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1 **Title Page:**

2 **Title:**

3 Efferocytosis of apoptotic HPV positive cervical cancer cells by human primary fibroblasts

4

5 **Short Title:**

6 Clearance of apoptotic cancer cells by fibroblasts

7

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22

23 **Abstract:**

24 Efficient clearance of dying cells is a fundamental physiological process for tissue development and
25 homeostasis. The contribution of amateur phagocytes like fibroblasts to apoptotic cell efferocytosis has been
26 established though the underlying mechanisms are not well understood. We recently demonstrated that
27 horizontal DNA transfer can occur through the uptake of apoptotic HPV positive cancer cells by human primary
28 fibroblasts (HPFs) leading to their transformation. In the present study, by using *in vitro* co-culture approaches,
29 we confirm the role of HPFs as non-professional phagocytes in the context of apoptotic cervical cancer cell
30 removal. Confocal microscopy imaging and flow cytometry analyses revealed that HPFs engulf more efficiently
31 late apoptotic cells than early apoptotic targets, but their phagocytic ability remains limited compared to
32 professional phagocytes as human monocytes-derived macrophages. Efferocytosis by HPFs occurs in a time-,
33 temperature- and calcium-dependent manner. Remodeling of actin-fibers contributes to the biogenesis of
34 macroendocytic vacuoles. Both morphological analyses and pharmacological approaches confirmed the
35 involvement of actin driven-phagocytosis and likely -macropinocytosis in apoptotic target internalization by
36 HPFs. The uptake of apoptotic cell requires phosphatidylserine recognition. In HPFs, immunofluorescence and
37 functional experiments using specific RNA interference assay suggested that this recognition is mainly mediated
38 by Brain-specific Angiogenesis Inhibitor-1 (BAI1) and that this receptor contributes to subsequent uptake
39 orchestration. Confocal microscopy analyses with organelle-specific markers revealed that internalized apoptotic
40 material traffics into late phagolysosomes and specific-features of Microtubule-associated protein 1 Light Chain
41 3 alpha (LC3)-associated phagocytosis are observed. We conclude that efferocytosis by HPFs is mediated by
42 phagocytosis/macropinocytosis involving phosphatidylserine recognition likely by the phosphatidylserine
43 receptor BAI1.

44

50 **Introduction:**

51 Clearance of apoptotic cells (AC), so-called efferocytosis, largely contributes to tissue homeostasis *in*
52 *vivo*. Although this fundamental physiological process is a property of most cell types [1-6], macrophages are
53 the main and most effective phagocytes for the removal of AC. They have been widely used to investigate the
54 molecular mechanisms underlying recognition, uptake, and degradation of AC [1, 2, 4, 6-10].

55 The recognition of AC by macrophages involves numerous receptors expressed on macrophage cell
56 surface that bind their specific ligands exposed on AC and referred to as "Eat me" signals [1, 2, 6, 11, 12]. To
57 date, various "Eat me" signals have been described including altered sugars, lipids and proteins [6] but
58 Phosphatidylserine (PtdSer) is the best-studied [13-17]. Previous publications support the importance of PtdSer
59 receptors, including T cell Immunoglobulin-and-mucin domain Molecule (TIM), Brain-specific Angiogenesis
60 Inhibitor 1 (BAI1), and stabilin-2, in the clearance of AC through the direct recognition of externalized PtdSer
61 on the AC surface [18-23]. TIM-4, highly expressed by macrophage cell lines, is a small transmembrane protein
62 that binds PtdSer *via* its immunoglobulin domain [19, 24]. Bone marrow-derived macrophages from TIM-4^{-/-}
63 mice were recently reported to have a highly reduced ability to engulf apoptotic bodies or PtdSer-bearing targets
64 [25, 26]. Such observations suggest that TIM-4 is the primary receptor for PtdSer in those professional
65 phagocytosis models. BAI1, another PtdSer receptor, is a seven transmembrane receptor that belongs to a
66 subfamily of adhesion G-protein-coupled receptors [27]. The first recognized function of BAI1 was its ability to
67 inhibit angiogenesis and tumor formation through the proteolytic processing of its N-terminal domain into
68 secreted vacuolostatins [28-30]. BAI1 is expressed in macrophage cell lines and immunological tissues such as
69 the bone marrow and spleen and is known to contribute to AC phagocytosis and myoblast fusion *via* its
70 thrombospondin (TSP) type 1 repeats [21, 31]. Other receptors [e.g. vitronectin receptor ($\alpha v\beta 3$ integrin), Mer
71 tyrosine kinase family (MerTK, Tyro3 and Axl)] are involved in indirect recognition of PtdSer and require
72 soluble bridging proteins [e.g. TSP 1 or Milk Fat Globule-EGF factor 8 (MFG-E8), Gas6 or protein S] [6].

73 Engagement of the PtdSer receptors initiates signaling events within the phagocyte. The Rho-family
74 GTPases are prominent players in the transmission and integration of signals and activate actin-related protein-
75 2/3 (Arp2/3) complex, formins and WH2 domain-containing nucleators [32-34]. This leads to actin cytoskeleton
76 remodeling, engulfment of dead cells into macroendocytic vacuole [1, 21, 35].

77 Regarding the internalization of dead cells by professional phagocytes, different mechanisms are now
78 being appreciated, even if information about their target cell specificity (apoptosis and necrosis) remains sparse

79 (reviewed in Ref. [36]). Indeed, several observations indicate that the interaction of macrophages with dead cells
80 initiates internalization of the targets, and that internalization can be preceded by phagocytosis "zipper"-like
81 mechanism (i.e. related to a formation of a close-fitting phagocytic cups) or macropinocytotic mechanism (i.e.
82 related to a formation of spacious macropinosomes accompanied by ruffling of the ingesting macrophages).

83 During macroendocytic maturation, their membrane fuses with intracellular organelles including early
84 endosomes, late endosomes and lysosomes [37-39]. Recently, Martinez and colleagues highlighted that efficient
85 clearance of AC by macrophages involves the recruitment and lipidation of Microtubule-associated protein 1
86 light chain 3 alpha (LC3) to the AC-containing phagosome membrane followed by an acidification of the
87 phagocytic vacuole [25]. Authors suggested that during this process named LC3-associated phagocytosis (LAP),
88 LC3 facilitates the phagosome maturation and the degradation of cellular corpses.

89 AC clearance by non-specialized phagocytic cells has been investigated in a limited number of studies
90 [40-45], the results of which suggest that mechanisms underlying prey cell recognition and clearance may differ
91 from those observed in professional phagocytes [1, 2, 5, 11].

92 Through the endocytic process, AC can act as a DNA vector that may confer a selective advantage to
93 the recipient cell [46, 47]. In a previous study, we provided evidence of an engulfment of apoptotic human
94 cervical cancer-derived cells by human primary fibroblasts (HPFs) [48]. Apoptotic HPV-positive cells ensured a
95 horizontal transfer of oncogenes to HPFs revealed by *in situ* hybridization and PCR analyses. HPFs subsequently
96 expressed HPV E6 mRNA and exhibited transformed cell properties such as increased proliferation, anchorage-
97 independent growth capacity and aneuploidy. The results highlighted an alternative mechanism of HPV-
98 associated carcinogenesis from mesenchymal cells that are not natural targets of HPV [48-50].

99 In the present study, we have analyzed mechanisms that drive non-professional phagocytes, namely
100 HPFs, to clear apoptotic HPV-positive cervical cancer cells. By using flow cytometry, confocal imaging and
101 functional studies, we show that HPFs are able to phagocytize dead tumor cell corpses by recognizing and
102 binding PtdSer thanks to the specific receptor BAI1 expressed by HFP, engaging actin-fibers remodeling to the
103 macroendocytic vacuole formation and translocating LC3 for its maturation.

104

105 **Results:**

106 **Characterization of apoptotic cell death**

107 The apoptosis of cervical carcinoma donor cells was induced by ultra-violet (UV)-B exposure followed
108 by staurosporine (STS) treatment for 6 h and 48 h as previously described [48]. To ensure that only dying and
109 dead cells were used for endocytosis assays, sloughed cells were collected and characterized by apoptosis assays.
110 By contrast, viable HeLa and Ca Ski used as untreated cell controls were harvested by trypsinisation. As shown
111 by Sub-G1 analysis, the percentage of cells with fragmented DNA increased in a time-dependent manner; no
112 obvious feature of apoptosis or hypodiploid cells was detected in untreated cells (Figure 1A). Apoptosis was
113 further confirmed by specific nuclear morphology changes evidenced by DAPI staining (Figure 1B). Flow
114 cytometry analysis demonstrates that treatment for 6 h resulted in an increase of early apoptotic cells (EAC)
115 defined as annexin V single-positive ($74.2\% \pm 3.8$ for HeLa; $80.8\% \pm 1.0$ for Ca Ski) and treatment for 48 h
116 resulted in a high rate of late apoptotic cells (LAC) defined as annexin V and PI double-positive ($97.0\% \pm 1.9$ for
117 HeLa; $88.6\% \pm 3.9$ for Ca Ski) (Figure 1C).

