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1 Arpin is critical for phagocytosis in macrophages and is targeted by human rhinovirus 16

2

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27

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29

30

31 **Abstract**

32

33 Human rhinovirus is a causative agent of severe exacerbations of chronic obstructive
34 pulmonary disease (COPD). COPD is characterized by an increased number of alveolar
35 macrophages with diminished phagocytic functions, but how rhinovirus affects macrophage
36 functions is still unknown. Here we describe that human rhinovirus 16 **impairs** bacterial
37 uptake and receptor-mediated phagocytosis in macrophages. The stalled phagocytic cups
38 **contain** accumulated F-actin. Interestingly, we **find** that human rhinovirus 16 **down-regulates**
39 the expression of Arpin, a negative regulator of the Arp2/3 complex. Importantly, re-
40 expression of the protein **rescues** the defective internalisation in human rhinovirus 16 treated
41 cells, demonstrating that Arpin is a key factor targeted to impair phagocytosis. We further
42 show that Arpin **is** required for efficient uptake of multiple targets, for F-actin cup formation
43 and for successful phagosome completion in macrophages. Interestingly, Arpin **is** recruited at
44 sites of membrane extension and phagosome closure. Thus, we identify Arpin as a central
45 actin regulator during phagocytosis. We reveal that it is targeted by human rhinovirus 16,
46 allowing the virus to perturb bacterial internalisation and phagocytosis in macrophages.

47

48

49 **Introduction**

50 Human rhinovirus (HRV) belongs to the *Picornaviradae* family. It is a small, non-enveloped
51 virus with a single stranded, positive sense RNA genome encased within an icosahedral
52 protein capsid with 60 copies each of four key viral proteins, VP1-VP4 [1]. Dependent on the
53 clades the viruses use either the low-density lipoprotein receptor (LDLR) family, the
54 intracellular adhesion molecular 1 (ICAM1) or cadherin related family member 3 to bind and
55 enter cells [2-5]. HRV is known to productively infect epithelial cells [6-12], but the response
56 in macrophages has received limited attention [7, 13]. Reports suggest that HRV can infect
57 monocytes/macrophages [7, 14, 15]. A recent study demonstrated that epithelial cells directly
58 enhance the replication of rhinovirus in monocytes [15]. Traditionally HRV is seen as an
59 upper respiratory tract pathogen [16]. However, mounting evidence shows that HRV can
60 infect the lower respiratory tract in patients with chronic inflammatory diseases including
61 chronic obstructive pulmonary disease (COPD) driving disease exacerbations [17-20].
62 Interestingly, in subjects experimentally infected with RV16, RV was ingested by recruited
63 CD68-positive and CD11b-positive macrophages in asthmatic humans, providing direct
64 evidence that tissue macrophages in the lower airways contribute to anti-RV responses *in vivo*
65 [21]. How HRV disrupts macrophage/monocyte functions remains unknown, but HRV was
66 reported to induce a defective secondary response in macrophages [13, 22-24].

67

68 An important arm of the innate immune responses is the phagocytic uptake by myeloid cells.
69 Phagocytosis is a mechanism of internalization of large particulate material, cell debris and
70 microorganisms [25-27]. It is strictly dependent on actin polymerization that represents the
71 major force driving plasma membrane deformation and engulfment. Actin polymerization is
72 induced by surface phagocytic receptors after ligation of the target and intracellular [signal](#)
73 transduction. Phagocytic receptors include receptors for host serum factors (opsonins) such as
74 immunoglobulin (Ig) and the complement fragment C3bi that engage Fc receptor (FcRs) and
75 complement receptors (CR3, α M β 2), respectively, and non-opsonic receptors such as the
76 Toll-like receptors (TLRs), the lectins and scavenger receptors [27-29]. Key players of the
77 signaling to actin polymerization are the small GTPases of the Rho family [30, 31]. In the
78 well-characterized FcR-mediated phagocytosis, Cdc42 activation in the nascent phagocytic
79 cup activates effectors like N-WASP, an actin nucleation-promoting factor (NPF) that acts on
80 the actin related protein 2/3 (Arp2/3) actin nucleation complex. Rac1 is then essential for F-
81 actin polymerization to complete extension and closure, through activation of another NPF,
82 the WAVE complex [26, 32, 33].

83

84 During phagosome formation, actin polymerization is transient and forms a specific F-actin
85 ring-like structure, called the phagocytic cup. The actin ring diameter progressively shrinks
86 until the membrane extensions eventually fuse, a step promoted by dynamin [26, 34]. Actin
87 filaments experience a high turnover, with intense polymerization in the tips of the membrane
88 folds and depolymerization at the base of the phagocytic cup [33, 35-38]. Several protein
89 activities have been reported to play a role in actin remodeling, including cofilin and
90 enzymatic activities leading to PIP(4,5)₂ hydrolysis or consumption [36, 38-42].

91

92 In addition, proteins inhibiting directly Arp2/3 have been described, namely Gadkin, PICK1
93 and Arpin [43-45], for which no role in phagocytosis has been reported yet. These inhibitors
94 are not found freely in the cytosol but localise to specific membranes like NPFs making them
95 ideal candidates to counteract NPF activity [46]. Arpin was found to bind to the Arp2/3
96 complex without activating it [45]. Instead Arpin exposes its COOH terminal acidic tail to
97 inhibit the Arp2/3 complex [47, 48]. In cells studied so far, Arpin localizes at lamellipodial
98 edges along with the WAVE complex [45]. The ability of Arpin to interact with Arp2/3 was
99 found to depend on Rac1 signalling [45]. In response to Rac1 signalling, Arpin inhibited
100 Arp2/3 at lamellipodial tips where Rac1 also stimulates actin polymerisation through WAVE
101 [45]. This placed Arpin downstream of Rac1 in a cycle with Rac inducing and inhibiting actin
102 polymerisation [45]. The major function of Arpin described to date is the inhibition of cell
103 migration [49] and a control of cell steering [45].

104

105 In this study, we demonstrate that HRV16 impairs macrophage phagocytosis of multiple
106 targets. We report that HRV16 induced a down-regulation of Arpin in macrophages. By re-
107 expressing Arpin in a model cellular system where HRV16 exposure led to decreased
108 internalisation, we could rescue this defect. Further analysis revealed that Arpin is required
109 for efficient internalisation. Thus, Arpin plays a critical role in coordinating and orchestrating
110 actin remodelling around internalised particles, necessary for efficient phagocytosis.
111 Therefore, we add phagocytosis to the growing list of functions being attributed to Arpin and
112 highlight a host cell factor specifically targeted by rhinovirus.

113

114 **Results**

115

116 **Human rhinovirus 16 impairs bacterial internalisation in human macrophages**

117 We used HRV16 for our studies because it is commonly isolated in COPD [19, 20]. We first
118 set out to determine if macrophages could internalise bacteria after HRV16 challenge. We
119 challenged human monocyte-derived macrophages (hMDMs) with HRV16, HRV16
120 inactivated by a UV treatment (HRV16^{UV}) or mock infected medium (MI) for 1 h at room
121 temperature followed by overnight rest. The next day, we exposed them to non-typeable
122 *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis*, *Staphylococcus aureus* or
123 *Pseudomonas aeruginosa* that are frequently associated with exacerbations of COPD [20].
124 We measured internalisation at 30 and 120 min after washing and incubation with antibiotics
125 to kill extracellular bacteria. We found that hMDMs challenged with HRV16 were
126 significantly impaired in their ability to internalise all four bacteria over 120 min compared to
127 hMDMs challenged with HRV16^{UV} or mock infected (Figure 1A-D). To determine if this
128 result was due to a high HRV16 tissue culture infective dose 50 (TCID₅₀), we repeated our
129 experiments over a range of TCID₅₀'s and measured internalisation of NTHi and *S. aureus*
130 over 120 min. We found that HRV16 impaired the internalisation of NTHi (Figure EV1A-E)
131 and *S. aureus* in hMDMs (Figure EV2A-E) from TCID₅₀'s as low as 1 x 10³ reaching a
132 maximal impairment at TCID₅₀ 1 x 10⁷. We also calculated the percentage inhibition of
133 internalisation of NTHi or *S. aureus* at 120 min for all TCID₅₀'s relative to mock infection
134 and found that on average the impairment in internalisation caused by HRV16 was greater for
135 *S. aureus* than NTHi at TCID₅₀ 1 x 10⁵ or 1 x 10⁶ but identical and maximal by TCID₅₀ 1 x
136 10⁷ reaching 80% (Figure EV1F and EV2F).

137
138 Then, because reduced internalization of bacteria could be due to a reduced initial binding to
139 the cell surface, we repeated our experiments over 30 min by incubating hMDMs with the
140 different bacteria on ice for 5, 15 or 30 min (Figure 1E-H). No differences in bacterial
141 attachment to hMDMs were observed in cells challenged with HRV16 or control conditions.
142 These results indicated that HRV16 impaired the response of hMDMs to bacteria at the level
143 of internalisation.

144
145 Finally, because HRV16 is a predominant cause of exacerbation in COPD (Caramori *et al.*,
146 2003, Wilkinson *et al.*, 2017), we decided to explore the effect of HRV16 challenge on
147 bacterial internalisation by human alveolar macrophages (AMs) (table 1). We found that non-
148 smoker, non-COPD AMs challenged with HRV16 were significantly impaired in their ability
149 to internalise NTHi over 120 min compared to AMs challenged with HRV16^{UV} or mock
150 infection (MI) (Figure 1I). When we repeated our experiments using healthy smoker AMs we

151 first found that they internalised less bacteria at baseline relative to non-smoker, non-COPD
152 AMs (Figure 1J). Interestingly, even if smoking itself impaired bacterial internalisation,
153 HRV16 challenge led to an additive impairment in NTHi internalisation by healthy smoker
154 AMs (Figure 1J).

155

156 Taken together, these results demonstrate that HRV16 challenge in *in vitro* derived
157 macrophages impaired their ability to internalise bacteria. Importantly, we observed the same
158 defect in human lung macrophages.

159

160 **Human rhinovirus 16 impairs zymosan, CR3- and FcR-mediated internalisation in** 161 **human macrophages**

162 We next wanted to determine if the impairment in internalisation caused by HRV16 was
163 specific to bacteria. Therefore, we exposed hMDMs post HRV16 challenge to zymosan for 60
164 min. We found that hMDMs challenged with HRV16 were significantly impaired in their
165 ability to internalise zymosan compared to MI or HRV16^{UV} treated hMDMs (Figure 2A),
166 internalising on average 70% less zymosan (Figure 2A). To allow us to better characterize the
167 defect in internalisation caused by HRV16 we decided to next analyse the internalisation of
168 opsonized particles that trigger only one type of phagocytic receptor. Therefore, post HRV16
169 challenge we exposed hMDMs to sheep red blood cells (SRBC) opsonized with either IgM-
170 iC3b or IgG for 60 min. hMDMs challenged with HRV16 were significantly impaired in their
171 ability to internalise either IgM-iC3b or IgG opsonized SRBC compared to control or
172 HRV16^{UV} treated hMDMs (Figure 2B-E), internalising on average 50% less of either particle
173 (Figure 2B-C and EV4A showing the internalisation index). Representative images of mock
174 infected or HRV16 treated hMDMs are shown in figure 2D-E, highlighting the internalized
175 SRBC in green. The images clearly demonstrate the impaired internalisation caused by
176 HRV16 towards IgM-iC3b opsonized SRBC (Figure 2D) or IgG opsonized SRBC (Figure
177 2E).

