Sanyoubio #AHA003, diluted 1:250). The cells were incubated with
118 primary antibodies in PBS supplemented with 5% GS for 30 minutes. They were washed three
119 times with PBS and then incubated for 30 minutes with Alexa-488-conjugated goat anti-human
120 IgG and cyanin-3-conjugated goat anti-mouse IgG secondary antibodies (Jackson
121 Immunoresearch) in 5% goat serum in PS supplemented with 1 µg/ml DAPI (4',6-diamidino-
122 2-phenylindole). Coverslips were then rinsed four times with PBS, once in water and mounted

123 on microscope slides in Mowiol 4-88-containing medium. Images were acquired on an Evos
124 M5000 imaging system (Thermo Fisher Scientific) equipped with light cubes for DAPI, GFP
125 and RFP, and a 10x objective. For each coverslip, six 8-bit images of each channel were
126 acquired. The total number of cells was determined by counting the nuclei. Infected cells,
127 defined as positive for dsRNA and S immunolabeling, were counted, and the percentage of
128 infected cells was calculated. The experiment was performed 3 times in duplicates. About 7,000
129 to 11,000 cells were counted per time point in each experiment using a homemade macro
130 running in ImageJ.

131 **Immunoblot**

132 Infected cells were lysed in non-reducing Laemmli loading buffer. Lysates were incubated at
133 95°C for 25 min before exit of the BSL3 facility. Then the samples were reduced by addition
134 of 40 mM dithiothreitol and incubated for 10 min at 70°C before separation of the proteins on
135 a 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred on a nitrocellulose
136 membrane (Amersham). Membrane-bound N and S proteins were detected using a rabbit
137 polyclonal antibody to SARS nucleocapsid (Novus) and a mouse polyclonal antibody to
138 SARS-CoV-2 S protein produced by Biotem, as primary antibodies, and horseradish
139 peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). Detection was carried
140 out by chemoluminescence (SuperSignal™ West Pico PLUS, Thermo Scientific). The signals
141 were recorded using a LAS 3000 apparatus (Fujifilm).

142 **Genome quantification**

143 Total RNA of infected cells was extracted by using the Nucleospin RNA kit (Macherey-Nagel)
144 as recommended by the manufacturer. Reverse transcription was performed using the high
145 capacity cDNA reverse transcription kit (Life Technologies) according to the manufacturer's
146 instructions. Then RNA were subjected to qRT-PCR using the SYBR green PCR master mix
147 (Life Technologies) with primers (GTGARATGGTCATGTGTGGCGG and
148 CARATGTTAAASACACTATTAGCATA) for RdRp fragment amplification. qRT-PCR was
149 performed with a Quantstudio 3 (Life Technologie). A standard curve was generated with *in*
150 *vitro* transcribed RNA corresponding to the RdRp fragment.

151 **Viral secretion**

152 The quantity of virus secreted in cell supernatant was assessed by the TCID50 method.

153 **Transmission electron microscopy**

154 Cells were infected at a MOI of 0.25 for one hour. Infected and mock-infected cells were then
155 fixed at various time points post-infection, by incubation for 24 h in 1% glutaraldehyde, 4%
156 paraformaldehyde, (Sigma, St-Louis, MO) in 0.1 M phosphate buffer (pH 7.2). Samples were
157 then washed in phosphate-buffered saline (PBS) and post-fixed by incubation for 1 h with 2%
158 osmium tetroxide (Agar Scientific, Stansted, UK). Cells were then fully dehydrated in a graded
159 series of ethanol solutions and propylene oxide. They were impregnated with a mixture of (1:1)
160 propylene oxide/Epon resin (Sigma) and left overnight in pure resin. Samples were then
161 embedded in Epon resin (Sigma), which was allowed to polymerize for 48 hours at 60°C. Ultra-
162 thin sections (90 nm) of these blocks were obtained with a Leica EM UC7 ultramicrotome

163 (Wetzlar, Germany). Sections were stained with 2% uranyl acetate (Agar Scientific), 5% lead
164 citrate (Sigma), and observations were made with a transmission electron microscope (JEOL
165 1011, Tokyo, Japan). Infected cells were observed 4 h, 6 h, 8 h, 10 h, 12 h and 24 h post-
166 infection, and compared to mock-infected cells. For quantitative analysis, ultrastructural
167 features were monitored in 100 cells (100 consecutive sections on the EM grid, except those
168 with no nucleus section), for each cell pellet.

169

170 **Results**

171 **Kinetics of SARS-CoV-2 infection in Vero cells**

172 We analyzed the kinetics of SARS-CoV-2 infection. First, we quantified the number of infected
173 cells based on the expression of the spike protein S and the presence of dsRNA, a replication
174 complex marker, by evaluating immunofluorescence over time in infected Vero cells (Fig. 1a).
175 At 4 hours post infection (hpi), a few cells displayed a weak dsRNA signal but no S staining.
176 The first clear sign of infection was observed at 6hpi, and a gradual increase in the number of
177 infected cells and in the intensity of labeling was observed up to 8 to 10 hpi. At12 hpi, the
178 number of infected cells increased again, and groups of cells corresponding to infection foci
179 appeared. These foci contained dsRNA-positive S-negative cells as observed in newly infected
180 cells at 6 hpi, indicating that a new round of infection had occurred. We counted the number of
181 dsRNA-positive cells and of S-positive cells, to measure the progression of the infection (Fig
182 1b). Our results suggest that the replication cycle of SARS-CoV-2 has a periodicity of about 8

183 h in Vero cells. To further confirm this result, we measured the secretion of virus at each time
184 point (Fig 1c). Viral secretion was detectable at 8h post-infection and increased over time. In
185 parallel we monitored viral replication by quantifying intracellular viral RNA (Fig. 1d). Viral
186 replication was detected at 4hpi and increases until 10hpi to reach a plateau at 10-12hpi. From
187 12hpi to 24hpi, a 0.7Log moderate increase of the quantity of viral RNA was observed. Finally,
188 the gradual increase of N and S viral proteins in infected cells was confirmed by immunoblot
189 (Fig 1.e).

190 **Ultrastructural features of SARS-CoV-2 infected cells**

191 At 4 hpi, we observed no perceptible difference between infected and mock-infected cells (Fig.
192 2a). The first virus-induced modifications were observed at 6 hpi, when discrete clusters of
193 single-membrane vesicles formed in the cytoplasm (Fig. 2b and 2c). Another change detectable
194 as early as 6 hpi was the frequent presence of annulate lamellae (AL) in the cell sections (Fig.
195 2d and 2e). AL are a particular type of intracellular membrane, consisting of stacks of highly
196 ordered ER-derived membranes, arranged in parallel, and containing nuclear pore complexes
197 (NPCs) [19]. In these SARS-CoV-2-infected Vero cells, they appeared in continuity with the
198 nuclear envelope (Fig. 2d) or within the cytoplasm (Fig. 2e).

199 At 8 hpi, massive changes were observed, with the presence of numerous DMVs
200 forming a replication network, frequently surrounded by mitochondria (Fig. 3a and 3b). At this
201 time point, the first virus particles assembled within the cells were detected in intracellular
202 vesicles probably related to the Golgi apparatus or ERGIC (Fig. 4a). In some sections, the

203 budding of viruses towards the lumen of the ERGIC/Golgi vesicles could be seen (Fig. 4b, 4c
204 and 4d). These budding viral particles or those fully assembled in the vesicle lumen (Fig. 4e)
205 had prominent surface projections, or spikes. These intracellular spiky viruses were detected in
206 small vesicles, each containing only a few particles, at 8 hpi. At 10 and 12 hpi, numerous
207 extracellular particles could be seen at the cell surface (Fig. 5a). Images of virus-carrying
208 vesicles that were presumably moving towards the plasma membrane (Fig. 5a), or merging with
209 the plasma membrane (Fig. 5b, 5c, 5d and 5e), suggested that the virions were released by
210 exocytosis mechanisms. The fact that these vesicles often contained several viral particles
211 suggested that they represent viral exit rather than cellular entry.

212 At 24 hpi, viruses were still detectable at the cell surfaces and were present in high
213 amount. Although these viruses could represent virions released by the imaged cells, we could
214 not exclude that some of them originate from adjacent cells before to be trapped at the plasma
215 membrane of these cells. Also, large numbers of viral particles were found to have accumulated
216 in very large intracellular vacuoles (Fig. 6a and 6b). Surprisingly, the viral particles present in
217 these large intracellular compartments had a smooth surface and bore no spikes (Fig. 7a and
218 7b). Their appearance was clearly different from that of viruses released at the cell surface,
219 which carried many spikes (Fig. 8a and 8b). At these later stages of the infection, most of the
220 Vero cells infected with SARS-CoV-2 contained an abundant reticulovesicular network of
221 DMVs, occupying an entire pole of the cytoplasm, in which viral replication and the assembly
222 of new virions occurred simultaneously (Fig. 9a). DMVs were still the main component of this

223 network, sometimes associated with other virus-induced structures, such as myelin-like
224 membrane whorls or autophagic-like packaged membranes (Fig. 9b). These membrane whorls
225 were sometimes also associated with the spike-less viral particles in the large intracellular
226 vacuoles (Fig. 6b and Fig. 7a).

227 The quantification of these ultrastructural features in 100 consecutive cell sections
228 showed that AL were present in only 1% of mock-infected cells, but increased up to 7% at 6
229 hpi, before to slowly decrease until 10 hpi and finally stabilize around 3% at 12 hpi (Fig. 10).
230 Clusters of single-membrane vesicles that appeared between 6 hpi and 8 hpi were observed in
231 40% of the cells at 8 hpi, before reaching 60% at 12 hpi and then dropping to less than 20% at
232 24 hpi. DMVs were observed in a constantly increasing number of cells between 4 hpi and 24
233 hpi, reaching 80% of the cells at 24 hpi (Fig.10). Membrane whorls and autophagic-like
234 structures, observed in 7% of mock-infected cells, increased slowly during the first 10 hpi and
235 then more rapidly to reach almost 60% of the cells at 24 hpi. Concerning the viral particles,
236 spiky virions found in golgian/ERGIC vesicles were first observed at 8 hpi, and the number of
237 cells with this feature regularly increased to reach 48% at 24 hpi. Large vacuoles containing
238 spike-less virions started to accumulate in cells from 10 hpi, and their number increased to also
239 reach a maximum at 24 hpi, being present in about 60% of the cells. Finally, the number of
240 cells surrounded by extracellular spiky virions increases exponentially from 10 hpi, to reach
241 80% of the cells at 24 hpi (Fig.10).

242

243 Discussion

244 As previously reported for coronaviruses and, more recently, for SARS-CoV-2 [20, 21, 22, 23],
245 our findings confirm that the SARS-CoV-2 viral cycle is supported by a reticulovesicular
246 network derived from endoplasmic reticulum (ER) membranes and consisting mostly of
247 double-membrane vesicles (DMVs). After the budding of the virions, presumably in the Golgi
248 apparatus stacks or the ER-Golgi intermediate compartment (ERGIC), the virus is released by
249 exocytosis at the plasma membrane. The kinetics of appearance of the DMVs and virions inside
250 and on the surface of the cells are globally similar to those observed in a recent study performed
251 with human pulmonary epithelial Calu-3 infected cells [21]. Thus, our findings confirm
252 previous results, but they also shed new light on early and late stages of the viral infectious
253 cycle.

