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1 Distinct timing of neutrophil spreading and stiffening
2 during phagocytosis

3

4 **CONDENSED RUNNING TITLE (<=40 CH. AND SPACES)**

5 Mechanics of neutrophil phagocytosis

6

7 **AUTHORS**

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9

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12

ABSTRACT (290≤300 WORDS)

13 Phagocytic cells form a first line of defense of the organism, engulfing microbial pathogens. Phago-
14 cytosis involves cell mechanical changes that are not yet well understood. Understanding these me-
15 chanical modifications promises to shed light onto the immune processes that trigger pathological
16 complications. Previous studies showed that phagocytes undergo a sequence of spreading event
17 around their target followed by an increase in cell tension. Seemingly in contradiction, other studies
18 observed an increase in cell tension concomitant with membrane expansion. Even though phago-
19 cytes are viscoelastic, few studies have quantified viscous changes during phagocytosis. It is also
20 unclear whether cell lines behave mechanically similarly to primary neutrophils. We addressed the
21 question of simultaneous versus sequential spreading and mechanical changes during phagocytosis
22 by using IgG-coated 8 and 20 μm-diameter beads as targets. We used a micropipette-based single-
23 cell rheometer to monitor viscoelastic properties during phagocytosis by both neutrophil-like PLB
24 cells and primary human neutrophils. We show that the faster expansion of PLB cells on larger beads
25 is a geometrical effect reflecting a constant advancing speed of the phagocytic cup. Cells become
26 stiffer on 20- than on 8-μm beads, and the relative timing of spreading and stiffening of PLB cells
27 depends on target size: on larger beads, stiffening starts before maximal spreading area is reached
28 but ends after reaching maximal area. On smaller beads, the stiffness begins to increase after cells
29 have engulfed the bead. Similar to PLB cells, primary cells become stiffer on larger beads but start
30 spreading and stiffen faster, and the stiffening begins before the end of spreading on both bead
31 sizes. Our results show that mechanical changes in phagocytes are not a direct consequence of cell
32 spreading and that models of phagocytosis should be amended to account for causes for cell stiff-
33 ening other than membrane expansion.

34

STATEMENT OF SIGNIFICANCE (107≤120 WORDS)

35 Some specialized immune cells form a first line of defense of the organism by engulfing microbial
36 pathogens, a process termed phagocytosis. During this process, immune cells become stiffer, but
37 we do not understand well the function and how these mechanical changes depend on their target.
38 Quantifying these mechanical events is important for understanding how elimination of microbes
39 works. It also represents opportunities for developing new diagnostic tools based on mechanical
40 states of leukocytes. We asked how human phagocytes adapt to the size of their target by feeding
41 them with antibody-coated microbeads and quantifying cell mechanical changes during phagocyto-
42 sis. Our results enhance our understanding of this complex defense mechanism.
43

45 Neutrophils are leukocytes of the innate immune system that are often first arrivers at a site of
46 infection. Neutrophils engulf microbial pathogens by performing phagocytosis, which is triggered
47 through the engagement of specific receptors present at the neutrophil surface (1, 2). A cell that
48 performs phagocytosis (called a phagocyte) contacts, spreads, engulfs and destroys its target inside
49 a phagosome, a specialized and transient organelle in which the target is attacked by reactive oxy-
50 gen species produced by NADPH oxidase (Nox) and aggressive biochemical agents delivered through
51 granule fusion (3). An abundant literature describes the biochemical reactions and signaling path-
52 ways involved during phagocytosis (2, 4–6), dissects the role of receptor engagement (7) and details
53 the formation of the phagosome and Nox assembly (8–10). Conversely, the literature on the me-
54 chanical events that occur during phagocytosis is scarcer, in part due to the difficulty of quantifying
55 mechanical properties in live leukocytes at the single cell level during processes that last only a few
56 minutes. Quantifying these mechanical events is necessary to fully understand the cellular process
57 of phagocytosis and to why it is sometimes less efficient, leading to evasion of the immune system
58 by some pathogens. More broadly, human neutrophil mechanical properties are thought to play an
59 important role in a number pathological settings including acute respiratory distress syndrome (11)
60 during which neutrophils stiffen and become trapped in the lung microvasculature. Improved un-
61 derstanding of leukocyte mechanical properties also provides an opportunity to develop new diag-
62 nostic tools based on mechanical states of leukocytes (12–15). Finally, understanding the mechanics
63 of phagocytosis is required for elucidating how a phagocyte reacts to its mechanical environment
64 and how it adapts to the shape and stiffness of its target (16–19) and to external forces (20), thereby
65 using the mechanical input to identify its target (21).

66 Micromanipulation techniques such as micropipettes, magnetic and optical tweezers, and
67 atomic force microscopy, have been used to quantify mechanical properties of phagocytes in a rest-
68 ing state or during phagocytosis (22–29). More than twenty years ago, Evans et al. (30) showed that
69 phagocytes stiffen during phagocytosis of opsonized yeast. A series of studies by Herant et al. (31,
70 32) represented a significant milestone in quantitative measurements and modelling of phagocyto-
71 sis, confirming that phagocytes stiffen during phagocytosis. They described an increase in cortical
72 tension induced by expansion of the membrane beyond a threshold of initially available membrane.
73 During expansion, the tension would progressively build up in proportion to the rate of membrane
74 expansion. Following this model, one would expect that once engulfment is complete, tension
75 would drop or at least not increase further, which is difficult to reconcile with Evans’s finding of a
76 rising cell tension after spreading. Kovari *et al.* (33) used traction force microscopy to measure con-
77 traction forces during spreading of macrophages on planar surfaces that were too large to be en-
78 gulfed, leading to so-called frustrated phagocytosis. They observed that upon reaching the maximal
79 spreading area, phagocytes contracted, exerting major stresses on their substrate. Using optical
80 tweezers, Masters et al. (34) showed that a peak in membrane tension signaled the transition from
81 spreading to contraction during frustrated phagocytosis, which appears to be consistent with a se-
82 quence of spreading followed by contraction. However, Masters et al. measured membrane tension,
83 as opposed to the cell tension including contributions of membrane, cortex, and membrane-cortex
84 adhesion (35), thereby making it difficult to compare with other studies. In light of the above, it
85 remains unclear why cell expansion and stiffening seems to be simultaneous in some studies, and
86 sequential in others.

87 Cell stiffening was measured in several studies, but leukocytes are viscoelastic, and few stud-
88 ies have quantified both elastic and viscous changes in phagocytes. Herant et al. (31) made the most
89 refined, albeit indirect, measurements of viscous effects. We have recently shown that activation of
90 a variety of types of leukocytes (T cells, B cells, and neutrophil-like PLB cells) induced a large increase
91 in both cell stiffness and viscosity, and that the viscous properties of leukocytes were intrinsically
92 linked to their elastic properties (29). Following our previous results, an increase in stiffness during
93 leukocyte activation occurs simultaneously with an increase in viscosity, with a modulation in the
94 ratio of elastic-to-viscous properties which evolves over time and is a signature of leukocyte type
95 (29).

96 In this study, we address the question of simultaneity or sequential occurrence of spreading
97 and mechanical changes in a phagocyte during the course of phagocytosis. We asked if the viscous
98 properties of both primary cells and cell lines evolve simultaneously with elastic properties during
99 phagocytosis and confronted this with the established model of Herant *et al.* To address these ques-
100 tions, we used a single-cell rheometer (29) that allowed us to monitor both elastic and viscous prop-
101 erties with a high temporal resolution, together with the monitoring of cell morphology using high
102 magnification transmitted-light microscopy.

103 MATERIALS AND METHODS

104 Cells and reagents

105 *PLB cells and reagents.* Cells of the human acute myeloid leukemia cell line PLB-985 (henceforth
106 referred to as PLB cells) were cultured in RPMI 1640 1X GlutaMax (Gibco) supplemented with 10%
107 heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were passaged twice
108 a week and differentiated into a neutrophil-like phenotype by adding 1.25 % (v/v) dimethyl sulfoxide
109 (DMSO, Sigma-Aldrich, St.-Louis, MO, USA) to the cell suspension the first day after passage and, in
110 a second time, three days later when changing the culture media (36). Twenty four hours before
111 experiments, 2000 U/ml of interferon- γ (IFN- γ ; Immuno Tools, Friesoythe, Germany) was added to
112 the cell culture flask (37), cells were centrifuged for 3 min at 300g and resuspended in complete
113 HEPES medium (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES pH 7.4, 1.8 mg/mL
114 glucose, and 1% heat-inactivated FBS). All solutions in contact with cells were filtered using 0.22- μ m
115 diameter pore filters (Merck Millipore).

116
117 *Primary human neutrophils and reagents.* Human blood samples were taken with the understanding
118 and written consent of each volunteer by the “Etablissement Français du Sang, Cabanel, Paris” (the
119 French blood transfusion service and national blood bank: <https://www.ints.fr/>). An agreement
120 (N°11/Necker/103) allowing us to use blood samples from volunteers for research purposes was
121 signed between this organization and the Institut de Chimie Physique. Neutrophils were isolated
122 from healthy donor whole blood by means of dextran sedimentation and Ficoll centrifugation as
123 previously described (38). Briefly, after sedimentation for 30 minutes in 2 % Dextran 50 G (Sigma-
124 Aldrich, ref. 31392) in physiologic serum (0.9 % NaCl in purified water), the leukocyte-rich yellow
125 part of the liquid was centrifuged for 8 minutes at 400g, then the supernatant was discarded and
126 leukocytes were resuspended in Dulbecco's Phosphate Buffer Saline (DPBS, Gibco). The leukocytes
127 were then isolated with density separation in 15 mL Ficoll (Ficollpaque-plus GE-Healthcare, ref.

128 GE17-144002, Sigma-Aldrich). Cells were counted, and before the experiment, cells were centri-
129 faged for 3 minutes at 300g at room temperature and resuspended in the same volume of complete
130 HEPES medium. Between experiments, the batch of tested primary neutrophils was maintained at
131 4°C.