178

179 These results along with the results in Figure 1 demonstrate that HRV16 challenge of hMDMs
180 impairs various phagocytic pathways, suggesting that HRV16 targets a global regulator of
181 phagocytosis in macrophages.

182

183 **Human rhinovirus 16 impairs phagocytic cup formation in human macrophages**

184 Having shown that HRV16 impaired internalisation by hMDMs, we next set out to analyse if
185 HRV16 altered membrane remodelling at the onset of phagocytosis. For this, we challenged
186 hMDMs with HRV16 or MI control and then exposed them to IgG-opsonised SRBC for 2-30
187 min. The samples were stained with phalloidin to analyse F-actin recruitment around the
188 internalised SRBC (Figure 3A, Z projections of stack images). We counted the number of F-
189 actin positive cups (Figure 3B) and calculated the enrichment of F-actin around the SRBC at
190 each phagocytic cup relative to the cell cortex (Braun 2007) (Figure 3C). Over the first 5 min
191 of internalisation, we did not observe any significant difference in the number of F-actin cups
192 that formed in mock infected or HRV16 treated hMDMs (Figure 3B). Despite this, there was
193 less internalisation in HRV16 challenged hMDMs over the first 5 min compared to control
194 hMDMs (Figure 3D). Importantly, there was no equivalent difference in SRBC association to
195 HRV16 challenged hMDMs over these first 5 min (Figure 3E). Despite the similarity in the
196 number of F-actin cups that formed at 5 min in both conditions, we could observe differences
197 in the F-actin make up around the internalised SRBC. The cups looked thinner and less
198 defined in HRV16 challenged hMDMs compared to control hMDMs, and the intensity of F-
199 actin staining around the cups was lower in the early time point (2 min, Figure 3C). At 15 and
200 30 min post-internalisation, we observed a sustained number of F-actin cups in HRV16
201 challenged hMDMs compared to mock infected hMDMs where only few F-actin cups were
202 still observed (Figure 3B) and this reached statistical significance at 15 min (Figure 3B). In
203 line with these results, we observed significantly less internalisation over these later time
204 points in HRV16 challenged hMDMs (Figure 3D), with no differences in association (Figure
205 3E). Importantly, internalisation was still inhibited at 60 min in HRV16 challenged hMDMs,
206 demonstrating that HRV16 does not just delay, but inhibits, internalisation (Figure EV4A).
207 When we next looked at F-actin cups at 15 min, we could see they were stalled and still
208 contained F-actin, similar to MI at 5 min, but the intensity of staining was lower (Figure 3A
209 and C). Very interestingly, as opposed to the control cells where the cups had disassembled
210 and only adhesion structures were detected in the images projected in Z, we observed a
211 sustained enrichment of F-actin around internalised SRBC that was similar from 5-30 minutes
212 and showed no clear decrease (Figure 3A,E).

213 Finally, to better visualise the membrane architecture in HRV16 challenged hMDMs, we
214 performed scanning electron microscopy on mock infected or HRV16 challenged hMDMs
215 after 5 min of internalisation of IgG-opsonised SRBC (Figure 3F). In mock infected hMDMs,
216 we often observed a lot of membrane folds in several layers progressing over the SRBC
217 (Figure 3F). In HRV16 challenged hMDMs, there were fewer membrane folds, the membrane

218 covering the SRBC was thinner and only partially covered of the SRBC (Figure 3F).
219 Combined with our fluorescence images, these results demonstrate that HRV16 impairs
220 internalisation in hMDMs by perturbing early phagosome formation and actin remodelling.

221

222 **Human rhinovirus does not affect the expression of F-actin in human macrophages but** 223 **down-regulates the expression of Arpin**

224 We next set out to determine more precisely whether HRV16 disrupted the actin cytoskeleton
225 in hMDMs. We challenged hMDMs with HRV16 or control medium for 1 h followed by
226 overnight rest and then stained the cells with phalloidin. The F-actin network did not appear
227 to be disrupted by HRV16, but the global F-actin intensity was lower in HRV16 challenged
228 hMDMs compared to mock infected hMDMs (Figure 4A). When we quantified our images
229 we first determined that the total F-actin intensity in each field imaged was significantly lower
230 in HRV16 challenged hMDMs compared to control hMDMs (Figure 4B). In addition, we
231 observed that the intensity of the punctate F-actin was significantly lower in HRV16
232 challenged *vs* mock treated hMDMs (Figure 4C). Because the primary cells are
233 heterogeneous, we also analysed the data in figure 4C on a per cell basis and determined that
234 the intensity of staining of punctate F-actin was significantly lower in individual cells in
235 HRV16 challenged hMDMs compared to control conditions (Figure 4D). Collectively, these
236 results suggested that HRV16 disrupts the F-actin network in hMDMs.

237 We therefore postulated that there might be globally less actin protein in our HRV16
238 challenged hMDMs. However, immunoblot analysis revealed no significant change in the
239 actin expression between HRV16 challenged (lane 3), HRV16^{UV} challenged (lane 2) or mock
240 infected cells (lane 1) (Figure 4E-F). Although the quantification across 10 donors revealed
241 variations in the global amount there was no significant trend in either direction towards more
242 or less actin in either condition (Figure 4F). This result suggested that HRV16 might be
243 targeting a regulator of the F-actin network in hMDMs rather than the G-actin pool. To test
244 this hypothesis, we challenged hMDMs to HRV16 or HRV16^{UV} or mock conditions and
245 screened for a range of actin-associated proteins. Of the actin regulators that we tested, we
246 found that the Arp2/3 inhibitor Arpin was consistently and significantly down-regulated on
247 average by 30% in HRV16 challenged hMDMs compared to control conditions (Figure 4E-
248 G). Of note, there was no effect on the global expression of Rac1, which is upstream of Arpin
249 and activates Arp2/3 via WAVE (Eden 2002) (Figure 4G). We found no significant decrease
250 in the expression of Cdc42 that activates Arp2/3 via WASP (Figure 4G). We then looked at
251 subunits of Arp2/3 including ArpC2 (p34-Arc) and ArpC5 (p16-Arc). We observed no

252 difference in the global expression of p34-Arc in HRV16 challenged hMDMs compared to
253 control conditions and a non-significant increase in the global expression of p16-Arc that was
254 not specific to the live virus (Figure 4G). We also analysed the expression of cofilin and
255 observed no difference in the total or phosphorylated forms (Figure 4H-I, ratio).

256

257 Finally, to further confirm that the disruption of Arpin expression by HRV16 was mediating
258 deficient internalisation caused by HRV16, we set out to re-express the protein and analyse if
259 internalisation is restored. To overcome the issues of transfecting primary human
260 macrophages, we made use of retinal pigment epithelial-1 (RPE-1) cells that were transduced
261 with lentivirus to express the phagocytic FcγRIIA/CD32. We first verified by flow cytometry
262 that the transduced cells expressed the receptor and found that 100% of cells were positive for
263 CD32 (Figure EV5A). The RPE-1-FcγRIIA cells were able to specifically phagocytose IgG-
264 opsonized SRBC as compared with non-opsonized particles, while the parental cells were not
265 competent for this phagocytosis (Figure EV5B-D). Next, we exposed these cells to HRV16 or
266 to mock infected medium for 1 h followed by overnight rest and then challenged them with
267 IgG-opsonised SRBC for 1 h. We found that RPE-1-FcγRIIA cells exposed to HRV16
268 internalised on average 40% less SRBC compared to a mock treatment (Figure EV5D).
269 Having confirmed that RPE-1-FcγRIIA cells responded like human macrophages to HRV16,
270 we exposed these cells to HRV16 or mock medium and, after the overnight rest, transfected
271 them with plasmids encoding either EGFP or EGFP-Arpin for 24 h (Figure 4J,K). Then we
272 performed a phagocytosis experiment using IgG-opsonised SRBC for 1 h. We demonstrated
273 that RPE-1-FcγRIIA cells exposed to HRV16 and then transfected with EGFP internalised
274 significantly fewer SRBC compared to mock infection controls, which averaged an
275 approximate 40% decrease (Figure 4K). Importantly, when RPE-1-FcγRIIA cells were
276 exposed to HRV16 and then transfected to express EGFP-Arpin, the efficiency of
277 internalisation of SRBC was restored (Figure 4K).

278

279 Taken together, our results show that live HRV16 impairs the F-actin network in hMDMs and
280 reveal that the Arp2/3 inhibitor Arpin is downregulated in live HRV16-treated macrophages.
281 Furthermore, these results demonstrate that HRV16 specifically targeted Arpin to decrease
282 internalisation and that transient expression of the protein restored the phagocytic capacities
283 of HRV16-treated cells. Therefore, Arpin is a crucial factor targeted by this rhinovirus to
284 impair phagocytosis.

285

286 **Arpin knockdown impairs bacterial internalisation**

287 Because the Arp2/3 inhibitor had never been implicated in phagocytosis, we next addressed
288 whether a decrease in Arpin expression could recapitulate the perturbations in phagosome
289 formation that we observed in macrophages challenged with HRV. We treated hMDMs with
290 two siRNA against Arpin [45] or luciferase as a control, or left them non-treated for 96 h.
291 Immunoblot analysis and quantification demonstrated significant Arpin reduction for both
292 siRNA sequences in 9 donors (Figure 5A and 5B). We then challenged the Arpin-depleted
293 macrophages with either NTHi, *M. catarrhalis*, *S. aureus* or *P. aeruginosa* for up to 120 min.
294 We found that hMDMs treated with Arpin siRNA were significantly impaired in their ability
295 to internalise all bacteria (Figure 5C-F) relative to non-treated hMDMs. Importantly, hMDMs
296 treated with siLuciferase demonstrated similar bacterial internalisation to non-treated hMDMs
297 (Figure 5C-F). Finally, we also assessed bacterial binding to hMDMs over 5-30 min of
298 infection post siRNA treatment and found no difference between the four conditions (Figure
299 EV3A-D). These results demonstrate that Arpin plays a crucial role in bacterial internalisation
300 in hMDMs.

