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1 Amoeboid swimming is propelled by molecular paddling in Lymphocytes

2

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14 **ABSTRACT**

15 Mammalian cells developed two main migration modes. The slow mesenchymatous mode, like
16 crawling of fibroblasts, relies on maturation of adhesion complexes and actin fibre traction, while the
17 fast amoeboid mode, observed exclusively for leukocytes and cancer cells, is characterized by weak
18 adhesion, highly dynamic cell shapes, and ubiquitous motility on 2D and in 3D solid matrix. In both
19 cases, interactions with the substrate by adhesion or friction are widely accepted as a prerequisite
20 for mammalian cell motility, which precludes swimming. We show here experimental and
21 computational evidence that leukocytes do swim, and that efficient propulsion is not fuelled by
22 waves of cell deformation but by a rearward and inhomogeneous treadmilling of the cell external
23 membrane. Our model consists of a molecular paddling by transmembrane proteins linked to and
24 advected by the actin cortex, whereas freely diffusing transmembrane proteins hinder swimming.
25 Furthermore, continuous paddling is enabled by a combination of external treadmilling and selective
26 recycling by internal vesicular transport of cortex-bound transmembrane proteins. This mechanism
27 explains observations that swimming is five times slower than the retrograde flow of cortex, and also
28 that lymphocytes are motile in non-adherent confined environments. Resultantly, the ubiquitous
29 ability of mammalian amoeboid cells to migrate in 2D or 3D, and with or without adhesion, can be
30 explained for lymphocytes by a single machinery of heterogeneous membrane treadmilling.

31 SIGNIFICANCE STATEMENT

32 Leukocytes have a ubiquitous capacity to migrate on or in solid matrices, and with or without
33 adhesion, which is instrumental to fight infections. The precise mechanisms sustaining migration
34 remain however arguable. It is for instance widely accepted that leukocytes cannot crawl on 2D
35 substrates without adhesion. In contrast, we showed that human lymphocytes swim on non-
36 adherent 2D substrates and in suspension. Furthermore, our experiments and modelling suggest that
37 propulsion rely hardly on cell body deformations and predominantly on molecular paddling by
38 transmembrane proteins protruding outside the cell. For physics, this study reveals a new type of
39 micro-swimmer, and for biology it suggests that leukocytes ubiquitous crawling may have evolved
40 from an early machinery of swimming shared by various eukaryotic cells.

41

42 INTRODUCTION

43 Individual living cells developed different strategies to migrate and explore their environment.
44 Bacteria, microalgae or mammalian gametes swim in a fluid under the propulsion of a flagellum¹ or
45 of shape deformations², whereas somatic mammalian cells crawl with adhesion on a solid tissue, via
46 a continuous sequence of forward pushing of the cell front, strengthening of adhesion at the leading
47 edge, and pulling of the cell rear^{3,4}. In vivo, mammalian cells crawl either on 2D substrates, like
48 leukocytes on inner blood vessels and epithelial surfaces, or in 3D environments within tissues. The
49 critical role of adhesion for crawling motility was recently revised in the case of amoeboid
50 mammalian cells, i.e. white blood cells and cancer cells. Amoeboid cells differ from mesenchymatous
51 cells (e.g. fibroblasts) by a significantly higher speed (typically 5-20 $\mu\text{m}\cdot\text{min}^{-1}$ vs 0.1-1 $\mu\text{m}\cdot\text{min}^{-1}$) and
52 by highly dynamic shape deformations. Both amoeboid and mesenchymatous cells crawl on adhering
53 substrates but only amoeboid cells remain motile without adhesion provided that they are confined
54 by a 3D environment⁵⁻⁸. Although different models exist, this motility in confined non-adherent
55 conditions was generally explained by a chimneying⁶ mode where cell-substrate interactions are
56 mediated by friction instead of adhesion⁸⁻¹¹. It is therefore widely accepted that amoeboid motility of
57 mammalian cells is strictly dependent on adhesion on 2D substrates and on adhesion/friction in 3D
58 media, while non-adherent 2D migration and swimming are precluded^{5-7,12-19}.

59 In contradiction with this paradigm of adhesive or frictional crawling, Barry and Bretscher²⁰ reported
60 in 2010 that human neutrophils do swim. They discussed that propulsion may result from membrane
61 treadmilling (rearward movement of the cell surface) or shape-deformation (protrusions and
62 contractions along the cell body) but provided no experimental or theoretical evidence supporting
63 either of these hypotheses. Swimming studies on non-mammalian eukaryotic cell, the amoeba
64 *Dyctyostellium discoideum*, have later defended a deformation-based propulsion²¹, whereas others

65 discarded both treadmilling and shape deformation²². For tumoral cells, a theoretical model of
66 blebbing mentioned the possibility of migration in suspension by shape changes²³, whereas other
67 modelling efforts validated a swimming mechanism based on shape deformation for the case of
68 amoeba^{24–26}, cyanobacteria²⁷ and microalgae². A recent study on leukocytes, a mesenchymatous
69 macrophages cell line RAW 264.7, reported that an amoeboid swimming mode could be artificially
70 triggered by optogenetic activation of actomyosin contractility in cell rear²⁸. In contrast to previous
71 studies, membrane treadmilling seemed mainly involved in propulsion, whereas the contribution of
72 deformations was not assessed. Altogether, swimming of cells without flagellum remains mostly
73 explained by shape deformation mechanisms. Moreover, swimming of mammalian cells without
74 flagellum remains widely discarded^{5,6,12,14,17–19,29}.

75 Here, we demonstrate the existence of mammalian amoeboid swimming on primary human T
76 lymphocytes and decipher its functioning experimentally and theoretically at the cellular and
77 molecular scales. T lymphocytes are known to crawl on 2D adhering substrates^{16,30–32} and in 3D
78 matrices via adhesion/friction^{11,33,34} at typical speeds of 20 $\mu\text{m}\cdot\text{min}^{-1}$. We observed swimming motility
79 with an average speed of 5 $\mu\text{m}\cdot\text{min}^{-1}$ and show that propulsion can be mainly explained by a
80 heterogeneous treadmilling of cell membrane. Actin-bound transmembrane proteins paddle by
81 retrograde transport at the membrane and are recycled from rear to front of cells by anterograde
82 vesicular transport, whereas non-actin-bound transmembrane proteins are diffusive and hinder
83 swimming. This molecular description is consistent with a speed significantly lower for swimming cell
84 than for crawling cells and cortex retrograde flow. Interestingly, heterogeneous membrane
85 treadmilling supports also ubiquitous cell motility within non-adhesive solid matrices because
86 environmental friction is necessarily larger with a solid than with a fluid.