132 **Oponization of beads and flat surfaces**

133 *20- μ m beads.* 20- μ m diameter polystyrene microbeads at 10^6 beads/mL (ref. 74491-5ML-F, Sigma-
134 Aldrich) were washed three times by centrifugation at 16000 g for 3 minutes and resuspended in
135 DPBS. Beads were then incubated overnight at room temperature with a 5% (w/v) solution of Bovine
136 Serum Albumin (BSA, Sigma-Aldrich) in DPBS. The beads were washed again three times by centrif-
137 ugation at 16000 g for 3 minutes, resuspended in DPBS, and incubated with 1:500 anti-BSA rabbit
138 antibody (Sigma-Aldrich, ref. B1520) in DPBS for an hour at room temperature. Microbeads were
139 again washed three times (16000g for 3 minutes in DPBS), stored at 4°C, and resuspended in DPBS
140 at 10^6 beads/mL before use.

141
142 *8- μ m beads.* 8- μ m diameter polystyrene BSA-covered microbeads (Spherotech, ref. BP-60-5, Lake
143 Forest, IL, USA) were opsonized with the same anti-BSA rabbit antibodies used for 20- μ m beads.
144 Control microbeads were non-opsonized BSA microbeads, washed three times and resuspended in
145 DPBS at the same concentration as the opsonized microbeads. In absence of the anti-BSA antibodies
146 on beads cells never spread around the bead as clearly as in the presence of antibodies.

147
148 *Flat surfaces.* Glass bottom Petri dishes (Fluorodish, WPI, Sarasota, FL, USA) were washed three
149 times with DPBS and incubated overnight at room temperature with 1 mL of 5% (w/v) Bovine Serum
150 Albumin (BSA; Sigma-Aldrich) in DPBS, after which they were washed three times with DPBS. Dishes
151 were then incubated with 1:500 anti-BSA rabbit antibody (ref. B1520, Sigma-Aldrich) in DPBS for an
152 hour at room temperature. The Petri dishes were finally washed three times with 1 mL of complete
153 HEPES medium (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES 448 pH 7.4, 1.8
154 mg/mL glucose, and 1% heat-inactivated FBS). Control surfaces were made on glass bottom Petri
155 dishes coated with BSA only. We never observed any cell spreading on these surfaces, while in the
156 presence of these anti-BSA antibodies every cell eventually spread on the surface.

157 **Micropipette preparation**

158 Stiff micropipettes were prepared as described previously (39–42) by pulling borosilicate glass ca-
159 pillaries (Harvard Apparatus, Holliston, MA, USA) with a P-97 micropipette puller (Sutter Instru-
160 ments, Novato, CA, USA) and cutting them with an MF-200 microforge (World Precision Instruments,
161 Sarasota, FL, USA) to get an inner diameter of 3- μ m at the tip of the micropipette. This micropipette
162 was then bent at a 45° angle with an MF-900 microforge (Narishige, Tokyo, Japan).

163 Flexible micropipettes were either connected to a pneumatic microinjector (IM-11-2,
164 Narishige) used to strongly hold a polystyrene bead with an aspiration pressure on the order of 1
165 kPa when used to indent phagocytes in the front (see below), or their extremity was melted to form
166 a (non-adherent) glass bead, when used to indent phagocytes in the back. The bending stiffness of
167 these flexible micropipettes was calibrated as previously described (40) by pressing them against
168 standard microindenters of known stiffness and by measuring the resulting bending of both micropi-
169 pettes. The stiffness of the standard microindenters was calibrated using a commercial force probe

170 (model 406A with a force range of 0–500 nN; Aurora Scientific, Aurora, ON, Canada). In the present
171 study, flexible micropipettes had a typical bending stiffness of 0.1-0.2 nN/ μm .

172 **Single-cell rheometer**

173 The rheometer was developed based on our microindentation setups (40, 41) and described in (29).
174 The principle of the rheometer is to apply a controlled force to a cell and to record the resulting cell
175 deformation during phagocytosis. To do so, we use an inverted microscope (Ti2, Nikon Instruments,
176 Tokyo, Japan) equipped with a SPARK camera (Hamamatsu Photonics, Hamamatsu City, Japan), a
177 100x oil immersion 1.3 NA objective (Nikon Instruments) placed on an air suspension table (Newport,
178 Irvine, CA, USA). The microscope is equipped with 2 or 3 micropipettes whose tips are plunged into
179 a glass-bottom Petri dish (Fluorodish, WPI) placed on the microscope stage. The force is applied to
180 the cell by compressing it with a flexible micropipette of known bending stiffness. This flexible mi-
181 cropipette is linked to a manual micropositioner (Thorlabs, Newton, NJ, USA) placed on top of a
182 single-axis translation stage controlled with a piezo actuator (TPZ001; Thorlabs). The force is applied
183 to the cell via a bead located at the tip of this flexible micropipette. When used for both activating
184 and probing the cell, this microbead is opsonized and maintained by a strong aspiration pressure
185 provided by the flexible micropipette. When used only for probing the cell, the bead is made of glass
186 and fused with the flexible micropipette.

187 By translating the piezo stage, the bead (activating bead or the bare glass bead) at the tip of
188 a flexible micropipette is pushed against the cell that is gently held by a stiff micropipette, which is
189 linked to a motorized micromanipulator (MP-285; Sutter Instruments). To control the force towards
190 the cell, a live feedback loop detects the position of the bead (at a rate of 400-500 Hz) by analyzing
191 a portion of each image acquired by the microscope camera which is controlled by the Microman-
192 ager software (43). The latter is controlled by a Matlab (Mathworks) custom code. The force applied
193 by the bead on the cell is the product of the deflection and the bending stiffness of the flexible
194 micropipette. The deflection is obtained as the difference between the position of the bead and the
195 position of the piezo stage. The feedback loop adapts the position of the base of the flexible mi-
196 cropipette in order to set a desired micropipette deflection, hence a desired force applied to the
197 cell. The cell deformation is determined by the position of the bead.

198 During each experiment, a first mechanical measurement is performed when the initial con-
199 tact is made between the bead and the cell, which is obtained by translating the base of the flexible
200 micropipette at a constant velocity of 1 $\mu\text{m/s}$ until an initial compressive force $F_0 = 180$ pN is attained.
201 This level of force was chosen to obtain an indentation that was not too large at early times (less
202 than 1 μm), when cells were soft, but not too small (more than 0.1 μm) when cell became stiffer.
203 We analyze the resulting force-indentation curve, where the indentation is obtained by analyzing
204 the position of the tip of the indenting bead, $x_{tip}(t)$, from which the position of the contact point is
205 subtracted. We use the Hertz model (44, 45) to extract the cell's Young's modulus from the force-
206 indentation curve (29). We did not correct for finite thickness of the cell, but we estimated that
207 although it would modulate the quantitative values of the Young's modulus, the trends would be
208 unaffected (increase in Young's modulus during activation). Once the 180-pN threshold F_0 is reached,
209 the setup automatically switches to a regime during which an oscillatory force $F(t)$ is applied to the
210 cell: $F(t) = \langle F \rangle + \Delta F \cos(\omega t)$, where the average of the force $\langle F \rangle = F_0 = 180$ pN, the amplitude of
211 the oscillations $\Delta F = 50$ pN, and $\omega = 2\pi f$ is the angular frequency of the oscillations at a frequency
212 $f = 1$ Hz. The amplitude ΔF was chosen large enough so that the induced modulation in indentation

213 would be detectable, but small enough so that it could be considered as a small perturbation. The
 214 1-Hz frequency was sufficiently faster than the timescale associated with the spreading process, but
 215 it had also to be low enough to consider that measurements performed at this frequency would be
 216 close to the zero-frequency values. As a result of this oscillatory force, the cell deforms in a sinusoi-
 217 dal manner, as reported by the position of the tip of the flexible micropipette, $x_{tip}(t) = \langle x_{tip} \rangle +$
 218 $\Delta x_{tip} \cos(\omega t - \varphi)$, where $\langle x_{tip} \rangle$ is the average of $x_{tip}(t)$, Δx_{tip} the amplitude of the oscillations
 219 (typically 100 nm, with a few-nanometer accuracy) and φ is a phase lag due to the cell's viscous
 220 properties (an applied force leads to a delayed deformation) (Suppl. Fig. S1). Note that the relative
 221 variation of $x_{tip}(t)$ is a measure of the relative variations of the cell length. As usually done in rhe-
 222 ological studies (27), we consider a complex numbers formalism where the complex force is $F^*(t) =$
 223 $\langle F \rangle + \Delta F e^{i\omega t}$ and the corresponding complex tip position is $x_{tip}^*(t) = \langle x_{tip} \rangle + \Delta x_{tip} e^{i(\omega t - \varphi)}$. We
 224 define the complex stiffness of the cell K^* by writing $F^*(t) - \langle F \rangle = K^*(x_{tip}^*(t) - \langle x_{tip} \rangle)$. The com-
 225 plex cell stiffness $K^* = K' + iK''$ has a real part K' characterizing the elastic properties of the cell,
 226 and an imaginary part K'' characterizing its viscous properties. Henceforth, for simplicity we call K'
 227 the cell stiffness, but we refer to our previous study where we addressed in detail why K' can be
 228 considered as reflecting changes in intrinsic elastic modulus of the cell, not only a change in cell
 229 geometry (29). Note that this stiffness is an effective parameter that does not allow identifying a
 230 specific contribution of different compartments of the cell. K' and K'' are deduced from experi-
 231 mental measurements through the relationship $K' = \frac{\Delta F}{\Delta x_{tip}} \cos \varphi$ and $K'' = \frac{\Delta F}{\Delta x_{tip}} \sin \varphi$, respectively
 232 (29). It is also relevant to monitor the ratio of K'' and K' , called the loss tangent η . From the above
 233 relationships, η is solely related to the phase lag φ by the relationship $\eta = \tan \varphi$. Experiments were
 234 performed at room temperature to avoid thermal drift. On top of the live detection of the flexible
 235 micropipette tip, one image of a region of interest showing the pipettes, cell and bead was recorded
 236 every second.