301

302 **Arpin knockdown impairs membrane extension around internalised particles**

303 Having shown that Arpin knockdown impaired internalisation of bacteria by human
304 macrophages, we next set out to analyse if it impaired F-actin dynamics at the phagocytic cup.
305 We treated hMDMs with Arpin siRNA or controls and after 96 h challenged them with IgG-
306 opsonised SRBC for 15-30 min (Figure 6A-D). Significantly fewer SRBC were internalized
307 in macrophages when Arpin was knocked down, as compared with control conditions (Figure
308 6B). In contrast, there was a non-significant trend towards less association of SRBC to
309 hMDMs treated with siRNA against Arpin (Figure 6C), indicating that depletion of Arpin
310 leads to a defective phagocytosis. Similar to what we observed with HRV16, internalisation
311 was not simply delayed in Arpin-depleted macrophages, as internalisation was still impaired
312 after 60 min (Figure EV4B). In addition, we observed significantly more F-actin cups in
313 Arpin-depleted macrophages as compared to control conditions at 15 min post internalisation
314 (Figure 6 A and D). We then examined the F-actin cups in Arpin-depleted *versus* non-treated
315 or siLuciferase treated hMDMs where the F-actin cups were complete, with homogeneous F-
316 actin organisation around the particles (Figure 6A). In contrast, Arpin knockdown not only
317 led to an increased number of cups visible in figure 6A, but also to cups that appeared
318 fragmented with puncta of staining (Figure 6A). By 30 min post-internalisation there were
319 very few cups in control conditions (Figure 6A, D). However, in Arpin knockdown hMDMs,

320 there were still significantly more F-actin cups compared to control conditions albeit the
321 global number had decreased (Figure 6D). We next calculated the enrichment of F-actin
322 around internalised SRBC relative to the cell cortex. As expected, the enrichment of F-actin
323 around internalised SRBC was similar for non-treated or siLuciferase treated hMDMs at 15
324 min and decreased by 30 min (Figure 6E). Strikingly, in Arpin-depleted hMDMs, we
325 observed significantly more F-actin enrichment around internalised SRBC at 15 and 30 min
326 relative to control conditions (Figure 6E), consistent with the increased number of F-actin
327 cups counted (Figure 6D). To better observe the membrane architecture when Arpin was
328 knocked down in human macrophages, we performed scanning electron microscopy on Arpin
329 siRNA or control siRNA-treated hMDMs after 15 min of internalisation of IgG-opsonised
330 SRBC (Figure 6F). In control hMDMs, we could see a very thin covering of host membrane
331 over the SRBC indicating near-complete internalisation (Figure 6F). In hMDMs treated with
332 siRNA targeting Arpin, we could observe accumulation of membrane folds around the SRBC
333 or thin membrane layers partially covering the particle (Figure 6F), which was reminiscent of
334 the phenotype obtained after HRV16 treatment. This indicated that Arpin knockdown was
335 impairing phagocytic cups progression and completion.

336

337 To further describe the role of Arpin in phagosome formation, we used the RAW264.7 murine
338 cells transfected to transiently express lifeact-mCherry to detect polymerized branched actin
339 and EGFP-Arpin. We performed the 3D phagosome formation and closure assay that we set
340 up using Total Internal Reflection Fluorescence Microscopy (TIRFM) [34, 36, 50, 51] (Figure
341 6G-H). In this assay, living cells phagocytose particles that are non-covalently attached to the
342 coverslips, allowing for observation of the base of the phagocytic cup in the epifluorescence
343 mode when the stage is moved 3 μm in z, the extending pseudopods, as well as the precise
344 site of phagosome scission in the TIRFM mode (Figure 6G). We observed that Arpin was
345 recruited at the site of phagosome formation with branched actin in the extending membrane
346 folds (Figure 6H). The epifluorescence images showed that Arpin was not accumulated at the
347 base of the phagosome in the region where actin is cleared. Interestingly, Arpin was enriched
348 at the site of phagosome closure detected in both epifluorescence and TIRFM modes. Arpin is
349 thus potentially playing a very local role in the actin polymerization/depolymerization cycle,
350 rather than a role in the actin clearance at the base of the phagocytic cup.

351

352 Together, these results show that Arpin is not required for the initial onset of actin
353 polymerization but regulates the local branched actin network for a successful phagocytic cup
354 extension and closure.

355

356

357 **Discussion**

358

359 In this study, we show that HRV16 impairs the internalisation of bacteria and other particles
360 by human macrophages. We reveal that Arpin is a host cell protein downregulated by the
361 virus that is necessary and sufficient for the internalisation defect driven by HRV16 as re-
362 expression rescued deficient internalisation in RPE-1 cells exposed to the virus. We further
363 demonstrate for the first time that this Arp2/3 inhibitor is critical for efficient receptor-
364 mediated phagocytosis and bacterial uptake in macrophages.

365

366 Our understanding of how macrophages, including differentiated tissue macrophages, deal
367 with HRV infections compared to epithelial cells is incomplete [52]. There are reports
368 indicating that HRV can infect monocytes/macrophages *in vitro* and *in vivo* [7, 14, 15, 20, 21,
369 23]. In our work, we studied how differentiated human macrophages and resident lung
370 derived macrophages are affected by HRV infection. We first found that post HRV16
371 exposure, macrophages did not internalise a range of gram-positive and gram-negative
372 bacteria that are frequently associated with exacerbations [20]. Interestingly, we observed an
373 innate impairment of bacterial internalisation in alveolar macrophages from healthy smokers
374 compared to non-smokers controls that was further exacerbated following exposure to
375 HRV16. The ability of smoking to worsen viral infections has been suggested, but the reports
376 are still conflicting (for a review, see [53]). Because reduced phagocytic capacities could be
377 due to reduced surface phagocytic receptor expression [54], we explored whether bacterial
378 binding to macrophages was affected. We found that this was not the case, further
379 emphasising that HRV16 specifically affected the internalisation step. These results are
380 important in the context of COPD where HRV is frequently isolated [20] and, together with
381 other defective functions like cell activation [13, 22-24], could be one of the explanations as
382 to why patients show bacterial outgrowths post HRV infection [13, 19].

383

384 Phagocytosis is strictly dependent on actin polymerization (for review, [26, 55], and upon
385 phagocytosis, actin polymerization at the site of particle binding is transient and intense and
386 corresponds to the formation of a phagocytic cup. We demonstrate here that, although HRV16
387 challenged macrophages were impaired in internalisation over 30 min, they could form F-
388 actin cups. However, there was an inherent delay to complete phagocytosis and to clear F-
389 actin from forming phagosomes. Importantly, we were able to demonstrate that, while in
390 mock infected macrophages actin disassembled after 30 min, there was a maintenance of F-

391 actin in phagocytic cups in HRV16 challenged macrophages. When we analysed the
392 membrane extension over particles by scanning electron microscopy, we first observed that in
393 contrast to monocytes [36], hMDMs seemed to have more folds of membrane around the
394 particles. While there were multiple ruffles and folds of membrane in uninfected macrophages
395 at 5 min, in HRV16 challenged macrophages, the membrane was a lot thinner and only
396 partially covered the particle. In Arpin knockdown macrophages, we observed two
397 phenotypes, either a very thin covering of membrane over the internalised particle, similar to
398 HRV16, or small partial folds of membrane below the particle. All these results suggested that
399 HRV16 challenged macrophages and Arpin-depleted macrophages show similar defects in
400 their ability to promote actin remodelling and to extend membranes for efficient phagosome
401 completion.

402

403 Arpin is a newly discovered inhibitor of the Arp2/3 complex that counteracts the activatory
404 signals provided by the WAVE/Rac1 complex [45]. In addition to its reported role in cell
405 steering and in controlling the lifetime of lamellipodia [45, 49], it was shown to be
406 dispensable for chemotactic migration of cancer cells and of *Dictyostelium discoideum* [56].
407 Of note, we found no significant difference in the spontaneous migration of macrophages
408 between HRV treated and control cells (Appendix Figure 1). As it has been documented that
409 the Arp2/3 complex is critical for phagocytosis [57], and despite a recent report [58], we
410 postulated that the ability of Arpin to deactivate the Arp2/3 complex at sites of phagosome
411 formation would also be important for efficient phagocytosis and that Arpin could play a role
412 in this. Indeed, siRNA knockdown of Arpin impaired the internalisation of multiple bacteria
413 as well as opsonized particles by macrophages without affecting their association. It has to be
414 noted that the reduction in FcR-mediated phagocytosis measured in RPE-1 cells or in
415 macrophages upon HRV16 treatment is lower than the reduction in initial uptake of bacteria
416 after viral challenge. Other factors might be implicated in the virus-induced defective uptake
417 of bacteria, in addition to Arpin. We found that Arpin was localized with branched actin in the
418 extending membrane folds in the nascent phagosome, as well as at sites of phagosome
419 scission, and not at the base of the phagosome where F-actin is cleared and where some other
420 enzymatic activities like PLC or OCRL, involved in the actin depolymerization, have been
421 reported to be preferentially localized (Marion et al, 2012, Schlam et al, 2015). These results
422 suggest that Arpin is playing a very local role in the actin polymerization/depolymerization
423 cycle and regulation of the Arp2/3 complex, rather than a role in the actin clearance at the
424 base of the phagocytic cup, which further expands our knowledge on the network of

425 regulatory elements required for the dynamic actin remodelling and polymerization/
426 depolymerisation cycles that are necessary for efficient phagosome building by macrophages
427 [26].

428

429 The Arpin protein level was decreased in HRV16-exposed human macrophages, which could
430 be the consequence of different downregulation mechanisms. We analysed the mRNA levels
431 of Arpin in HRV16 exposed hMDMs and there was a non-significant trend towards less
432 expression also (Appendix Figure 2), indicating that HRV16 could affect Arpin expression at
433 mRNA level in hMDMs. The virus could regulate transcription factors binding in the
434 promoter region of the Arpin gene like NF- κ B RelA, c-Rel and En1, as it has been shown for
435 different promoters in HRV16 infected epithelial cells [59-61]. In addition, there could be
436 miRNA regulation by the virus controlling mRNA stability and/or protein translation, as well
437 as mechanisms of protein degradation. Interestingly, interrogation of public transcriptomic
438 dataset [62] of alveolar macrophages from COPD-smokers, healthy smokers and non-
439 smokers, revealed many genes differentially expressed in COPD-smokers vs healthy smokers.
440 Pathway analyses indicated a downregulation of the mechanisms of actin polymerization or
441 cytoskeletal reorganization, which further emphasizes that actin regulation is key in COPD.

442

443 Our results demonstrate that Arpin is both necessary and sufficient for the impairment in
444 internalisation driven by HRV16. This provides key insights into a major molecular player to
445 explain how HRV16 is able to impair internalisation in hMDMs, which significantly builds on
446 previous research (Oliver et al., 2008). Of note, the impairment of phagocytosis might be
447 secondary to the establishment of viral factories hijacking cellular machineries and
448 intracellular trafficking in the infected macrophages. In addition, the inhibition of bacterial
449 uptake together with the inhibition of the cell inflammatory activity probably provides a
450 beneficial environment for the virus itself.

451 In conclusion, our results demonstrate that Arpin is crucial for successful phagocytosis in
452 macrophages and adds phagocytosis as a new function we can now attribute to this protein.

453 We reveal that Arpin is a major target of HRV16 infection, which opens new avenues for
454 strategies to improve clearance of apoptotic cells and reduce inflammation and to increase
455 bacterial uptake and suppress colonisation in the airways.

456

457

458 **Methods**

459

460 **Antibodies and reagents**

461 The following primary antibodies were used: mouse anti-actin (clone AC-40; Sigma, A3853),
462 mouse anti-Cdc42 (BD Bioscience, 610929), mouse anti-Rac1 (BD Bioscience, 610650),
463 rabbit anti-Arpin (kind gift from Alexis Gautreau), mouse anti-p16 (Synaptic Systems,
464 305011), rabbit anti-p34 (Merck Millipore, 07-227), rabbit anti-phospho cofilin (Cell
465 Signalling #3313), mouse anti-total cofilin (Cell Signalling, Clone D3F9, #5175), mouse anti-
466 tubulin alpha (clone DM1A, Sigma, T9026) and purified rabbit anti-SRBCs (IGN
467 Biochemicals). DAPI was from Sigma (D9542) and phalloidin-Cy3 from Life Technologies
468 (A22283). Zymosan A (Sigma-Aldrich) was coupled to Cy2 (GE Healthcare). Secondary
469 antibodies were all from Jackson ImmunoResearch and were as follows: Cy5 or 488-labeled
470 F(ab')₂ anti-rabbit IgG, and mouse or rabbit anti-IgG-HRP. siRNA sequences were as
471 follows: 5'-GGAGAACUGAUCGAUGUAUCU-3' (Arpin sequence 1) and 5'-
472 GCUUCCUCAUGUCGUCCUACA-3' (Arpin sequence 2), [45]. The control siRNA-
473 targeting Luciferase was 5'-CGUACGCGGAAUACUUCGA-3'.