118 **Late apoptotic cells are preferentially engulfed by HPFs**

119 To establish the extent to which HPFs could engulf apoptotic cervical carcinoma HeLa and Ca Ski cells
120 regardless of various stages of apoptosis, HPFs were co-cultured with PKH67-labeled EAC or LAC, washed
121 twice to remove adherent cells, fixed in PFA, and labeled with phalloidin-TRITC and DAPI. Based on confocal
122 microscopy analysis, unloaded HPFs could be clearly distinguished from HPFs having ingested early or late
123 PKH67-labeled apoptotic HeLa or Ca Ski cells. Figure 2A is representative of confocal microscopy images of
124 co-culture. It has been established that late apoptotic/necrotic cells induce phagocyte activation, whereas early
125 apoptotic cells do not [3]. As might be expected, the engulfment of EAC analyzed by confocal microscopy was
126 much less efficient than that of LAC (Figure 2A and B). Indeed, although EAC and LAC were co-cultured with
127 HPFs at similar ratio (10:1), most of EAC were released following washing and the remaining cells mostly
128 displayed cell-cell contact but were not phagocytosed as shown in Figure 2A, left panel. The 6 h co-culture
129 duration was chosen for further experiments in order to maintain transient nature of the early apoptotic signature
130 throughout period of co-culture and to avoid appearance of LAC [51]. LAC were mostly engulfed by HPFs, as
131 soon as 2 h of co-culture (Figure 2B), as demonstrated by the presence of scattered yellow/green AC within the
132 red HPFs (Figure 2A, middle panel). This result demonstrates that cell fragments (1 to 10 μm in diameter) were

133 unequivocally inside the HPFs. Moreover, the human monocytes-derived macrophages ($M\phi$), serving as
134 professional phagocyte controls, exhibited their high capacity for the uptake of LAC compared to HPFs (Figure
135 2A, right panel).

136 Using the quantitative flow cytometry approach, we assessed the percentage of PKH26-stained HPFs
137 that engulfed PKH67-stained apoptotic cancer cells at early time periods (2 h and 6 h) of co-culture. As shown
138 by the appearance of PKH67- and PKH26 double- positive populations (Figure 2C), the cytometry analyses
139 confirmed the rate of AC uptake by HPFs observed by microscopy (Figure 2C and D). Taken together, these
140 results show that HPFs clearly distinguished EAC and LAC and that their phagocytic capacity was limited to
141 apoptotic subcellular fragments (LAC), which is consistent with their non-professional nature [40].

142 We next studied the kinetics of AC internalization by HPFs and $M\phi$ by varying the exposure time (2 -
143 72 h) of LAC to the phagocytic cells. Again, the phagocytic capacity was defined as the percentage of HPFs and
144 $M\phi$ that engulf LAC. Figure 2E indicates that HPFs and $M\phi$ reached their maximum phagocytic capacity at 24 h
145 post-exposure. Nevertheless, the percentage of phagocytosing HPFs was less compared to $M\phi$ (Figure 2E). In
146 total HPFs engulfed AC with a lower efficiency than $M\phi$, an observation that further substantiates their amateur
147 phagocyte nature [52].

148 Moreover, we studied in detail morphological aspects of dead cell engulfment by HPFs using confocal
149 microscopy (Figure 2F). The reorganization of the actin cytoskeleton observed during internalization of double-
150 PKH67/DAPI-labeled LAC suggests both phagocytosis and macropinocytotic process. During the initial step of
151 endocytosis, LAC bound to the plasma membrane. Actin polymerization was localized underneath the plasma
152 membrane in contact with LAC to be ingested, and dense network of actin filaments appeared in this region
153 (Figure 2Fa). Then membranous expansions extended around the dead cell (Figure 2Fb) to ultimately engulf it
154 (Figure 2Fd). Macropinocytosis-like movements were also observed (Figure 2Fc). The ingested LAC was
155 completely enclosed into HPFs within 6 h, in a membrane-bound vacuole (Figure 2Fe and f).

156 In addition, the monitoring of AC clearance was evaluated by position-referenced microscopy as we
157 published [53]. By tracking the fluorescent dyes during co-culture between PKH26-labeled HPFs and CFSE-
158 labeled HeLa LAC at different time periods, we observed that apoptotic corpses quickly adhered (1 h) to the
159 plasma membrane of HPFs and were efficiently internalized by HPFs within 6 h after first tethering (Figure 2G
160 and Figure S1). These time-lapse events have been already described for other non-professional phagocytes [42,
161 52].

162

163 **Clearance of LAC by HPFs is inhibited by low temperature incubation, EDTA, cytochalasin D and**
164 **dimethylamiloride**

165 The surface morphology as visualized by confocal microscopy revealed dynamic actin-based membrane
166 protrusions of HPFs that induce phagocytic cup formation during the process of AC internalization. To better
167 understand the type of endocytosis used by HPFs to engulf AC, we tested physical and chemical inhibitors.
168 When recipient cells were co-incubated with HeLa and Ca Ski AC at 4°C or in the presence of 2 mM EDTA, the
169 percentage of internalization significantly dropped to 2% suggesting that internalization requires energy and
170 divalent ions (Ca^{2+} , Mg^{2+}) (Figure 3A). Cytochalasin D (a F-actin depolymerizing agent) or dimethylamiloride
171 (DMA; an inhibitor of Na^+/H^+ exchange that prevents membrane ruffling and macropinocytosis) have been used
172 to treat HPFs prior to the addition of LAC. These treatments resulted in significant decreases of LAC uptake by
173 HPFs in a dose-dependent manner, suggesting the involvement of phagocytosis and/or macropinocytosis in the
174 internalization process (Figure 3A). A partial inhibition of LAC uptake was seen using chlorpromazine (an
175 inhibitor of clathrin-dependent endocytosis) at a concentration of 20 μM . By contrast nystatin (an inhibitor of
176 caveolin-dependent endocytosis) had no effect on the uptake of LAC whatever the concentration used (Figure
177 3A). Taken together, these data suggest the main contribution of phagocytosis and macropinocytosis in AC
178 clearance.

179 It is noteworthy that 6 h treatment with high concentrations of cytochalasin D (> 4 μM), DMA (> 80
180 μM), chlorpromazine (> 20 μM) and nystatin (> 20 μM) interfered with HPFs viability, as monitored by MTT
181 test (Figure S2), and were not considered for internalization assays.

182 **Efferocytosis of LAC by HPFs is partially mediated by phosphatidylserine and BAI1**

183 Phosphatidylserine (PtdSer) exposure is widely considered as a canonical marker of apoptosis. The
184 contribution of PtdSer recognition by HPFs was evaluated by measuring the ability of HPFs to engulf PKH67-
185 AC incubated with an excess of annexin V. Pretreatment with 40 and 160 $\mu\text{g}/\text{mL}$ of annexin V efficiently block
186 PtdSer recognition (Figure 3B) and resulted in a dose-dependent decrease of LAC uptake by HPFs (Figure 3C).
187 Four main PtdSer receptors have been shown to be involved in the uptake of AC by professional phagocytes:
188 BAI1, TIM-1, TIM-4 and Stabiline 2 [1, 5]. Among these 4 different PtdSer receptors, we noted that 2 were
189 expressed by $\text{M}\phi$, namely BAI1 and TIM-4 as shown in RT-qPCR analysis (Figure 3D). The receptors involved
190 in endocytosis of AC by amateur phagocytes have not been clearly identified. Here, HPFs expressed only *BAI1*
191 that displayed lower levels of mRNA compared to $\text{M}\phi$ (Figure 3D). Moreover, immuno-localization studies with

192 the H270 antibody indicated that BAI1 was not uniformly distributed but showed a variable and patchy
193 membrane staining in HPFs, as well as in M ϕ , in absence of apoptotic cells (Figure 3E). During co-culture
194 between HPFs and AC, BAI1 showed a tendency to accumulate within the phagocytic cup (Figure 3F). We
195 raised the hypothesis that fibroblastic BAI1 may subserve an engulfment function of apoptotic corpses. To
196 assess the function of BAI1 in this system we performed short interference RNA (siRNA) transfection in HPFs
197 prior to co-culture as described in detail in material and methods. This resulted in a significant decrease of
198 human BAI1 mRNA levels (Figure 3G). Compared to siRNA negative control, HPFs transfected with BAI1
199 siRNA demonstrated a lower ability to internalize PKH67-labeled LAC, either HeLa or Ca Ski LAC (Figure
200 3H). Thus we believe BAI1 to be a surrogate molecule to engulf apoptotic targets.

201 **Recruitment of LC3 onto the phagocytic vacuole occurs after LAC efferocytosis by HPFs**

202 Some components of the autophagy machinery being involved in the maturation of macroendocytic
203 vacuoles [25, 54, 55], we further sought to assess the recruitment of LC3 and LAMP-1 to macroendocytic
204 vacuoles after AC engulfment by HPFs. Western blotting analyses revealed that HPFs co-cultured with LAC
205 contained higher levels of the lipidated LC3 form (LC3-II) compared to control cells suggesting that LAC
206 uptake led to LC3-II conversion in HPFs (Figure 4A). Confocal microscopy analyses of LC3 immunostaining
207 after cell fixation showed that LC3 could be recruited to AC-containing vacuole (Figure 4B). Thus, LC3-II
208 conversion after AC uptake might be at least partly associated to the direct LC3 lipidation onto macroendocytic
209 vacuole. Taken together, these results indicate that AC uptake by HPFs can initiate LC3-associated phagocytosis
210 as previously described in macrophages [25, 54, 55]. Furthermore, a positive staining of some macroendocytic
211 vacuoles with Lysosomal-associated membrane protein 1 (LAMP-1), a classical marker of lysosomal
212 compartments [56, 57], suggest a fusion of AC-containing vacuoles with lysosomes (Figure 4B). Further work is
213 necessary to establish fully the significance of these observations.

214

215 **Materials and Methods:**

216 **Cell culture**

217 HeLa (HPV18 DNA-positive) and Ca Ski (HPV16 DNA-positive) cervical cancer cell lines were
218 obtained from American Type Culture Collection (Rockville, MD, USA) and used to generate early (EAC) or
219 late (LAC) apoptotic cells. They were maintained as previously described [58].