87

88 **RESULTS**

89 **Leukocytes are motile on a solid substrate without adhesion or friction**

90 Upon recruitment from the blood stream toward inflammation zones, leukocytes arrest and crawl on
91 the inner surface of blood vessels. This 2D migration, called crawling, was here mimicked in vitro with
92 human primary effector T lymphocytes on glass substrates coated with ICAM-1 molecules, a specific
93 ligand for the integrin adhesion molecules LFA-1 ($\alpha\text{L}\beta\text{2}$). Effector T lymphocytes developed a stable
94 polarization in suspension, with a front pole forming protrusions (lamellipods) under the influence of
95 actin polymerization and a rear pole (uropod) undergoing contraction cycles enforced by acto-myosin
96 contractility. When introduced into a chamber coated with ICAM-1, lymphocytes sedimented,
97 adhered to, and migrated on the substrates. They crawled with a random walk pattern (Figure 1-A

98 and Suppl. Mat. Movie 1) of curvilinear speed $14.7 \pm \text{SD } 7.5 \mu\text{m}\cdot\text{min}^{-1}$, with a wide spread lamellipod
99 in front, the nucleus positioned in the cell central zone, and a narrow uropod in rear. To challenge
100 the idea that adhesion is necessary for amoeboid migration on a 2D substrate, we then replaced the
101 ICAM-1 surface treatment by an anti-adhesive coating of Pluronic[®] F127 (Figure 1-B). Without
102 adhesion, cells were highly sensitive to residual flows, which must be dampened to allow observation
103 and characterization of self-propulsion. After sedimentation, cells displayed a random walk with an
104 apparent average curvilinear speed of $5.5 \pm \text{SD } 2.2 \mu\text{m}\cdot\text{min}^{-1}$ (Figure 1-B-i and Suppl. Mat. Movie 1).
105 To assess the adhesion state of cells versus surface treatment, we performed contrast
106 interferometric imaging, RICM (Figure 1-A,B-iii and Suppl. Mat. Movie 1). A dark contact zone of cells
107 on ICAM-1 substrates corresponded to a cell-substrate distance around 50 nm, which is characteristic
108 of tightly adhered cells with extracellular proteins and polysaccharides confined between the
109 substrate and the adherent plasma membrane^{35,36}. In contrast, a bright contact zone of cells on
110 Pluronics in RICM corresponded to a distance around 100 nm, which is typical of the presence of a
111 thin liquid film separating the substrate from the fluctuating plasma membrane of a cell settling by
112 gravity. According to the RICM assays, 100% of polarized and motile cells were non adherent on
113 Pluronics (Suppl. Mat **Erreur ! Source du renvoi introuvable.**). Non-adherent Cells migrating in the
114 vicinity of a non-adherent substrate were further imaged in 3D by spinning-disk microscopy (Suppl.
115 Mat. Movie 2). Although strong phototoxicity hampered long-term 3D imaging, migration of
116 polarized cells with dynamic 3D shape deformations could be monitored on tens of micrometres.
117 Altogether, these data show that polarized lymphocytes do migrate on a 2D surface in the absence of
118 adhesion.

119 **Cells can switch between adherent and non-adherent migration**

120 To shed light on the transition between adherent and non-adherent migration, we then presented
121 lymphocytes to substrates patterned with alternated stripes of adhesive and anti-adhesive coatings
122 (Figure 1-C and Suppl. Mat. Movie 3). While mesenchymatous cells are known to be strictly confined
123 to adherent stripes in similar experiment³⁷, lymphocytes showed here a unique capability to transfer
124 repeatedly between adherent and non-adherent zones. The stripes width of $40 \mu\text{m}$ was chosen large
125 enough to allow cells to travel on homogeneous stripes for tens of seconds, and small enough to
126 favour the occurrence of transitions between different stripes. Interference microscopy attested that
127 cells travelled across the adhesive zones with adhesive contact to the substrate (green signal) and
128 across anti-adhesive zones with a lubrication film separating the cells from the substrate (no green
129 signal). Cells were even found to have the same probability of presence on adherent and anti-
130 adherent stripe³⁸. These observations suggest that the transition between adherent and non-

131 adherent motility modes is fast, or that the two phenotypes share the same machinery, which
132 altogether cancels the idea of switch between two modes.

133

134 **Diffusive versus active motion in non-adherent migration**

135 The average speed of cells measured using the average displacements of cell mass centre every 30s
136 was significantly different between adherent and non-adherent cells (Figure 2-A). However, this
137 estimation of the active motion of cells was biased by several effects for non-adherent cells. First, our
138 system of effector lymphocytes in suspension comprised two populations, one of round and inactive
139 cells with a typical circularity > 0.8 , and the other of polarized and active cells with a typical
140 circularity < 0.8 . The average fraction of active cells in suspension is around $48 \pm \text{SEM } 5\%$. Figure 2-B
141 presents the histogram of raw speeds for individual cells in the case of live and fixed lymphocytes (all
142 cells shape are frozen by paraformaldehyde in the latter case). While fixed cells were not motile, they
143 displayed an apparent raw curvilinear speed of $2.8 \pm 0.3 \mu\text{m}\cdot\text{min}^{-1}$ due to Brownian diffusion. Live
144 cells displayed two populations, one with low speed corresponding to round and inactive cells, and
145 one with higher speed corresponding to polarized and active cells. The apparent speed of inactive
146 cells was close to the one of fixed cells, meaning that their movement was close to Brownian motion.
147 In contrast, active cells had a significantly larger average raw curvilinear speed of $5.9 \pm 4.2 \mu\text{m}\cdot\text{min}^{-1}$.
148 However, this new estimated speed does not strictly characterize active motility because it still
149 comprises diffusion effects, as evidenced by fixed cells or round inactive cells. We therefore
150 performed a more detailed analysis of cell trajectories to discriminate the diffusive contribution from
151 the effective active motion. First, we investigated the mean square displacement averaged over all
152 cells in the population as a function of time. Second, we fitted the mean square displacements as a
153 function of time interval by a random-walk law, which combines 2D Brownian-like diffusion with
154 persistent motion, and we analysed the distribution of speeds v_s and diffusion coefficients D_t
155 obtained by the fitting procedure:

156 Equation 1
$$\langle [r(t) - r(0)]^2 \rangle = v_s^2 t^2 + 4D_t t,$$

157 We then separated all cells in the population into two groups. We considered as active the cells that
158 travelled at least a distance of $25 \mu\text{m}$ (about two cell diameters) during the acquisition time of 13 min
159 and the rest of the cells being referred to as inactive. **Figure 2-C** shows the mean square
160 displacement as a function of time for fixed and live cells, and we also report inactive and active cells
161 separately for live cells. Both inactive and fixed cells had a diffusive behaviour, with an average
162 diffusion coefficient of respectively $2.3 \mu\text{m}^2\cdot\text{min}^{-1}$ and $1.1 \mu\text{m}^2\cdot\text{min}^{-1}$, whereas active cells had a
163 super-diffusive behaviour. A satisfactory fit could not be obtained with Equation 1 and we extended

164 the model by adding rotational diffusion (with a rotational diffusion coefficient D_r), which accounted
165 for gradual changes in the direction of the cells (see Suppl. Mat.). The fitting procedure gave here $v_s =$
166 $4.3 \mu\text{m}\cdot\text{min}^{-1}$, $D_r = 0.19 \text{ min}^{-1}$ and $D_t = 7.28 \mu\text{m}^2\cdot\text{min}^{-1}$ (black curve in Figure 2-C). To obtain the
167 distribution of speeds of active cells, we then simplified the analysis and considered only
168 displacements for time intervals of 2 min, which is 3 times smaller than $1/D_r$ and therefore allowed
169 us to neglect rotational diffusion. As can be observed in Figure 2-D, most of active cells had a speed
170 around 3 to 5 $\mu\text{m}\cdot\text{min}^{-1}$ and root mean square speed extracted of individual active cells was finally
171 $4.9 \mu\text{m}\cdot\text{min}^{-1}$ in cell culture medium. This precise determination of cell speed without adhesion will
172 later be instrumental to propose a consistent mechanism.

173 **Active lymphocytes swim in free suspension**

174 While active lymphocytes migrated in the vicinity of a surface without adhesion, one may argue that
175 propulsion close to a substrate may be favoured by hydrodynamic coupling between cells and
176 substrate. We therefore performed experiments with cells in bulk suspension to test the existence of
177 swimming without hydrodynamic coupling to any solid wall. It appeared quite challenging to cancel
178 artefactual cell displacements in suspension. Conditions of negligible flow were difficult to achieve
179 due to several perturbative effects such as temperature gradients, pressure imbalance between
180 channel outlets, or gravimetric imbalance resulting from cell dispersion inhomogeneity. In the end,
181 we used a microfluidic channel connected to high precision pressure controller and highly resistant
182 tubing connections to slow down pressure-driven flows. Besides, cell sedimentation was decreased
183 using Ficoll supplemented medium to match the average density of cells. Still, cells had dispersed
184 densities and they exchanged material with the medium, so that sedimentation drifts were changing
185 with each cell and with time. As a consequence, flow and sedimentation drifts could not be totally
186 cancelled and we decided to merge their combined artefactual effects on a single axis by tilting the
187 microscope by 90° and setting the main axis of the microfluidic channel along the vertical axis (Figure
188 3-A and Suppl. Mat. for details). In this configuration, the vertical z-axis cumulated artefacts due to
189 pressure-driven flow across the microchannel main direction, which was low but non null, and due to
190 the gravity-induced sedimentation, which was different from one cell to another. Swimming prowess
191 was then measured on the two other axes x and y (Figure 3-B and suppl. Mat. Movie 4). Cell position
192 on y-axis was determined from selection of the best focused images on y-stack scans for each time
193 point. In line with the results on a non-adherent substrate, round cells (circularity > 0.8) were used as
194 negative control for inactive and non-swimming cells, whereas polarized cells (circularity < 0.8) were
195 tested as the active and potentially swimming cells. Figure 3-C shows that inactive cells displayed a
196 curvilinear speed of $2.1 \pm \text{SD } 0.6 \mu\text{m}\cdot\text{min}^{-1}$, which is close to the value found for diffusive cells in 2D.
197 In contrast, active polarized cells displayed an apparent average curvilinear speed of $8.9 \pm \text{SD } 2.4$

198 $\mu\text{m}\cdot\text{min}^{-1}$. These data confirm the intrinsic capability of polarized lymphocytes to swim in suspension
199 at a several $\mu\text{m}\cdot\text{min}^{-1}$. Furthermore, experiments showed that the vicinity of a wall within a few
200 micrometre range had no significant effect on swimming speed (**Erreur ! Source du renvoi**
201 **introuvable.**). We performed theoretical calculations to get quantitative insight on the influence of
202 substrate on swimming speed down to a distance 100 nm range, which corresponds to the case of
203 sedimented cells migrating over non-adherent substrates in Figures 1 and 2. Our calculations support
204 that the vicinity of a single wall has a negligible effect on swimming speed (see Suppl Mat. “Vicinity of
205 a wall is negligible”). In what follows, systematic measurements to characterize swimming properties
206 were therefore performed with the more convenient set-up of cells close to a non-adherent
207 substrate.

208 **Swimming propulsion is oriented along polarization axis toward lamellipod edge**

209 Swimming appears to be a specific property of polarized and active cells, while inactive and round
210 cells are just passively diffusing. We further analysed the angular difference between the direction of
211 polarization of active cells and their instant displacement direction (Movie 5). Active cells showed a
212 maximum probability for swimming direction collinear to cell polarization, whereas fixed cells
213 displayed no preferential orientation of motion (See suppl. Mat. **Erreur ! Source du renvoi**
214 **introuvable.**). This analysis supports further that the process of swimming is specific to active and
215 polarized cells, and that swimming propulsion is oriented along the cells polarization axis in the
216 direction of lamellipod.