474

475 **Cell culture**

476 Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of
477 healthy donors (Etablissement Français du Sang Ile-de-France, Site Trinité, Inserm agreement
478 #15/EFS/012 and #18/EFS/030 ensuring that all donors gave a written informed consent and
479 providing anonymized samples) by density gradient sedimentation using Ficoll-Plaque (GE
480 Healthcare). This was followed by adhesion on plastic at 37°C for 2 h and culture in the
481 presence of adhesion medium (RPMI 1640 (Life Technologies) supplemented with 100 µg/ml
482 streptomycin/penicillin and 2 mM L-glutamine (Invitrogen/Gibco). Then, the adhered cells
483 were washed once with warm adhesion medium and left to rest in macrophage medium
484 (RPMI 1640 supplemented with 10% FCS (Eurobio), 100 µg/ml streptomycin/penicillin, and
485 2 mM L-glutamine). On day 1, the cultures were washed with adhesion medium and then
486 supplemented every 2 days with fresh macrophage medium. The adherent monocytes were
487 left to differentiate into macrophages as described previously [63] and used for experiments at
488 day 10.

489

490 Human alveolar macrophages (AMs) were obtained by bronchoalveolar lavage fluid (BALF)
491 of lung specimens from non-smokers or healthy smokers (Protocole de recherche non
492 interventionnelle, Number ID RCB 2015-A01809) ensuring that all donors signed a consent
493 form. The health condition of the patient was registered before the samples were treated
494 anonymously. The sample was initially centrifuged at $290 \times g$ for 5 minutes, the pellet
495 resuspended in the original volume in adhesion medium and the cell count obtained. Cells
496 were then plated onto plastic and incubated for 4 h in adhesion medium at 37°C . They were
497 then washed thoroughly with adhesion medium and rested overnight in AM media (X-VIVO
498 10 without phenol red and gentamicin (Lonza) supplemented with $50 \mu\text{g/ml}$
499 streptomycin/penicillin, 1 Mm L-glutamine and $20 \mu\text{g/ml}$ amphotericin B (Sigma)) and
500 experiments were performed the next day.

501

502 HeLa Ohio cells were purchased from the European Collection of Authenticated Cell Cultures
503 (ECACC) and were cultured in DMEM GlutaMax containing 25 mM D-glucose and 1 mM
504 sodium pyruvate (Life Technologies) supplemented with 10% FCS, $100 \mu\text{g/ml}$
505 penicillin/streptomycin and 2 mM L-glutamine. They were passaged every 3 days.

506

507 RAW264.7 macrophages were grown in complete medium, consisting of RPMI1640-
508 glutamax supplemented with 10 mM HEPES, 1mM sodium pyruvate, $50\mu\text{M}$ β -eta-
509 mercaptoethanol, 2 mM L-Glutamine and 10 % FCS (all from Gibco). Cells were transfected
510 by electroporation with the Electrobuffer kit (Cell project). Routinely, one 100 mm plate of
511 cells was grown to sub-confluence and $10 \mu\text{g}$ of each plasmid was used for co-transfections.
512 The cells were electroporated in 0.4 cm cuvettes (Biorad) at 250 V, $900 \mu\text{F}$ in an
513 electroporation apparatus (X Pulser Bio-Rad Laboratories), then immediately resuspended in
514 complete culture medium. Efficiency of transfection was 10–40%.

515

516 **Generation of RPE-1-FcγRIIA cells.**

517 FcγRIIA gene was amplified by PCR from the pRK5-FcγRIIA plasmid (E. Caron, Imperial
518 College, London) using the following oligos: forw: 5'-
519 CCGGCGGCCGCTCTCCAAGGTGTCC-3', and rev: 5'-
520 GGCCCGACCGTTTATTACTGTTGACATGG-3' carrying respectively NotI and
521 AgeI restriction site. The NotI/AgeI digested amplicons were inserted in a pLEX MCS
522 plasmid (Open Biosystems) digested as well and dephosphorylated. Lentiviral particles were

523 produced by co-transfection of HEK293T cells with packaging plasmids (pCMV 8.91 and
524 pEnv_{VSVG}) and pLEX-FcγRIIA plasmid. After 48 h of culture at 37°C, the virus-containing
525 supernatant was filtered and ultracentrifuged at 60,000 × g for 90 min at 4°C on a PEG
526 cushion (50 %). The virion-enriched pellet was resuspended in PBS and aliquoted for storage
527 at -80°C. hTERT RPE-1 cells (ATCC^(R) n° CRL-4000TM, BIOPHENICS facility, Institut
528 Curie, Paris) were infected with lentiviral particles from MOI 1 to MOI 10. FcγRIIA-
529 expressing hTERT RPE-1 cells were cultured in Dubelcco's modified Eagle medium
530 (DMEM) F-12 (Thermo Fisher Scientific) supplemented with 10 % Fetal Calf Serum (FCS,
531 Gibco), 10 µg/ml hygromycin B and 2.5 µg/ml puromycin (Sigma). They were passaged
532 every two days.

533

534 **RPE-1 cell transfection**

535 RPE1 cells at 80 % confluence were washed with PBS and detached using 0.25 %
536 Trypsin/EDTA (Life Technologies). After centrifugation, the cell count was determined and
537 the cells seeded on coverslips at a density of 15, 000 per coverslip and allowed to adhere
538 overnight. The next day, the coverslips were exposed to HRV16 or mock infected as
539 described above. Then they were transferred to a 6 well plate and transfected. The plasmid
540 solution was prepared in OptiMEM medium (GlutaMAX supplemented, Gibco), containing
541 Fugene reagent (Invitrogen) and each plasmid at a concentration of 3 µg. Plasmid solution
542 was added to each well and cultures left for 24 h at 37°C before being treated for FcR
543 phagocytosis.

544

545 **Bacterial strains and culture**

546 NTHi strain RdKW20 [64, 65] and *Moraxella catarrhalis* strain 25293 [66] were purchased
547 from the American Type Culture Collection (ATCC). *Staphylococcus aureus* strain
548 160201753001 and *Pseudomonas aeruginosa* strain 160601067201 from blood culture were
549 provided by Professor Claire Poyart (Cochin Hospital). NTHi, *S. aureus* and *P. aeruginosa*
550 were cultured on chocolate agar plates and *M. catarrhalis* was cultured on brain-heart
551 infusion (BHI) agar plates. Plates were incubated for 24 h at 37°C until colonies appeared. All
552 strains were grown in LB medium but for NTHi, this was also supplemented with 10 µg/ml
553 hemin and 1 µg/ml nicotinamide adenine dinucleotide (NAD).

554

555 **Human rhinovirus production**

556 Human Rhinovirus 16 (HRV16) (VR-283, strain 11757, lot 62342987) was purchased from
557 the ATCC and stocks were produced by infecting HeLa Ohio cells in virus medium (DMEM
558 GlutaMax containing 25 mM D-glucose and 1 mM sodium pyruvate supplemented with 10%
559 FCS and 2 mM L-glutamine) as described previously [67]. Briefly, HeLa Ohio cells were
560 grown to 80% confluence and infected with 5 ml HRV16 or control media for 1 h at room
561 temperature with agitation. The remaining solution was made to 10 ml and the cells with
562 HRV16 left for 48 h to allow for 90% CPE to develop. Supernatants were then clarified by
563 centrifugation and filtration [67] and 1 ml stocks were produced and stored at -80°C. To UV
564 inactivate HRV16 it was treated with UV light (1000mJ/cm²) for 20 minutes. Inactivation was
565 confirmed by adding the inactivated virus to HeLa Ohio cells and checking for CPE.

566

567 **Quantification of the tissue culture infective dose 50 (TCID₅₀) of HRV16**

568 HeLa Ohio cells were cultivated in 96 well plates at 1 x 10⁵ cells/well for 24 h. HRV16 was
569 diluted 10-fold from undiluted to 10⁻⁹ in virus medium 50 µl of each dilution was added to the
570 cells in 8 replicate wells. 50 µl of virus medium was added to 2 groups of control wells in 8
571 replicate wells per group. Cultures were incubated for 4 days at 37°C until CPE was observed
572 in 50% of wells. TCID₅₀ was calculated using the Spearman-Kärber formula as previously
573 outlined [67].

574

575 **HRV16 and bacterial infection of human macrophages**

576

577 Macrophages were washed once in PBS and rested in virus medium. HRV16, HRV16^{UV} or
578 MI supernatants were added to the macrophages and placed at room temperature for 1 h with
579 agitation to achieve a TCID₅₀ of 1 x 10⁷/ml. Cultures were then washed with virus medium
580 and rested in macrophage medium overnight.

581

582 NTHi, *M. catarrhalis*, *S. aureus* or *P. aeruginosa* were grown until mid-log growth phase,
583 centrifuged at 1692 x g for 5 min and re-suspended in 1 ml phagocytosis medium (RPMI
584 supplemented with 2 mM L-glutamine). Bacteria was added to macrophages pre-treated with
585 HRV16, HRV16^{UV} or MI to achieve a multiplicity of infection (MOI) of 10/cell. Cultures
586 were then centrifuged at 602 x g for 2 min and placed at 37°C, 5% CO₂ for 30 or 120 min. At

587 each time point, cultures were washed with PBS and treated with 100 µg/ml gentamicin
588 (NTHi, *S. aureus*, *P. aeruginosa*) or 20 µg/ml (*M. catarrhalis*) for 20 min. Cultures were
589 washed and lysed in saponin as previously described [63] and colony forming units (CFU)
590 estimated using the Miles-Misra technique [68].

591

592 **Measurement of bacterial binding**

593

594 Macrophages were challenged with HRV16 or controls as described above and bacteria was
595 prepared in the same manner. Cultures were washed with PBS and bacteria were added to
596 macrophages to achieve an MOI of 10 bacteria per cell. They were placed on ice for 5, 15 or
597 30 min. At each time point the extracellular supernatant was taken for CFU determination.
598 Cultures were then washed with PBS and the final wash again taken for CFU determination to
599 determine no residual bacteria remained. Cultures were then treated with saponin and lysed
600 and CFU estimated as described previously [63].

601

602 **FcR, CR3 or zymosan phagocytosis**

603

604 Macrophages were challenged with either IgM-iC3b or IgG-opsonised SRBC or zymosan for
605 up to 60 min as described [36]. Briefly, for CR3-mediated phagocytosis, SRBCs were washed
606 in PBS/BSA 0.1% and incubated for 30 min with rotation in rabbit IgM anti-SRBCs. They
607 were washed and incubated in complement C5-deficient serum without rotation for 20 min at
608 37°C. SRBCs were further washed, re-suspended in phagocytosis medium and added to
609 macrophages to give approximately 10 SRBCs per cell. The plates were centrifuged at room
610 temperature at 502 x g for 2 min and then placed at 37°C for various time points. At each time
611 point, cells were washed with room temperature phagocytosis medium and fixed in warm 4%
612 paraformaldehyde (Sigma-Aldrich) at room temperature for 15 min and then treated with
613 0.05M NH₄Cl/PBS1X for 10 min. For FcR-mediated phagocytosis, SRBCs were washed as
614 above and opsonized for 30 min with rotation in rabbit IgG anti-SRBCs. They were further
615 washed, re-suspended in phagocytosis medium and added to macrophages as above. All
616 subsequent steps were as for CR3. For zymosan uptake, zymosan was washed twice in
617 PBS/BSA 0.1% and then re-suspended in phagocytosis medium. Macrophages were
618 challenged with zymosan for 60 min and all subsequent steps were as described above.