254 We show that, at early stages of infection, the cells contained only discrete single-
255 membrane vesicle clusters (Fig. 2b and 2c), potentially acting as the precursors of DMVs, as
256 previously suggested for other viral models. This hypothesis is consistent with our quantitative
257 analysis (Fig. 10), showing that the number of positive cells for these single-membrane vesicle
258 clusters was decreasing in the late phase of the infection while the number of DMVs positive
259 cells increased (Fig. 10). In hepatitis C virus (HCV), for example, it has been suggested that
260 single-membrane vesicle clusters act as the precursors of the DMVs characterizing the
261 membranous web induced by this virus [24, 25]. A similar process has also been described for
262 poliovirus, for which early viral organelles are gradually transformed into double-membrane

263 structures by the extension of membranous walls and/or the collapse of the luminal cavity of
264 the single-membrane structure [26]. Recent investigations by high-resolution electron
265 tomography analysis of SARS-CoV-2 infected Calu-3 cells have confirmed that DMVs are
266 closely associated to the ER, being linked by smooth ER connectors or sometimes embedded
267 into the rough ER such as the DMV outer membrane was contiguous to the ER membrane [21].
268 However, the mechanism underlying DMV biogenesis is still poorly understood and the
269 potential role of these single-membrane vesicle clusters in their formation will certainly need
270 further investigation.

271 We also report the presence of annulate lamellae (AL) in Vero cells infected with SARS-
272 CoV-2 (Fig. 2d and 2e). These structures are not specific of the viral infection as they were
273 observed in mock-infected cells in few cells (around 1%), but their presence increased
274 following infection (peak at 7% 6 hpi) (Fig. 10). Similar observations were reported by another
275 group, but little comment was made on their possible meaning [27]. AL have no obvious
276 function, other than as a reservoir of nuclear envelope components. AL were initially thought
277 to be an ultrastructural feature characteristic of rapidly growing germ and tumor cells, but they
278 have since been characterized as a marker of infection for various viruses, including hepatitis
279 A virus [28], Japanese encephalitis virus [29] and human herpes virus 6 [30]. However, their
280 role in the infection process is unknown. Interestingly, in the HCV model, infected cells display
281 an increase in the amount of cytoplasmic nuclear pore complex proteins (Nups), which
282 accumulate in or close to cytoplasmic membranes enriched in HCV proteins [31]. It has,

283 therefore, been suggested that cytoplasmic nuclear pore complexes (NPCs), or potentially
284 derivatives of these structures, such as AL, may facilitate establishment of the HCV-induced
285 membranous web [31, 32]. It will be interesting to investigate, whether such a mechanism is
286 also involved in the biogenesis of the SARS-CoV-2 replication network.

287 As for AL, membrane whorls and autophagic-like structures were not specific of the
288 viral infection as they were observed in mock-infected cells. However, if they were detected in
289 7% of uninfected cells, the number of cells containing these structures slowly increased during
290 the first 10 hpi, and then more rapidly to reach almost 60% of the cells at 24 hpi (Fig. 10). Once
291 again, these data are in favor of virus-induced mechanisms stimulating the formation of these
292 host cell membranes derived structures.

293 At later stages of infection, we observed large intracellular vacuoles containing viral
294 particles that appeared to be completely devoid of spikes. Such images have been reported by
295 other groups, for SARS-CoV-2 propagated in cell culture [22, 23 27, 33] or detected in the lung
296 tissue of infected patients [34]. Similar findings were also reported in the very first electron
297 microscopy investigations of SARS-CoV in cultured Vero cells [35, 36]. In the present study,
298 a comparative analysis, at high EM magnifications, of the viral particles present on the cell
299 surface and those present in these large intracellular vacuoles at these time points late in
300 infection clearly showed differences in terms of the presence or absence of spikes (Fig. 7 and
301 Fig. 8). The reason for the absence of spikes on these intracellular viral particles is currently
302 unknown. This does not seem to be related to a technical problem leading to a loss of the spikes

303 of the intracellular viruses since spikes can be well visualized on the viruses present in the
304 smaller vesicles of ERGIC or Golgi origin (Fig. 4). These large vacuoles containing spike-less
305 viral particles may be intracellular compartments in which degradation through an
306 autophagolysosomal process occurs. This hypothesis is supported by the presence of myelin-
307 like membrane whorls in these compartments (Fig. 7a), as also previously reported for SARS-
308 CoV [36]. However, these spike-less virions did not appear to be degraded in these
309 compartments, although they were sometimes somewhat distorted (Fig. 7b), presenting a
310 “doughnut shape” and a more pronounced electron-dense edge, as previously discussed by
311 another group [27]. Alternatively, these spike-less viral particles may be formed by a defective
312 virion assembly pathway, leading to immature and, thus, non-infectious particles. This would
313 be consistent with previous findings showing that S protein is dispensable for the budding and
314 assembly of coronaviruses [37, 38], whereas N protein seems to be essential for these
315 mechanisms and to drive them [39]. Further investigations will be required to determine the
316 potential role of these spike-free viral particles in the pathophysiology of SARS-CoV-2
317 infection, particularly given the huge numbers of such particles in infected cells.

318 A recent study by Ogando *et al.*, in which Vero cells were also used to propagate SARS-
319 CoV-2, suggested that this virus may carry fewer spikes than SARS-CoV [20]. We did not
320 directly compare the two viruses in our study, but our EM images show that SARS-CoV-2 has
321 many “club-shaped” projections at its surface (Fig. 8), as also previously reported for SARS-
322 CoV. The underlying reasons for this remain unclear, but may reflect the ultimate release of

323 spike-less viral particles from the intracellular vacuoles at very late stages of the infectious
324 cycle in the study conducted by Ogando et al. [20], possibly due to cell lysis. This may explain
325 why SARS-CoV-2 was found to be less infectious than SARS-CoV in this study, despite
326 generating larger amounts of intracellular viral RNA.

327 It will certainly be important to continue this type of investigation with other relevant
328 cellular models. Recent studies have shown the possibility of propagating the virus in polarized
329 organoids of human airway epithelial [40]. Here also, although this has not been particularly
330 commented by the authors, it seems that spike-free virus particles may accumulate
331 intracellularly. In any case, our ultrastructural study provides new insight into the early and late
332 steps of the SARS-CoV-2 infectious cycle, which merit further investigation in future studies
333 of this important new pathogen.

334

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342

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346 analysis, manuscript editing; SB: conceptualization, investigation, formal analysis, writing
347 original draft, manuscript editing, supervision; PR: conceptualization, investigation, formal
348 analysis, writing original draft, manuscript editing, supervision.

349

350 **References**

- 351 1. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJM, Wolthers KC,
352 Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B (2009) Identification of
353 a new human coronavirus. *Nat Med* 10:368-773.
- 354 2. Woo PCY, Lau SKP, Tsoi H-W, Huang Y, Poon RWS, Chu C-M, Lee RA, Luk WK, Wong
355 GK, Wong BH, Cheng VC, Tang BS, Wu AK, Yung RW, Chen H, Guan Y, Chan KH,
356 Yuen KY (2005) Clinical and molecular epidemiological features of coronavirus HKU1-
357 associated community-acquired pneumonia. *J Infect Dis* 192:1898-1907.
- 358 3. Edridge AWD, Kaczorowska J, Hoste ACR, Bakker M, Klein M, Loens K, Jebbink MF,
359 Matser A, Kinsella CM, Rueda P, Ieven M, Goossens H, Prins M, Sastre P, Deijs M, van
360 der Hoek L (2020) Seasonal coronavirus protective immunity is short-lasting. *Nat Med* :
361 doi: 10.1038/s41591-020-1083-1, online ahead of print.

- 362 4. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Ong S, Urbani C,
363 Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J,
364 Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini
365 WJ, Anderson LJ (2003) A novel coronavirus associated with severe acute respiratory
366 syndrome. *N Engl J Med* 348:1953-1966.
- 367 5. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM (2012).
368 Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J*
369 *Med* 367:1814–1820.
- 370 6. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P,
371 Zhan F, Ma X, Wang D, Xu W, Wu G, Gao GF, Tan W (2019) A Novel Coronavirus from
372 Patients with Pneumonia in China. *N Engl J Med* 382:727-733.
- 373 7. Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, Zhou P, Yang X-L, Wang X-
374 G. (2020) A pneumonia outbreak associated with a new coronavirus of probable bat origin.
375 *Nature* 579:270-273.
- 376 8. Verity R, Okell LC, Dorigatti I, Winskill P, Whittaker C, Imai N, Cuomo-Dannenburg G,
377 Thompson H, Walker PGT, Fu H, Dighe A, Griffin JT, Baguelin M, Bhatia S, Boonyasiri
378 A, Cori A, Cucunubá Z, FitzJohn R, Gaythorpe K, Green W, Hamlet A, Hinsley W, Laydon
379 D, Nedjati-Gilani G, Riley S, van Elsland S, Volz E, Wang H, Wang Y, Xi X, Donnelly
380 CA, Ghani AC, Ferguson NM (2020). Estimates of the severity of coronavirus disease
381 2019: a model-based analysis. *Lancet Infect Dis* 20:669-677.

- 382 9. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, Lau YC, Wong JY, Guan Y, Tan X, Mo
383 X, Chen Y, Liao B, Chen W, Hu F, Zhang Q, Zhong M, Wu Y, Zhao L, Zhang F, Cowling
384 BJ, Li F, Leung GM (2020) Temporal dynamics in viral shedding and transmissibility of
385 COVID-19. *Nat Med* 26:672-675.
- 386 10. Chen Y, Liu Q, Guo D (2020) Emerging coronaviruses: Genome structure, replication, and
387 pathogenesis. *J Med Virol* 92: 418-423.
- 388 11. Maier HJ, Neuman BW, Bickerton E, Keep SM, Alrashedi H, Hall R, Britton P (2016)
389 Extensive coronavirus-induced membrane rearrangements are not a determinant of
390 pathogenicity. *Sci Rep* 6:1-12.
- 391 12. Netherton CL, Wileman T (2011) Virus factories, double membrane vesicles and viroplasm
392 generated in animal cells. *Curr Opin Virol* 1:381-387.
- 393 13. Blanchard E, Roingard P (2015) Virus-induced double-membrane vesicles. *Cell*
394 *Microbiol* 17:45-50.
- 395 14. Knoops K, Kikkert M, Worm SHE van den, Zevenhoven-Dobbe JC, van der Meer Y,
396 Koster AJ, Mommaas AM, Snijder EJ (2008) SARS-coronavirus replication is supported
397 by a reticulovesicular network of modified endoplasmic reticulum. *PLoS Biol* 6:e226.
- 398 15. Stertz S, Reichelt M, Spiegel M, Kuri T, Martínez-Sobrido L, García-Sastre A, Weber F,
399 Kochs G (2007) The intracellular sites of early replication and budding of SARS-
400 coronavirus. *Virology* 361:304-315.