217 **Viscosity hardly affects swimming speed**

218 Swimming relies necessarily on the coupling between a cell motility machinery and the surrounding
219 fluid. One may therefore expect the viscosity of the fluid to have an effect on the coupling efficiency.
220 Swimming speed was measured versus the different viscosities of the external medium by addition of
221 dextran of molecular weight 2,000 kDa. Viscosity was increased by 100 folds as compared to normal
222 medium. Higher dextran concentrations were inaccessible because cells were strongly pressed to the
223 substrate by depletion force and consequently immobilized. For a 10-fold viscosity increase, there
224 was a slight decrease of speed by 25 %, however this tendency was not confirmed for a 100-fold
225 viscosity increase, for which speed was similar to control. Hence, counterintuitively, cell swimming
226 speed was hardly affected by a change of viscosity of two orders of magnitude (Suppl. Mat. Table 1
227 and **Erreur ! Source du renvoi introuvable.**). The insensitivity of cell speed to external viscosity is
228 traced back to the fact that dissipation due to swimming occurs predominately in the cell cortex
229 which has a much higher viscosity compared to the suspending medium. This means that we expect
230 an effect of external viscosity only when it becomes comparable to that of the cortex.

231 **Actin mediates swimming propulsion by polymerization and to a lesser extent contractility**

232 Actin cytoskeleton is widely accepted as the molecular engine propelling cell crawling. Effector T
233 lymphocytes are characterized by a strongly polarized state with actomyosin contractility mainly in
234 the cell rear (uropod), and actin polymerization mainly in the cell front (lamellipod). We first
235 perturbed cells with Blebbistatin, a potent inhibitor of actomyosin contractility. The uropod was not
236 distinguishable anymore and the cell body looked devoid of contractile activity, but motile cells
237 displayed a small lamellipod, which attested a conserved front-rear polarization. (Figure 4-A,B and
238 Suppl. Mat. Movie 6). The dampening of cells deformations due to contractility inhibition induced a
239 significant increase of the circularity index (Figure 4-B, Right), while swimming speed decreased by
240 25% as compared to control cells (Figure 4-C and Table 1). Swimming direction and cell polarization
241 were still markedly correlated, even more than for control cells (See suppl. Mat. **Erreur ! Source du**
242 **renvoi introuvable.**). These results suggest that actin contractility and contractile cell deformations
243 are not required for swimming, and conversely that frontal actin polymerization play a dominant
244 role. We then perturbed actin polymerization in the cell front with Latrunculin (Figure 4-A Suppl.
245 Mat. Movie 6). The dose was chosen low enough to inhibit the lamellipod formation at the cell front
246 while preserving contractility in the cell rear. Latrunculin-treated cells were therefore deprived of
247 lamellipod but conserved a uropod, which is the opposite situation to blebbistatin-treated cells. The
248 fraction of swimming cells was significantly lower than for both control and blebbistatin-treated cells
249 (Figure 4-C and Table 1), which suggests that actin polymerisation plays a more preponderant role
250 than contractility in propulsion. We then treated cells with CK666, an inhibitor of the protein Arp2/3
251 that mediates branching of the actin network in lamellipods (Figure 4-A Suppl. Mat. Movie 6). While
252 the front of migrating leukocytes usually displayed lamellar shaped protrusions³⁹, CK666-treated cells
253 formed filopodia and blebs in cell front. The average effect of CK666 on swimming speed was found
254 intermediate between the blebbistatin and Latrunculin cases in terms of fraction of migrating cells.
255 Altogether, swimming was more efficient with a perturbed lamellipod (CK666) than without
256 lamellipod (Latrunculin). This correlation between a stronger perturbation of the lamellipod and
257 slower swimming is in line with the lamellipod being important for propulsion. Besides, swimming
258 was more efficient for totally inhibited uropod (Blebbistatin) than for partially inhibited lamellipod
259 (CK666), which corroborates the idea that swimming would be mediated to a larger extent by
260 lamellipod rather than by uropod. Finally, swimming was fully abrogated with a combination of
261 blebbistatin and Latrunculin (Figure 4-A,C and Suppl. Mat. Movie 6), which suggests that actin
262 network is the main engine of lymphocyte swimming.

263 **Rearward travelling of membrane protrusions alone seems not efficient enough to propel**
264 **lymphocyte swimming**

265 Swimming propulsion arises from the interactions of the cell external membrane with the
266 surrounding fluid, therefore the dynamic properties of the cell external membrane is the key of
267 swimming mechanism. Like all amoeboid cells, lymphocytes display highly dynamic shape
268 deformations, which are good candidates for generating propulsion. To precisely monitor cell
269 deformations and circumvent the strong photosensitivity issues, we resorted to light-sheet soSPIM
270 microscopy on cells transfected with RFP-Lifeact. The 3D dynamics of cell cytoskeleton (Figure 5-A-
271 left and Suppl. Mat. Movie 7) revealed waves of lamellar protrusions that formed with different
272 orientations in the cell front^{39,40} and travelled backwards until they vanished in cell rear. Similar
273 propagating waves of cell membrane were also visible in transmission microscopy (Figure 5-A-right,
274 and Suppl. Mat. Movie 8)^{18,41,42} together with constriction rings provoking sudden nucleus movement
275 and important reorganizations of cells contours toward the direction of cell motility (See also Suppl.
276 Mat. Movie 8). This dynamics of cell contours is qualitatively reminiscent of the shape-deformation
277 cycles analysed in theoretical modelling of amoeboid swimming². To get more quantitative insight,
278 we developed a direct numerical simulation of normal active forces applied to the cell membrane
279 (with force-free and torque-free conditions) that generated deformations travelling backwards along
280 the cell body (Figure 5-B, Suppl. Mat Movie 9, and suppl. Mat **Erreur ! Source du renvoi introuvable.**,
281 **Erreur ! Source du renvoi introuvable.**). Our model presents the rare advantage of using only
282 parameters accessible experimentally such as the amplitude and speed of deformation waves. Waves
283 of a few microns amplitude and 10 $\mu\text{m}\cdot\text{min}^{-1}$ speed observed experimentally yielded in our
284 simulation a swimming speed 1000 times slower than the experimental swimming speed (Suppl. Mat.
285 **Erreur ! Source du renvoi introuvable.**). This quantitative analysis suggests strongly that
286 deformations are not efficient enough to propel swimming of lymphocytes. To support this result
287 further, we performed perturbations of cell shapes by osmotic swelling. Addition of 50% of water in
288 medium resulted in an average cell volume increase by 65%, as assessed by the size increase of
289 round cells, and imposed a more roundish shape on motile cells, as assessed by the increase of their
290 circularity index (Figure 5-C and Suppl. Mat. Movie 10). Nevertheless, swimming speed was not
291 significantly affected. This result supports that average shapes are not crucial for swimming, but it
292 says nothing about the role of propagating deformations. We then performed a spatio-temporal
293 analysis of cells contours using wavelet transforms (Figure 5-D,E and suppl. Mat **Erreur ! Source du**
294 **renvoi introuvable.** and **Erreur ! Source du renvoi introuvable.**). As compared to control cells, the
295 dynamic mode of protrusion that propagated backwards was found hardly altered by osmotic
296 swelling, but strongly affected by blebbistatin treatment. For osmotic swelling, protrusions paddling
297 and swimming are both hardly perturbed, which yields little information. In contrast, with
298 blebbistatin treatment, the amplitude of propagative protrusions was decreased by a factor larger
299 than two (see also Suppl. Mat. Movie 6) so that swimming speed should decrease by more than 80%