237

238 **Setup configuration to indent the cell in the front or in the back.**

239 The automation of the setup was used in three different ways, in order to indent activating leuko-
 240 cytes: in the “front”, in the “back”, and in the “back” but by cyclically retracting the indenter and
 241 forming a new contact at the beginning of each ~30-s cycle. Micropipette positioning and shape
 242 were adapted accordingly (see below, Figure 1, and movie S1).

243

244 *Indentation in the front of the cell.* To indent the cell in the front, a stiff micropipette holds the cell
 245 gently and the flexible micropipette holds an activating bead with a strong aspiration (movie S1).
 246 Before testing a new cell, a new activating microbead is picked up from the bottom of the Petri dish
 247 and aspirated by the flexible micropipette. A cell is then also picked up from the bottom of the dish
 248 by micromanipulation the stiff micropipette.

249

250 *Indentation in the back of the cell.* Indenting the cell in the front allows monitoring only frustrated
 251 phagocytosis due to the high aspiration pressure (several kPa) used to aspirate the activating mi-
 252 crobead at the tip of the flexible micropipette. The seal between the pipette and the bead surface
 253 is not perfect (probably due to nanometer-scale imperfections), so that cell membrane can get as-

254 pirated by the flexible micropipette if it reaches its tip, thus making the phagocytic cup closure im-
255 possible. In order to study complete phagocytosis, we developed a more complex setup using three
256 micropipettes: a stiff pipette that holds an activating bead, with a low (~ 20 Pa) aspiration, while the
257 cell is gently positioned on the other side of the bead (Fig. 1B) using an auxiliary micropipette hold-
258 ing the cell also with a low aspiration (~ 20 Pa). The auxiliary pipette is withdrawn once the cell ad-
259 heres to the activating bead. To indent the cell, a flexible micropipette is used as in the case of front
260 indentation, but this flexible micropipette now has a glass bead fixed (through melting the pipette
261 tip) at its extremity. This glass bead can indent the cell on its “back”, while it is phagocytosing the bead
262 on its “front” (movie S1). In the presence of serum, we very rarely saw any clear adhesion between
263 the glass bead and the cell within the timescale of an experiment. The principle used to measure
264 the viscoelastic properties of the cell during phagocytosis remains the same as for front indentation.
265 Representative examples for 8- and 20- μm bead phagocytosis are shown in Supplementary Movie
266 S1.

267
268 *Cyclic indentation in the back of the cell.* During the indentation in the back described above, the
269 microindenter (the flexible micropipette with a fused bead at its tip) stays in contact with the cell
270 throughout phagocytosis. In a previous study, we addressed the question of whether the changes
271 in K' and K'' reflected intrinsic changes in cell stiffness and viscosity, respectively (29). This requires
272 measuring the Young’s modulus of the cell over time in order to correlate it with K' . To do so, the
273 setup is the same as for indentation in the back, but the automation is changed. The microindenter
274 follows a sequence of repeated operations (called cycles) during which it: (i) is brought into contact
275 with the cell as done at the beginning of experiments when indenting in the front or in the back, (ii)
276 applies an oscillatory force to the cell following the preceding compressive phase, and (iii) is re-
277 tracted away from the cell, which ends one cycle (movie S1). Fifteen cycles are repeated consecu-
278 tively, allowing fifteen consecutive measurements of the Young’s modulus of each cell, assuming a
279 Poisson’s ratio of the cell equal to 0.5 (29). We took into account the cell curvature that we updated
280 over time while the cell was spreading over the bead, based on video images. A complete cyclic-
281 indentation for a cell lasts ~ 7.5 minutes (Fig S1).

282
283 *Measuring curvilinear abscissa.* The curvilinear abscissa of the cup front position was measured us-
284 ing a kymograph (Fig. S2). To determine when the spreading slowed down, and transitioned from
285 linear to slower spreading, a linear trend was fitted over the first 5 μm of the curvilinear abscissa s ,
286 which led to a measurement of $v_0 = ds/dt$. Departure from this linear trend was defined as the time
287 at which s was more than 0.5 μm away from the linear trend (Fig. 2E and Fig. S2). The maximum of
288 s , s_{max} , was also determined based on the kymograph.

289
290 *Measuring total cell surface area.* When indenting PLB cells in the back while they phagocyte 20- μm
291 beads, we also estimated the total cell surface area when the phagocytic cup attained its maximal
292 area. To do so, we modeled the outer cell area as a spherical cap whose height was measured di-
293 rectly on video for each cell, and we added to the surface area of this spherical cap the surface area
294 of another spherical cap defined by the curvature of the 20- μm bead.

295

296 **Frustrated phagocytosis on flat surfaces**

297 A glass bottom Petri dish (Fluorodish, WPI) was placed in the focal plane of an inverted microscope
298 under bright field or DIC illumination (TiE or Ti2, Nikon Instruments, Tokyo, Japan) equipped with a
299 40X or a 60X objective (Nikon Instruments) and a CMOS camera (Flash 4.0 or SPARK, Hamamatsu
300 Photonics, Hamamatsu City, Japan). A few thousands cells were then injected into the Petri dish.
301 Time-lapse recordings were acquired at the rate of one image per second. The focus was adjusted
302 manually, and experiments were performed at room temperature (movie S3). The spreading dy-
303 namics were assessed using a kymograph based on a line oriented radially across the cell (Fig. S2).
304 The radius of the cell's phagocytic cup was analyzed the same way as the curvilinear abscissa s on
305 beads in order to measure the velocity of the cell's front during spreading, v_0 , and the value of the
306 radius $r = s_{\text{slowdown}}$ ($r = s$ on a flat surface) at which the spreading began to slow down, as well as the
307 maximum value of r , s_{max} .

308 **Micropipette aspiration to determine maximal cell surface area.**

309 A glass bottom Petri dish (Fluorodish, WPI) was placed on a TE300 inverted microscope (TE300, Ni-
310 kon Instruments, Tokyo, Japan) placed on an air suspension table (CVI Melles Griot, Netherlands).
311 The microscope was equipped with a 100 \times oil immersion objective (Nikon Instruments) and a Flash
312 4.0 CMOS camera (Hamamatsu). Epifluorescence illumination using a mercury lamp (Intensilight,
313 Nikon Instruments) was used simultaneously with a faint brightfield illumination provided by a LED
314 placed on a custom-made holder. Exposure time was typically set to 80 ms and a neutral density
315 filter 4 or 8 in the fluorescence illumination path was used. The fluorescence aperture diaphragm
316 was adjusted to limit exposure in the field of view. The Petri dish was filled with complete HEPES
317 medium supplemented with 160 $\mu\text{g}/\text{mL}$ of propidium iodide (Sigma-Aldrich). A stiff micropipette
318 filled with HEPES medium at its tip (the rest being filled with distilled water) was connected to a
319 water-filled glass tube whose height was adjusted to control hydrostatic pressure inside the mi-
320 cropipette. The reservoir level was set at 2 mm below zero pressure level at which the flow through
321 the tip of the micropipette vanishes, leading to an aspiration pressure in the micropipette of ap-
322 proximately 20 Pa. Once a cell was picked up at the bottom of the Petri dish and aspirated for a few
323 seconds, we shifted aspiration level from the previous -2 mm to a high aspiration level of -50 mm
324 (i.e. about 500 Pa). Time-lapse image series were acquired at one image per second using Mi-
325 cromanager software (43). Cell area measurements during passive aspiration were made by approx-
326 imating cell shape with simple geometrical shapes as described previously (41). Briefly, the cell body
327 inside the micropipette was modeled as a cylinder, while the part of the cell at the tip inside the
328 micropipette and out of the micropipette was modeled as a spherical cap.

329

330 **Quantifying cell spreading and stiffening.**

331 To quantify changes in cell shape and mechanics during phagocytosis, we defined a set of parame-
332 ters to be monitored over time.

333

334 *Quantifying cell spreading.* Following the contact between the cell and the activating surface (bead
335 or plane) at a time taken as $t = 0$, the phagocytic cup starts spreading at time $t = t_{\text{cup}}$ determined by
336 visual inspection of the movies. Spreading stops at time $t = t_{\text{Amax}}$, when the phagocytic cup reaches
337 its maximal area A_{max} , either because the phagocytosis is frustrated, or because the bead has been
338 engulfed. The duration of cup spreading is the difference $t_{\text{Amax}} - t_{\text{cup}}$. The average rate of spreading is

339 $\langle \frac{dA}{dt} \rangle = \frac{A_{max} - A_{init}}{t_{Amax} - t_{cup}}$, where A_{init} is the cup area when its formation is detected (A_{init} is non-zero as the
340 initial contact zone hides the cup at its very early moments), and $A_{max} - A_{init}$ is its area expansion.

341
342 *Quantifying cell stiffening.* The cell stiffness is characterized by the elastic part K' of the complex
343 stiffness K^* (see Single-cell rheometer section in Materials and Methods). After cell-bead contact,
344 K' remains close to its initial value K'_{init} , until it starts increasing quickly at $t = t_{K'start}$. To set $t_{K'start}$
345 systematically, we defined it as the time at which K' overtakes K'_{init} by 0.5 nN/ μm . Following this
346 time point, K' increases until it peaks at a value $K' = K'_{max}$ at time $t = t_{K'max}$, after which K' decreases
347 (Suppl. Fig. S3). We also measured an average rate of increase of K' , $\langle \frac{dK'}{dt} \rangle = \frac{K'_{max} - K'_{start}}{t_{K'max} - t_{K'start}}$ (Suppl.
348 Fig. S4).