- 401 16. Roingard P (2008) Viral detection by electron microscopy: past, present and future. *Biol*
402 *Cell* 100:491-501.
- 403 17. Matsuyama S, Nao N, Shirato K, Kawase M, Saito S, Takayama I, Nagata N, Sekizuka T,
404 Katoh H, Kato F, Sakata M, Tahara M, Kutsuna S, Ohmagari N, Kuroda M, Suzuki T,
405 Kageyama T, Takeda M. (2020) Enhanced isolation of SARS-CoV-2 by TMPRSS2-
406 expressing cells. *Proc Natl Acad Sci USA* 117:7001-7003.
- 407 18. Pizzorno A, Padey B, Julien T, Trouillet-Assant S, Traversier A, Errazuriz-Cerda E, Fouret
408 J, Dubois J, Gaymard A, Lescure FX, Dulière V, Brun P, Constant S, Poissy J, Lina B,
409 Yazdanpanah Y, Terrier O, Rosa-Calatrava M (2020) Characterization and treatment of
410 SARS-CoV-2 in nasal and bronchial human airway epithelia. *Cell Rep Med* 1:100059.
- 411 19. Walther TC, Askjaer P, Gentzel M, Habermann A, Griffiths G, Wilm M, Mattaj IW, Hetzer
412 M (2003) RanGTP mediates nuclear pore complex assembly. *Nature* 424:689-694.
- 413 20. Ogando NS, Dalebout TJ, Zevenhoven-Dobbe JC, Limpens RW, van der Meer Y, Caly L,
414 Druce J, de Vries JJC, Kikkert M, Bárcena M, Sidorov I, Snijder EJ (2020) SARS-
415 coronavirus-2 replication in Vero E6 cells: replication kinetics, rapid adaptation and
416 cytopathology. *J Gen Virol* 101:925-940.
- 417 21. Cortese M, Lee JY, Cerikan B, Neufeldt CJ, Oorschot VMJ, Köhrer S, Hennies J, Schieber
418 NL, Ronchi P, Mizzon G, Romero-Brey I, Santarella-Mellwig R, Schorb M, Boermel M,
419 Mocaer K, Beckwith MS, Templin RM, Gross V, Pape C, Tischer C, Frankish J, Horvat
420 NK, Laketa V, Stanifer M, Boulant S, Ruggieri A, Chatel-Chaix L, Schwab Y,

- 421 Bartenschlager R (2020) Integrative Imaging Reveals SARS-CoV-2-Induced Reshaping of
422 Subcellular Morphologies. *Cell Host Microbe*, in press. doi: 10.1016/j.chom.2020.11.003
- 423 22. Mendonça L, Howe A, Gilchrist JB, Sun D, Knight ML, Zanetti-Domingues LC, Bateman
424 B, Krebs AS, Chen L, Radecke J, Sheng Y, Li VD, Ni T, Kounatidis I, Koronfel MA,
425 Szykiewicz M, Harkiolaki M, Martin-Fernandez ML, James W, Zhang P (2020) SARS-
426 CoV-2 Assembly and Egress Pathway Revealed by Correlative Multi-modal Multi-scale
427 Cryo-imaging. *BioRxiv*, preprint. doi: 10.1101/2020.11.05.370239. Preprint.
- 428 23. Neil D, Moran L, Horsfield C, Curtis E, Swann O, Barclay W, Hanley B, Hollinshead M,
429 Roufosse C (2020) Ultrastructure of cell trafficking pathways and coronavirus: how to
430 recognise the wolf amongst the sheep. *J Pathol*, in press. doi: 10.1002/path.5547
- 431 24. Ferraris P, Blanchard E, Roingard P (2010) Ultrastructural and biochemical analyses of
432 hepatitis C virus-associated host cell membranes. *J Gen Virol* 91:2230-2237.
- 433 25. Ferraris P, Beaumont E, Uzbekov R, Brand D, Gaillard J, Blanchard E, Roingard P (2013)
434 Sequential biogenesis of host cell membrane rearrangements induced by hepatitis C virus
435 infection. *Cell Mol Life Sci* 70:1297-1306.
- 436 26. Belov GA, Nair V, Hansen BT, Hoyt FH, Fischer ER, Ehrenfeld E (2012) Complex
437 dynamic development of poliovirus membranous replication complexes. *J Virol* 86:302-
438 312.

- 439 27. Brahim Belhaouari D, Fontanini A, Baudoin J-P, Haddad G, Le Bideau M, Yaacoub Bou
440 Khali J, Raoult D, La Scola B (2020) The strengths of scanning electron microscopy in
441 deciphering SARS-CoV-2 infectious cycle. *Front Microbiol* 11:2014.
- 442 28. Marshall JA, Borg J, Coulepis AG, Anderson DA (1996) Annulate lamellae and lytic HAV
443 infection in vitro. *Tissue Cell* 28:205-214.
- 444 29. Wang JJ, Liao CL, Chiou YW, Chiou CT, Huang YL, Chen LK. (1997) Ultrastructure and
445 localization of E proteins in cultured neuron cells infected with Japanese encephalitis virus.
446 *Virology* 238:30-39.
- 447 30. Cardinali G, Gentile M, Cirone M, Zompetta C, Frati L, Faggioni A, Torrisi MR (1998)
448 Viral glycoproteins accumulate in newly formed annulate lamellae following infection of
449 lymphoid cells by human herpesvirus 6. *J Virol* 72:9738-9746.
- 450 31. Neufeldt CJ, Joyce MA, Levin A, Steenbergen RH, Pang D, Shields J, Tyrrell DL, Wozniak
451 RW (2013) Hepatitis C virus-induced cytoplasmic organelles use the nuclear transport
452 machinery to establish an environment conducive to virus replication. *PLoS Pathog*
453 9:e1003744.
- 454 32. Bonamassa B, Ciccarese F, Antonio VD, Contarini A, Palù G, Alvisi G (2015) Hepatitis C
455 virus and host cell nuclear transport machinery: a clandestine affair. *Front Microbiol*
456 6:e619.
- 457 33. Goldsmith CS, Miller SE, Martines RB, Bullock HA, Zaki SR (2020) Electron microscopy
458 of SARS-CoV-2: a challenging task. *Lancet* 395:e99.

- 459 34. Martines RB, Ritter JM, Matkovic E, Gary J, Bollweg BC, Bullock H, Goldsmith CS,
460 Silva-Flannery L, Seixas JN, Reagan-Steiner S, Uyeki T, Denison A, Bhatnagar J, Shieh
461 WJ, Zaki SR (2020) Pathology and pathogenesis of SARS-CoV-2 associated with fatal
462 coronavirus disease, United States. *Emerg Infect Dis*, in press. doi:
463 10.3201/eid2609.202095
- 464 35. Ng ML, Tan SH, See EE, Ooi EE, Ling AE (2003) Early events of SARS coronavirus
465 infection in Vero cells. *J Med Virol* 71:323-331.
- 466 36. Ng ML, Tan SH, See EE, Ooi EE, Ling AE (2003) Proliferative growth of SARS
467 coronavirus in Vero E6 cells. *J Gen Virol* 84:3291-3303.
- 468 37. Rottier PJ, Horzinek MC, van der Zeijst BA (1981) Viral protein synthesis in mouse
469 hepatitis virus strain A59-infected cells: effect of tunicamycin. *J Virol* 40:350-357.
- 470 38. Ujike M, Taguchi F (2015) Incorporation of Spike and Membrane Glycoproteins into
471 Coronavirus Virions. *Viruses* 7:1700-1725.
- 472 39. McBride R, van Zyl M, Fielding BC (2014) The Coronavirus Nucleocapsid Is a
473 Multifunctional Protein. *Viruses* 6:2991-3018.
- 474 40. Zhu N, Wang W, Liu Z, Liang C, Wang W, Ye F, Huang B, Zhao L, Wang H, Zhou W,
475 Deng Y, Mao L, Su C, Qiang G, Jiang T, Zhao J, Wu G, Song J, Tan W (2020)
476 Morphogenesis and cytopathic effect of SARS-CoV-2 infection in human airway epithelial
477 cells. *Nat Commun* 11:3910.

478

479

Figure Legends

480

481 **Fig. 1** Kinetics of infection analyzed by different methods. **(a)** Vero cells grown on glass
482 coverslips were infected, fixed at the indicated time points and processed for
483 immunofluorescence labeling of S (green) and dsRNA (red). Nuclei were stained with DAPI
484 (blue). **(b)** Cells positive for S and dsRNA were counted and the percentage of infected cells
485 was plotted against time. **(c)** The quantity of virus secreted in the cell supernatant was assessed
486 by the TCID50 method. **(d)** Intracellular genome quantification was performed by qRT-PCR.
487 **(e)** Intracellular N and S viral proteins were detected by immunoblot.

488

489 **Fig. 2** Early ultrastructural changes encountered in Vero cells at 6h post-infection with SARS-
490 CoV-2. **(a)** At 4 hpi, no ultrastructural modifications relative to mock-infected cells, shown
491 here, were visible. **(b, c)** At 6 hpi, the first discrete virus-induced structures appeared, consisting
492 of clusters (delimited by the white arrows) of several single-membrane vesicles. These clusters
493 contained 5 to 10 single-membrane vesicles per cell section, and were frequently observed in
494 the perinuclear area (n=nucleus). **(d, e)** At 6 hpi, annulate lamellae (AL) were frequently
495 observed in infected cells, either as nuclear (n) expansions (**d**, white arrows) or isolated in the
496 cytoplasm (**e**, white arrows), suggesting that the formation of these structures may have been
497 promoted by virus infection. AL consist of stacks of highly ordered endoplasmic reticulum-

498 derived membranes, arranged in parallel and containing nuclear pore complexes, that can be
499 visualized in tangential (**e**) or cross (**d**) sections.

500

501 **Fig. 3** Replication network established in Vero cells at 8h post-infection with the SARS-CoV-
502 2. (**a, b**) A massive replication network consisting of large clusters of numerous double-
503 membrane vesicles (DMVs, presented at high magnification in the insets) was observed. These
504 clusters of DMVs were frequently surrounded by mitochondria (m).

505

506 **Fig. 4** Morphogenesis of intracellular SARS-CoV-2 virions in Vero cells at 8h post-infection.
507 (**a**) Virus particles (white arrows) were observed in intracellular vesicles related to the Golgi
508 apparatus or the endoplasmic reticulum/Golgi intermediate compartment (ERGIC). No virus
509 was released at the plasma membrane (pm) at this time point. (**b, c, d**) The budding of the
510 viruses (white arrows) towards the lumen of the ERGIC/Golgi vesicles was visualized in some
511 cell sections. (**e**) At this time point, the intracellular viruses were detected in small vesicles,
512 each containing only a few viral particles. These viral particles displayed prominent spikes,
513 clearly visible at the surface (black arrowhead).

514

515 **Fig. 5** Release of the SARS-CoV-2 virions by exocytosis from the Vero cells at 10h post-
516 infection. (**a**) Virions (thin black arrows) were frequently observed at the plasma membrane
517 (pm). Numerous virus-carrying vesicles (white arrows) presumably in transit to the plasma

518 membrane were also visualized. **(b, c, d, e)** These virus-carrying vesicles fused with the plasma
519 membrane to release their contents into the extracellular space by exocytosis. Similar
520 ultrastructural events were observed at 12 hpi.

521

522 **Fig. 6** Intracellular accumulation of SARS-CoV-2 particles in large vacuoles within Vero cells
523 at 24 hpi. **(a, b)** Viruses were still detected at the cell surface (thin black arrows), but large
524 numbers of viral particles were observed accumulated in very large intracellular vacuoles
525 (white arrows) of various sizes, predominantly located in the perinuclear region. At this time
526 point, an accumulation of myelin-like membrane whorls or autophagic-like packaged
527 membranes (white asterisk in **b**) was also observed in the cells, separated from or associated
528 with the viral particles.

529

530 **Fig. 7** The SARS-CoV-2 particles accumulating in large intracellular vacuoles in Vero cells at
531 24 hpi have no spikes. **(a)** The numerous viral particles accumulating in these large intracellular
532 compartments were often electron-dense and presented a smooth surface. Membrane whorls
533 (white asterisks) were sometimes also associated with the viral particles within these large
534 intracellular vacuoles. **(b)** At high magnification, the viral particles present in these large
535 intracellular vacuoles were clearly observed to have no spikes (bar = 50 nm).

536

537 **Fig. 8** The SARS-CoV-2 particles released from the Vero cells at 24 hpi carry spikes. **(a)** All
538 the viral particles released at the cell surface (thin black arrows) were surrounded by
539 characteristic, “club-shaped”, spikes. **(b)** This was confirmed by an analysis of these viral
540 particles at high magnification, revealing their distinctive crown-like appearance (bar = 50 nm).