220 Human primary fibroblasts (HPFs) were isolated from surgical residues which are not subject to
221 validation from an ethics committee and patient's consent in accordance with the law L.1245-2 of the “Code de
222 la santé publique” applied in France. However, the laboratory that provided HPFs has documents stating the
223 patient's non-opposition to the use of surgical residues for medical research in accordance with the law L.1211-2.
224 HPFs were grown at 37°C (5% CO₂) in complete DMEM L-glutamine (Lonza, Basel, Switzerland)
225 supplemented with 10% fetal bovine serum (FBS; Lonza) and 10 × 10⁴ U/L penicillin/streptomycin (Lonza).

226 Human monocytes were isolated and purified from peripheral blood mononuclear cells (PBMC) of
227 buffy coat from healthy volunteers (Etablissement Français du Sang) using Ficoll-Hypaque (GE Healthcare,
228 Orsay, France). After isolation from PBMC using a CD14⁺ selection kit (Miltenyi Biotec, Paris, France),
229 monocytes were differentiated into human monocyte-derived macrophages (Mφ) by culturing cells at 1 × 10⁶
230 cells/mL in a 24-well plate for 7 days in RPMI (Gibco, Life Technologies) supplemented with 10% FBS (Gibco)
231 and 50 ng/mL macrophage colony-stimulating factor (M-CSF; Miltenyi Biotec). Non-adherent cells were
232 removed by washing prior experiments.

233 Cells were routinely checked for mycoplasma contamination.

234 **Generation of apoptotic cells**

235 Twenty-four hours prior to apoptosis induction, cancer cells were seeded at 5 × 10⁴ cells/cm² and
236 apoptosis was induced with UV-B irradiation (UV cross-linker, Ultra-Violet Products Ltd., Cambridge, UK) (20
237 mJ/cm²) followed by 300 nM staurosporine (STS; Sigma-Aldrich, St. Quentin Fallavier, France) treatment for
238 the indicated time periods. Cells (viable and apoptotic) were then washed twice thoroughly with Phosphate
239 Buffered Saline (PBS; Lonza) before apoptosis assays and endocytosis assays described thereafter.

240 **Confocal microscopy**

241 *Apoptosis detection.* For imaging analysis, apoptotic cells were dropped on polysine® microscope
242 slides (ThermoScientific, Illkirch, France), washed with PBS and fixed with 3.7% (w/v) paraformaldehyde

243 (PFA; Sigma-Aldrich) for 10 min at room temperature (RT). DNA from apoptotic cells was stained with 300 nM
244 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen, Life Technologies, St. Aubin, France) for 5
245 min at RT. After an ultimate washing with PBS, glass coverslips of 1.9 cm² (Dutscher, Brumath, France) were
246 mounted on slides in Vectashield Mounting Media for Fluorescence (VectorLabs, Burlingame, California,
247 USA). Cells were finally examined by fluorescent confocal microscopy using the Olympus FluoView 1000
248 (Olympus, Tokyo, Japan) with appropriate filter settings and 40X/1.0 or 100X/1.0 numeric aperture oil
249 objectives. Images were captured and data were generated using the Olympus FluoView10-ASW (Version 4.2)
250 software.

251 *Endocytosis assay.* HeLa and Ca Ski cells were fluorescently labeled with PKH67 (green) prior to
252 apoptosis induction according to the manufacturer's instructions (PKH67-Fluorescent Cell Linker Kits for
253 General Cell Membrane Labeling; Sigma-Aldrich). In brief, 10⁷ HeLa or Ca Ski cells/mL were washed in serum-
254 free culture medium and resuspended in 2 mL PKH67-containing Diluent C (2 × 10⁻⁶ M) for 4 min at RT. Non-
255 labeled HPFs and Mφ (10⁴ and 10⁵ cells/cm² respectively) were seeded on coverslips of 1.9 cm² in a 24-well
256 plate. Then PKH67-labeled apoptotic cells were co-cultured with HPFs or Mφ at a 10:1 ratio for different time
257 periods at 37°C in 500 μL DMEM according to our previous data [48]. Coverslips were washed twice with PBS
258 to remove the non-ingested apoptotic cells. HPFs or Mφ were fixed with 3.7% (w/v) PFA. F-actin was stained
259 with phalloidin-tetramethylrhodamine-isothiocyanate (TRITC) (Sigma-Aldrich) (2.5 × 10⁻² g/L) for 30 min at
260 4°C and DNA was stained with 300 nM DAPI for 5 min at RT. Cells were finally examined and images captured
261 as described above. For each condition, more than 200 HPFs or Mφ were randomly observed and scored by two
262 independent blinded observers.

263 Uptake of apoptotic cells by HPFs was also investigated by position-referenced microscopy to track the
264 fate of HPFs exposed to apoptotic cells, as we previously described in detail [53]. Briefly, (5-(and 6-)-
265 carboxyfluoresceine diacetate succinimidyl ester- (CFDA, SE; Invitrogen) labeled apoptotic cells were incubated
266 with PKH26-labeled HPFs for 11 h with an observation every hour. Images recorded at different instants in time
267 are superimposed in a common coordinate system with subpixel accuracy.

268 *Fluorescent staining.* Fluorescence analyses were performed as described above using Cell Proliferation
269 Dye eFluor® 450 (eBioscience, San Diego, CA, USA), phalloidin-TRITC, Rabbit monoclonal anti-BAI1 [SC-
270 66815 _ Anti-Mouse, Rat, Human, BAI-1 (H270), Santa Cruz Biotechnology, Inc., Dallas, Texas, USA], rabbit
271 polyclonal anti-LC3A/B (#4108, Cell Signalling Technology, Danvers, MA, USA), mouse anti-LAMP1
272 (CD107a, BD Pharmingen, BD Biosciences, Le Pont de Claix, France) primary antibodies, and DAPI according

273 to the manufacturer's recommendations. For immunostaining, primary antibodies and goat-anti-rabbit-Alexa488-
274 and donkey-anti-mouse-CyTM3- (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA)
275 conjugated secondary antibodies were used diluted at 1:100 and 1:250 respectively.

276 **Flow cytometry**

277 *Apoptosis detection.* For sub-G1 analysis, cells were fixed overnight in ice-cold 70% ethanol. After
278 washing with PBS, cells were incubated with propidium iodide (PI; BD Pharmingen) (10 µg/mL) in PBS
279 supplemented with DNase-free RNase A (Thermo Fisher Scientific, Waltham, USA) (1 mg/mL) in darkness for
280 15 min at RT. The intracellular PI fluorescence intensities of 20,000 cells were measured for each sample using
281 the FC500 flow cytometer (Beckman Coulter, Orange County, CA, USA). The cytometry analyses were
282 performed using CXP software (Beckman Coulter). For the annexin V/PI apoptosis assay, the cells were stained
283 with fluorescein isothiocyanate (FITC) annexin V from Apoptosis Detection Kit I (BD Pharmingen) and PI in
284 darkness for 15 min at RT, resuspended in binding buffer and analyzed by flow cytometry according to the
285 manufacturer's instructions.

286 *Endocytosis assay.* HeLa and Ca Ski cells were fluorescently labeled with PKH67 (green) prior to
287 apoptosis and HPFs were labeled with PKH26 (red) according to the manufacturer's instructions (PKH26-
288 Fluorescent Cell Linker Kits for General Cell Membrane Labeling; Sigma-Aldrich). HPFs were seeded at 10⁴
289 cells/cm² in a 24-well plate. Then PKH67-labeled HeLa or Ca Ski were co-cultured with 20,000 PKH26-labeled
290 adherent HPFs at a 10:1 ratio for different time periods in the conditions described previously [48]. HPFs
291 incubated with apoptotic cells were harvested by trypsinization and engulfment was assayed using 2-color flow
292 cytometry. The percentage of PKH26-positive cells stained positive for PKH67 was considered as the proportion
293 of HPFs having ingested apoptotic cells. Appropriate controls were performed to set the cytometer for each
294 color. When indicated, culture or co-culture conditions were modified prior to endocytosis assays. A PtdSer
295 masking assay was performed to assess the contribution of the PtdSer exposed by LAC in their uptake by HPFs.
296 Briefly, late apoptotic cells (LAC) were pre-incubated in binding buffer with 40 or 160 µg/mL unlabeled
297 purified recombinant annexin V (BD Pharmingen) at RT for 30 min, before being used in flow cytometry
298 endocytosis assays. To quantify the saturation rate of annexin V specific binding sites, a fraction of pre-
299 incubated cells were incubated with phycoerythrin (PE) annexin V (BD Pharmingen) at RT for 30 min. Titration
300 of blocked annexin V binding sites was performed by flow cytometry analysis. HPFs were pre-incubated with
301 Ethylene diamine tetraacetic acid (EDTA) disodium salt (Lonza) (up to 2 mM), cytochalasin D (Sigma-Aldrich)
302 (up to 4 µM), 5-(N,N-dimethyl)amiloride hydrochloride (DMA; Sigma-Aldrich) (up to 80 µM), chlorpromazine

303 (Sigma-Aldrich) (up to 20 μ M) and nystatin (Sigma-Aldrich) (up to 20 μ M) at 37°C for 1 h before performing
304 flow cytometry endocytosis assays at 37°C [59]. In additional experiments, HPFs were co-incubated with
305 apoptotic targets at 4°C. Moreover, BAI1 siRNA (On-TargetPlus SMART pool; Dharmacon, Lafayette, CO,
306 USA) and siRNA negative control (Eurogentec, Angers, France) were introduced into HPFs by transfection
307 (Lipofectamine® 2000; Life Technologies) 48 h before exposure to LAC. In brief, 50 μ M siRNA were added to
308 2×10^4 cells incubated in Opti-MEM (Life Technologies). RNA was prepared to assess the level of BAI1
309 expression by Real-time RT-PCR (RT-qPCR) analysis.