350 **Influence of setup configuration on these parameters.**

351 The protocol used to probe cell stiffness did influence cell behavior: front indentation led to a de-
352 layed onset of cup formation ($t_{cup} = 128$ s vs 72 s on 20- μm beads, median values, $p < 0.05$, two-
353 tailed Mann Whitney test, Fig. 2A and 3A, inset on top), a longer spreading time ($t_{Amax} - t_{cup}$, about 3
354 instead of 2 minutes, Fig. 3B). However, some parameters were independent of the protocol: back
355 and front indentation led to the same maximal cup area (A_{max} , Fig. 2A and 3A, inset on the right) and
356 $t_{K'start}$ (Fig. 2A and 3A, inset below). Being aware of this influence of the protocol, we compared
357 dynamics on 8 and 20- μm beads using the same protocol.

358 **RESULTS**

359 **The rate of membrane expansion is larger on 20- μm beads due to a constant velocity of the front** 360 **of the spreading lamellum.**

361 Back indentation on phagocytes fed with 8- and 20- μm beads and flat surfaces led to the same delay
362 t_{cup} to start forming the phagocytic cup (Fig. 2A). The spreading duration $t_{Amax} - t_{cup}$ was also the same
363 for 8- and 20- μm beads with back indentation and on flat surfaces ($t_{Amax} - t_{cup} \sim 2$ minutes, Fig. 2B).

364 One notable difference among the targets of different sizes was the average spreading rate
365 $\langle dA/dt \rangle$. It was significantly larger on 20- than on 8- μm beads (Fig. 2D). To test if this difference was
366 a sign of adaptation by the cell to the size of the bead, we measured the advancing speed of the cell
367 front on beads and flat surfaces by quantifying the curvilinear abscissa s of the cell front over time
368 (see Materials and Methods). This curvilinear abscissa s first increased linearly with time, meaning
369 that the speed $v_0 = \frac{ds}{dt}$ was constant over time ($v_0 \approx 0.1 \mu\text{m/s}$, Figure 2E). After this first constant-
370 speed spreading phase, the abscissa s reached a value $s_{slowdown}$ above which its increase slowed
371 down (Fig. 2E, *inset above*), until reaching a local maximum. Afterwards, it usually exhibited oscilla-
372 tory movement of small amplitude, similar to that observed in macrophages on flat substrates (33).
373 Given a constant v_0 , one can follow purely geometrical considerations to quantitatively predict a
374 rate of increase of the phagocytic cup area that depends on the bead radius (Suppl. Mat. 1). For a
375 v_0 independent of bead size, one predicts that the larger the bead, the larger the spreading rate
376 dA/dt . Our predictions of the ratio of average rates $\langle dA/dt \rangle$ on large and small beads are consistent

377 with our experimental observation (Suppl. Mat. 1), suggesting that instead of an adaptation to cur-
378 vature, the observed increasing spreading rate (in surface area per unit time) is a direct consequence
379 of a conserved velocity of the advancing front of the cell lamellum during phagocytosis.

380

381 **The surface area deployed during frustrated phagocytosis is the same as deployed by micropi-**
382 **pette aspiration.**

383 The maximal surface area reached by the phagocytic cup of PLB cells during frustrated phagocytosis
384 was the same on 20- μm beads as on planar surfaces ($A_{max} \sim 300 \mu\text{m}^2$, Fig. 2A, inset on the right). On
385 8- μm beads, this maximum area was set by the bead surface area ($A_{max} \sim 200 \mu\text{m}^2$). Phagocytosis,
386 frustrated or not, requires the deployment of membrane stores such as those present in surface
387 folds and microvilli (31, 46). To test if the maximal cup area reached during frustrated phagocytosis
388 corresponded to the mobilization of these membrane stores, we aspirated PLB cells in a micropi-
389 pette until their membrane broke (see Materials and Methods). This micropipette aspiration assay
390 led to the same measured area as the one reached during spreading on 20- μm beads (Fig. 2F).

391

392 **Cells become stiffer during frustrated phagocytosis on 20- μm beads than during complete phago-**
393 **cytosis of 8- μm beads.**

394 The delay between the onset of increase in K' and the time when K' attains its maximum value,
395 $t_{K'_{max}} - t_{K'_{start}}$, was the same on 8- and 20- μm beads, but the peak value of K' , K'_{max} , was larger on 20-
396 than on 8- μm beads (Fig 2C and G). The stiffness depends on the geometry of the cell: for a given
397 cell Young's modulus, which is the intrinsic elasticity of the cell material, a thinner cell will have a
398 larger K' . To exclude a potential geometrical contribution to the measured differences in K' , we pro-
399 ceeded as in our previous study (29) and measured the effective cell Young's modulus E_{Young} using
400 cyclic indentation in the back on the cell (see Material and Methods). Confirming the trend in K' ,
401 E_{Young} was significantly higher during back indentation on 20- μm than on 8- μm beads (Fig. 4A).

402

403 **Cell stiffness reaches its maximum after spreading has ended. In some cells, stiffening starts after**
404 **total engulfment of 8- μm beads.**

405 During most cases (73%) of frustrated phagocytosis of 20- μm beads by PLB cells, K' reached its max-
406 imum after spreading had stopped (i.e. $t_{K'_{max}} - t_{A_{max}} > 0$, Fig. 2H): stiffness continued increasing even
407 after completion of cell spreading. With some 20- μm beads (23% of cases) and more often with 8-
408 μm beads (75% of cases), K' started increasing after spreading had already stopped (Fig. 2I and 2J,
409 $t_{K'_{start}} - t_{A_{max}} > 0$), meaning that K' rose after any new mobilization of membrane was needed. Later we
410 discuss that this would not be predicted to happen by the model of Heinrich *et al.* but appears to be
411 consistent with the older observations by Evans *et al.* (30) mentioned earlier.

412 **Primary neutrophils spread faster and become stiffer than PLB cells during phagocytosis**

413 Primary neutrophils (see examples in movie S2) formed their phagocytic cup quicker than PLB cells
414 on 8- μm beads (shorter t_{cup} delay, Fig 3A). Primary neutrophils were quicker to reach their maximal
415 cup area on both 20- and 8- μm beads (Fig. 3B). The spreading rate $\langle \frac{dA}{dt} \rangle$ was larger for primary neu-
416 trophils than for PLB cells on 8- μm beads (Fig. 3D), which was a direct consequence of the fact that
417 primary neutrophils spread with a front speed v_0 that was larger than the front speed of PLB cells
418 on 8- μm beads (Fig. 3E). The maximal cup area of primary cells was significantly smaller than that of
419 PLB cells, which might only be coincidental with the fact that resting primary neutrophils are smaller

420 in radius than resting PLB cells (Suppl. Fig. S5). This maximal cup area of about $200 \mu\text{m}^2$ is compara-
421 ble with the one obtained by Herant *et al.* for primary neutrophils (32). Primary neutrophils became
422 stiffer than PLB cell (larger K'_{max} , Fig. 3C), and on $20\text{-}\mu\text{m}$ beads this stiffening occurred faster for
423 primary neutrophils (Fig. 3F). The average stiffening rate of primary neutrophils was larger than that
424 of PLB cells (front/back indentation, Suppl. Fig. S4). To sum up, primary cells exhibited “exacerbated”
425 and faster reactions, leading to more pronounced mechanical changes.
426

427 **For primary neutrophils, stiffness started increasing before maximal area was reached.**

428 As opposed to the case of PLB cells, even on $8\text{-}\mu\text{m}$ beads, primary neutrophils started stiffening
429 before reaching their maximum area (Fig. 3G).

430

431 **During phagocytosis by both PLB cells and primary neutrophils, elastic and viscous properties in-
432 crease, and the ratio of viscous to elastic properties decreases.**

433 For each cell, our rheometer allows quantifying the cell stiffness K' , the viscous part of the complex
434 stiffness K'' (which reports viscous properties of the cell), and the loss tangent $\eta = \frac{K''}{K'}$ (see Materials
435 and Methods). Given that $t_{K'start}$ varied considerably among cells, we synchronized K' curves before
436 averaging them by using $t = t_{K'start}$ as the time origin for each curve; we used this same time origin to
437 average K'' and η (Fig. 4). We had shown previously that elastic and viscous properties of leukocytes
438 follow parallel evolutions during activation (29), and that the loss tangent η characterizes the type
439 of leukocyte during its activation. Here, besides confirming that η decreases during activation of PLB
440 cells, i.e. that cell stiffness increases faster than cell viscous properties, we report the same behavior
441 for primary neutrophils, at least at early times (see in Fig. 4G: η increases again ~ 90 s after $t=t_{K'start}$
442 for back-indentation and $8\text{-}\mu\text{m}$ beads). K' , K'' and η follow a remarkably similar evolution for PLB
443 cells in front and back indentation on small and large beads during the first ~ 50 seconds following
444 $t=t_{K'start}$. (Fig. 4B, D, and F). Our measurements show that primary neutrophils and PLB cells have a
445 similar mechanical signature during activation that consists of a decrease in η at early times.

446 DISCUSSION

447 Phagocytes generate force during phagocytosis, change their mechanical properties, and adapt to
448 the shape and stiffness of their target (16–19, 47). However, the mechanical aspects of phagocytosis
449 are not fully understood (21, 48–50). To better characterize these aspects, we used a micropipette-
450 based single-cell rheometer to monitor the elastic and viscous properties and morphology of neu-
451 trophils and PLB cells during the phagocytosis of IgG-coated microbeads. We compared dynamics in
452 spreading and mechanical changes, depending on target size, and we used micropipette aspiration
453 to quantify the area of membrane stores available in microvilli and ruffles at the surface of resting
454 PLB cells. We were able to show that bead size does not have a significant impact on the response
455 time of PLB cells but leads to a rate of area expansion that increases with bead size. We have shown
456 that this is a geometrical effect due to the fact that the advancing speed of the phagocytic cup is
457 independent of the target size.