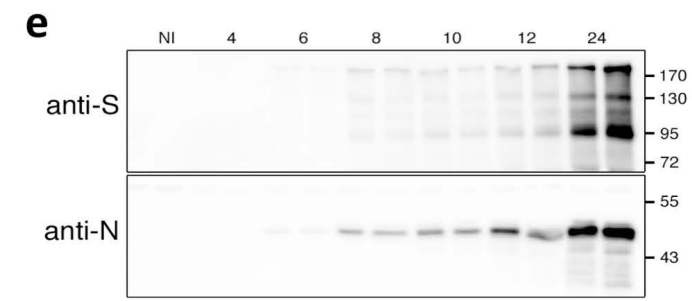
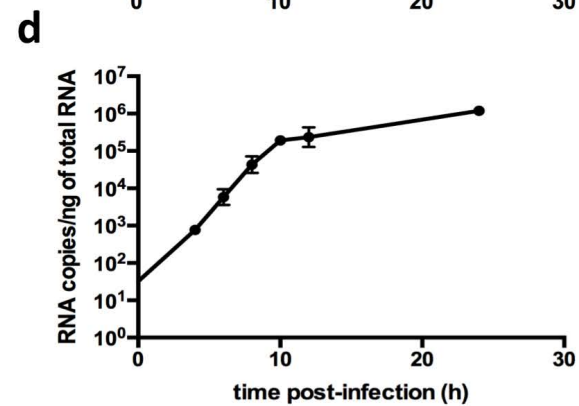
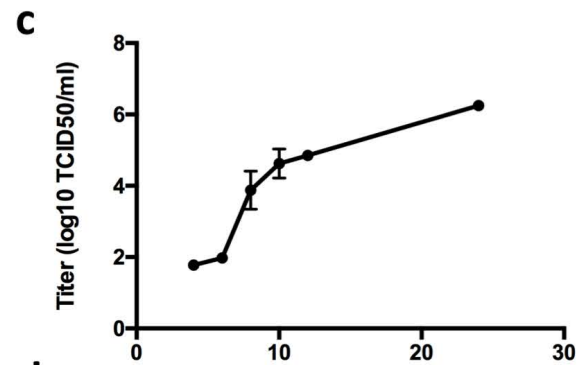
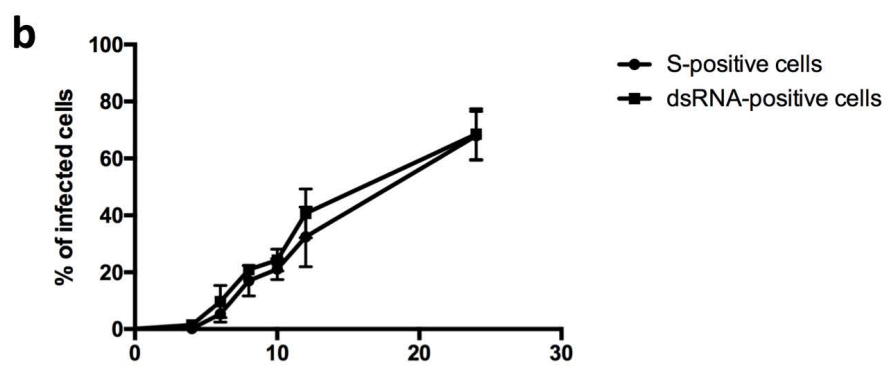
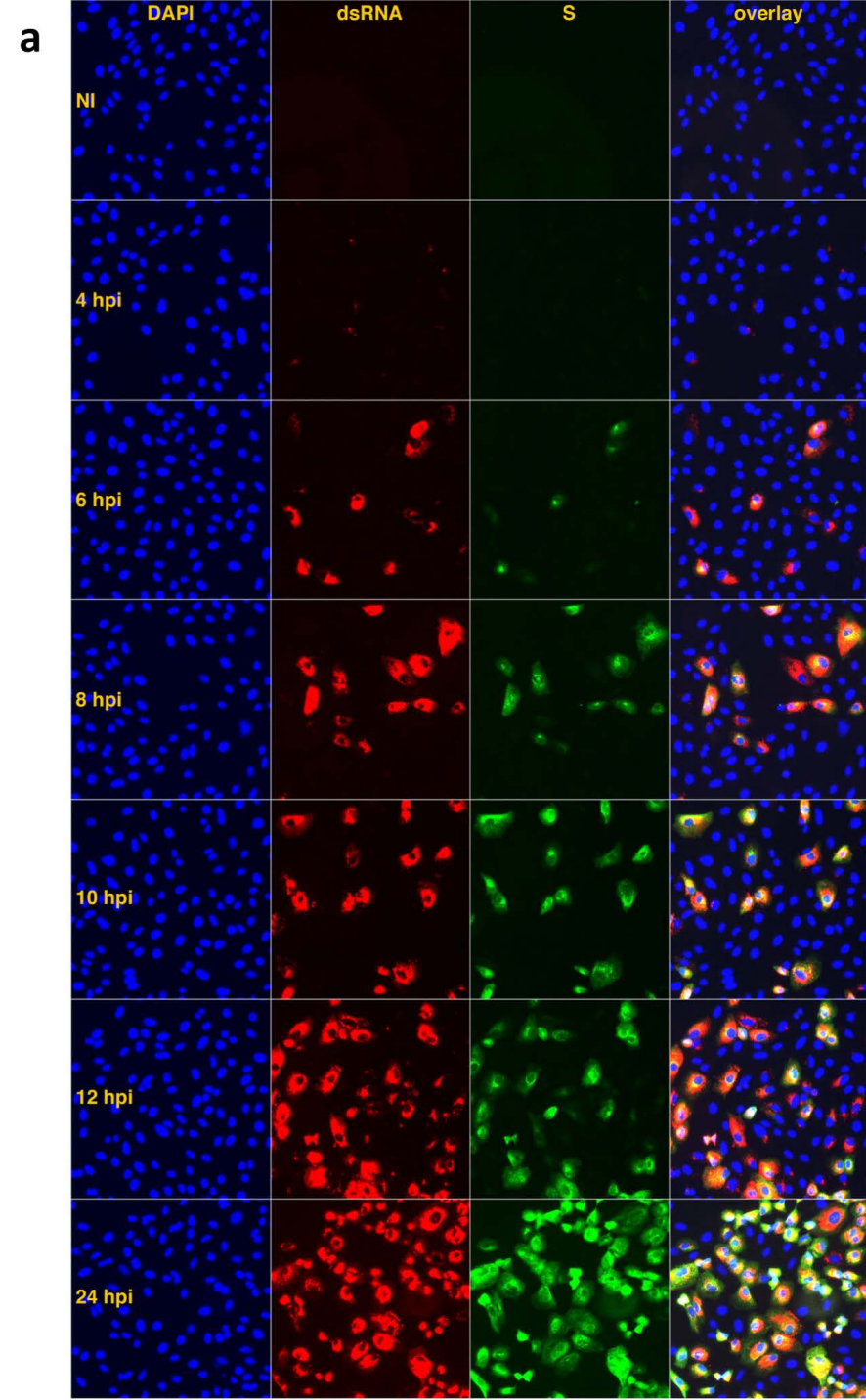
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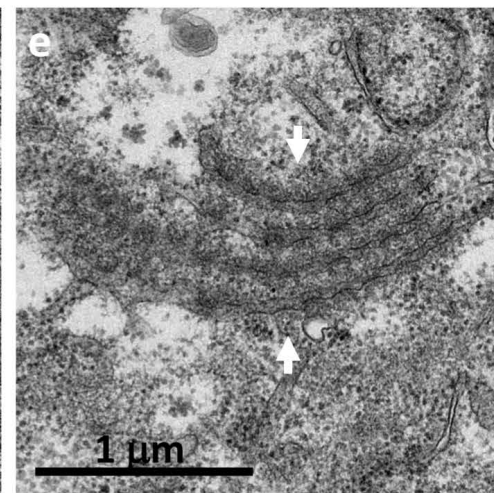
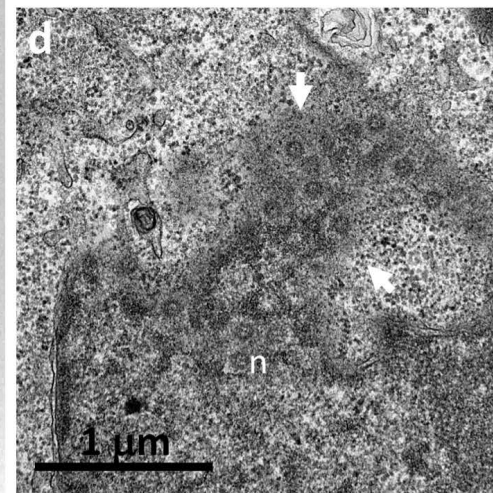
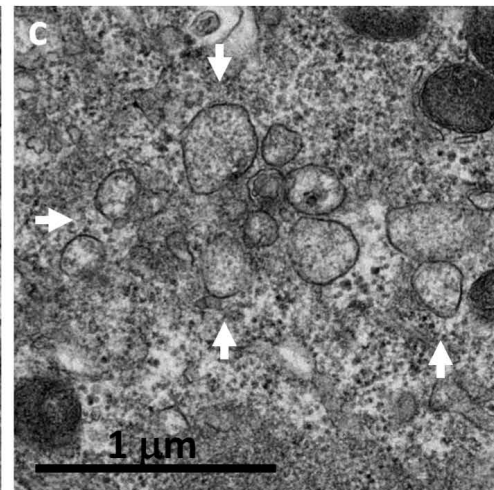
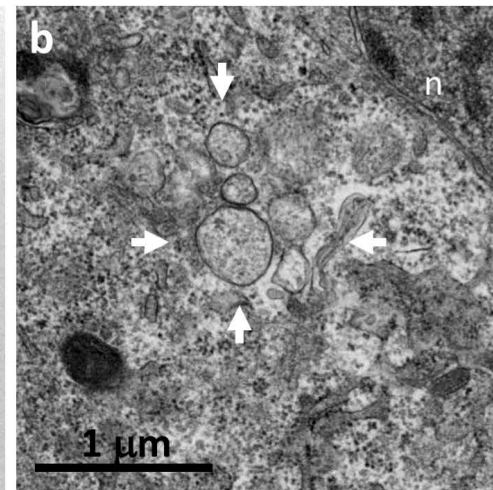
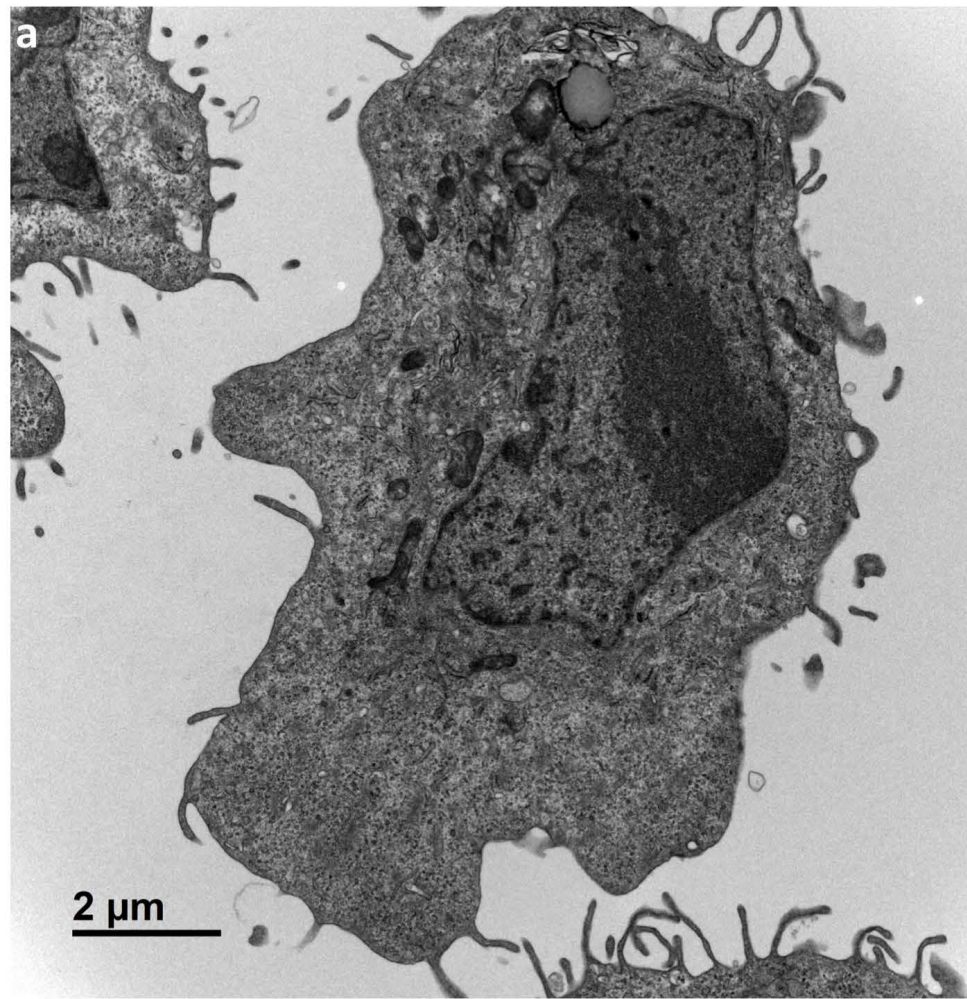
542 **Fig. 9** Invasive replication network in Vero cells at 24 h post-infection with SARS-CoV-2.