619 **Fluorescence Microscopy and phagocytosis quantification**

620 For FcR or CR3 mediated phagocytosis cultures were washed in 1XPBS/2% FCS and
621 external SRBCs were labelled for 30 min with F(ab')₂ anti-rabbit IgG Alexa Fluor 488 in
622 PBS/2% FCS. Cells were then washed with 1XPBS/2%FCS and re-fixed in 4% PFA for 15
623 min at room temperature and then treated with 0.05M NH₄Cl for 10 min before being
624 permeabilized in 1XPBS/2%FCS/0.05% saponin. Intracellular SRBCs were then detected
625 using a Cy5-labeled F(ab')₂ anti-rabbit IgG and F-actin was stained using phalloidin-Cy3 in
626 1XPBS/2%FCS/0.05% saponin for 30 min. After washing in 1XPBS/2%FCS/0.05% saponin
627 cells were stained with DAPI for 5 min and mounted using Fluormount G (Interchim). For
628 zymosan uptake, cultures were washed in 1XPBS/2% FCS and external zymosan was
629 detected with an anti- zymosan antibody for 30 min followed by Cy5-labeled F(ab')₂ anti-
630 rabbit IgG for 30 min. Cultures were washed in 1XPBS/2% FCS and permeabilized in
631 1XPBS/2%FCS/0.05% saponin before labelling with phalloidin Cy3 to detect F-actin. After
632 washing as above, cultures were treated with DAPI for 5 min and mounted using Fluormount
633 G. To quantify phagocytosis, the number of internalized SRBCs/zymosan per cell was
634 counted in 30 cells randomly chosen on the coverslips corresponding to the phagocytic index.
635 The index obtained was divided by the index obtained for control cells and was expressed as a
636 percentage of control cells. To determine the index of association, the total number of bound
637 and internalized SRBCs in a cell was divided by the total number of macrophages counted.
638 Image acquisition was performed on an inverted wide-field microscope (Leica DMI6000)
639 with a 100× (1.4 NA) objective and a MicroMAX camera (Princeton Instruments). Z-series of
640 images were taken at 0.3-µm increments. Analyses were performed using custom-made
641 ImageJ (National Institutes of Health) routines.

642

643 **Quantification of F-actin recruitment in phagocytic cups and in macrophages**

644

645 Quantification was performed as described previously [69]. Briefly, quantification was
646 performed on ImageJ 64bit software (NIH libraries) on a selected region in 1 place of a 16-bit
647 stack that was acquired. Primary fluorescence intensities through the phagocytic cup and in
648 the cell cortex were measured and background corrected. Ratio values were calculated by
649 dividing the fluorescence intensities in the phagocytic cups by the fluorescence intensities in
650 the cell cortex's and plotted. To quantify the F-actin intensity in HRV16 exposed
651 macrophages two macros were used. The first macro was written to quantify the intensity of
652 staining in the entire field of cells. Quantification was performed using ImageJ 64bit software

653 on entire 16-bit Z stacks. The macro automatically decided which plane of the Z stack to use
654 and when in focus it divided the field into 4 sections and quantified the fluorescence of the
655 punctate F-actin and the total F-actin in each section. The second macro was written to
656 quantify the intensity of the punctate F-actin per cell. This was done in the same way as for
657 macro 1 apart from instead of calculating the intensity in the section, the macro quantified it
658 in each individual cell within the field selected by the macro. To quantify the F-actin intensity
659 each macro used the FindFocussedSlices filter and two automatic ImageJ thresholding. The
660 first was Percentile Dark for the total F-actin and the second was Default Dark for the
661 punctate F-actin. The results from each output were reported as a summary for each cell or
662 field in ImageJ. All results for both macro 1 and 2 were transferred to Graphpad Prism and
663 plotted.

664

665 **Phagosome closure assay using Total Internal Reflection Fluorescence Microscopy** 666 **(TIRFM)**

667 IgG-RBCs were centrifuged onto 35mm glass bottom dishes (MatTek Corporation) pre-
668 treated with 0.01% poly-L-Lysine in PBS for 30 min at RT. The dishes were then washed
669 once with a 10% BSA in PBS and incubated for 30 min with 10% BSA in PBS. Then the
670 dishes were incubated with pre-warmed serum-free microscopy medium. RAW264.7
671 macrophages were resuspended and allowed to sediment onto opsonized SRBC-coated dishes
672 at 37°C.

673 TIRFM was performed using a Till PHOTONICS iMIC microscope equipped with an oil-
674 immersion objective (Apo N 100x, NA1.49 Olympus America Inc.), a heating chamber, and
675 two cameras: a cooled iXonEM camera and an iXon3 897 Single Photon Detection EMCCD
676 Camera (Andor Technology). The critical angle was verified at the beginning of each session
677 by scanning through incident angles of 0-5° to maximise evanescent wave induced
678 fluorescence. Excitation was performed with a 491 nm and a 561 nm laser. For the
679 phagosome closure assay, streams of 550 frames were acquired at 50 ms per frame in TIRF
680 mode, and 50 ms per frame in epifluorescence mode with Polychrome illumination at a 3 µm
681 increment every 2 s. TIRFM image streams were processed using ImageJ Color Profiler
682 software (NIH).

683

684 **Migration assay**

685 hMDMs were plated in IBIDI (15 microns) microscopy slides in 8 wells, treated or not with
686 the virus for 1 h, before washing and overnight rest in the incubator. They were then
687 incubated with sirDNA at 0.33 μ M and observed with a 20x, 1.4 NA, PH DIC objective with
688 a spinning disk confocal (Yokogawa CSU-X1M1) inverted microscope (Leica DMI6000)
689 equipped with a CoolSnap HQ² camera (Photometrics) and a heated chamber with CO₂ in a
690 BSL3 laboratory. Acquisition was performed with MetaMorph 7.5.5 (Molecular Devices) in
691 the bright field (BF) and CY5 channel excited with a 635 nm laser.

692

693 **Western blots**

694 Macrophages were lysed with lysis buffer (20 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5 %
695 NP-40, 50 mM NaF, and 1 mM sodium orthovanadate, supplemented with complete protease
696 inhibitor cocktail (Roche Diagnostic)) for 15 min. Lysates were centrifuged at 16,100 \times g for
697 10 min at 4°C. The supernatants were removed and stored at -20 °C and an equal
698 concentration of protein (BCA dosage kit, Pierce) was analyzed by SDS-PAGE. Proteins were
699 transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) at 4°C for 100
700 min and incubated in blocking solution TBS/0.1% Tween-20 supplemented with 5% milk or
701 BSA for 2 h. Blots were rinsed with TBS/0.1% Tween-20 and primary antibodies were
702 incubated in the blocking solution overnight or for 2 h as required. The membrane was further
703 washed and incubated with HRP-coupled secondary antibodies in blocking buffer for 45 min.
704 Detection was performed using ECL Dura substrate (GE Healthcare) and bands imaged by
705 Fusion (Vilber Lourmat) and quantified in ImageJ.

706 **Scanning electron microscopy**

707 Cells were fixed with 2.5 % glutaraldehyde (Sigma Aldrich) in 0.1M HEPES, pH 7.2 in
708 phagocytosis medium at 37°C for 1 h. They were then fixed in 2.5% glutaraldehyde in 0.1M
709 HEPES buffer in PBS at 4°C overnight. Post fixation was done with 1 % osmium tetroxide
710 (Merck) and 1.5 % ferrocyanide (Sigma Aldrich) in 0.1M HEPES. After dehydration by a
711 graded series of ethanol, the samples were transferred to a Leica EM CPD300 and dried
712 according to standard procedures. Samples were mounted on aluminium stubs and sputter
713 coated with 7 nm of gold palladium in a Gatan ion beam coater. Samples were examined at
714 5kV in an Jeol 6700F scanning electron microscope.

715

716 **siRNA Treatment**

717 Macrophages at day 7 were washed twice with macrophage medium and kept in macrophage
718 medium at 37°C. The siRNA solution was prepared in OptiMEM medium (GlutaMAX
719 supplemented, Gibco), containing Lipofectamine RNAiMAX reagent (Invitrogen) and siRNA
720 at a concentration of 100 µM. siRNA was added to each well and cultures left for 96 h at
721 37°C before being used. siRNA sequences were as follows: CGU ACG CGG AAU ACU
722 UCG A55 (siLuciferase_pGL2), GGA GAA CUG AUC GAU GUA UCU 55 (siArpin.1) and
723 GCU UCC UCA UGU CGU CCU ACA 55 (siArpin.2).

724

725 **qPCR**

726 hMDMs were exposed to HRV16 or MI control as described above. After overnight rest,
727 cultures were washed with PBS and RNA was extracted as previously described [70]. Briefly,
728 hMDMs were washed with PBS at room temperature and lysed using Trizol reagent
729 (ThermoFischer Scientific). Proteins (organic phase) and RNA and DNA (aqueous phase)
730 were separated using chlorophorm for 2 min at room temperature followed by 15 min
731 centrifugation at 4°C at 12,000 x g. The aqueous phase was collected and isopropanol was
732 added to precipitate RNA and incubated for 10 min at room temperature. Samples were
733 centrifuged for 20 min at 4°C at 15,000 x g and the pellet of RNA washed with 75% ethanol
734 and centrifuged for a further 5 min at 4°C at 10,000 x g. The pellet was dried at room
735 temperature and resuspended in pure water and warmed at 55°C for 5 min. The total amount
736 of RNA was quantified using nanodrop. For reverse transcription 1 µg of mRNA was retro-
737 transcribed into DNA using SuperScript II Reverse Transcriptase (ThermoFischer Scientific).
738 qPCR was performed using the LightCycler 480 SYBR Green I Master (Roche) with specific
739 oligos to detect Arpin with 18S RNA as control (Table 2). The relative quantification of Arpin
740 gene expression was assessed using the $\Delta\Delta C_t$ method [71].

741

742 **Statistical analysis**

743 Statistical tests were performed using Graphpad prism® version 6 software. All statistical
744 tests are listed in the figure legends and significance was determined if $p < 0.05$.

745

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747

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757

758 **Author contributions**

759

760 Conceptualization: JJ, NK and FN

761 Methodology: JJ, FH, EI, LO, PB and FN

762 Investigation: JJ, KAG, FH, AM, PB

763 Resources: PB, PRB

764 Writing: original draft: JJ, KAG, FH and FN

765 Writing: review and editing: GM, DMC, NK, FN

766 Supervision: NK and FN

767 Project administration: GM, DMC, NK, FN

768 Funding acquisition: GM, DMC, NK and FN

769

770 **Competing interests**

771 NK, DMC, LO, EI and GM are employed by the commercial company AstraZeneca and
772 AstraZeneca provided salaries for JJ and KAG as part of a collaborative grant with FN. All
773 authors have no competing interests to declare.