458 In a previous study, we used scanning electron microscopy to estimate the microscopic area
459 contained in microvilli and folds present on the surface of Jurkat cells, primary human CD4 T lymphocytes,
460 and human lymphoblasts (41). We showed that this surface microscopic area closely
461 matched the membrane area obtained using the same micropipette aspiration experiment assay as
462 used here on PLB cells. This suggested that passive extension of membrane is an effective readout
463 of membrane surface reservoirs. Assuming that this is also true for PLB cells, our micropipette aspiration
464 experiments show that their total surface area is about 1.8 fold larger than their apparent
465 surface area (547 vs 309 μm^2 , respectively, Figs. 2F and S5). As we obtained a maximal surface area
466 during frustrated phagocytosis that is consistent with this total area (Fig. 2F), we speculate that
467 frustrated phagocytosis does not require the mobilization of large inner membrane stores, but
468 mainly of membrane reservoirs present at the surface of PLB cells. Note that a small amount of inner
469 membrane recruitment is not excluded and may occur even during micropipette aspiration, as it has
470 been shown that other means of passive extension of cell membrane area such as osmotic swelling
471 can provoke some exocytosis in human cells over a timescale of seconds to tens of seconds (51).

472 Cells became stiffer on 20- vs 8- μm beads, over similar timescales. The relative timing of
473 spreading and stiffening of PLB cells depended on target size: on larger beads, stiffening started
474 before the maximal spreading area was reached. However, stiffening ended after this maximal area
475 was reached. On 8- μm beads, this trend was more pronounced as in some PLB cells, stiffening
476 started even after they had already engulfed the bead. In primary neutrophils, we did not observe
477 this sequence, as stiffening started before the end of spreading even on smaller beads. Primary cells
478 responded and stiffened faster, but behaved similar to PLB cells by also becoming stiffer on larger
479 beads.

480 Herant *et al.* characterized the tension of neutrophils as a function of the total apparent
481 surface area A of the cell (31). Following their model, once a certain amount of membrane slack has
482 been extended, the tension can be written as the sum of two terms that increase linearly with $\frac{A-A_0}{A_0}$
483 and $\frac{dA}{dt}$, respectively, where A_0 is the apparent surface area of the resting spherical neutrophil. This
484 model allowed understanding of why phagocytosis of beads of 2 μm -diameter did not evoke any
485 increase in tension (expanded surface area was lower than the slack), while larger beads led to an
486 increase in tension that increased with the size of the target. In the present study, during back-
487 indentation of PLB cells on 20- μm beads, in most cases we observed that the phagocytic cup started
488 spreading and cell stiffness K' started increasing at a similar moment, consistent with the observa-
489 tions and model of Herant *et al.*. However, we also observed that K' reached its maximum after the
490 maximum area A_{max} was reached. Once A_{max} is reached, $\frac{dA}{dt}$ vanishes, so following Herant's model,
491 K' is not expected to evolve any further if cortical tension is the only contributor to K' . Even more
492 puzzling, we observed that on 20- μm beads, a minority of cells reached their maximum cup area
493 before K' started increasing. These cases of late increase in K' occurred more often when cells phag-
494 ocyted 8- μm beads. In the latter cases, again, Herant's model cannot account for the increase in K' ,
495 as the cell's apparent area is constant, and its derivative is null. Our observation is consistent with
496 earlier observation by Evans *et al.* of a sequence of spreading followed by an increase in tension in
497 primary granulocytes phagocytosing yeast (30). However, it is not clear why Evans *et al.* seem to have
498 only seen spreading preceding an increase in stiffness. The different nature (opsonized yeast) might
499 be a reason, as Lee *et al.* later showed that the mechanical response of phagocytes is different on
500 zymosan vs. antibody-coated targets (52).

501 An alternative to Herant's model is keeping the concept of membrane slack to deploy before
502 inducing a signal leading to an increase in K' , but with a time delay between reaching the threshold
503 in membrane expansion and triggering the increase in stiffness. K' starts increasing earlier on larger
504 beads on which membrane expands faster, supporting this idea of a threshold reached earlier. As K'
505 takes the same amount of time to reach its maximum on both large and small beads, we shall con-
506 sider a constant delay independent of target size, which would reflect an inner timescale to turn on
507 cell contractility. As a consequence of a delayed increase in stiffness, the evolution of K' would not
508 be directly linked to spreading through a term proportional to either A or dA/dt . Another alternative
509 to amend the Herant model would be to consider that during phagocytosis, an important contribu-
510 tion to effective cell stiffness comes not from an increase in cell tension due to membrane expansion,
511 but rather from an increased active cell contraction, not directly correlated to cell surface area. To
512 account for the fact that the maximum in stiffness K' is higher when a larger surface area has been
513 deployed by a PLB cell, we speculate that the rate of increase of K' scales with the total amount of
514 mobilized surface membrane stores. Irmscher et al. (28) measured a stiffening in phagocytic cups of
515 THP-1 macrophages phagocytosing 4.5- μm IgG-coated magnetic particles. The stiffness reached its
516 maximum during particle engulfment. Interestingly, some phagocytoses that were interrupted led
517 to a larger increase in stiffness, supporting the idea that stiffening is not only a function of mem-
518 brane expansion. Other studies suggested that membrane tension instead of global cell tension is
519 the signal triggering cell contraction via myosins (34). Cell tension is a compound parameter that
520 encompasses membrane tension, actomyosin cortex tension, and energetic contributions from
521 membrane-cortex adhesion (35). In unperturbed cells, Masters *et al.* measured a peak in tension at
522 the onset of cell contraction after spreading, but this peak in tension was typically $\sim 10\%$ in magni-
523 tude (34). Based on measurements of cell tension in the literature and on our measurements of cell
524 stiffness, it appears that long before reaching its maximum area, the cell builds up a massive in-
525 crease in stiffness or (cell) tension that can be of up to $\sim 1000\%$ (29, 31). This build-up might well
526 end with a small peak in membrane tension, but it is hard envisioning that this massive increase in
527 stiffness does not also play a role *per se* in the regulation of cell spreading. Further work should
528 investigate how both cell and membrane tension regulate the dynamics of cells during phagocytosis.

529 In this study, we measured no difference in the time to start forming the phagocytic cup on
530 8- and 20- μm beads (using back indentation), and on planar surfaces; the maximal phagocytic cup
531 surface area was also the same on 20- μm beads and on planar surfaces, suggesting that for targets
532 of more than 8 μm , PLB cells are not sensitive to target size or curvature (we restricted our study to
533 beads, we could not vary curvature and size independently). It is not clear why others observed that
534 phagocytes respond faster on larger targets (31, 32), although comparison is not straightforward
535 when the effect of target was explored on targets smaller than our 8- μm beads and kinetics of the
536 onset of phagocytic cups were not systematically studied (53, 54). Other studies suggest that phag-
537 ocytes might sense the two local main curvatures (19), reacting differently to elongated ellipsoids
538 vs flat prolate ellipsoids (16). For beads, sensitivity to the target remains seemingly controversial.
539 Montel et al. (55) obtained results with fluid droplets that are consistent with the prediction of
540 Simon and Schmid-Schönbein (22) that the critical parameter is the surface area reservoir of the
541 cell and not the target size *per se*. In our study, phagocytes do not seem to adapt to target size. We
542 saw a conserved spreading velocity of the phagocytic cup front v_0 of about 0.1 $\mu\text{m/s}$, comparable to
543 velocities measured during phagocytosis of yeasts by granulocytes (30), to the spreading velocity of

544 macrophages (33), and to the faster internalization velocity of IgG-opsonized particles by macro-
 545 phages (16). This velocity is also compatible with polymerization velocity of actin filaments by the
 546 Brownian ratchet model (56). Interestingly, Champion *et al.* (57) reported no difference in the aver-
 547 age speed of cup progression in macrophages, which was about 4 $\mu\text{m}/\text{min}$ (i.e. about 0.07 $\mu\text{m}/\text{s}$),
 548 again consistent with our measured speed v_0 of about 0.1 $\mu\text{m}/\text{s}$. Champion *et al.* used beads of
 549 diameters ranging from 3 to 9 μm , suggesting that the v_0 that we measured could be constant even
 550 for targets as small as 3 μm . Jaumouill e *et al.* also measured cup protrusion speeds of 0.1 $\mu\text{m}/\text{s}$, in
 551 RAW 264.7 macrophages (49). This constant front velocity v_0 raises the possibility of considering a
 552 mechanism for “feeling” the local curvature of a target because at constant velocity, the rate of
 553 increase in surface area $\frac{dA}{dt}$ scales with the radius of the target. Furthermore, at a constant ligand
 554 density on the target surface, $\frac{dA}{dt}$ is proportional to $\frac{dN}{dt}$, where N is the number of ligated receptors
 555 on the phagocyte surface. Thus, a phagocyte could “measure” the local curvature of its target
 556 by “sensing” the rate of receptor engagement. However, this model remains insufficient because $\frac{dA}{dt}$
 557 would also depend on the ligand being homogeneously distributed, as well as its average density:
 558 the phagocyte could not decipher a difference in curvature and in ligand density, although Kovari *et al.*
 559 showed that this density did not influence spreading dynamics once cells were committed to
 560 phagocytosis (33).

561 When compared to indentation in the back of PLB cells, indentation in the front led to a
 562 delayed onset of cup formation and a longer spreading time although the same maximal phagocytic
 563 cup area was reached (Fig. 2). Thus, the front indentation protocol can be interpreted as perturbing
 564 the cells. One possibility is that during front indentation, the cell has to be held in the pipette with
 565 some aspiration during the entire experiment (although experiments usually end with the cell ex-
 566 tracting itself from the pipette because of its increased tension), which might perturb the cell. Fu-
 567 ture studies will require careful control of the influence of the protocol with the single-cell rheom-
 568 eter, and possibly favor the more challenging but less perturbing back indentation of the phagocyte.