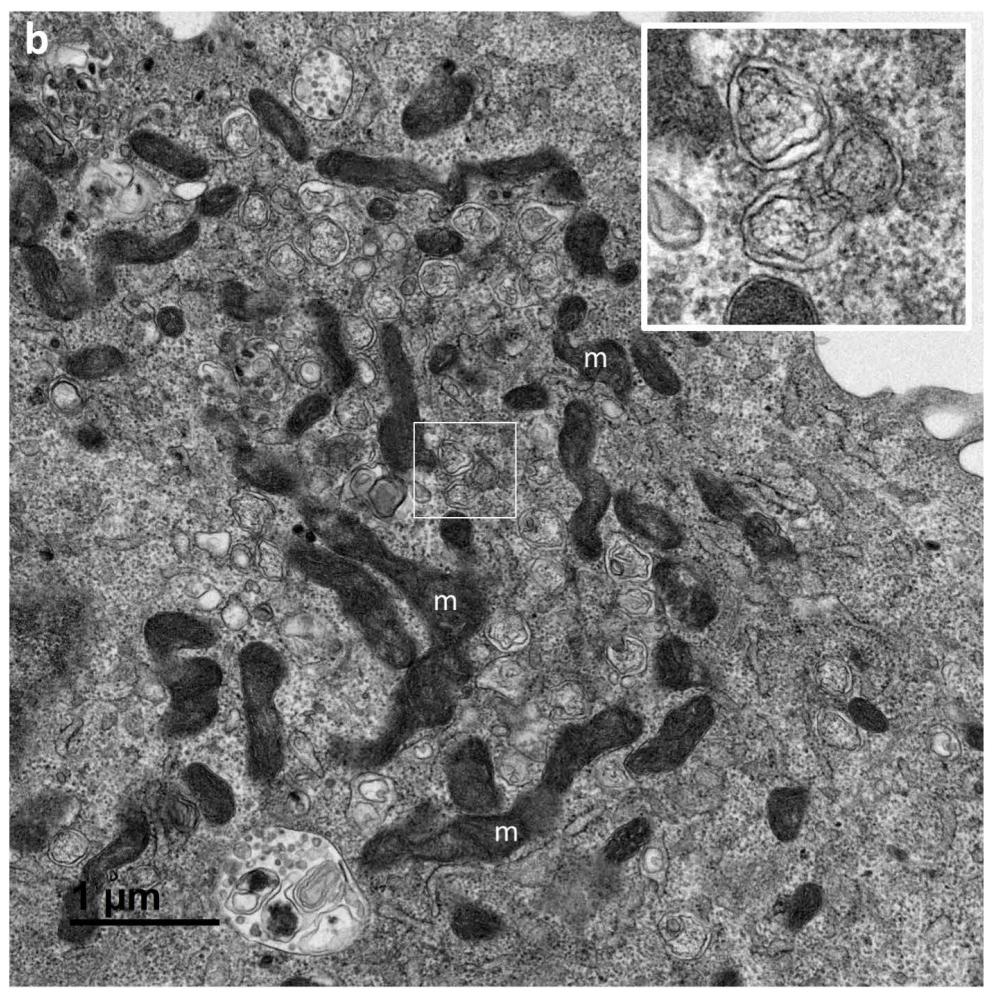
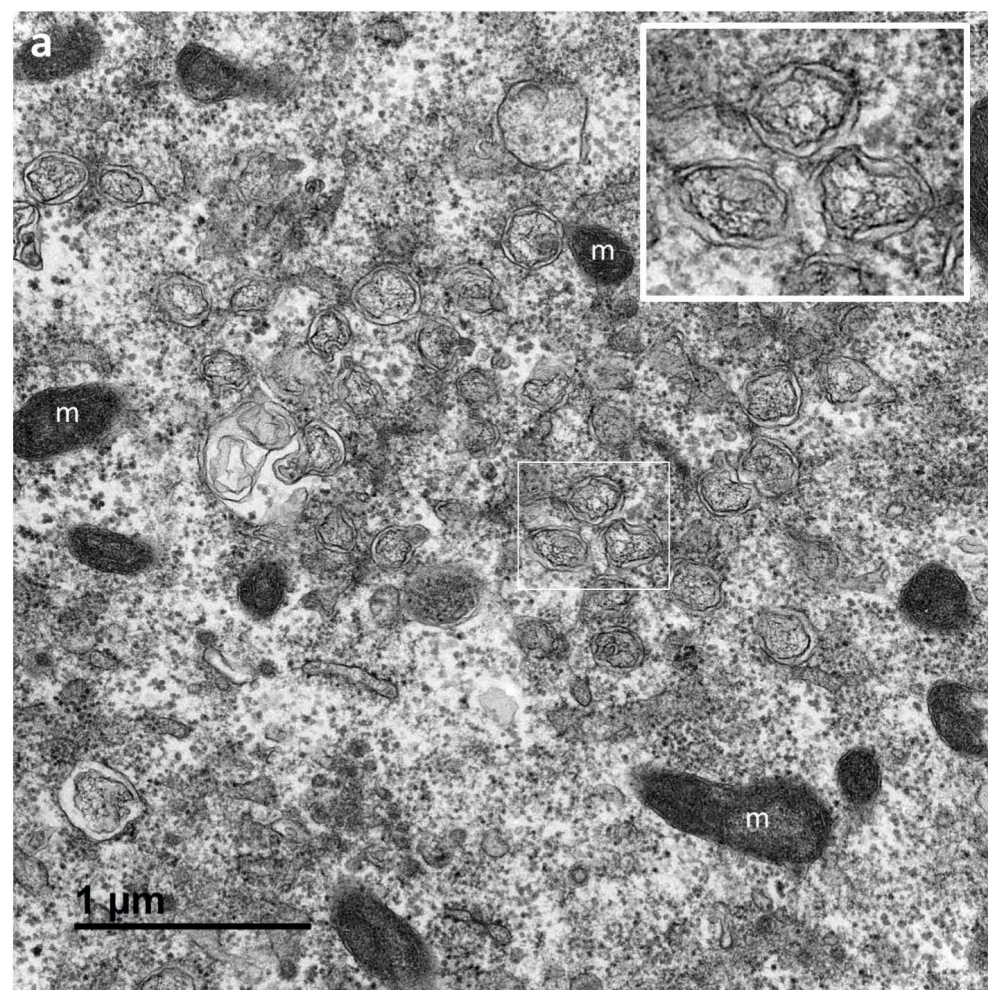
543 **(a, b)** At this time point, SARS-CoV-2-infected cells displayed an intense reticulovesicular
544 network consisting mostly of large numbers of DMVs, which occupied almost all the cytoplasm
545 and seemed to push against the nuclear compartment (n), with many mitochondria (m) recruited
546 at the edge of this network. Virion assembly continued next to this network (white arrows in
547 **a**), leading to release at the plasma membrane (thin black arrows in **a**). DMVs were often
548 associated with myelin-like membrane whorls or autophagic-like packaged membranes (white
549 asterisk in **b**).