310 **RNA isolation and real-time RT-PCR analysis**

311 RNA was extracted from 10^6 cells using the QIAamp® RNA Blood Mini Kit (Qiagen, Courtaboeuf,
312 France) according to the manufacturer's recommendations. Contaminant genomic DNA was removed by a
313 treatment with 0.2 U/ μ L of DNase I Amplification Grade (Invitrogen, Cergy Pontoise, France) before reverse
314 transcription with 500 ng of RNA using the SuperscriptVILO cDNA Synthesis Kit (Life Technologies).

315 Quantitation of BAI1, TIM-4, and human β -2-microglobulin (B2M) transcripts was performed with a
316 7500 Real Time PCR System (Applied Biosystems, Life Technologies) in the 1X TaqMan Gene Expression
317 Master Mix (Applied Biosystems), using respectively the Hs01105174_m1 and Hs00293316_m1 Assays-on-
318 Demand™ (Applied Biosystems) or 500 nM of each primer (forward primer:
319 GATGAGTATGCCTGCCGTGTG; reverse primer: CAATCCAAATGCGGCATCT, Eurogentec) and 200 nM
320 of probe (6-FAM-CCT-CCA-TGA-TGC-TGC-TTA-CAT-GTC-TCG-ITC-CC-BHQ-1, Eurogentec) targeting
321 the B2M. RT-qPCR were performed in 20 μ L of a reaction mixture containing 4 μ L of diluted cDNA (1:5) with
322 45 amplification cycles (95°C for 15 sec, 60°C for 1 min). Relative quantitation of mRNA was performed
323 according to the $2^{-\Delta\Delta C_t}$ method algorithm with B2M serving as housekeeping control and 5 serial dilutions (1:10)
324 cDNA from M ϕ as calibrator.

325 **Western blotting**

326 Western blotting was performed as described previously [48]. Cells were collected in RIPA lysis buffer
327 (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% Na deoxycholate, 1 mM EDTA) containing
328 anti-proteases (30 μ g/mL) (Roche) and proteins were extracted from whole cells. Protein concentrations were
329 quantified using the Biorad Protein Assay® (BioRad Laboratories, Hercules, CA, USA) according to the
330 manufacturer's instructions. Thirty μ g of proteins were boiled in Laemmli's buffer (BioRad Laboratories)
331 supplemented with 5% β -mercaptoethanol and run on 15% SDS-polyacrylamide gel. Proteins were then
332 electrotransferred onto Hybond™-P PVDF membranes (Amersham Pharmacia Biotech Europe GmbH, Saclay,

333 France), probed overnight with rabbit polyclonal anti-LC3A/B (#4108) and mouse monoclonal anti- β -actin
334 (clone AC15, Sigma-Aldrich) diluted at 1:1000 or 1:10,000 respectively according to the manufacturer's
335 instructions. After washing, immune complexes were revealed with horseradish peroxidase-(HRP) conjugated-
336 goat-anti-rabbit Ig and goat-anti-mouse Ig antibodies (BD Biosciences) for 1 h diluted at 1:8000 or 1:20,000
337 respectively. The reaction was visualized using ECL reagents (Pierce ECL2 Western Blotting Substrate,
338 ThermoScientific) according to the manufacturer's instructions and images were captured with a charge-coupled
339 device (CCD) camera of the imaging system Chemidoc XRS+ (Bio-Rad Laboratories). Band density was
340 quantified by Image Lab software (Bio-Rad Laboratories) and normalized to the loading control (β -actin). The
341 resulting values from three different experiments were expressed as LC3II/LC3I ratio respect to the control value
342 and reported under the western blotting as mean values \pm standard deviation (SD).

343 **Statistical analysis**

344 Data are expressed as means \pm SD of at least nine measurements (corresponding to experiments
345 performed independently at least three times in triplicate). P values were analyzed using the Wilcoxon test.
346 Statistical analysis was done using GraphPad Prism (GraphPad Software, San Diego, CA). Significant
347 differences between the groups are indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

348 **Cell viability assay (supplementary method)**

349 HPFs were seeded in 96-well plates (3000 cells/well) with 100 μ L of complete medium/well 24 h prior
350 to EDTA or chemical inhibitors of endocytosis treatment and incubated for 6 h at 37°C. Then 10 μ L (5 mg/mL)
351 of MTT (Cell Proliferation Kit I, Roche Diagnostics, Meylan, France) solution was added to each well. After 4
352 h, 100 μ L of solubilization solution (10% SDS in 0.01 M HCl) were added to each well, and cells were
353 incubated overnight. Absorbance at 570 nm wavelength was measured with a 665 nm reference wavelength
354 using the scanning multiwell spectrofluorimeter EnVision 2012 Multilabel Reader (PerkinElmer, Courtaboeuf,
355 France).

356

357 **Discussion:**

358 *In vivo*, the rapid and efficient clearance of dying cells is fundamental for tissue development and
359 homeostasis as well as protection against chronic inflammation and autoimmunity. As typical professional
360 phagocytes, macrophages play a central role in the efferocytosis process. Over the last few decades, it has
361 become clear that epithelial cells, endothelial cells, smooth muscle cells, and fibroblasts, namely non-
362 professional or amateur phagocytes may contribute to dying cell clearance [1, 2, 40-45, 52]. With regard to
363 fibroblasts, they are known to be involved in a variety of physiological (*e.g.* deposition of extracellular matrix,
364 regulation of epithelial differentiation) or pathological processes (*e.g.* regulation of inflammation, engagement in
365 wound healing) [60-65]. They are also able to remove apoptotic corpses [41, 48, 66]. Professional phagocytes
366 possess a battery of apoptotic-cell surface ligands and intermediate molecules that can lead to uptake of
367 apoptotic cells (AC). Although amateur phagocytes like fibroblasts contribute to AC efferocytosis, mechanisms
368 are not well understood.

369 We have recently demonstrated that horizontal DNA transfer can occur through the uptake of apoptotic
370 HPV positive cancer cells by human primary fibroblasts (HPFs) leading to their transformation [48]. In the
371 current study, by using the same *in vitro* co-culture approach, we confirm that HPFs take up late apoptotic cancer
372 cells and we demonstrate that internalization depends to a significant extent on BAI1. Interestingly, HPFs
373 discriminate precisely between viable (data not shown), dying apoptotic targets (EAC) and dead cells (LAC) and
374 mainly engulf LAC and not dying whole cells in contrast to professional phagocytes as macrophages [67, 68].
375 This is in line with observations having reported that other amateur phagocytes also preferentially engulf
376 apoptotic bodies [40, 42-44, 52]. As reported earlier [43, 44, 52], the AC uptake by HPF phagocytes occurs more
377 slowly and less efficiently than by professional phagocyte macrophages (Figure 2E). These data corroborate
378 those obtained with endothelial and peritoneal mesothelial cells, as non-professional phagocytes, exhibiting a
379 limited internalization potential compared to macrophages [42-44]. In addition, the relatively long-time
380 sequential events observed using position-referenced microscopy, suggest that upon first tethering, AC uptake by
381 HPFs requires a critical extended recognition and transduction period that triggers actin cytoskeleton remodeling
382 for efferocytosis. Accordingly, this hypothesis has ever been suggested from other amateur phagocytes [42, 52].
383 To our knowledge, the molecular mechanisms underlying this delay remain elusive.

384 Confocal microscopy analyses reveal that the internalization process involves the typical sequence of
385 phagosome formation (cup-shaped invaginations of the plasma membrane, that subsequently close at their distal

386 margins to form intracellular membrane-bounded organelles) largely mediated by an active actin cytoskeleton
387 remodeling (Figure 2F) even if macropinocytosis cannot be excluded as observed by specific restructuring of the
388 actin cytoskeleton such as membrane ruffles (Figure 2Fc) [35, 69]. Indeed the partial inhibition of AC uptake by
389 dimethylamiloride (Figure 3A) that affects neither phagocytosis nor clathrin or raft-dependent endocytosis
390 suggests that macropinosomes might proceed to AC clearance which agrees with several authors [15, 68-70]. In
391 addition, the data obtained with cytochalasin D confirm the involvement of actin driven-phagocytosis and likely
392 -macropinocytosis in LAC internalization (Figure 3A). In macrophages typically driving phagocytosis of
393 apoptotic bodies, it has been demonstrated that macropinocytosis could participate in AC uptake in a PtdSer-
394 dependent manner (reviewed in Ref. [36]). Thus, in our model, two engulfment mechanisms, phagocytosis and
395 macropinocytosis, might interplay for the removal of apoptotic material. Participation of clathrin-mediated
396 endocytosis might contribute to LAC uptake as documented by the results of our chlorpromazine experiments.
397 However, the low reduction of LAC internalization might also be explained by side effects of that chemical on
398 other endocytic pathways. Indeed, chlorpromazine can interfere with the formation of phagosomes or
399 macropinosomes (reviewed in Ref. [71]), likely because of the clathrin-actin crosstalk [72].

400 The first step to remove AC requires to distinguish viable cells from dying cells. The flipping of
401 membrane lipids that occurs during early stages of apoptosis results in their exposure on the outer leaflet of the
402 plasma membrane where they can be recognized by phagocytes [14]. Here, the use of annexin V, an inhibitor for
403 PtdSer-mediated efferocytosis, demonstrates that the exposure of PtdSer on the outer leaflet of the AC-plasma
404 membrane is a key event for AC recognition and subsequent uptake by HPFs (Figure 3B,C). Nevertheless, EAC
405 which also expose PtdSer (Figure 1C) remain weakly engulfed (Figure 2A,B, C, D). It seems clear that PtdSer
406 exposure is required but not sufficient for the target cells to be engulfed by macrophages [67, 73, 74]. As
407 suggested by Borisenko *et al.*, levels of exposed PtdSer above the phagocyte sensitivity threshold might be
408 necessary to allow an efficient apoptotic cell engulfment [75]. When the plasma membrane of AC becomes
409 permeabilized (i.e., in late apoptotic cells), additional molecules are revealed to shape a new combination of ‘eat-
410 me’ and ‘don’t-eat-me’ signals [76], which might favor an optimal apoptotic cell engulfment by HPFs.