569 Our technique allows direct quantification of viscous effects through the measurement of
 570 K'' , the imaginary part of the complex stiffness K^* , which is intimately linked to cell stiffness K' . An
 571 increase in cell phagocyte stiffness is paralleled by an increase in phagocyte viscosity. This is difficult
 572 to account for by a model where viscous effects emanate mainly from surface expansion during
 573 phagocyte spreading, because in cases when K' increased only after an 8- μm bead had been en-
 574 gulfed, there was no additional membrane expansion. Heinrich *et al.* provided a clear explanation
 575 (58) of how the ratio of the leukocyte tension γ to its effective viscosity ν (different from the surface
 576 viscosity introduced by Herant *et al.* mentioned above), i.e. the capillary velocity $v_c = \frac{\gamma}{\nu}$, is the rel-
 577 evant scale for the cell deformation speed. Heinrich *et al.* argued that comparing the capillary ve-
 578 locity v_c to the spreading velocity v_0 during phagocytosis allows understanding why a phagocyte
 579 rounds up after spreading a thin lamellum around its target. It is worth noting that the loss tangent
 580 that we measured here, $\eta = \frac{K'}{K''}$ (29), scales as $\frac{1}{v_c}$ following dimensional analysis, so that our experi-
 581 ments allow us to estimate v_c with high temporal resolution. We have shown that η decreases dur-
 582 ing phagocytosis (Fig. 4); hence, v_c increases, consistent with the fact that rounding up of the phag-
 583 ocyte requires a higher value of v_c than during spreading (58). Further work should make more sys-
 584 tematic use of this high-temporal resolution measurement of the loss-tangent in order to better
 585 understand the physical determinants of cell geometrical changes during activation.

586 As our observations suggest that increase in cell stiffness during phagocytosis is not a direct
587 consequence of surface expansion, we shall speculate on the origin and role of these mechanical
588 changes. The fact that on smaller beads cell stiffness increased after cup closure suggests that the
589 stiffening that we measure is not directly due to myosin-II generated contractile force considered
590 to be at play during cup closure (33, 48). The role of myosins during phagocytosis is debated (48),
591 and myosin-II could have a role in disassembling F-actin rather than only in exerting contractile
592 forces (50). It is possible that the recently described myosin-dependent “teeth” that indent a phag-
593 ocytic target are part of an actin-recruiting machinery that leads to cell-scale stiffening (50). The
594 formation of the phagosome might also explain cell stiffening, since it requires actin polymerization
595 (3), and a larger target requiring a larger phagosome, might thus explain a larger increase in K' . A
596 transient accumulation of F-actin on phagosomes has been observed during phagocytosis of bacte-
597 ria and IgG opsonized particles. In a recent study, Poirier *et al.* study these so-called ‘F-actin flashes’
598 in macrophages. The flashes appear on phagosomes shortly after internalization and coincide with
599 deformation of phagocytosed red blood cells (59). F-actin flashes recruit myosin II, whose activity leads
600 to phagosome content deformation. Interestingly, the rigidity of the phagosome contents influ-
601 enced F-actin flashing dynamics, suggesting that this ‘chewing’ might help probing the target me-
602 chanical properties. This process might also help severing a target into two subtargets, as has been
603 observed in *Dictyostelium discoideum* (19) and occasionally in macrophages (59). We speculate that
604 increasing tension around the phagosome could also decrease the phagosomal volume and thus
605 increase local concentration of aggressive species aimed at attacking a phagocytosed target. Poirier *et*
606 *al.*’s observations of deformed phagosomes occurring during F-actin flashes are consistent with ob-
607 servations by Vorselen *et al.* that macrophages induce a large reduction in soft gel particles after
608 complete internalization (50). It would be very interesting to directly correlate potential F-actin
609 flashes in neutrophils and direct measurements of cell stiffness.

610 We showed that understanding mechanical aspects of phagocytosis can benefit from a direct
611 measurement of viscoelastic properties of cells with a high temporal resolution, and simultaneous
612 monitoring of cell morphology with high spatial resolution. We have shown that not all mechanical
613 changes can be considered as direct consequences of cell spreading. Further work is required to
614 understand the function of these mechanical changes. One possible direction is to slow phagocyto-
615 sis dynamics and better separate the timescales of spreading and stiffening by performing experi-
616 ments at a temperature lower than room temperature (30).

617 **AUTHORS CONTRIBUTIONS**

618 J.H. designed experiments; A.Z. and J.H. performed experiments; A.Z., S.D.-C., E.H., O.N. and J.H.
619 analyzed the data; A.B., S.D.-C., E.H., A.I.B., and O.N. contributed material; A.Z. and J.H. wrote the
620 manuscript with helpful critical reading from the other authors.

621 **DECLARATION OF INTERESTS**

622 The authors declare no competing interests.

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MOVIE LEGENDS

769 Movie Legends

770

771 **Movie S1.** Activation of PLB cells using different beads and indentation modes. Time is in
 772 minutes:seconds. Top row/ left: 20- μm bead and indentation in the front; right: control non-acti-
 773 vating 20- μm bead and indentation in the front. Middle row/ left: 20- μm bead and indentation in
 774 the back; right: 8- μm bead and indentation in the back. Bottom row/ left: 20- μm bead and cyclic
 775 indentation in the back; right; 8- μm bead and cyclic indentation in the back.

776

777 **Movie S2.** Activation of primary human neutrophils. Left: 20- μm bead, front indentation. Center: 8-
 778 μm bead, indentation in the back. Right: control non-activating 20- μm bead, indentation in the front.

779

780 **Movie S3.** Frustrated phagocytosis of PLB cells on a flat surface. Left: large field of view. Right: zoom
 781 on the cell located in the upper right part in the large field of view. This cell was also shown as
 782 example in Suppl. Fig. S2. Time is in minutes:seconds.

783

784

FIGURE LEGENDS

785 **Figure 1.** Experimental techniques. (A) Setup to indent cells at the ‘front’. Left Inset: a compressive
 786 force (red arrow) is exerted against the cell while it is phagocytosing a 20- μm activating bead (repre-
 787 sented in green) tightly held by a flexible micropipette. The cell length is recorded during the exper-
 788 iments. (B) Setup to indent cells at the ‘back’. Left inset: a 20- μm activating microbead is held by a
 789 stiff micropipette. A compressive force is exerted at the back of the cell with a non-adherent glass
 790 bead located at the tip of a flexible micropipette. Right inset: an 8- μm activating microbead is held
 791 by a stiff micropipette with a low aspiration pressure $\Delta P \sim 20$ Pa. With this setup and bead size cells
 792 can perform complete phagocytosis. (C) Frustrated phagocytosis on IgG-coated flat surfaces seen
 793 from the side. Inset: top view of a cell during frustrated phagocytosis. (D) Passive aspiration of a
 794 neutrophil into a stiff micropipette. A cell is first gently held (with a low aspiration pressure $\Delta P \sim 20$
 795 Pa) and then aspirated under high aspiration pressure ($\Delta P \sim 500$ Pa) until the cell membrane breaks
 796 as reported by an increase in fluorescence level of propidium iodide (red star).

797

798 **Figure 2.** (A) Phagocytic cup area as a function of time following cell-substrate contact for PLB cells.
 799 Dotted lines are a guide to the eye, but circles on the lines are median values, and error bars on top
 800 and right are interquartile ranges. Raw data are shown in Figures S6-11. Each condition is repre-
 801 sented by a drawing: back indentation of PLB cells during phagocytosis of 20- μm beads (red; $N=3$,
 802 $n=15$ cells) and 8- μm beads (pink, $N=3$, $n=12$ cells), and frustrated phagocytosis on flat surfaces
 803 (green; $N=3$, $n=16$ cells, a condition where K' was not quantified). Inset on the right: Maximal phag-
 804 ocytic cup area A_{max} . Inset on top: time t_{cup} (relative to cell-substrate contact) when cells start form-
 805 ing a phagocytic cup. (B) Delay between the beginning of the cup formation (t_{cup}) and the time at
 806 which the phagocytic cup reaches its maximal area ($t_{A_{\text{max}}}$). (C) Cell stiffness K' as a function of time