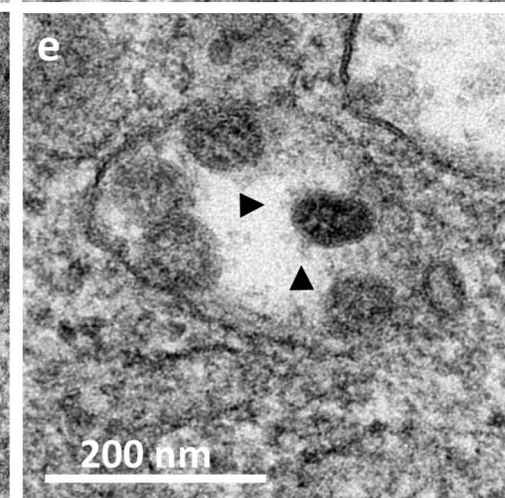
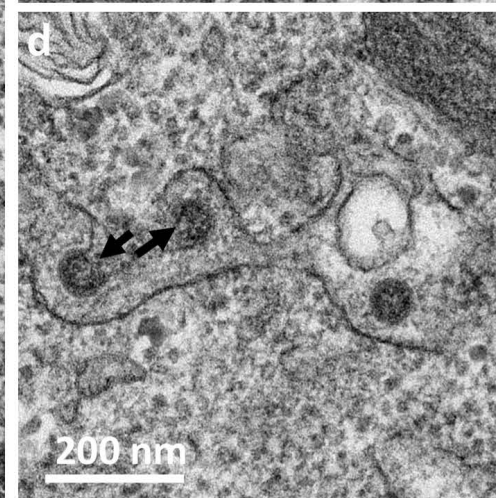
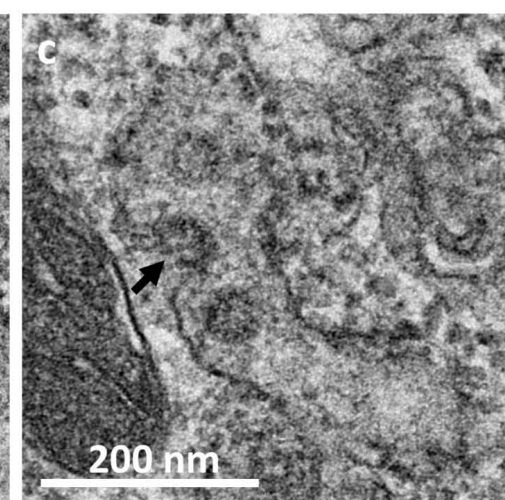
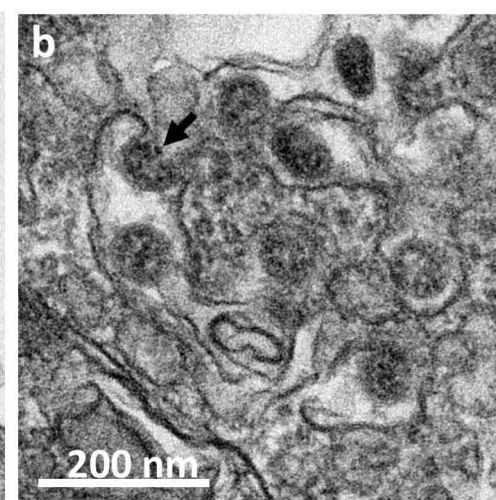
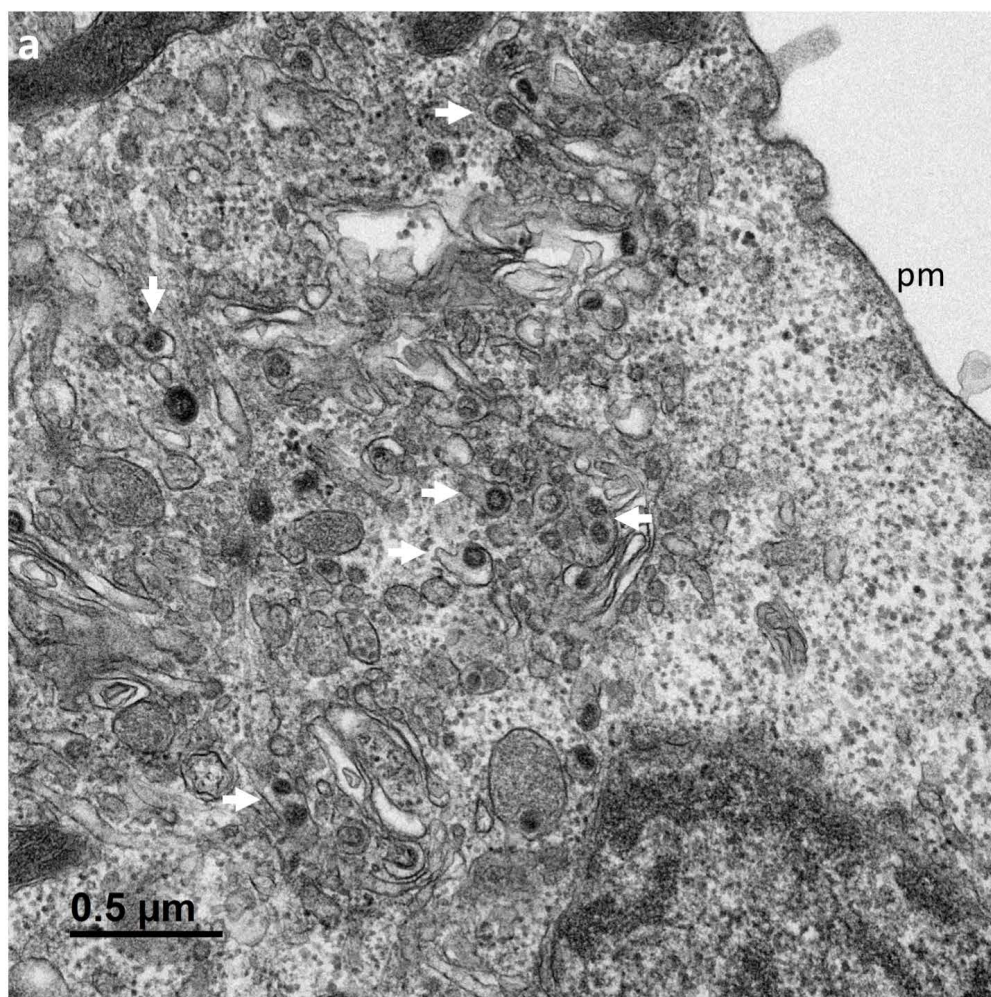
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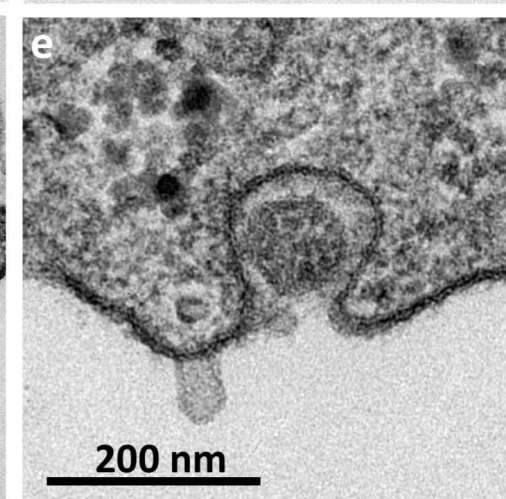
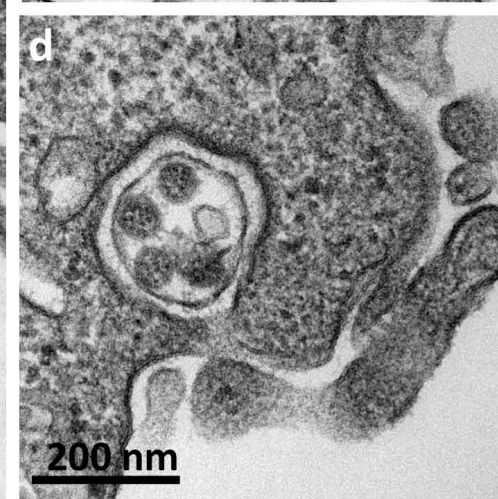
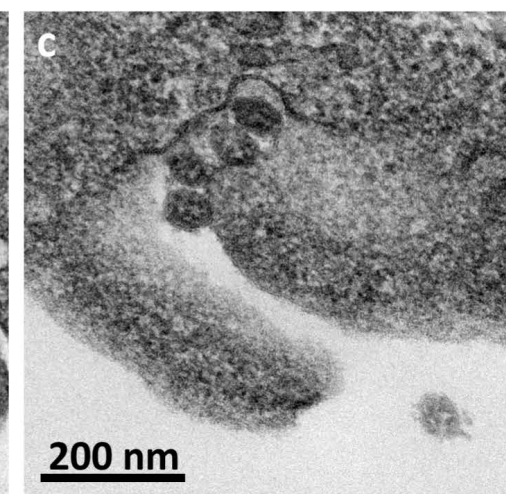
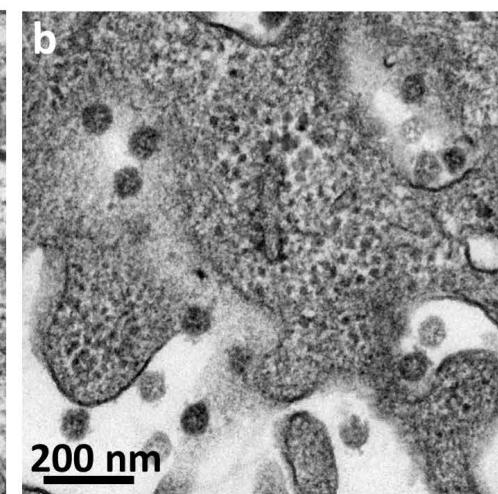
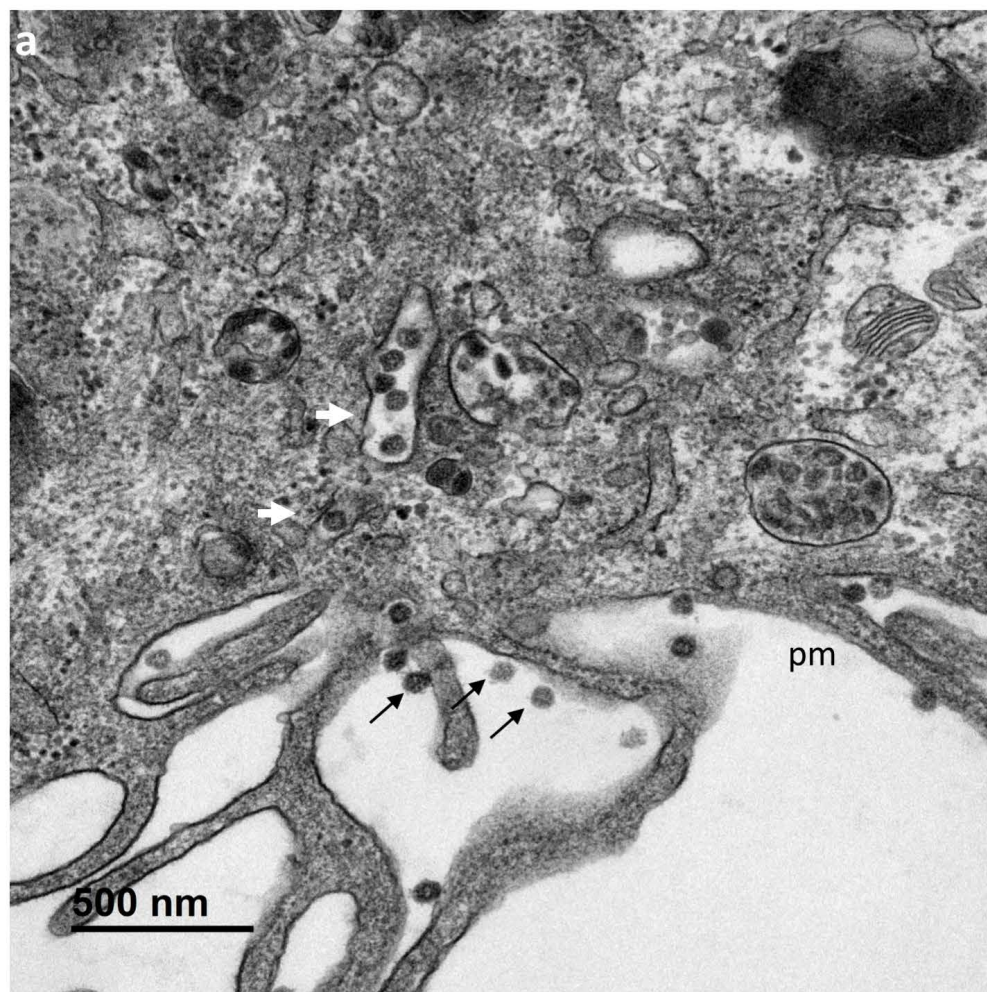
551 **Fig. 10** Quantitative analysis of the ultrastructural features in infected Vero cells, compared to
552 mock-infected cells. For each point of the kinetics, 100 cell sections have been analyzed.
553 Identification of at least one element in a cell section counted as one in the total account. For
554 the annulate lamellae graph, due to the rarity of these structures and therefore the low associated
555 cell-percentage, Y axis displays only a 10% value. * at 4 hpi, 170 cell sections have been
556 analyzed to find one cell containing annulate lamellae, leading to a < 1% result.