411 Our data also demonstrate that engulfment of apoptotic cancer cells is Ca²⁺-dependent, since this
412 process is inhibited following the chelation of extracellular Ca²⁺ by EDTA. They also indicate that engulfment is
413 significantly inhibited at 4°C. It is noteworthy that these two conditions were reported to inhibit AC-tethering
414 step that subsequently prevent AC uptake by macrophages [77]. Since integrins are known to be inactive at 4°C,

415 we may suggest a role for these cell adhesion receptors in the tethering of apoptotic cells by our amateur
416 phagocytes likely by the MFG-E8-TSP1- α v β 3 or α v β 5 through indirect interaction with PtdSer [41, 78, 79].
417 In 2007, TIM-4 and BAI1 have been identified in macrophages as direct phagocytic receptors for PtdSer-
418 exposing apoptotic corpses [19, 21]. Interestingly, our data demonstrate here that BAI1 receptor was expressed
419 by both HPFs and macrophages contrary to TIM-4 which expression is restricted to macrophages (Figure D).
420 Moreover, siRNA-mediated knockdown of BAI1 in our HPFs cause a significant reduction of BAI1 mRNA
421 associated with a reduction of efferocytosis ability (Figure 3H), as already shown in a model of astrocytes [21].
422 Together, the present results highlight the involvement of PtdSer recognition in AC efferocytosis and the role of
423 BAI1 as a key PtdSer receptor in our amateur phagocytes. The actin cytoskeletal remodeling observed by
424 confocal microscopy during LAC engulfment (Figure 4F) is likely the consequence of an activation of the
425 ELMO/Dock180/Rac1 pathway by interaction with the intracellular domain of BAI1 [21]. However, our
426 experiments highlight that both PtdSer-masking and BAI1 siRNA lead to partial reduction of AC clearance,
427 suggesting that recognition of apoptotic cells by HPFs may also be triggered by other molecules. One of the
428 potentially involved molecules may be the fibroblast vitronectin receptor [41]. While vitronectin receptor is
429 defined as an integrin receptor triggering thrombospondin-mediated clearance of AC [41, 80], it has also been
430 reported that this phagocytic receptor is attracted to PtdSer through indirect interactions *via* the bridge molecules
431 MFG-E8 [78, 79]. The formed protein complex has been shown to bind the ELMO/Dock180/Rac1 signaling
432 complex. Thus BAI1 and vitronectin receptor signaling seem intimately interconnected. This apparent
433 redundancy supports the idea of a complex recognition network that may explain why the identification of cell
434 surface proteins involved in the removal of apoptotic cells by non-professional phagocytes has been found to be
435 challenging.

436 Ingested cellular material can be degraded following maturation of macroendocytic vacuoles through
437 fusion of early endosomes, late endosomes and lysosomes [81]. Here, we further evidence that maturation of
438 AC-containing vacuole is associated with changes in the composition of their membranes as shown by the
439 detection of LAMP-1, a specific marker of lysosomes (Figure 4B). Interestingly, LAMP-1 colocalizes with the
440 key autophagy-related protein LC3 (Figure 4B). Florey *et al.* have recently uncovered a non-canonical activity of
441 this autophagy protein that is associated with macroendocytic engulfment [55, 82]. During this process, so-called
442 LC3-associated phagocytosis (LAP), LC3 is directly lipidated onto a variety of single-membrane compartments
443 such as AC-containing vacuoles [25, 55]. In professional phagocytes, Martinez *et al.* have suggested that direct
444 LC3 recruitment onto the phagosome membrane promotes phagosome maturation and subsequent degradation of

445 the engulfed AC [25]. In our knowledge, it is the first time that LAP is observed in amateur phagocytes.
446 However its significance in the clearance of LAC and the possible altered intracellular processing, especially in
447 the context of horizontal gene transfer, remain to be clarified. The identification of other elements of the
448 engulfment machinery expressed by HPFs and the characterization of their function in clearance of AC are
449 beyond the scope of our future investigations.

450

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458 **References:**

- 459 1. Flannagan RS, Jaumouille V, Grinstein S. The cell biology of phagocytosis. *Annu Rev Pathol.*
460 2012;7:61-98. doi: 10.1146/annurev-pathol-011811-132445. PubMed PMID: 21910624.
- 461 2. Underhill DM, Goodridge HS. Information processing during phagocytosis. *Nat Rev Immunol.*
462 2012;12(7):492-502. doi: 10.1038/nri3244. PubMed PMID: 22699831.
- 463 3. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. *Nature.* 2000;407(6805):784-8.
464 doi: 10.1038/35037722. PubMed PMID: 11048729.
- 465 4. Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and
466 consequences. *J Clin Invest.* 2001;108(7):957-62. doi: 10.1172/JCI14122. PubMed PMID: 11581295; PubMed
467 Central PMCID: PMC200959.
- 468 5. Erwig LP, Henson PM. Clearance of apoptotic cells by phagocytes. *Cell Death Differ.* 2008;15(2):243-
469 50. doi: 10.1038/sj.cdd.4402184. PubMed PMID: 17571081.
- 470 6. Hochreiter-Hufford A, Ravichandran KS. Clearing the dead: apoptotic cell sensing, recognition,
471 engulfment, and digestion. *Cold Spring Harb Perspect Biol.* 2013;5(1):a008748. doi:
472 10.1101/cshperspect.a008748. PubMed PMID: 23284042; PubMed Central PMCID: PMC3579390.
- 473 7. Wermeling F, Chen Y, Pikkarainen T, Scheynius A, Winqvist O, Izui S, et al. Class A scavenger
474 receptors regulate tolerance against apoptotic cells, and autoantibodies against these receptors are predictive of
475 systemic lupus. *J Exp Med.* 2007;204(10):2259-65. doi: 10.1084/jem.20070600. PubMed PMID: 17893199;
476 PubMed Central PMCID: PMC2118440.
- 477 8. Fadok VA, Bratton DL, Guthrie L, Henson PM. Differential effects of apoptotic versus lysed cells on
478 macrophage production of cytokines: role of proteases. *J Immunol.* 2001;166(11):6847-54. PubMed PMID:
479 11359844.
- 480 9. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have
481 ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine
482 mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest.* 1998;101(4):890-8. doi: 10.1172/JCI1112.
483 PubMed PMID: 9466984; PubMed Central PMCID: PMC508637.
- 484 10. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of
485 apoptotic cells. *Nature.* 1997;390(6658):350-1. doi: 10.1038/37022. PubMed PMID: 9389474.

- 486 11. Lauber K, Blumenthal SG, Waibel M, Wesselborg S. Clearance of apoptotic cells: getting rid of the
487 corpses. *Mol Cell*. 2004;14(3):277-87. PubMed PMID: 15125832.
- 488 12. Mevorach D, Mascarenhas JO, Gershov D, Elkon KB. Complement-dependent clearance of apoptotic
489 cells by human macrophages. *J Exp Med*. 1998;188(12):2313-20. PubMed PMID: 9858517; PubMed Central
490 PMCID: PMC2212421.
- 491 13. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of phospholipid asymmetry
492 and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and
493 fibroblasts. *J Biol Chem*. 2001;276(2):1071-7. doi: 10.1074/jbc.M003649200. PubMed PMID: 10986279.
- 494 14. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of
495 phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by
496 macrophages. *J Immunol*. 1992;148(7):2207-16. PubMed PMID: 1545126.
- 497 15. Hoffmann PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, et al.
498 Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic
499 cells. *J Cell Biol*. 2001;155(4):649-59. doi: 10.1083/jcb.200108080. PubMed PMID: 11706053; PubMed Central
500 PMCID: PMC2198875.
- 501 16. Krahling S, Callahan MK, Williamson P, Schlegel RA. Exposure of phosphatidylserine is a general
502 feature in the phagocytosis of apoptotic lymphocytes by macrophages. *Cell Death Differ*. 1999;6(2):183-9. doi:
503 10.1038/sj.cdd.4400473. PubMed PMID: 10200565.
- 504 17. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in
505 recognition of apoptotic cells by phagocytes. *Cell Death Differ*. 1998;5(7):551-62. doi: 10.1038/sj.cdd.4400404.
506 PubMed PMID: 10200509.
- 507 18. Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. A receptor for
508 phosphatidylserine-specific clearance of apoptotic cells. *Nature*. 2000;405(6782):85-90. doi: 10.1038/35011084.
509 PubMed PMID: 10811223.
- 510 19. Kobayashi N, Karisola P, Pena-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, et al. TIM-1 and TIM-4
511 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity*. 2007;27(6):927-40. doi:
512 10.1016/j.immuni.2007.11.011. PubMed PMID: 18082433; PubMed Central PMCID: PMC2757006.
- 513 20. Park D, Hochreiter-Hufford A, Ravichandran KS. The phosphatidylserine receptor TIM-4 does not
514 mediate direct signaling. *Curr Biol*. 2009;19(4):346-51. doi: 10.1016/j.cub.2009.01.042. PubMed PMID:
515 19217291.