774

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974

975 **Figure legends**

976

977 **Figure 1**

978 **HRV16 impairs bacterial internalisation by macrophages.**

979 **A-H** hMDMs were challenged with HRV16, HRV16^{UV} or MI and then exposed to either
980 (A, E) NTHi, (B, F) *Moraxella catarrhalis*, (C, G) *Staphylococcus aureus*, or (D, H)
981 *Pseudomonas aeruginosa* to assess internalisation (A-D) or binding (E-H). n=3 biological
982 replicates on different donors **p<0.01, ****p<0.0001 Two Way Anova with Dunnett's Post
983 Test vs MI.

984 **I, J** (I) Non-smoker, non-COPD or (J) Healthy smoker, non-COPD AM were challenged
985 with HRV16 or controls and then exposed to NTHi to assess internalisation. n=4 biological
986 replicates on different donors **p<0.01, ***p<0.001, ****p<0.0001 Two Way Anova with
987 Dunnett's Post Test vs MI.

988 Data information: Error bars represent standard error of the mean (SEM).

989

990 **Figure 2**

991 **HRV16 impairs receptor-mediated internalisation by macrophages.**

992 **A-C** hMDMs were challenged with HRV16 or controls and then exposed to (A) zymosan
993 for 60 min, n=3 donors, 30 random cells/coverslip, (B) IgM-iC3b-opsonised SRBC for 60
994 min, n=5 donors, 30 random cells/coverslip, or (C) IgG-opsonised SRBC for 60 min, n=5
995 donors, 30 random cells/coverslip. ***p<0.001, ****p<0.0001 One Way Anova with
996 Bonferonni's Post Test vs MI. Error bars represent standard error of the mean (SEM).

997 (D) Representative images of MI or HRV16 challenged hMDMs at 60 min post IgM-iC3b
998 SRBC internalisation. Intracellular particles are labeled with Cy5-coupled F(ab')₂ anti-rabbit
999 Ig and DAPI. Scale bar, 15 µm.

1000 (E) Representative images of MI or HRV16 challenged hMDMs at 60 min post IgG-SRBC.
1001 Intracellular particles are labeled with Cy5-coupled F(ab')₂ anti-rabbit Ig and DAPI. Scale
1002 bar, 15 µm.

1003

1004 **Figure 3**

1005 **HRV16 impairs phagocytic cup extension.**

1006 hMDMs were challenged with HRV16 or MI and then exposed to IgG-opsonized SRBC for
1007 2-30 min.

1008 (A) Representative images of a Z projection of phalloidin staining at 5 and 15 min post
1009 internalisation in MI and HRV16 challenged hMDMs. Scale bar, 15 μ m.
1010 (B) Quantification of the number of actin cups in hMDMs, n=3 donors, at least 80 cups at the
1011 early time points and 8-80 cups at later time points depending on the time
1012 (C) Quantification of the initial F-actin enrichment around internalized particles in hMDMs,
1013 [on n=3 different donors.](#)
1014 (D) Quantification of internalisation of SRBC over 30 min, n=3 donors, 30 random cells/
1015 coverslip.
1016 (E) Quantification of association of SRBC to hMDMs, n=3 donors, 30 random cells/coverslip.
1017 (F) Scanning electron microscopy images after 5 min of contact with IgG-opsonised SRBC
1018 showing differences in cup formation. Scale bar, 1 μ m.
1019 Data information: **p<0.01, ***p<0.001, ****p<0.0001 One Way Anova with Bonferonni's
1020 Post Test vs MI. Error bars represent standard error of the mean (SEM).

1021

1022 **Figure 4**

1023 **HRV16 downregulates Arpin expression in macrophages.**

1024 hMDMs were challenged with HRV16 or controls and then stained or lysed.

1025 (A) Representative images of MI or HRV16 challenged hMDMs stained with phalloidin-Cy3
1026 at 24 h post infection. Scale bar, 15 μ m.
1027 (B) Quantification of the total F-actin intensity per field, n=3 donors, 20 random fields per
1028 coverslip
1029 (C) Quantification of the punctate F-actin intensity per field, n=3 donors, 20 random fields
1030 par coverslip.
1031 (D) Quantification of the punctate F-actin intensity per cell in 20 cells per donor per
1032 condition, n=3 different donors.
1033 (E) Immunoblot of lysates from hMDMs challenged with MI, HRV16^{UV} or HRV16 to detect
1034 Arpin or actin.
1035 (F) Quantification of immunoblots normalized to MI control, n=10 experiments [on different](#)
1036 [donors.](#)
1037 (G) Quantification of Rac1, Cdc42, p16-Arc, and p34-Arc, n=10 experiments [on different](#)
1038 [donors.](#)
1039 (H) Quantification of total and phosphorylated cofilin expression, n=9 experiments [on](#)
1040 [different donors.](#)
1041 (I) Relative activation of cofilin vs total cofilin, n=9 experiments [on different donors.](#)

1042 (J) RPE-1-FcγRIIA cells challenged with mock medium (MI) and transfected with plasmids
1043 encoding EGFP (left) or EGFP-Arpin (right).

1044 (K) Quantification of FcR phagocytosis in RPE-1-FcγRIIA cells challenged with mock
1045 medium (MI) or HRV16 and then transfected to express EGFP or EGFP-Arpin, n=4
1046 experiments, between 30 and 50 random cells.

1047 Data information: In (B-D) **p<0.01, ***p<0.001, ****p<0.0001 Paired T test vs MI. In (F)
1048 *p<0.05 One Way Anova with Bonferonni's Post Test vs MI. In (K) **p<0.01, ****p<0.0001
1049 unpaired T test. Error bars represent standard error of the mean (SEM).

1050

1051 **Figure 5**

1052 **Arpin knockdown impairs bacterial internalisation by macrophages.**

1053 hMDMs were non-treated, treated with siLuciferase or 2 different Arpin siRNA sequences for
1054 96 h.

1055 (A) Immunoblot against Arpin and tubulin in siRNA-treated hMDMs.

1056 (B) Quantification of the expression of Arpin normalized to tubulin and presented relative to
1057 non-treated as a percentage, n=9 donors, ****p<0.0001 One Way Anova with Bonferonni's
1058 Post Test vs non-treated.

1059 C-F (C) NTHi internalisation, (D) *Moraxella catarrhalis* internalisation, (E)
1060 *Staphylococcus aureus* internalisation, and (F) *Pseudomonas aeruginosa* internalisation, n=3
1061 donors, **p<0.01, ***p<0.001, ****p<0.0001 Two Way Anova with Dunnett's Post Test vs
1062 non-treated.

1063 Data information: Error bars represent standard error of the mean (SEM).

1064

1065 **Figure 6**

1066 **Arpin knockdown impairs efficient phagocytic cups formation, is recruited in** 1067 **membrane extensions and at the closure site.**

1068 hMDMs were non-treated, treated with siLuciferase or 2 different Arpin siRNA sequences for
1069 96 h, then allowed to internalize IgG-opsonised SRBC for up to 30 min and F-actin structures
1070 around phagocytosed particles was assessed using phalloidin-Cy3.

1071 (A) Representative images at 15 and 30 min post internalisation. Scale bar, 15µm.

1072 (B) Quantification of internalisation of SRBC over 30 min, n=3 donors, 30 random
1073 cells/coverlip.

1074 (C) Quantification of association of SRBC to hMDMs over 30 min, n=3 donors, 30 random
1075 cells/coverlip.

1076 (D) Quantification of the number of F-actin cups in hMDMs over 30 min, n=3 donors, at least
1077 60 cups in the early time point and between 15-60 depending on time point.

1078 (E) Quantification of the initial F-actin enrichment around internalized particles in hMDMs
1079 over 30 min, n=3 donors, at least 60 cups in the early time point and between 15-60
1080 depending on time point,

1081 (F) Scanning electron microscopy images following Arpin knockdown or control and 15 min
1082 IgG-opsonised SRBC internalisation showing differences in membrane recruitment. Scale
1083 bar, 1 μ m.

1084 (G) Phagosome closure assay to detect the site of phagosome closure in the TIRFM mode
1085 (left) and the base of the phagosome in the epifluorescence mode after shifting the stage 3 μ m
1086 in z.

1087 (H) Phagosome closure assay was performed using RAW264.7 macrophages transiently
1088 expressing both Lifeact-mCherry and EGFP-Arpin. Lifeact-mCherry (upper panels) and EGFP-
1089 Arpin (lower panels) imaging by TIRFM and epifluorescence were performed alternatively at
1090 37°C every 2 s for 10 min. Transmitted light images are presented at the bottom.

1091 Data information: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 One Way Anova with
1092 Bonferonni's Post Test vs MI. Error bars represent standard error of the mean (SEM).

1093

1094 **Figure EV1**

1095 **HRV16 impairs NTHi internalisation by macrophages from TCID₅₀ 1 x 10³.**

1096 A-E hMDMs were challenged with HRV16, HRV16^{UV} or MI at various doses and then
1097 exposed to NTHi for up to 2 h. (A) TCID₅₀ 1 x 10¹; (B) TCID₅₀ 1 x 10³; (C) TCID₅₀ 1 x 10⁵;
1098 (D) TCID₅₀ 1 x 10⁶; (E) TCID₅₀ 3 x 10⁷. n=3 donors ** p<0.01, ****p<0.0001 Two Way
1099 Anova with Dunnett's Post Test vs MI.

1100 (F) Quantification of the percentage inhibition in NTHi uptake vs MI at 120 min in HRV16
1101 exposed hMDMs, n=3 donors, ****p<0.0001 One Way Anova with Dunnett's Post Test vs
1102 TCID₅₀ 1 x 10¹.

1103 Data information: Error bars represent standard error of the mean (SEM).

1104

1105 **Figure EV2**

1106 **HRV16 impairs *Staphylococcus aureus* internalisation by macrophages from TCID₅₀ 1 x
1107 10³.**

1108 A-E hMDMs were challenged with HRV16, HRV16^{UV} or MI at various doses and then
1109 exposed to *Staphylococcus aureus* for up to 2 h. (A) TCID₅₀ 1 x 10¹; (B) TCID₅₀ 1 x 10³; (C)

1110 TCID₅₀ 1 x 10⁵ ; (D) TCID₅₀ 1 x 10⁶ ; (E) TCID₅₀ 3 x 10⁷, n=3 donors *p<0.05,
1111 ****p<0.0001 Two Way Anova with Dunnett's Post Test vs MI.

1112 (F) Quantification of the percentage inhibition in *Staphylococcus aureus* uptake vs MI at 120
1113 min in HRV16 exposed hMDMs, n=3 donors, ****p<0.0001 One Way Anova with Dunnett's
1114 Post Test vs TCID₅₀ 1 x 10¹.

1115 Data information: Error bars represent standard error of the mean (SEM).

1116

1117 **Figure EV3**

1118 **Arpin knockdown does not impair bacterial binding to hMDMs.**

1119 **A-D** hMDMs were non-treated, treated with siLuciferase or 2 different Arpin siRNA
1120 sequences for 96 h and bacterial binding at 4°C assessed over 30 min. (A) NTHi ; (B)
1121 *Moraxella catarrhalis* ; (C) *Staphylococcus aureus* ; (D) *Pseudomonas aeruginosa*, n=3
1122 donors. Error bars represent standard error of the mean (SEM).