300 according to our model of propulsion by deformations (See suppl. Mat. **Erreur ! Source du renvoi**
301 **introuvable.**). In fact, the experimental speed decreased by less than 25%, which further supports
302 that protrusion may not be the main propelling source of swimming. Finally, we reasoned that if
303 micron-scale deformations were not sufficient to propel swimming, sub-micron-scale deformations,
304 typically undetected by optical microscopy, might mediate efficient propulsion. We therefore
305 simulated the effect of submicronic waves by varying their number, shapes and organization, and
306 found an optimum for an idealized case of trains of 3 waves travelling in phase (see Suppl. Mat.
307 **Erreur ! Source du renvoi introuvable.** and **Erreur ! Source du renvoi introuvable.**). Still, swimming
308 speed was more than 10 times slower than the protrusions travelling backwards at $10 \mu\text{m}\cdot\text{min}^{-1}$.
309 Altogether, quantitative analysis and perturbations experiments support that cell deformations are
310 not sufficient to propel lymphocytes swimming. This conclusion motivates the search of alternative
311 and more plausible sources of propulsion.

312 **Membrane rearward treadmilling correlates with swimming speed**

313 Normal motion of cell membrane seems to yield insufficient propulsion, but the membrane of
314 amoeboid cells displays also tangential movement triggered by the retrograde flow of the inner actin
315 cortex. To evidence the motion of cells external membrane, we incubated swimming cells with ICAM-
316 1 coated beads. When cells encountered beads along their path, beads attached to cell front via the
317 transmembrane integrins LFA-1 and were carried backwards (Figure 6-A and Movie 11). The average
318 speed of beads in the reference frame of the cell was $12 \pm 3 \mu\text{m}\cdot\text{min}^{-1}$, which is significantly larger
319 than swimming speed. Similar measurements with inhibitors Blebbistatin, CK666 and Latrunculin
320 showed a decrease of beads speed as compared to control (Figure 6-B and Movie 11). More
321 precisely, the speed changes with Blebbistatin, CK666 and Latrunculin correlated with the respective
322 changes in swimming speed (Figure 6-B), which suggests a direct role of tangential membrane
323 motion in swimming.

324 **Retrograde flow of cell membrane can propel swimming**

325 To analyse quantitatively the propulsion strength induced by tangential movements of cells
326 membrane, we developed a basic model of retrograde flow taking into account an internal actin
327 cortex, a cytoplasmic lipidic membrane and transmembrane proteins protruding outside the cell
328 (**Figure 6-C**). The transfer of movement from the inner cortex to the fluid surrounding the cell can be
329 either total or partial depending on the coupling mechanism between the actin cortex and the lipids
330 and proteins of the membrane, as discussed below. We first considered that the cell surface was
331 made of a homogeneous membrane with an average treadmilling velocity proportional to the

332 velocity of the actin cortex, v_a , and a transmission coefficient denoted β . In the laboratory frame,
333 the velocity of the fluid adjacent to the cell membrane, denoted as v_f , is:

334 Equation 2
$$v_f(r) = \beta v_a(r) + v_s + \omega_s r$$

335 where v_s and ω_s are respectively the translation and rotation swimming velocities of the cell. They
336 were obtained by solving the Stokes equations (see Suppl. Mat.) in the fluid outside the cell, taking as
337 boundary conditions that the flow vanished at large distances away from the cell, and that it obeyed
338 Equation 2 at the cell surface. The obtained flow field was parametrized by the still unknown
339 quantities v_s and ω_s , which allowed us to express the viscous forces acting on the cell. The system
340 was closed by imposing that the total force and torque acting on the swimmer vanished, yielding the
341 values of v_s and ω_s . A remarkable observation is that the swimming velocity does not depend on the
342 viscosity of the suspending medium for a given cortex velocity (see Suppl. Mat. for a proof), which is
343 consistent with experimental observations. The problem could be solved analytically for a sphere
344 (**Figure 6-D**) and the swimming velocity was given by $v_s = \beta v_0$, where v_0 is the retrograde flow
345 velocity at the equator. β is found equal to 1 for a complete transmission between actin retrograde
346 flow and external membrane. A similar result has been obtained by Lighthill for a model ciliated
347 swimmer (squirmers) which yielded $v_s = 2/3 v_0$ for the swimming speed⁴³, the difference of
348 proportionality coefficient being due to the choice of the surface flow. For other shapes, we solved
349 numerically the problem using the boundary integral formulation for the Stokes equations (see
350 Suppl. Mat.). In particular, we discretized a shape of T-lymphocyte obtained by 3D soSPIM
351 microscopy (**Figure 6-E**) and introduced an actin source in a small region at the front of the cell and
352 an actin sink at its rear. The overall flow pattern was similar to the spherical case and the swimming
353 velocity was close to the velocity of the cell membrane in the central region of the cell (in the cell
354 frame), like for a spherical shape (Suppl. Mat. Movie 12). Besides, since most experimental data were
355 obtained for cells close to a rigid wall, we used our numerical simulations to confirm that the
356 swimming velocity was largely insensitive to the distance to the wall (see Suppl. Mat. **Erreur ! Source
357 du renvoi introuvable.**). Altogether, modelling confirms that swimming speed has the same
358 magnitude as the speed of the external membrane treadmilling, supporting that cortex retrograde
359 flow contributes significantly to lymphocytes swimming and could be the sole propelling source.