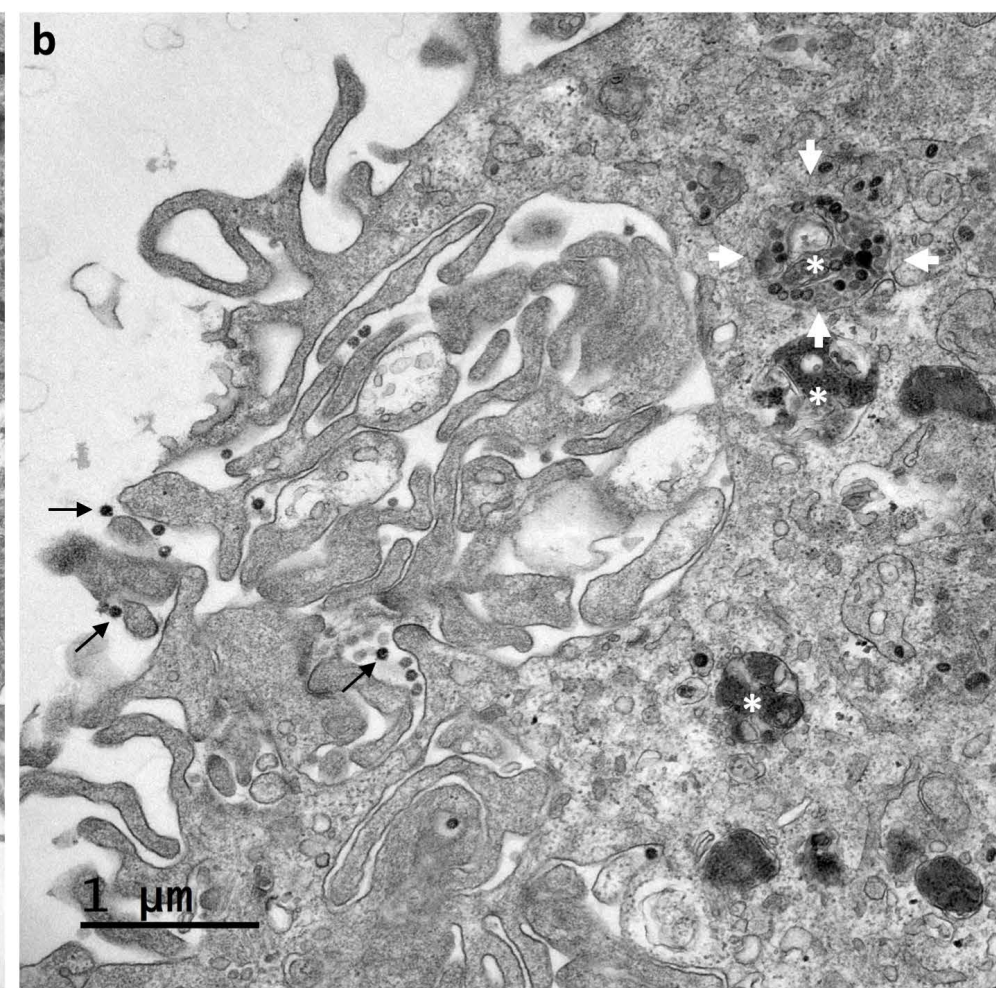
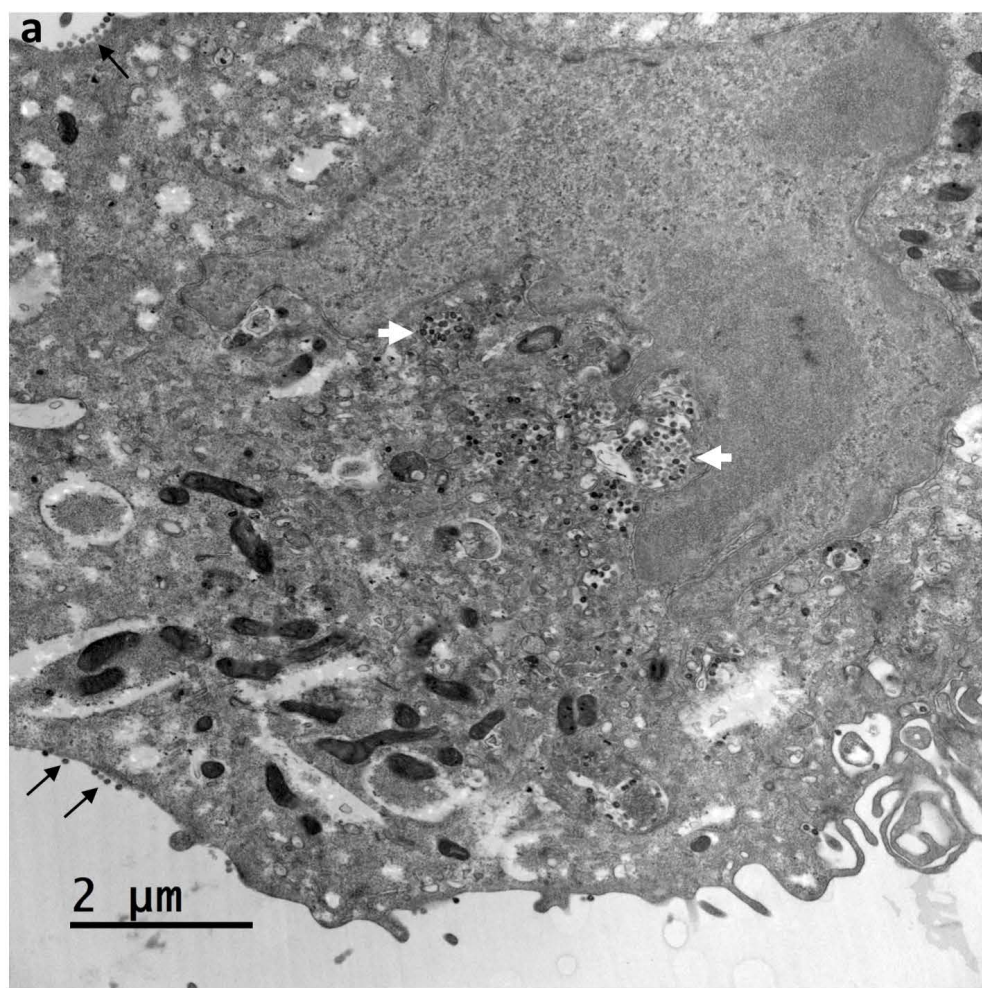


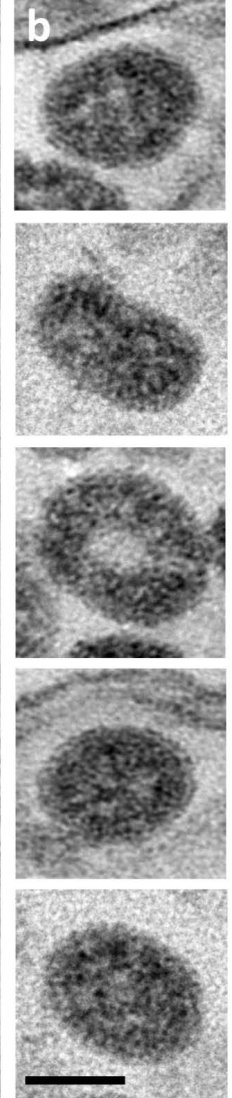
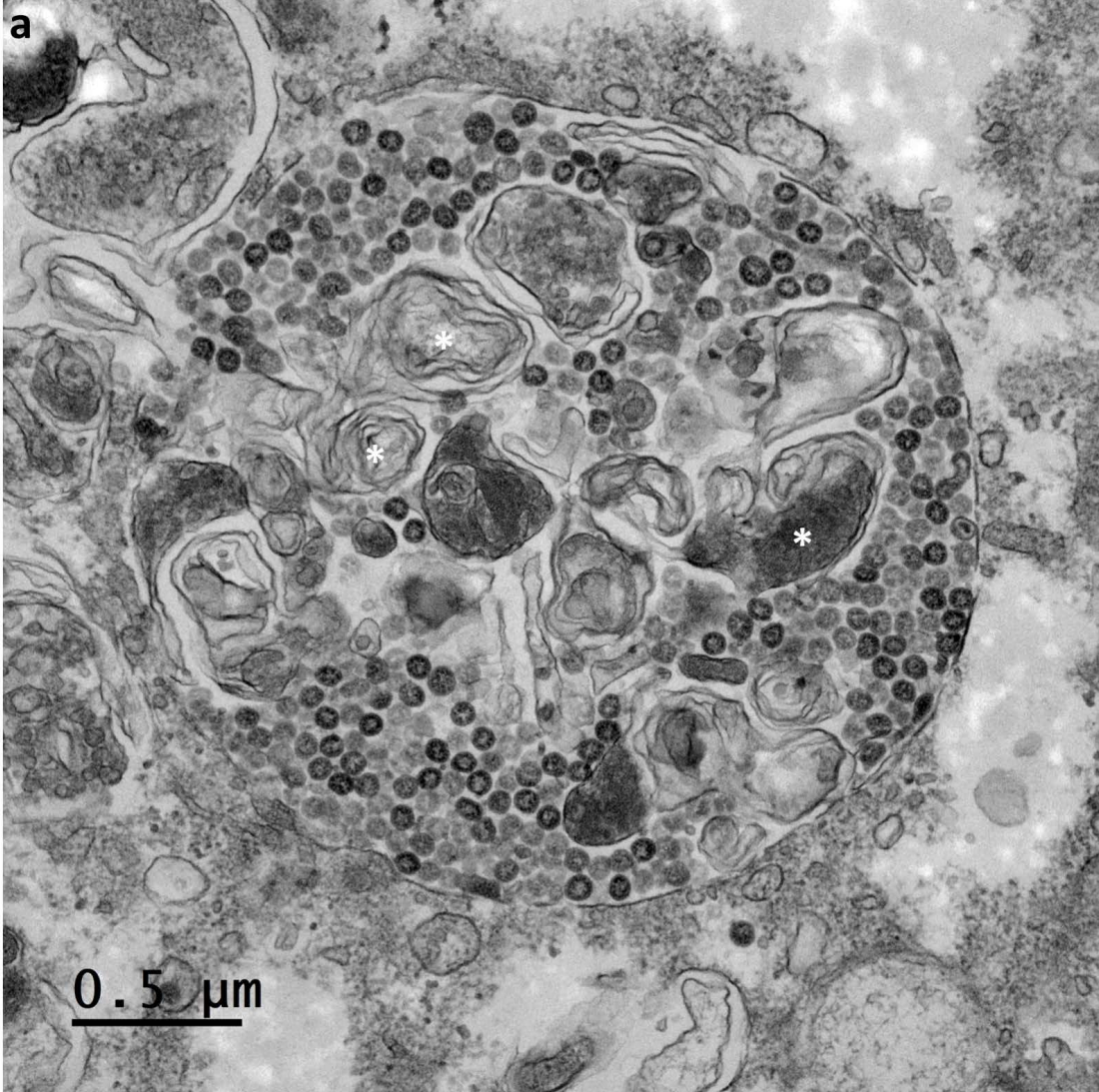


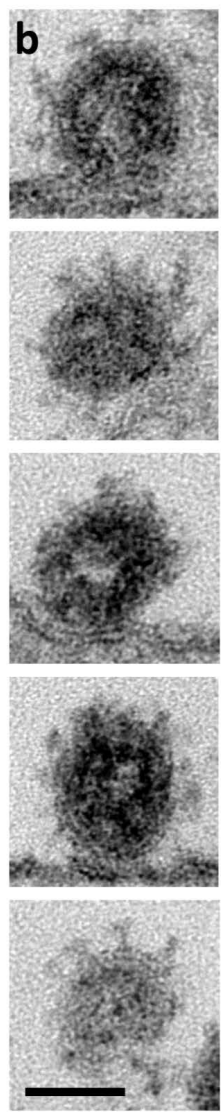
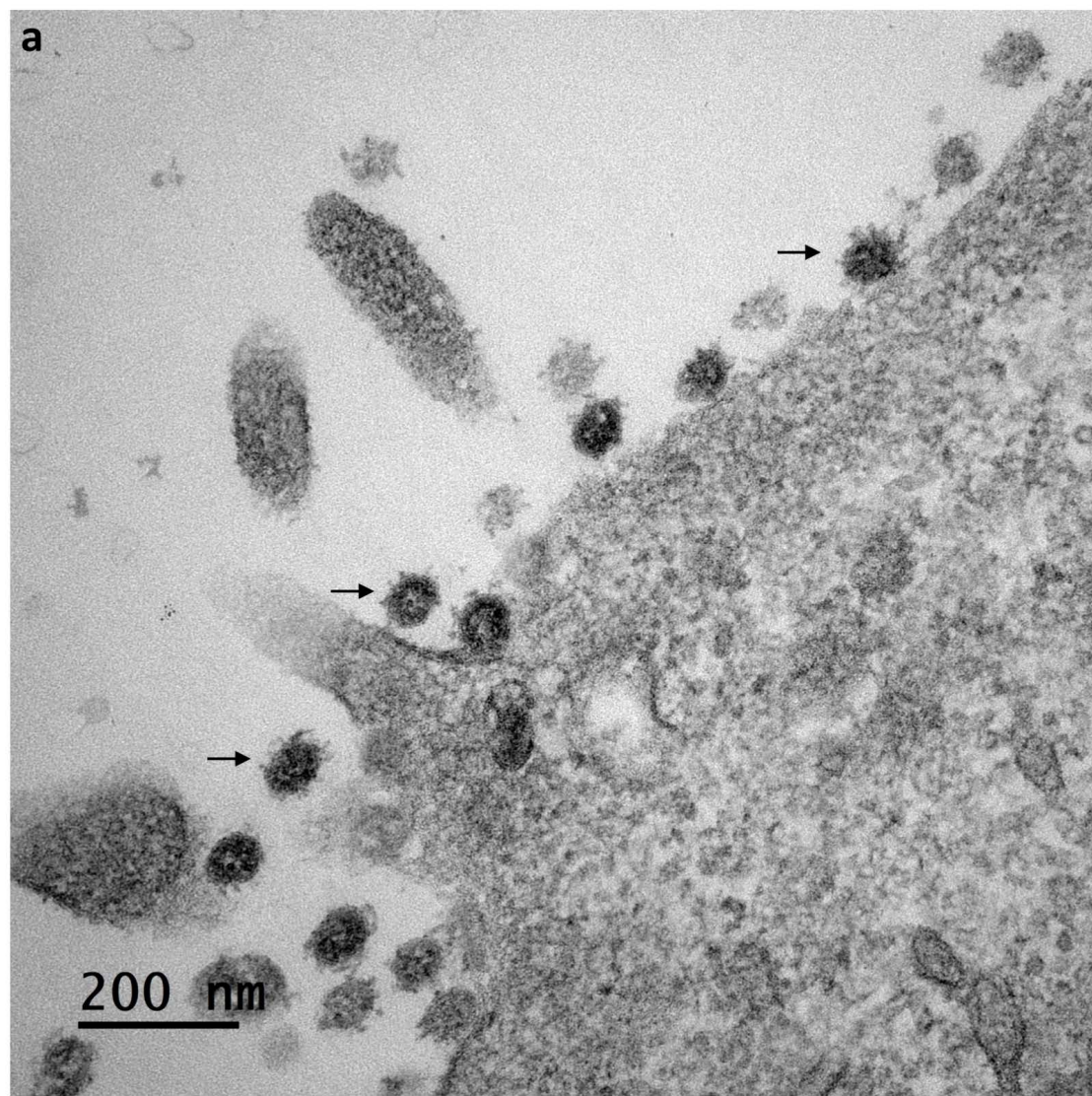