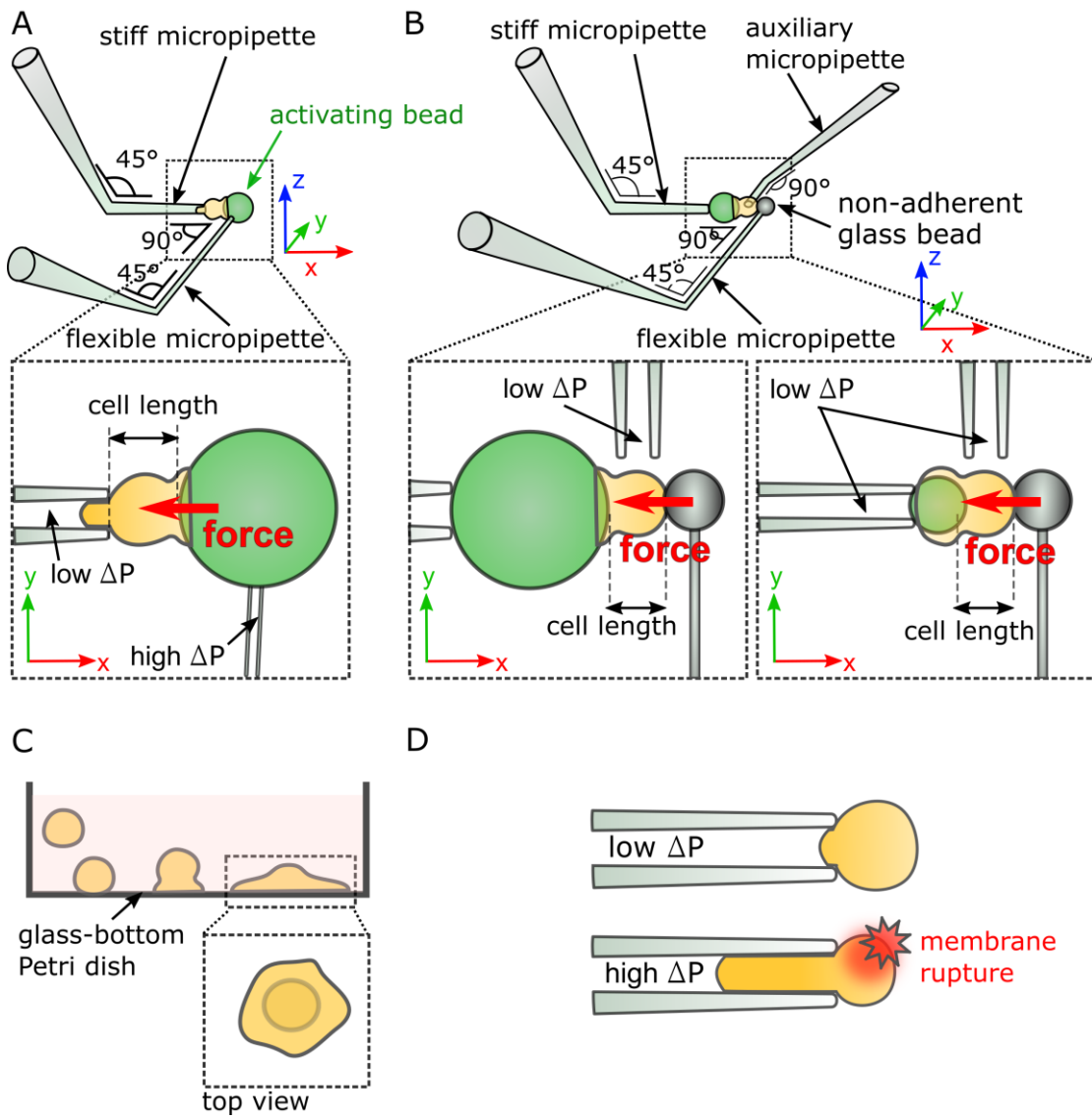
807 following cell-substrate contact for PLB cells. Curves are a guide to the eye, but dots on the curves
808 are median values. Each condition is represented by a drawing: back indentation of PLB cells during
809 phagocytosis of 20- μm beads (red; N= 3, n= 15 cells) and 8- μm beads (pink, N=3, n=12 cells). Inset
810 on the right: Maximal value of K' , K'_{max} . Inset at the bottom: time $t_{K'start}$ (relative to cell-substrate
811 contact) when K' starts increasing faster. (D) Average spreading rate of the phagocytic cup $\langle \frac{dA}{dt} \rangle =$
812 $\frac{A_{max}-A_{init}}{t_{Amax}-t_{cup}}$, where A_{init} is the initial area of the phagocytic cup. (E) Initial speed $v_0 = \frac{ds}{dt}$ of the cup
813 front during spreading. Inset on top: example of time evolution of the curvilinear abscissa s of the
814 front of a PLB cell during the phagocytosis of a 20- μm bead, during indentation in the back of the
815 cell: s slows down when reaching a level $s = s_{slowdown}$. (F) Maximal total cell area as measured using
816 micropipette aspiration (purple squares) and during frustrated phagocytosis on 20- μm beads during
817 back indentation (red squares), both for PLB cells. (G) Duration of cell stiffening, $t_{K'max}-t_{K'start}$. (H)
818 Difference between $t_{K'max}$ and t_{Amax} . This difference is positive if K' reaches its maximum after the
819 phagocytic cup had already reached its maximal area. (I) Difference between $t_{K'start}$ and t_{Amax} . This
820 difference is positive if K' starts increasing after the phagocytic cup had already reached its maximal
821 area. (J) Examples of different relative time of cell spreading (phagocytic cup area in thick blue line)
822 and stiffening (cell stiffness K' in red thin line) during phagocytosis (indentation in the back of PLB
823 cells phagocytosing 8- μm beads). In A-I, lines are median values and error bars are interquartile ranges.
824 Statistical tests in A and F: Kolmogorov-Smirnov test (* p<0.05, ** p<0.01). Statistical tests in B-E
825 and G-I: two-tailed Mann-Whitney (* p<0.05, ** p<0.01).

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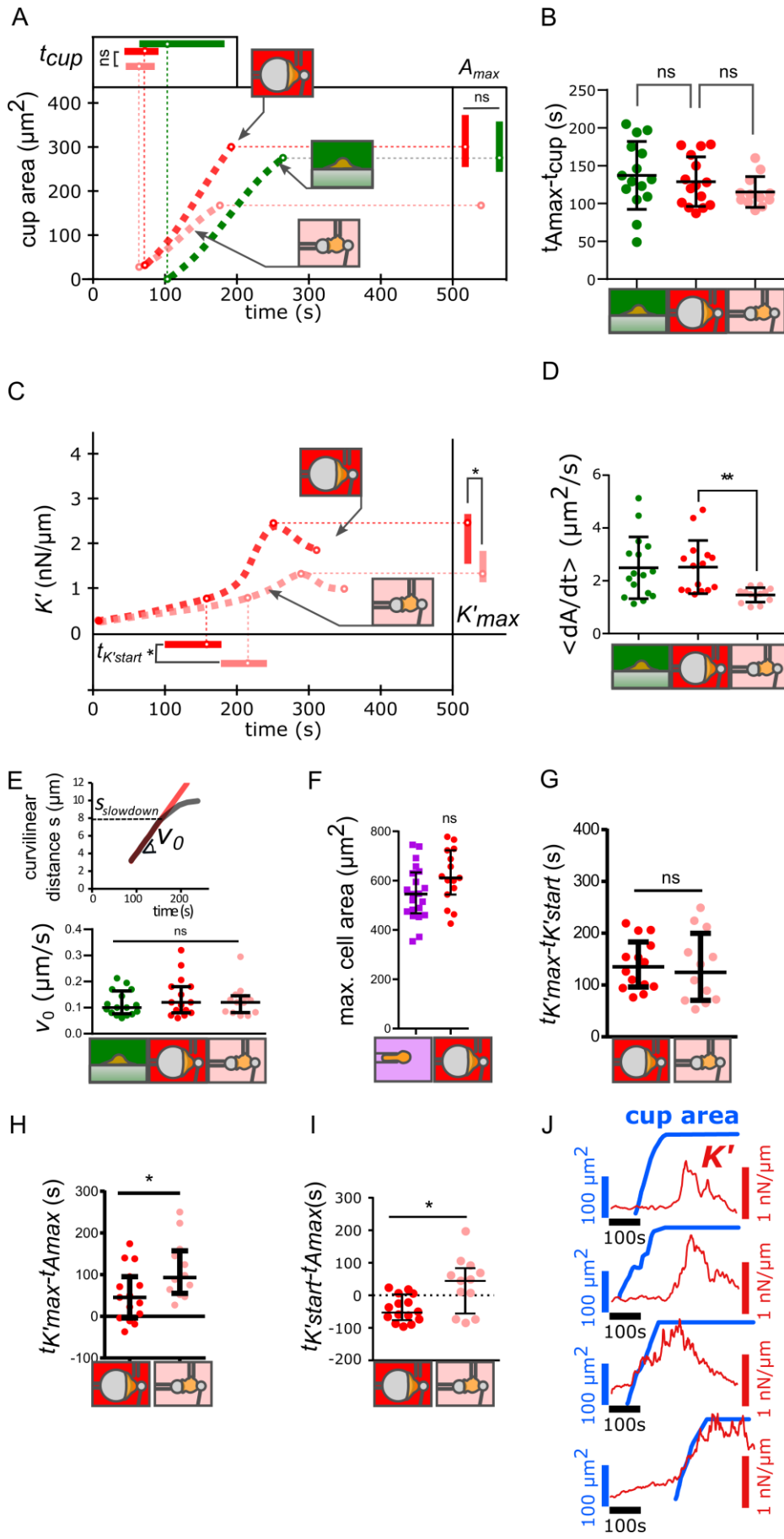
827 **Figure 3.** Comparison of PLB cells and primary neutrophils. (A) Phagocytic cup area as a function of
828 time following cell-substrate contact. Dotted lines are a guide to the eye, but circles on the lines are
829 median values, and error bars on top and right are interquartile ranges. Added data for primary
830 neutrophils (blue) are back indentation during phagocytosis of 20- μm beads (dark blue in A-G; N=2,
831 n= 15 cells) and 8- μm beads (light blue in A-G; N=2, n= 13 cells). Inset on the right: Maximal phago-
832 cytic cup area A_{max} . Inset on top: time t_{cup} (relative to cell-substrate contact) at which cells start
833 forming a phagocytic cup. Raw data are shown in Figures S6-11. (B) Delay $t_{Amax} - t_{cup}$ between the
834 beginning of the cup formation (t_{cup}) and the time at which the phagocytic cup reaches its maximal
835 area (t_{Amax}). (C) Cell stiffness K' as a function of time following cell-substrate contact. Curves are a
836 guide to the eye, but dots on the curves are median values. Each condition is represented by a
837 drawing: back indentation of 20- and 8- μm beads for PLB cells and primary neutrophils. Inset on the
838 right: Maximal value of K' , K'_{max} . Inset at the bottom: time $t_{K'start}$ (relative to cell-substrate contact)
839 when K' starts increasing faster. (D) Average spreading rate of the phagocytic cup $\langle \frac{dA}{dt} \rangle = \frac{A_{max}-A_{init}}{t_{Amax}-t_{cup}}$,
840 where A_{init} is the initial area of the phagocytic cup. (E) Initial speed $v_0 = \frac{ds}{dt}$ of the cup front during
841 spreading. (F) Duration of cell stiffening, $t_{K'max}-t_{K'start}$. (G) Difference between $t_{K'start}$ and t_{Amax} . This
842 difference is positive if K' starts increasing after the phagocytic cup had already reached its maximal
843 area. In A-G, median values are shown with interquartile ranges. Statistical tests in A, and C: Kolmo-
844 gorov-Smirnov test (* p<0.05). Statistical tests in B, D-G: two-tailed Mann-Whitney (* p<0.05, **
845 p<0.01, *** p<0.001, **** p<0.0001).