360 **Membrane treadmilling is heterogeneous at the molecular scale**

361 The propulsion model by a treadmilling membrane considered a total coupling between the external
362 fluid and a homogeneous membrane. This model yielded equal speeds between cell swimming and
363 membrane treadmilling, however the swimming speed ($v_s \sim 5 \mu\text{m}\cdot\text{min}^{-1}$) was found 2 times smaller

364 than the membrane treadmilling speed ($> 10 \mu\text{m}\cdot\text{min}^{-1}$), as revealed by beads attached to the
365 membrane. This difference suggests that the coupling between cell membrane and surrounding fluid
366 is not total and/or that the membrane is not homogeneous. Both the composition and the dynamics
367 of a cell cytoplasmic membrane are indeed highly heterogeneous at molecular level. The external
368 fluid is therefore in contact with numerous lipids and transmembrane proteins, and the
369 hydrodynamic coupling between a fluid and such a complex surface is a delicate and hardly
370 considered problem. Also the molecular dynamics of cell membrane itself is not fully characterized. If
371 the retrograde flow of actin cortex and actin-bound transmembrane proteins is well attested, the
372 circulation of lipids and of non-actin-bound transmembrane proteins is less documented. To get
373 more insight into the molecular dynamics of the cell membrane at the time scale of tens of seconds
374 and at the spatial scale of the entire cell, which are the relevant scales for cell swimming, we
375 performed live FRAP-TIRF measurements on non-adherent cells maintained in the vicinity of the
376 probing glass/fluid interface using depletion force induced by addition of dextran in the medium. On
377 RFP-actin transfected cells, we observed the motion of actin clusters that displayed no detectable
378 diffusion. Actin cortical cytoskeleton behaved like a solid gel (**Figure 7-A**, and Suppl. Mat. **Movie 13**
379 and **Movie 14**) flowing backwards at $24 \pm 9 \mu\text{m}\cdot\text{min}^{-1}$ (Figure 7-E). This result for human lymphocytes
380 is in the top range of literature for other cellular systems (between 6 and $20 \mu\text{m}\cdot\text{min}^{-1}$)^{12,19,34,44,45}. For
381 transmembrane proteins, different dynamics could be expected whether proteins could bind or not
382 to the actin cortex. To shed light on this issue, we used fluorescent specific antibodies against an
383 actin-bound protein, the integrin LFA-1 in high affinity state, and a non-actin-bound protein, the
384 ligand of T lymphocyte receptor, MHC-1. Like actin, actin-bound proteins LFA-1 formed clusters that
385 persistently flowed backwards (**Figure 7-B** and Suppl mat. **Movie 13**) with an average speed of 25 ± 5
386 $\mu\text{m}\cdot\text{min}^{-1}$ (Figure 7-E). Absence of diffusion and similarity of speed with actin retrograde flow are
387 consistent with a strong attachment rate of high-affinity integrins to subcortical actin. By contrast,
388 non-actin-bound proteins MHC-1 displayed a diffusive dynamics in FRAP experiments (**Figure 7-C** and
389 **Movie 13**) with a characteristic diffusion coefficient of $D = 0.26 \pm 0.22 \mu\text{m}^2\cdot\text{s}^{-1}$ (Figure 7-E). Similarly,
390 for the lipidic layer, FRAP experiments with Vybrant® DiO lyophilic molecules inserted in the
391 cytoplasmic membrane (**Figure 7-D** and **Movie 13**) yielded a diffusion coefficient of $3.1 \pm 1.8 \mu\text{m}^2\cdot\text{s}^{-1}$
392 (Figure 7-E). Altogether, these results confirm that the molecular dynamics of the plasma membrane
393 is heterogeneous. Actin-bound transmembrane proteins display ballistic backwards motion whereas
394 lipids and non-actin-bound transmembrane display diffusion. This heterogeneity was not taken into
395 account in simulations and may explain the difference between the speed of cell swimming and actin
396 treadmilling.

397 **Model of retrograde flow transmission by a heterogeneous membrane**

398 To quantify the effect of partial coupling between the actin retrograde flow and the external fluid
399 through a heterogeneous membrane, we considered the layers of transmembrane proteins
400 protruding in the external fluid as a brush of polymeric molecules, and analysed the flow inside the
401 brush composed of either advected or diffusing molecules. Based on polymer science developments,
402 the brush of proteins was considered as a Brinkman medium⁴⁶ with a hydrodynamic penetration
403 length, denoted as $\lambda^{-1} \sim r / [2(\varphi_i^{2D})^{1/2}]$, where φ_i^{2D} is the area fraction occupied by advected
404 transmembrane proteins (e.g. integrins) and r is the typical lateral extent of the proteins on the
405 membrane. The transmission coefficient can be expressed as:

406 Equation 3
$$\beta = 1 - 2g / (1 + g^2) \quad \text{with} \quad g = e^{\lambda h}$$

407 where h is the brush thickness. We first considered the effect of advected proteins linked to actin.
408 Integrins LFA-1 and VLA-4, dominant in lymphocytes, were measured with an average occurrence of
409 25,000 and 15,000 molecules per cell respectively (Suppl. Mat. **Erreur ! Source du renvoi**
410 **introuvable.**). Considering a radius $r = 5$ nm for each integrin and an excess of membrane in
411 microvilli of 150 %, the surface occupancy of LFA-1/VLA-4 corresponds to a mere 0.1 %. The fact that
412 integrins are not in high affinity state at the same time tends to decrease further this value.
413 Conversely, T lymphocytes express other integrins and other transmembrane proteins that link actin
414 (e.g. TCR or CD44), which tends to increase the surface occupancy by advected molecules. All in all,
415 exhaustive experimental data are lacking to determine the exact value of φ_i^{2D} . However assuming
416 φ_i^{2D} of 2% and h of 20 nm, the theoretical coupling value was found at $\beta = 0.2$, which is in agreement
417 with the experimental result of a swimming speed 5 times smaller than the cortex retrograde flow.
418 The effect of diffusing transmembrane proteins was also taken into account by considering that they
419 were indirectly advected by the drag of the external fluid and by viscous interactions within the
420 membrane. Modelling of fluid drag and estimation of membrane viscosity from FRAP measurements
421 allowed us to show that membrane viscosity dominates over the external fluid drag (Suppl. Mat) and
422 that the presence of diffusing transmembrane proteins reduces the value of β (Suppl. Mat **Erreur !**
423 **Source du renvoi introuvable.**). Altogether, membrane treadmilling can participate to swimming,
424 and heterogeneity of the membrane can significantly modulate the coupling efficiency.

425 **Actin-bound proteins are advected backwards at cell membrane whereas non-actin-bound**
426 **proteins are not**

427 Simulations show that swimming speed decreases when a fraction of cell membrane has no
428 backwards motion. Assessing the average motion of lipids was not possible experimentally due to
429 their fast diffusion. However, for transmembrane proteins, we managed to get more insight by
430 performing line-shaped FRAP experiments on cells with double staining for LFA-1 and MHC-1. On

431 Figure 7-F, clusters of LFA-1 and the centre of mass of the FRAP zone for LFA-1 moved backwards at
432 $22 \mu\text{m}\cdot\text{min}^{-1}$, in line with previous measurements. In contrast, the centre of mass of the FRAP zone
433 for diffusing MHC-1 was on average immobile. These data are a direct support that a fraction of the
434 membrane is purely diffusive and hinders the propulsion effect of paddling molecules.

435 **Actin-bound proteins are recycled by internal vesicular extrusion and non-actin-bound proteins are**
436 **not**

437 A sustainable retrograde flow at the surface of an axisymmetric object requires a source of material
438 at the front and a sink at the back, which therefore implies the existence of an internal anterograde
439 transport to loop a cycle. Conversely, when molecules are not advected backwards (and are only
440 diffusive), there must be no internal frontward recycling, because a source at cell front would
441 generate an external retrograde flow. Our swimming model implies therefore the existence and the
442 absence of an internal anterograde recycling of respectively actin-bound and non-actin-bound
443 proteins. To support further the relevance and consistency of our model, we then intended to verify
444 these two properties by performing square-shaped FRAP experiments on cells front (Figure 8-A and
445 Suppl. Mat Movie 16). In the case of LFA-1, a source of fluorescence at the cell leading edge after
446 photobleaching (Figure 8-A and Suppl. Mat Movie 16) was consistent with the extrusion of integrins
447 originated from unbleached cell rear and carried undetected by TIRF by internal vesicular transport.
448 Interestingly, the cycle of extrusion/treadmilling of integrins occurred within a minute, which is
449 consistent with the treadmilling and swimming time scales. These observations validate the existence
450 of a complete cycling of integrins by treadmilling and vesicular transport, whose timescale is
451 consistent with swimming speed. In the case of MHC-1, fluorescence after photobleaching recovered
452 only by diffusion from the cell back (Figure 8-B and Suppl. Mat. Movie 16) and there was no source at
453 cell front. This confirms that internal recycling is not occurring for this non-actin-binding protein.
454 Altogether, FRAP-TIRF data support that lymphocytes membrane does not treadmill as a whole.
455 Actin-bound proteins undergo retrograde ballistic motion externally and anterograde vesicular
456 transport internally, whereas non-actin-bound proteins undergo diffusion at the cell surface without
457 internal recycling. Some surface molecules treadmill and paddle, whereas others diffuse and hinder
458 motion.

459 **Treadmilling of a heterogeneous membrane can propel ubiquitous locomotion**

460 Cell swimming by molecular paddling of a heterogeneous membrane is illustrated in Figure 9-A with
461 the treadmilling proteins exerting propulsion (orange), the diffusing proteins hindering propulsion
462 (blue), and the inner recycling of paddling proteins by vesicular transport. To test further the
463 mechanism, we perturbed the molecular cycling machinery without affecting actin treadmilling or

464 actin-driven cell deformations by using inhibitors of endocytosis. Figure 9-B shows results with
465 Primaquin 100 μM ⁴⁷ and with a cocktail of inhibitors including a clathrin inhibitor (pitstop2 50 μM), a
466 dynamin inhibitor (dynasore 200 μM), and an inhibitor of the Arf6 GEF cytohesin (secinH3 20 μM)²⁸.
467 Swimming was significantly slowed down with doses low enough to preserve cell morphological
468 dynamics. The fact that osmotic swelling did not affect swimming speed (Figure 5) is consistent with
469 heterogeneous treadmilling propulsion because swelling does not affect protein cycle and cell shape
470 has a marginal influence on propulsion by treadmilling (Figure 6-E,D). Beyond swimming, it is
471 interesting to underline that the heterogeneous treadmilling mechanism can also foster propulsion
472 on adherent 2D substrates and 3D matrices coated with integrin ligands. In these cases, integrin-
473 mediated clutch mechanism promotes optimal momentum transfer between internal cortex and
474 external medium, and cell speed is indeed close to actin treadmilling speed. This corresponds to
475 actin-integrin clutch mechanism⁴⁸. Furthermore, heterogeneous treadmilling mechanism can also
476 propel cells through non-adherent confined environments because friction of the cell membrane
477 with a solid substrate is expected to be stronger than with a fluid. We found that cells confined
478 between two plates and in microfluidic tubes were motile (Figure 9-C, Suppl. Mat Movie 17 and
479 Movie 18). Between plates, two adjacent zones with adherent and non-adherent coatings were
480 prepared by optical patterning³⁸ and tube surfaces were treated with Pluronic F107¹⁴. Cells are
481 slightly faster between plates and significantly faster in tubes as compared to swimming mode.
482 Literature data reported the case of cells in a microfluidic channel displaying treadmilling of
483 membrane but no motion, which was interpreted as an example of null friction and null transmission
484 between membrane and substrate¹⁴. However, an ideal solid/cell interface with perfect slippage or
485 null friction is not physically relevant because hydrodynamics of physical contact always yield friction.
486 The mechanism of heterogeneous membrane treadmilling supports therefore ubiquitous lymphocyte
487 migration in fluid and solids, with and without adhesion.