1123

1124 **Figure EV4**

1125 **HRV16 or Arpin knockdown does not cause a delay in internalisation in hMDMs.**

1126 **A,B** hMDMs were challenged with HRV16, HRV16^{UV} or MI, or non-treated, treated with
1127 siLuciferase or 2 different Arpin siRNA sequences for 96 h and internalisation of IgG-
1128 opsonised SRBC assessed over 60 min. (A) HRV16 exposure, n=5 donors. (B) Arpin
1129 knockdown, n=3 donors, ****p<0.0001 One Way Anova with Bonferonni's Post Test vs non-
1130 treated. Error bars represent standard error of the mean (SEM).

1131

1132 **Figure EV5**

1133 **FcgRIIA-expressing RPE-1 cells phagocytose IgG-opsonized SRBC.**

1134 (A) RPE-1 or RPE-1-FcgRIIA cells were labeled with an anti-CD32 antibody or an isotype
1135 control antibody coupled to PE. Fluorescence was analyzed by flow cytometry (BD Accuri™
1136 C6, 10 000 cells per condition).

1137 **B-D** (B) Phagocytosis of IgG-opsonized or non-opsonized SRBC was assessed in RPE-1 or
1138 RPE-1 FcgRIIA cells, and quantified for (C) SRBC association at 0 min (between 13 and 16
1139 cells). Scale bar, 30 μm. One representative experiment was quantified. ****p<0.0001,
1140 Unpaired T test, and (D) internalization at 60 min (between 10 and 12 cells) ***p=0.0002,
1141 Unpaired T test. Error bars represent standard error of the mean (SEM).

1142

1143

1144 **Table 1**

1145 **Patient demographics for AM samples used in figure 1**

	Non-smoker	Healthy smoker
Male/female	3/1	3/1
Age	44 (average)	46 (average)
Ex/current smoker	N/A	0/4

1146

1147 **Table 2: List of qPCR primers used in this study**

Gene	Sequence	Reference
18S histone	Forward: AGGAATTGACGGAAGGGCAC Reverse: GGACATCTAAGGGCATCACA	Génin <i>et al.</i>, 2015 Plos Pathogens 11: e1004971
Arpin	Forward: CTTCCTCATGTCGTCCTACAAGGT G Reverse: CTGTCAGCGCGAGCAGCTCT	Dang <i>et al.</i>, 2013 Nature Letters 503: 281