- 516 21. Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, et al. BAI1 is an engulfment
517 receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature*. 2007;450(7168):430-4. doi:
518 10.1038/nature06329. PubMed PMID: 17960134.
- 519 22. Park SY, Jung MY, Lee SJ, Kang KB, Gratchev A, Riabov V, et al. Stabilin-1 mediates
520 phosphatidylserine-dependent clearance of cell corpses in alternatively activated macrophages. *J Cell Sci*.
521 2009;122(Pt 18):3365-73. doi: 10.1242/jcs.049569. PubMed PMID: 19726632.
- 522 23. Park SY, Jung MY, Kim HJ, Lee SJ, Kim SY, Lee BH, et al. Rapid cell corpse clearance by stabilin-2, a
523 membrane phosphatidylserine receptor. *Cell Death Differ*. 2008;15(1):192-201. doi: 10.1038/sj.cdd.4402242.
524 PubMed PMID: 17962816.
- 525 24. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a
526 phosphatidylserine receptor. *Nature*. 2007;450(7168):435-9. doi: 10.1038/nature06307. PubMed PMID:
527 17960135.
- 528 25. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, et al. Microtubule-associated
529 protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells.
530 *Proc Natl Acad Sci U S A*. 2011;108(42):17396-401. doi: 10.1073/pnas.1113421108. PubMed PMID:
531 21969579; PubMed Central PMCID: PMC3198353.
- 532 26. Flannagan RS, Canton J, Furuya W, Glogauer M, Grinstein S. The phosphatidylserine receptor TIM4
533 utilizes integrins as coreceptors to effect phagocytosis. *Mol Biol Cell*. 2014;25(9):1511-22. doi:
534 10.1091/mbc.E13-04-0212. PubMed PMID: 24623723; PubMed Central PMCID: PMC4004599.
- 535 27. Stephenson JR, Purcell RH, Hall RA. The BAI subfamily of adhesion GPCRs: synaptic regulation and
536 beyond. *Trends Pharmacol Sci*. 2014;35(4):208-15. doi: 10.1016/j.tips.2014.02.002. PubMed PMID: 24642458;
537 PubMed Central PMCID: PMC4029589.
- 538 28. Cork SM, Van Meir EG. Emerging roles for the BAI1 protein family in the regulation of phagocytosis,
539 synaptogenesis, neurovasculature, and tumor development. *J Mol Med (Berl)*. 2011;89(8):743-52. doi:
540 10.1007/s00109-011-0759-x. PubMed PMID: 21509575; PubMed Central PMCID: PMC3152611.
- 541 29. Kaur B, Brat DJ, Devi NS, Van Meir EG. Vasculostatin, a proteolytic fragment of brain angiogenesis
542 inhibitor 1, is an antiangiogenic and antitumorigenic factor. *Oncogene*. 2005;24(22):3632-42. doi:
543 10.1038/sj.onc.1208317. PubMed PMID: 15782143.
- 544 30. Kaur B, Cork SM, Sandberg EM, Devi NS, Zhang Z, Klenotic PA, et al. Vasculostatin inhibits
545 intracranial glioma growth and negatively regulates in vivo angiogenesis through a CD36-dependent mechanism.

546 Cancer Res. 2009;69(3):1212-20. doi: 10.1158/0008-5472.CAN-08-1166. PubMed PMID: 19176395; PubMed
547 Central PMCID: PMC2659670.

548 31. Hochreiter-Hufford AE, Lee CS, Kinchen JM, Sokolowski JD, Arandjelovic S, Call JA, et al.
549 Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion. Nature.
550 2013;497(7448):263-7. doi: 10.1038/nature12135. PubMed PMID: 23615608; PubMed Central PMCID:
551 PMC3773542.

552 32. Kuhn S, Geyer M. Formins as effector proteins of Rho GTPases. Small GTPases. 2014;5:e29513. doi:
553 10.4161/sgtp.29513. PubMed PMID: 24914801; PubMed Central PMCID: PMC4111664.

554 33. Pollard TD. Regulation of actin filament assembly by Arp2/3 complex and formins. Annu Rev Biophys
555 Biomol Struct. 2007;36:451-77. doi: 10.1146/annurev.biophys.35.040405.101936. PubMed PMID: 17477841.

556 34. Campellone KG, Welch MD. A nucleator arms race: cellular control of actin assembly. Nat Rev Mol
557 Cell Biol. 2010;11(4):237-51. doi: 10.1038/nrm2867. PubMed PMID: 20237478; PubMed Central PMCID:
558 PMC2929822.

559 35. Swanson JA. Shaping cups into phagosomes and macropinosomes. Nat Rev Mol Cell Biol.
560 2008;9(8):639-49. doi: 10.1038/nrm2447. PubMed PMID: 18612320; PubMed Central PMCID: PMC2851551.

561 36. Krysko DV, D'Herde K, Vandenabeele P. Clearance of apoptotic and necrotic cells and its
562 immunological consequences. Apoptosis. 2006;11(10):1709-26. doi: 10.1007/s10495-006-9527-8. PubMed
563 PMID: 16951923.

564 37. Henry RM, Hoppe AD, Joshi N, Swanson JA. The uniformity of phagosome maturation in
565 macrophages. J Cell Biol. 2004;164(2):185-94. doi: 10.1083/jcb.200307080. PubMed PMID: 14718518;
566 PubMed Central PMCID: PMC2172341.

567 38. Kinchen JM, Ravichandran KS. Phagosome maturation: going through the acid test. Nat Rev Mol Cell
568 Biol. 2008;9(10):781-95. doi: 10.1038/nrm2515. PubMed PMID: 18813294; PubMed Central PMCID:
569 PMC2908392.

570 39. Rogers LD, Foster LJ. The dynamic phagosomal proteome and the contribution of the endoplasmic
571 reticulum. Proc Natl Acad Sci U S A. 2007;104(47):18520-5. doi: 10.1073/pnas.0705801104. PubMed PMID:
572 18006660; PubMed Central PMCID: PMC2141809.

573 40. Dini L, Lentini A, Diez GD, Rocha M, Falasca L, Serafino L, et al. Phagocytosis of apoptotic bodies by
574 liver endothelial cells. J Cell Sci. 1995;108 (Pt 3):967-73. PubMed PMID: 7622623.

- 575 41. Hall SE, Savill JS, Henson PM, Haslett C. Apoptotic neutrophils are phagocytosed by fibroblasts with
576 participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin. *J*
577 *Immunol.* 1994;153(7):3218-27. PubMed PMID: 7522254.
- 578 42. Monks J, Rosner D, Geske FJ, Lehman L, Hanson L, Neville MC, et al. Epithelial cells as phagocytes:
579 apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator
580 release. *Cell Death Differ.* 2005;12(2):107-14. doi: 10.1038/sj.cdd.4401517. PubMed PMID: 15647754.
- 581 43. Patel VA, Lee DJ, Feng L, Antoni A, Lieberthal W, Schwartz JH, et al. Recognition of apoptotic cells
582 by epithelial cells: conserved versus tissue-specific signaling responses. *J Biol Chem.* 2010;285(3):1829-40. doi:
583 10.1074/jbc.M109.018440. PubMed PMID: 19910463; PubMed Central PMCID: PMC2804341.
- 584 44. Wagner BJ, Lindau D, Ripper D, Stierhof YD, Glatzle J, Witte M, et al. Phagocytosis of dying tumor
585 cells by human peritoneal mesothelial cells. *J Cell Sci.* 2011;124(Pt 10):1644-54. doi: 10.1242/jcs.078907.
586 PubMed PMID: 21525033.
- 587 45. Viegas MS, Estronca LM, Vieira OV. Comparison of the kinetics of maturation of phagosomes
588 containing apoptotic cells and IgG-opsonized particles. *PLoS One.* 2012;7(10):e48391. Epub 2012/11/03. doi:
589 10.1371/journal.pone.0048391
590 PONE-D-12-19932 [pii]. PubMed PMID: 23119002; PubMed Central PMCID: PMC3485219.
- 591 46. Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL, et al. Horizontal transfer of
592 oncogenes by uptake of apoptotic bodies. *Proc Natl Acad Sci U S A.* 2001;98(11):6407-11. doi:
593 10.1073/pnas.101129998. PubMed PMID: 11353826; PubMed Central PMCID: PMC33481.
- 594 47. Ehnfors J, Kost-Alimova M, Persson NL, Bergsmedh A, Castro J, Levchenko-Tegnebratt T, et al.
595 Horizontal transfer of tumor DNA to endothelial cells in vivo. *Cell Death Differ.* 2009;16(5):749-57. doi:
596 10.1038/cdd.2009.7. PubMed PMID: 19219067.
- 597 48. Gaiffe E, Pretet JL, Launay S, Jacquin E, Saunier M, Hetzel G, et al. Apoptotic HPV positive cancer
598 cells exhibit transforming properties. *PLoS One.* 2012;7(5):e36766. doi: 10.1371/journal.pone.0036766.
599 PubMed PMID: 22574222; PubMed Central PMCID: PMC3344932.
- 600 49. Grayson W, Taylor LF, Cooper K. Carcinosarcoma of the uterine cervix: a report of eight cases with
601 immunohistochemical analysis and evaluation of human papillomavirus status. *Am J Surg Pathol.*
602 2001;25(3):338-47. PubMed PMID: 11224604.

603 50. Unger ER, Vernon SD, Hewan-Lowe KO, Lee DR, Thoms WW, Reeves WC. An unusual cervical
604 carcinoma showing exception to epitheliotropism of human papillomavirus. *Hum Pathol.* 1999;30(4):483-5.
605 PubMed PMID: 10208473.

606 51. Silva MT. Secondary necrosis: the natural outcome of the complete apoptotic program. *FEBS Lett.*
607 2010;584(22):4491-9. doi: 10.1016/j.febslet.2010.10.046. PubMed PMID: 20974143.