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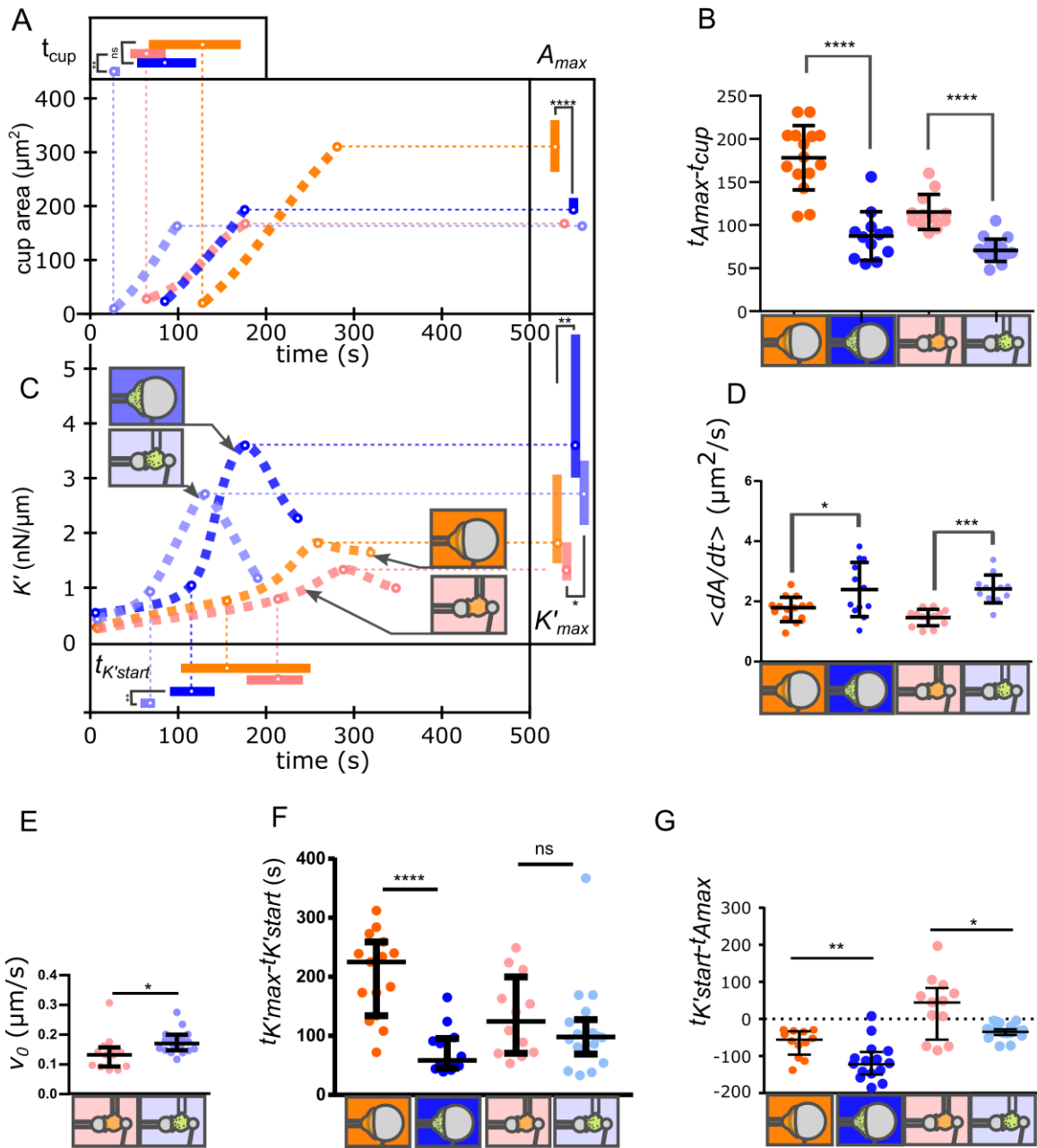
847 **Figure 4.** Viscoelastic behavior during both complete and frustrated phagocytosis when indenting
848 PLB cells (A, B, D, F) and primary neutrophils (C, E, G). Indentation in the front, back and on 20- or
849 8- μm beads is indicated with schematics of the protocol used. In B-G, for each individual curve, time
850 was shifted so that time origin corresponds to the moment at which K' reaches a level of 0.5 nN/ μm
851 above the initial level ($t=t_{K'start}$). (A) Young's modulus E_{Young} of PLB cells measured by cyclic indenta-
852 tion in the back (see Materials and Methods and Suppl. Fig. S1). E_{Young} is significantly higher during
853 back indentation on 20- than on 8- μm beads at $t = 178$ s (*). Median values (dots) and interquartile
854 ranges (error bars) are shown. (B-C) Median cell stiffness K' over time. In B, at $t=78$ s, K' is larger
855 during indentation of 20- than 8- μm beads (* and **, Mann). (D-E) Median K'' over time. (F-G) Bot-
856 tom: loss tangent $\eta = \frac{K''}{K'}$ over time. Different experimental conditions are shown: PLB cells with 20-
857 μm (red; N= 3, n= 15 cells) and 8- μm beads (pink; N= 3 experiments; n= 12 cells); control condition
858 with non-opsonized 8- μm beads with back-indentation of PLB cells (black, N = 3 experiments, n= 17
859 cells); human primary neutrophils with 20- μm (dark blue; N = 2 experiments, n= 12 cells) and 8- μm
860 (light blue; N = 2 experiments, n= 18 cells) beads. Each solid line represents the median value; for
861 readability the interquartile range is not shown. η decreases during phagocytosis (comparing $t-t_{K'start}$
862 = -100s with $t-t_{K'start} = 50$ s or 80 s in F, and comparing $t-t_{K'start} = -60$ s with $t-t_{K'start} = 50$ s in G, star
863 symbols, two-tailed Mann-Whitney test). Raw data are shown on Figures S6-11.
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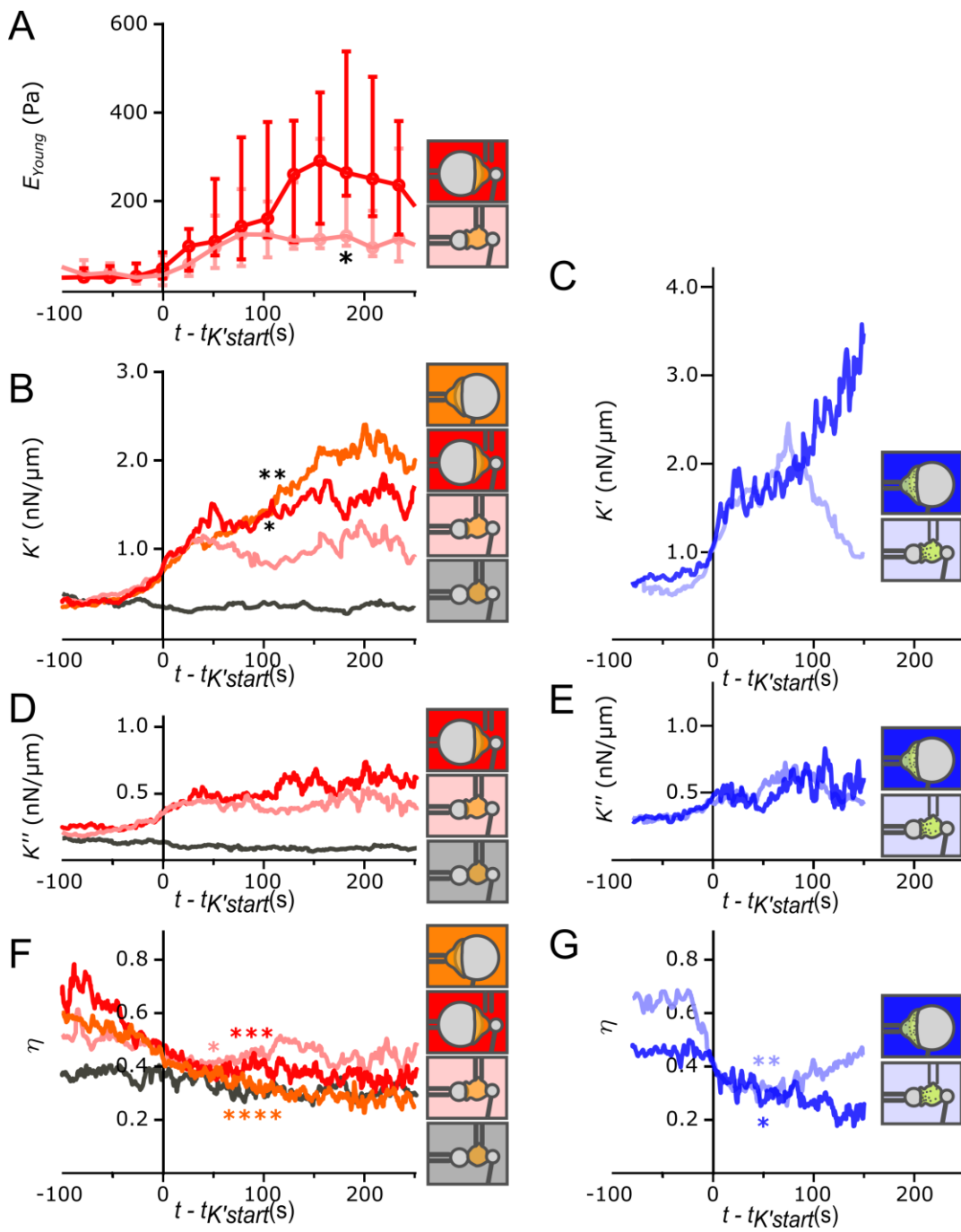
865
866 Figure 1
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873 Figure 4