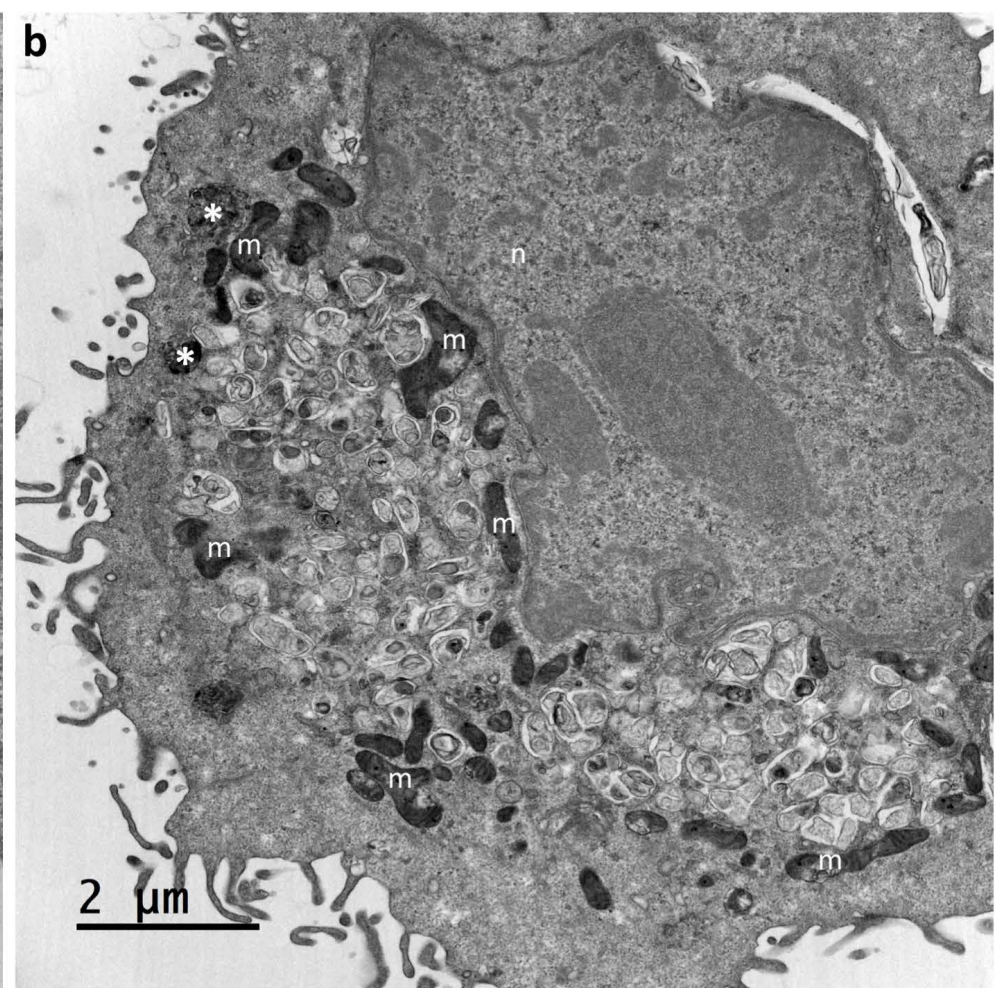
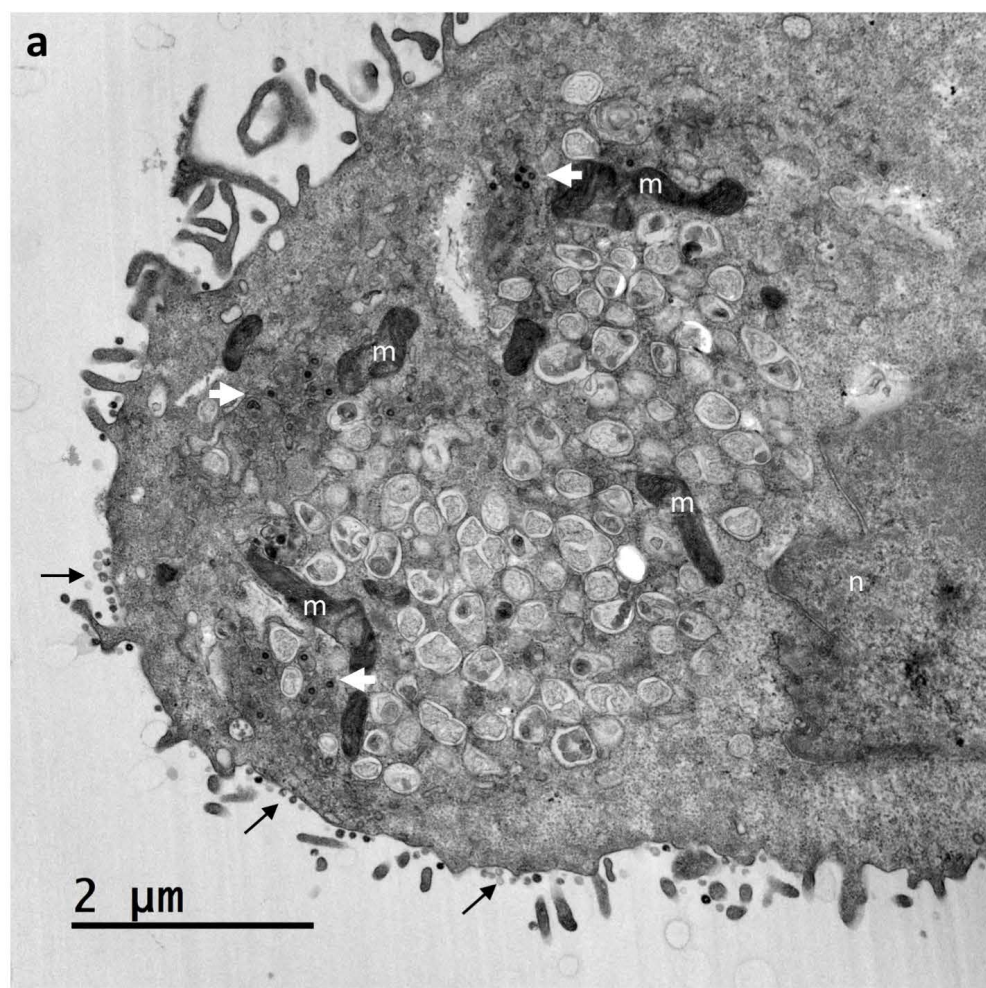




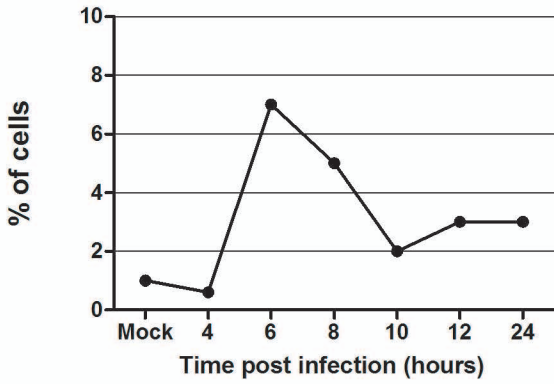




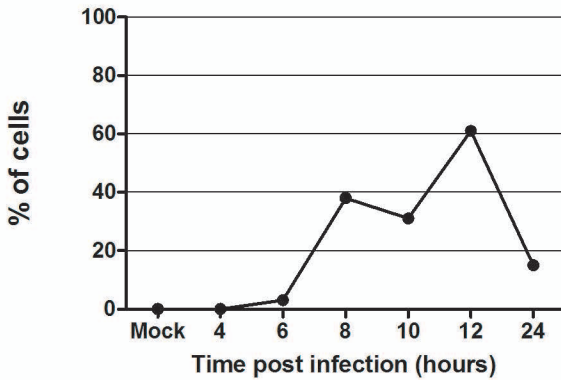




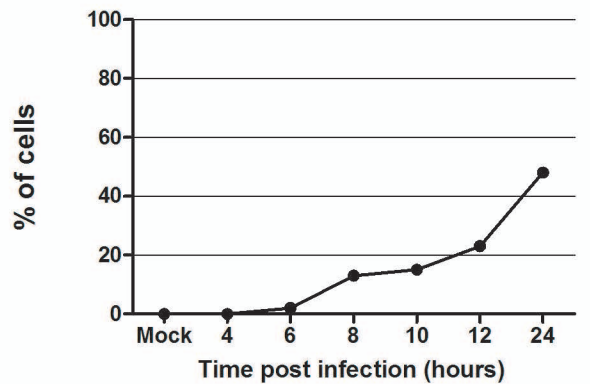
Annulate lamellae



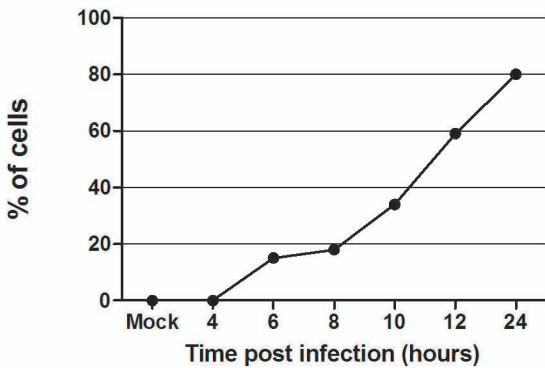
Single membrane vesicles



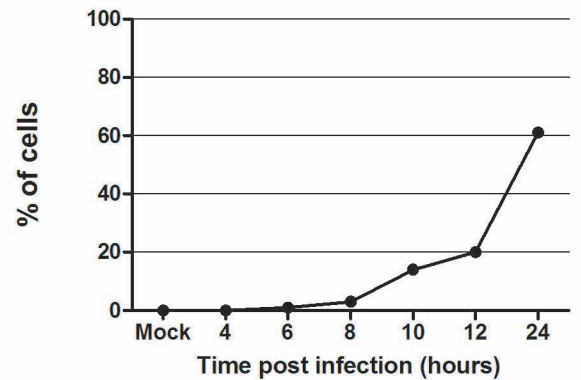
Golgian/ERGIC vesicles with spiky virions



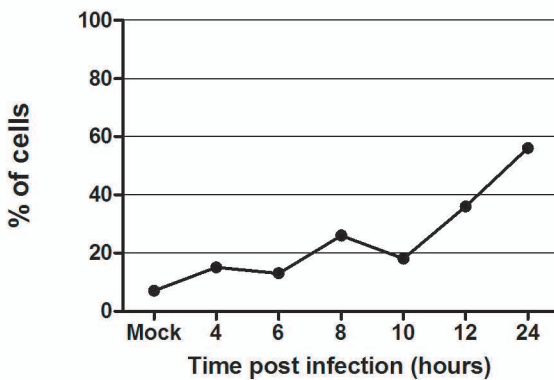
Double membrane vesicles



Large vacuoles with spike-less virions



Membrane whorls



Virions at cell surface (with spikes)