1148

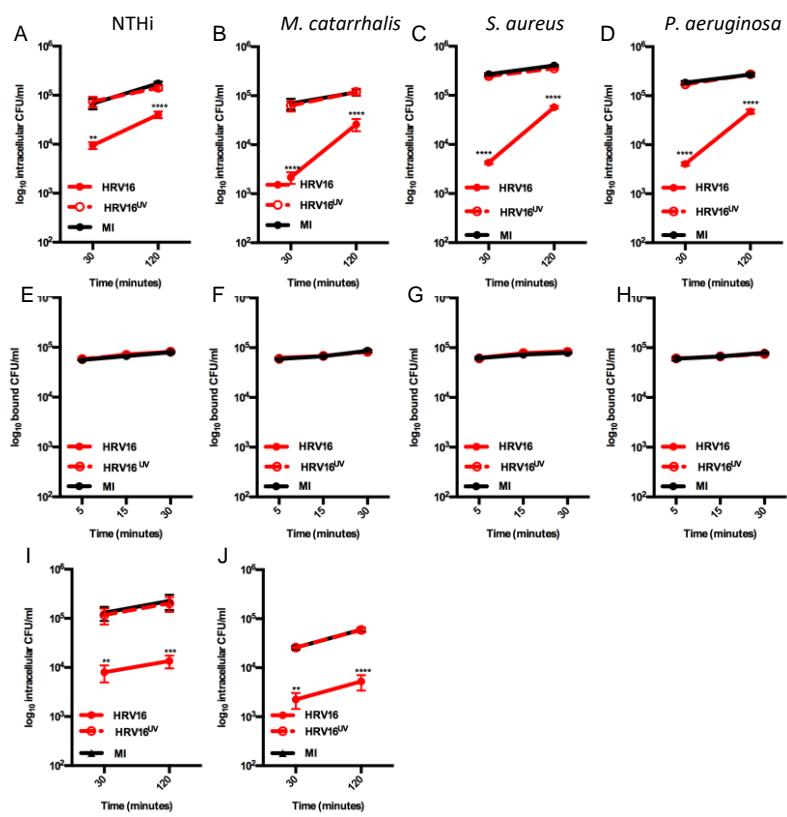


Figure 1

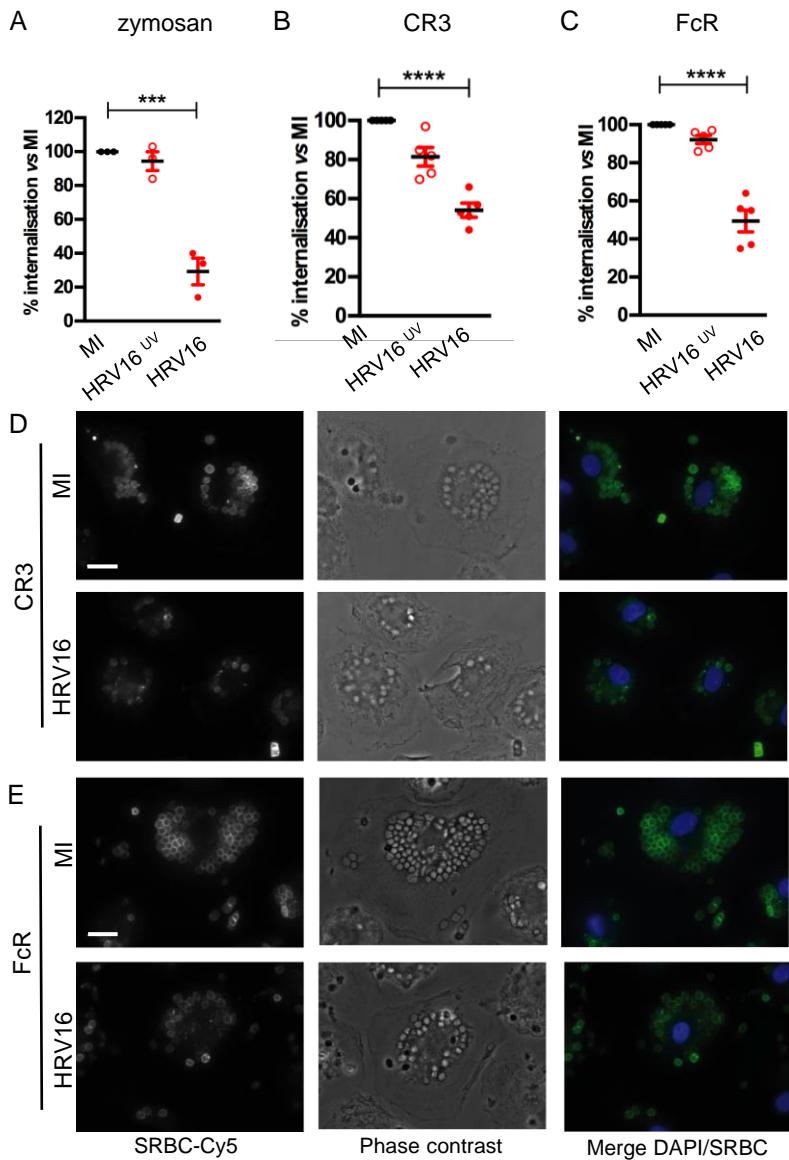


Figure 2

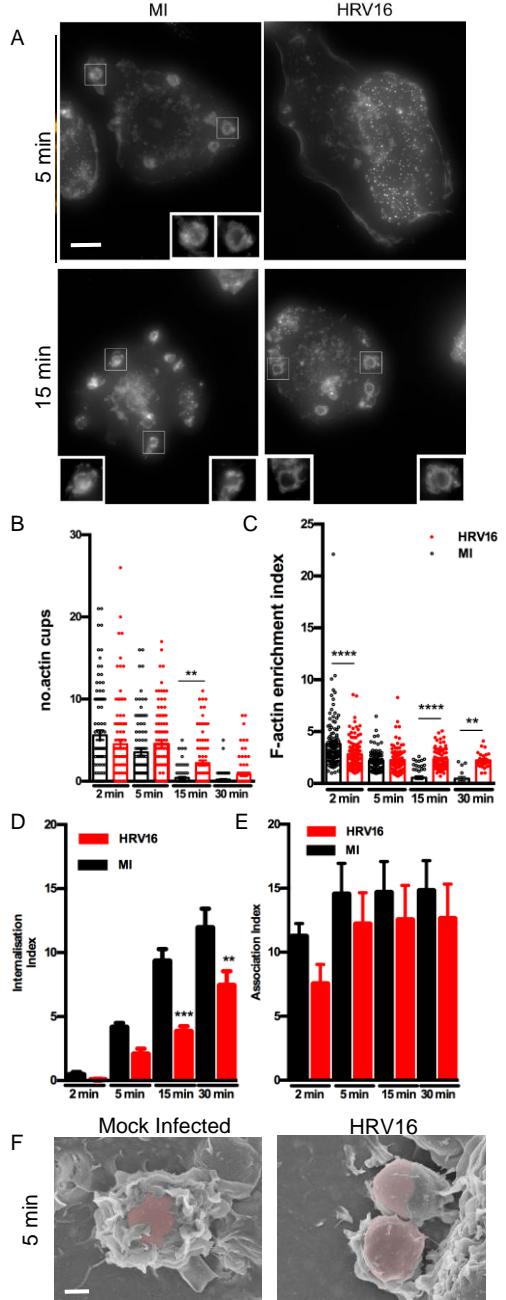


Figure 3

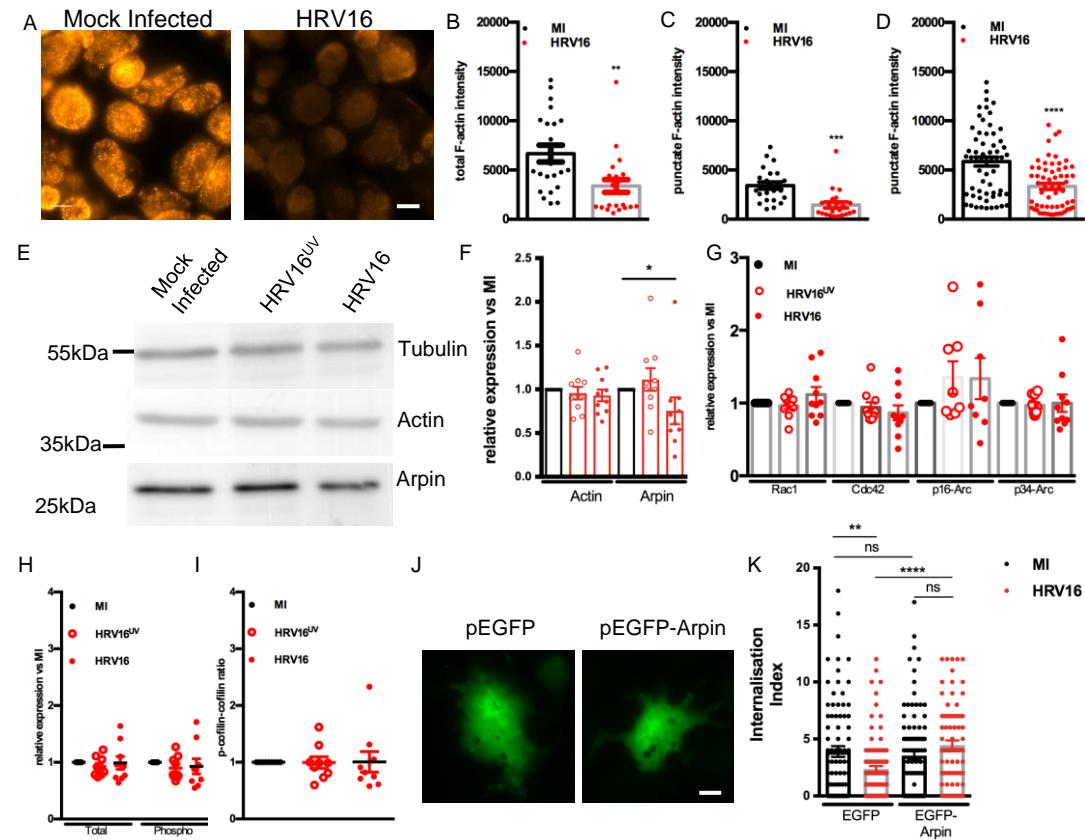


Figure 4

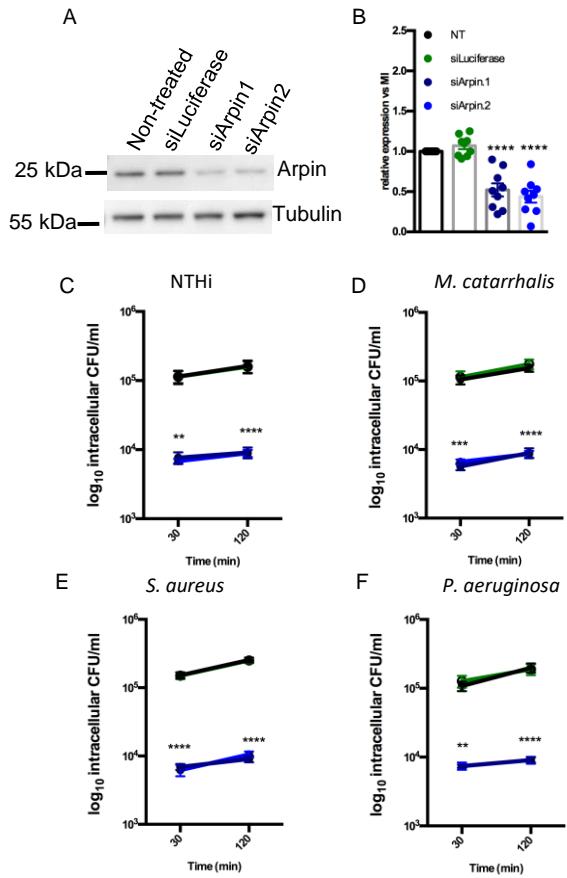


Figure 5

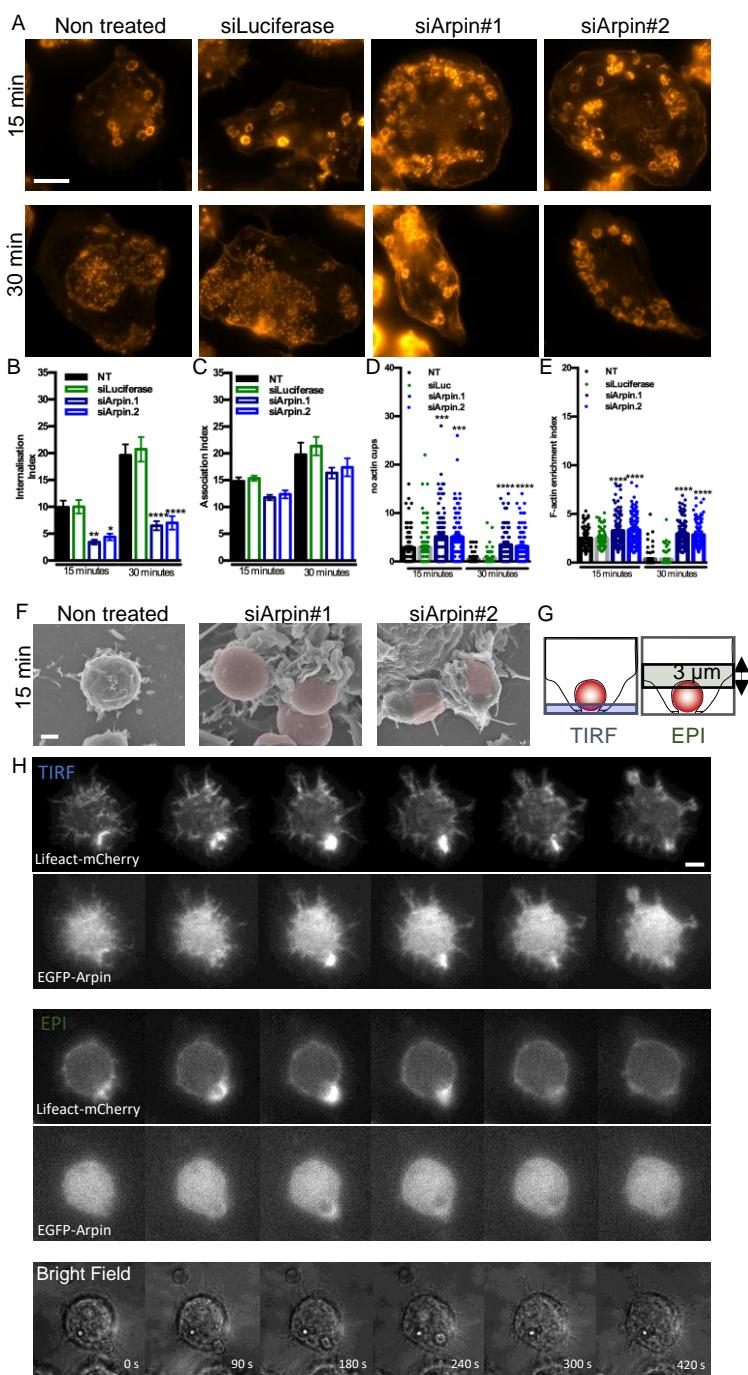


Figure 6

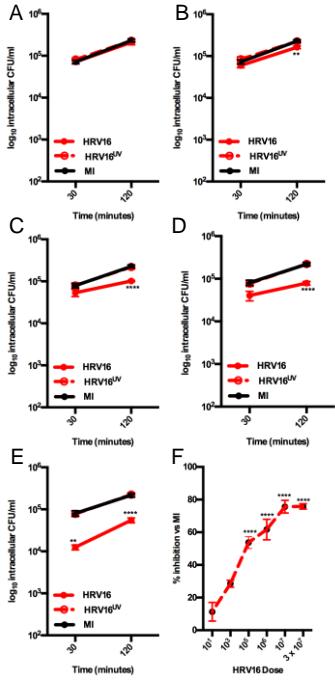


Figure EV1

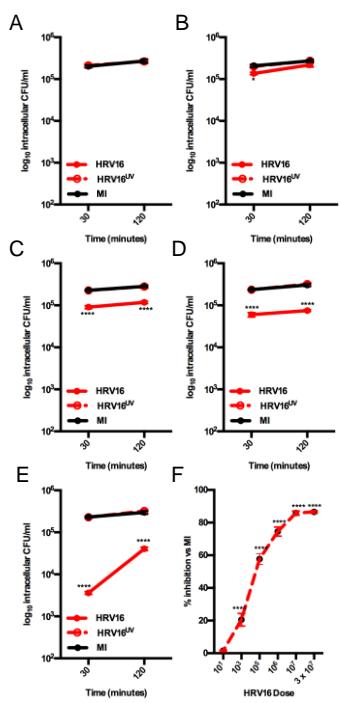


Figure EV2

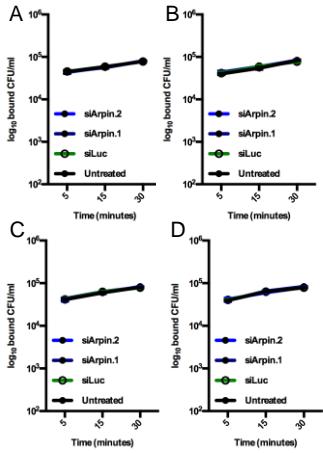


Figure EV3

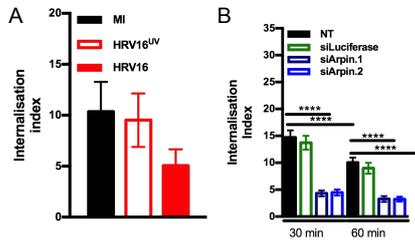


Figure EV4

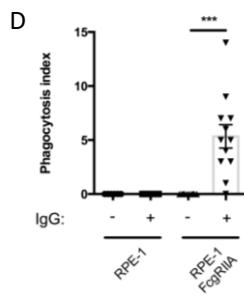
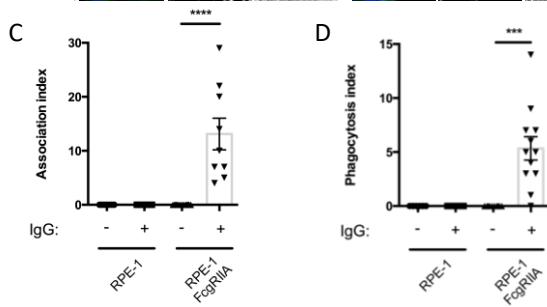
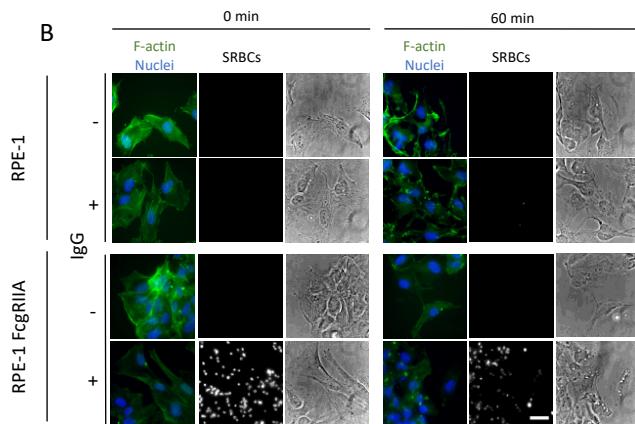
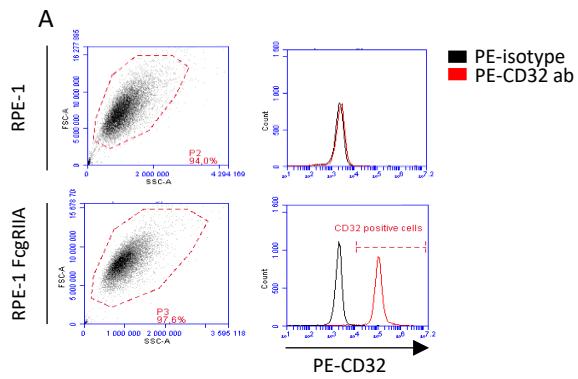


Figure EV5