608 52. Parnaik R, Raff MC, Scholes J. Differences between the clearance of apoptotic cells by professional and
609 non-professional phagocytes. *Curr Biol.* 2000;10(14):857-60. PubMed PMID: 10899007.

610 53. Galeano ZJ, Sandoz P, Gaiffe E, Launay S, Robert L, Jacquot M, et al. Position-referenced microscopy
611 for live cell culture monitoring. *Biomed Opt Express.* 2011;2(5):1307-18. doi: 10.1364/BOE.2.001307. PubMed
612 PMID: 21559142; PubMed Central PMCID: PMC3087587.

613 54. Sanjuan MA, Dillon CP, Tait SW, Moshiah S, Dorsey F, Connell S, et al. Toll-like receptor signalling
614 in macrophages links the autophagy pathway to phagocytosis. *Nature.* 2007;450(7173):1253-7. doi:
615 10.1038/nature06421. PubMed PMID: 18097414.

616 55. Florey O, Kim SE, Sandoval CP, Haynes CM, Overholtzer M. Autophagy machinery mediates
617 macroendocytic processing and entotic cell death by targeting single membranes. *Nat Cell Biol.*
618 2011;13(11):1335-43. doi: 10.1038/ncb2363. PubMed PMID: 22002674; PubMed Central PMCID:
619 PMC3223412.

620 56. Alvarez-Dominguez C, Roberts R, Stahl PD. Internalized *Listeria monocytogenes* modulates
621 intracellular trafficking and delays maturation of the phagosome. *J Cell Sci.* 1997;110 (Pt 6):731-43. PubMed
622 PMID: 9099947.

623 57. Tjelle TE, Lovdal T, Berg T. Phagosome dynamics and function. *Bioessays.* 2000;22(3):255-63. doi:
624 10.1002/(SICI)1521-1878(200003)22:3<255::AID-BIES7>3.0.CO;2-R. PubMed PMID: 10684585.

625 58. Hirchaud F, Hermetet F, Ablise M, Fauconnet S, Vuitton DA, Pretet JL, et al. Isoliquritigenin induces
626 caspase-dependent apoptosis via downregulation of HPV16 E6 expression in cervical cancer Ca Ski cells. *Planta*
627 *Med.* 2013;79(17):1628-35. doi: 10.1055/s-0033-1350956. PubMed PMID: 24214831.

628 59. Ivanov AI. Pharmacological inhibition of endocytic pathways: is it specific enough to be useful?
629 *Methods Mol Biol.* 2008;440:15-33. doi: 10.1007/978-1-59745-178-9_2. PubMed PMID: 18369934.

630 60. Driskell RR, Watt FM. Understanding fibroblast heterogeneity in the skin. *Trends Cell Biol.*
631 2015;25(2):92-9. doi: 10.1016/j.tcb.2014.10.001. PubMed PMID: 25455110.

- 632 61. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006;6(5):392-401. doi:
633 10.1038/nrc1877. PubMed PMID: 16572188.
- 634 62. Parsonage G, Filer AD, Haworth O, Nash GB, Rainger GE, Salmon M, et al. A stromal address code
635 defined by fibroblasts. *Trends Immunol*. 2005;26(3):150-6. doi: 10.1016/j.it.2004.11.014. PubMed PMID:
636 15745857; PubMed Central PMCID: PMC3121558.
- 637 63. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of
638 connective tissue remodelling. *Nat Rev Mol Cell Biol*. 2002;3(5):349-63. doi: 10.1038/nrm809. PubMed PMID:
639 11988769.
- 640 64. Chiarugi P. Cancer-associated fibroblasts and macrophages: Friendly conspirators for malignancy.
641 *Oncoimmunology*. 2013;2(9):e25563. doi: 10.4161/onci.25563. PubMed PMID: 24319632; PubMed Central
642 PMCID: PMC3850271.
- 643 65. Madar S, Goldstein I, Rotter V. 'Cancer associated fibroblasts'--more than meets the eye. *Trends Mol*
644 *Med*. 2013;19(8):447-53. doi: 10.1016/j.molmed.2013.05.004. PubMed PMID: 23769623.
- 645 66. Holmgren L, Szeles A, Rajnavolgyi E, Folkman J, Klein G, Ernberg I, et al. Horizontal transfer of DNA
646 by the uptake of apoptotic bodies. *Blood*. 1999;93(11):3956-63. Epub 1999/05/26. PubMed PMID: 10339505.
- 647 67. Cocco RE, Ucker DS. Distinct modes of macrophage recognition for apoptotic and necrotic cells are not
648 specified exclusively by phosphatidylserine exposure. *Mol Biol Cell*. 2001;12(4):919-30. Epub 2001/04/11.
649 PubMed PMID: 11294896; PubMed Central PMCID: PMC32276.
- 650 68. Xu W, Roos A, Schlagwein N, Woltman AM, Daha MR, van Kooten C. IL-10-producing macrophages
651 preferentially clear early apoptotic cells. *Blood*. 2006;107(12):4930-7. Epub 2006/02/25. doi: 2005-10-4144 [pii]
652 10.1182/blood-2005-10-4144. PubMed PMID: 16497970.
- 653 69. Krysko DV, Denecker G, Festjens N, Gabriels S, Parthoens E, D'Herde K, et al. Macrophages use
654 different internalization mechanisms to clear apoptotic and necrotic cells. *Cell Death Differ*. 2006;13(12):2011-
655 22. doi: 10.1038/sj.cdd.4401900. PubMed PMID: 16628234.
- 656 70. Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, et al. C1q and
657 mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake
658 of apoptotic cells. *J Exp Med*. 2001;194(6):781-95. PubMed PMID: 11560994; PubMed Central PMCID:
659 PMC2195958.
- 660 71. Dutta D, Donaldson JG. Search for inhibitors of endocytosis: Intended specificity and unintended
661 consequences. *Cell Logist*. 2012;2(4):203-8. Epub 2013/03/30. doi: 10.4161/cl.23967

662 2012CELLULARLOG0030R [pii]. PubMed PMID: 23538558; PubMed Central PMCID: PMC3607622.

663 72. Shen Q, He B, Lu N, Conrads B, Grant BD, Zhou Z. Phagocytic receptor signaling regulates clathrin
664 and epsin-mediated cytoskeletal remodeling during apoptotic cell engulfment in *C. elegans*. *Development*.
665 2013;140(15):3230-43. Epub 2013/07/19. doi: 10.1242/dev.093732
666 140/15/3230 [pii]. PubMed PMID: 23861060; PubMed Central PMCID: PMC3931732.

667 73. Devitt A, Pierce S, Oldreive C, Shingler WH, Gregory CD. CD14-dependent clearance of apoptotic
668 cells by human macrophages: the role of phosphatidylserine. *Cell Death Differ*. 2003;10(3):371-82. Epub
669 2003/04/18. doi: 10.1038/sj.cdd.4401168
670 4401168 [pii]. PubMed PMID: 12700637.

671 74. Segawa K, Suzuki J, Nagata S. Constitutive exposure of phosphatidylserine on viable cells. *Proc Natl*
672 *Acad Sci U S A*. 2011;108(48):19246-51. Epub 2011/11/16. doi: 10.1073/pnas.1114799108
673 1114799108 [pii]. PubMed PMID: 22084121; PubMed Central PMCID: PMC3228483.

674 75. Borisenko GG, Matsura T, Liu SX, Tyurin VA, Jianfei J, Serinkan FB, et al. Macrophage recognition of
675 externalized phosphatidylserine and phagocytosis of apoptotic Jurkat cells--existence of a threshold. *Arch*
676 *Biochem Biophys*. 2003;413(1):41-52. Epub 2003/04/23. doi: S0003986103000833 [pii]. PubMed PMID:
677 12706340.

678 76. Poon IK, Hulett MD, Parish CR. Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell*
679 *Death Differ*. 2010;17(3):381-97. Epub 2009/12/19. doi: 10.1038/cdd.2009.195
680 cdd2009195 [pii]. PubMed PMID: 20019744.

681 77. Dransfield I, Zagorska A, Lew ED, Michail K, Lemke G. Mer receptor tyrosine kinase mediates both
682 tethering and phagocytosis of apoptotic cells. *Cell Death Dis*. 2015;6:e1646. Epub 2015/02/20. doi:
683 10.1038/cddis.2015.18
684 cddis201518 [pii]. PubMed PMID: 25695599.

685 78. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that
686 links apoptotic cells to phagocytes. *Nature*. 2002;417(6885):182-7. Epub 2002/05/10. doi: 10.1038/417182a
687 417182a [pii]. PubMed PMID: 12000961.

688 79. Borisenko GG, Iverson SL, Ahlberg S, Kagan VE, Fadeel B. Milk fat globule epidermal growth factor 8
689 (MFG-E8) binds to oxidized phosphatidylserine: implications for macrophage clearance of apoptotic cells. *Cell*
690 *Death Differ*. 2004;11(8):943-5. Epub 2004/03/20. doi: 10.1038/sj.cdd.4401421
691 4401421 [pii]. PubMed PMID: 15031725.

692 80. Savill J, Hogg N, Ren Y, Haslett C. Thrombospondin cooperates with CD36 and the vitronectin
693 receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest.* 1992;90(4):1513-22.
694 Epub 1992/10/01. doi: 10.1172/JCI116019. PubMed PMID: 1383273; PubMed Central PMCID: PMC443198.

695 81. Zhou Z, Yu X. Phagosome maturation during the removal of apoptotic cells: receptors lead the way.
696 *Trends Cell Biol.* 2008;18(10):474-85. Epub 2008/09/09. doi: 10.1016/j.tcb.2008.08.002
697 S0962-8924(08)00211-0 [pii]. PubMed PMID: 18774293; PubMed Central PMCID: PMC3125982.

698 82. Florey O, Overholtzer M. Autophagy proteins in macroendocytic engulfment. *Trends Cell Biol.*
699 2012;22(7):374-80. doi: 10.1016/j.tcb.2012.04.005. PubMed PMID: 22608991; PubMed Central PMCID:
700 PMC3383932.

701

702

703 **Figure Legends:**

704 **Figure 1: Generation of early and late apoptotic cancer cells.**

705 HeLa and Ca Ski cells were exposed to 20 mJ/cm² dose of UV irradiation and 300 nM staurosporine for 6 or 48
706 h. **(A)** For Sub-G1 analysis, cells were fixed, washed and incubated with PI for 15 min. The intracellular PI
707 fluorescence intensities of 20,000 cells were measured in each sample using flow cytometry. Data shown are
708 representative of 3 independent assays in triplicate. **(B)** Nuclear fragmentation was observed by fluorescent
709 microscopy after DAPI (*blue*) staining. All panels were photographed at X40 and scaled to the same size.
710 Fluorescent microscopic images are representative of 3 independent assays. Bar = 30 μm. **(C)** Early apoptosis
711 and late apoptosis were respectively assessed by a simple cell staining with annexin V and a double cell staining
712 with annexin V and PI detected by flow cytometry. The ratio of EAC and LAC (%) are indicated in the given
713 quadrant. Data shown are representative of 3 independent assays in triplicate.

714

715 **Figure 2: Uptake of apoptotic cells by HPFs occurs in a time- and stage-dependent manner.**

716 (A) The binding and uptake of PKH67-labeled HeLa EAC and LAC (*green*) by HPFs (left and middle panel
717 respectively) or M ϕ (right panel) was investigated by confocal microscopy after incubation for 6 h at 37°C. Non-
718 labeled HPFs or M ϕ were co-cultured with PKH67-labeled apoptotic cells (AC) at a phagocyte cell/target ratio
719 of 1:10, followed by washing to remove the non-ingested apoptotic cells. The nuclei and actin cytoskeletons of
720 fixed HPFs or M ϕ and apoptotic targets were respectively stained with DAPI (*blue*) and TRITC-conjugated
721 phalloidin (*red*). All panels were photographed at a X40 magnification and scaled to the same size. Fluorescent
722 microscopic images are representative of 3 independent assays. Bar = 60 μ m. (B) Based on the fluorescent
723 microscopic analyses after 2 h and 6 h of co-culture, more than 200 HPFs were scored as described in "Material
724 and Methods" to determine the percentage of engulfed HeLa AC- (left panel) or Ca Ski AC- (right panel)
725 phagocytosing (means \pm SD). (C) For analysis by flow cytometry, PKH67-labeled HeLa EAC and LAC (*green*)
726 and PKH26-labeled (*red*) adherent HPFs were cultured separately (upper row) or in a 10:1 ratio for 6 h at 37°C
727 (lower row). Flow cytometry acquisition was performed as described in "Material and Methods". Co-culture
728 assays show that HPFs became positive for PKH67. The percentage of HPFs that were phagocytosing was
729 determined as described in "Material and Methods". Representative dot plots are shown for HPFs and HeLa AC.
730 (D) As depicted in C, quantitative analyses (means \pm SD) of apoptotic HeLa cell (left panel) or Ca Ski cell (right
731 panel) phagocytosis by HPFs after 2 and 72 h of co-culture at a 10:1 ratio were performed from 3 experiments in
732 triplicate. (E) Uptake kinetics of LAC by HPFs and M ϕ were investigated by confocal microscopy starting from
733 2 h of co-culture and phagocytosis was evaluated as depicted in B. (F) The morphological analysis of the uptake
734 of HeLa LAC by HPFs was investigated by confocal microscopy as depicted in A. All panels were photographed
735 at a X60 magnification and scaled to the same size. Bar = 10 μ m. (G) To illustrate engulfment of a HeLa LAC
736 (*arrow*) by HPFs, a sequence of images were obtained by position-referenced microscopy from a co-culture
737 between CFSE-labeled (*green*) HeLa LAC and PKH26-labeled (*red*) adherent HPFs observed at time intervals
738 of one hour over 11 h. A time-lapse version of G is given in the Supplemental Figure 1.

739

740 **Figure 3: Involvement of phosphatidylserines and BAI1 receptor in the uptake of late apoptotic cells by**
741 **HPFs.**

742 **(A)** PKH26-labeled HPFs were incubated at 4°C or preincubated or not with EDTA, cytochalasin D, DMA,
743 chlorpromazine or nystatin at 37°C for 1 h before the co-culture with PKH67-labeled apoptotic cells for 6 h at
744 4°C or 37°C. Uptake was quantified by flow cytometry as depicted in Figure 2C. These data (means ± SD)
745 represent the relative uptake against the control (untreated cells). **(B)** PKH67-labeled apoptotic or viable cells
746 were preincubated with or without unlabeled annexin V at RT for 30 min before incubation with PE annexin V
747 in darkness at RT for 20 min and cytometry analysis. Data show the dose-dependent PtdSer masking of HeLa
748 (left panel) or Ca Ski (right panel) late apoptotic cells by annexin V preincubation. The graphs are representative
749 of 3 independent experiments in triplicate. **(C)** PKH67-labeled apoptotic cells were preincubated with or without
750 unlabeled annexin V at RT for 20 min before the co-culture with PKH26-labeled HPFs. Based on cytometry
751 analyses, the data (means ± SD) represent the relative uptake against the control (untreated cells) and show the
752 inhibitory effect of PtdSer masking on HeLa (left panel) or Ca Ski (right panel) apoptotic cell engulfment. **(D)**
753 The relative expression of BAI1 and TIM-4 PtdSer mRNA in HPFs and Mφ was assessed by RT-qPCR. **(E)** For
754 analysis of the subcellular localization of BAI1 by confocal microscopy, HPFs were cultured separately (upper
755 row). Mφ that demonstrate strong membranous staining for BAI1 were used as positive control cells (lower
756 row). Indirect immunofluorescence studies using anti-BAI1 antibody (H270; Alexa Fluor 488 signal, *green*),
757 TRITC-conjugated phalloidin staining of F-actin (*red*) and DAPI staining (*blue*) were performed. All panels
758 were photographed at a X40 magnification and scaled to the same size. Bar = 50 μm. **(F)** HPFs were cultured
759 with PKH67-labeled HeLa LAC (*green*) in a 1:10 ratio for 6 h at 37°C. Indirect immunofluorescence studies
760 using anti-BAI1 antibody (H270; Cy3 signal, *red*) and DAPI staining (*blue*) were performed. All panels were
761 photographed at a X60 magnification and scaled to the same size. Bar = 30 μm. **(G) (H)** PKH26-labeled HPFs
762 were transfected with BAI1 siRNA or control siRNA. Two days after siRNA transfection, **(G)** relative RNA
763 quantification of the relative BAI1 contents in siRNA-treated cells were performed, and PKH26-labeled HPFs
764 were co-cultured with late apoptotic HeLa or Ca Ski cells. **(H)** Based on cytometry analyses, the data (means ±
765 SD) represent the relative uptake of siRNA BAI1 and siRNA control transfected HPFs against the control
766 (untreated HPFs) and show that HPFs transfected with BAI1 siRNA demonstrated a lower ability to internalize
767 PKH67-labelled LAC compared to the control.

768

769 **Figure 4: The uptake of late apoptotic cells by HPFs induces LC3 lipidation onto the phagocytic vacuoles.**

770 (A) Immunoblot of LC3 and beta actin was performed to evaluate LC3 lipidation in HPFs after 24 h co-culture at
771 a ratio of 10:1 at 37°C with LAC HeLa and Ca Ski. HPFs were used as controls. The values of the densitometric
772 analysis were normalized and expressed as fold increase. (B) The colocalisation of LC3 (Alexa Fluor 488 signal,
773 *green*) and LAMP1 (Cy3 signal, *red*) to the membrane of Cell Proliferation Dye eFluor® 450-labeled (*violet*)
774 HeLa LAC -containing vacuole in HPFs after 24 h of co-culture at a ratio of 10:1 at 37°C was analyzed by
775 confocal microscopy. HPF images were captured as controls. All panels were photographed at a X40
776 magnification and scaled to the same size. Fluorescent microscopic images are representative of 3 independent
777 assays. Bar = 20 µm. (C) The graphs display the colocalisation of the fluorescence signals, given in arbitrary
778 fluorescence units (AFU), for the HeLa LAC (*violet AFU*) and the phagosome membrane proteins (LC3, *green*
779 *AFU*; LAMP1, *red AFU*). The white bars in the pictures show the localization of the cross-section corresponding
780 to the fluorescence diagram within the HPFs. Bar = 20 µm.

781

782 **Figure S1: HPF delays ingesting late apoptotic cell for hours after recognizing it.**

783 The video combines a sequence of images - obtained by confocal fluorescence microscopy and phase contrast

784 imaging - performed from a co-culture between CFSE-labeled (*green*) HeLa LAC and PKH26-labeled (*red*)

785 adherent HPFs observed at time intervals of one hour over 11 h.

786

787 **Figure S2: The drug-dependent effect on late apoptotic cell uptake by HPFs is independent of cytotoxic**
788 **effect.**

789 HPFs were treated with or without increasing concentrations of cytochalasin D, DMA, chlorpromazine or
790 nystatin at 37°C for 7 h, a time condition that mimicks 1 h of preincubation and 6 h of co-culture with apoptotic
791 cells. Based on MTT assay, these data (means \pm SD) represent the relative viability against the control (untreated
792 cells) and show that the drug-dependent effect on late apoptotic cell uptake by HPFs is independent of cytotoxic
793 effect.