488

489 **DISCUSSION**

490 Amoeboid migration of mammalian cells, i.e. leukocytes and cancer cells, has attracted intensive
491 interest in the last decade for their ubiquitous ability to migrate at high speed in various 2D and 3D
492 environments. The requirement of adhesion with a substrate remains a widely accepted hallmark for
493 2D migration^{12,14,18 19 5-7,15,16,17}, whereas two studies reported non-adherent motility and swimming
494 with leukocytes^{28,49}. However, Barry and Bretcher did not investigate the mechanisms of neutrophils
495 swimming, and O'Neil et al. considered an original but artificial swimming mode induced by
496 optogenetic activation of contractility in cell rear. In this context, we provided here direct

497 experimental evidences and quantifications of swimming by primary lymphocytes, together with an
498 original theoretical model of molecular paddling that can explain amoeboid swimming at speeds
499 compatible with precise experimental measurements, as well as ubiquitous migration in/on adherent
500 and non-adherent substrates.

501 In principle, cell swimming without flagella can be propelled either by normal (protrusion) and/or
502 tangential (treadmilling) motion of the cell membrane. Mechanistic studies of swimming by
503 eukaryotic cells have mostly focused on amoeba *Dictyostelium discoideum* and favoured propulsion
504 by shape deformation rather than treadmilling^{21,24–26}. However, most models involve parameters
505 difficult to link to experimental data²⁶ or yield swimming speed smaller than the experimental
506 ones^{24,25}. There is therefore no consensus for swimming propelled by deformation. Interestingly, a
507 recent study by O’Neil et al²⁸ put forward original evidence that membrane treadmilling could be
508 involved in propulsion however without assessing the contributions of deformations in propulsion.
509 Here, we proposed theoretical and experimental evidences that swimming of lymphocytes is barely
510 relying on cell deformations. Modelling based only on parameters accessible experimentally like the
511 micron-scale amplitudes and 10-20 $\mu\text{m}\cdot\text{min}^{-1}$ speed of protrusions, yielded that cell deformations
512 cannot propel swimming at a speed as high as 5 $\mu\text{m}\cdot\text{min}^{-1}$. Experimental perturbations of the average
513 and time-dependant cell deformations were also hardly perturbing swimming prowess. In contrast,
514 experiments and modelling both validated a high efficiency of propulsion by membrane treadmilling,
515 as proposed by O’Neil et al.²⁸, and we further show that membrane does not treadmill as a whole.
516 Altogether, the effect of shape deformations seems negligible in lymphocytes swimming whereas
517 membrane treadmilling would be the main propelling machinery.

518 Actin dynamics acts a priori as a hybrid motor of membrane treadmilling, polymerization at the cell
519 front pushing the nascent crosslinked cortical network backwards, and contractility at the cell rear
520 pulling the network backwards. It is not clear whether the two mechanisms combine their action on a
521 continuous actin gel or if they act independently on disconnected actin networks at cell front and
522 rear. However, we found here that lymphocytes swimming relies more on frontal actin
523 polymerization than on rear myosin-II contractility. This diverges from the Rho-A induced swimming
524 mode described by O’Neil et al²⁸, as well as from several recent reports on adherent crawling^{15,18,50–54}
525 that attribute a dominant propelling role to a gradient of contractility along cells front-rear
526 polarization axis. Our results are more in line with studies proposing that contractility is marginally
527 involved in propulsion and mostly relevant for detachment of cell rear (for *Dictyostellium amoebae*)⁵⁵
528 or for squeezing of cell nucleus through pores (for dendritic cells)⁵. Hence, depending on cell types,
529 actin treadmilling may be powered more by actin network contractility²⁸ or by actin polymerization,

530 but in any case our results corroborate the idea that membrane treadmilling is an essential player or
531 lymphocytes motility, either by swimming or crawling.

532 Hydrodynamic coupling between a treadmilling membrane and a surrounding fluid is the key of
533 amoeboid swimming, and we analysed here its molecular basis experimentally and theoretically.
534 Studies considering that cellular membranes treadmill as a whole yield theoretical ratio between
535 swimming and membrane speeds ranging between $2/3$ and 1^{56} . O'Neil et al ²⁸ measured swimming
536 speeds $2/3$ of membrane speeds and observed gradients of several membrane components (lipids,
537 and proteins) along cell rear-front axis. This led them to conclude that cell membrane was
538 treadmilling as a whole and propelling swimming. One may however argue that their correlation
539 between speeds of membrane and speed of cell swimming relied on a narrow set of data. Besides,
540 proteins gradients do not strictly imply that proteins treadmill because gradients can also result from
541 a balance between diffusion and drag by surrounding molecules. In contrast, the analysis of
542 lymphocytes swimming motion showed here that swimming speed was $1/3$ of membrane
543 treadmilling speed (measured with a bead attached to the membrane) and $1/5$ of the actin-bound
544 proteins speed (measured by TIRF), and therefore too low to arise from the treadmilling of a
545 homogeneous membrane. Moreover, direct microscopy experiments showed that membrane
546 dynamics was heterogeneous at molecular level and that only a fraction of transmembrane protein
547 treadmilled backwards. Hence some membrane molecules treadmill backwards, paddle and promote
548 swimming, whereas others are diffusive and hinder swimming. Simulations of propulsion by a
549 heterogeneous treadmilling support further that a fraction of treadmilling proteins is efficient
550 enough to support swimming at speed close to internal cortex speed, and also that a fraction of
551 diffusing protein can significantly slow down speed to the observed swimming speed.

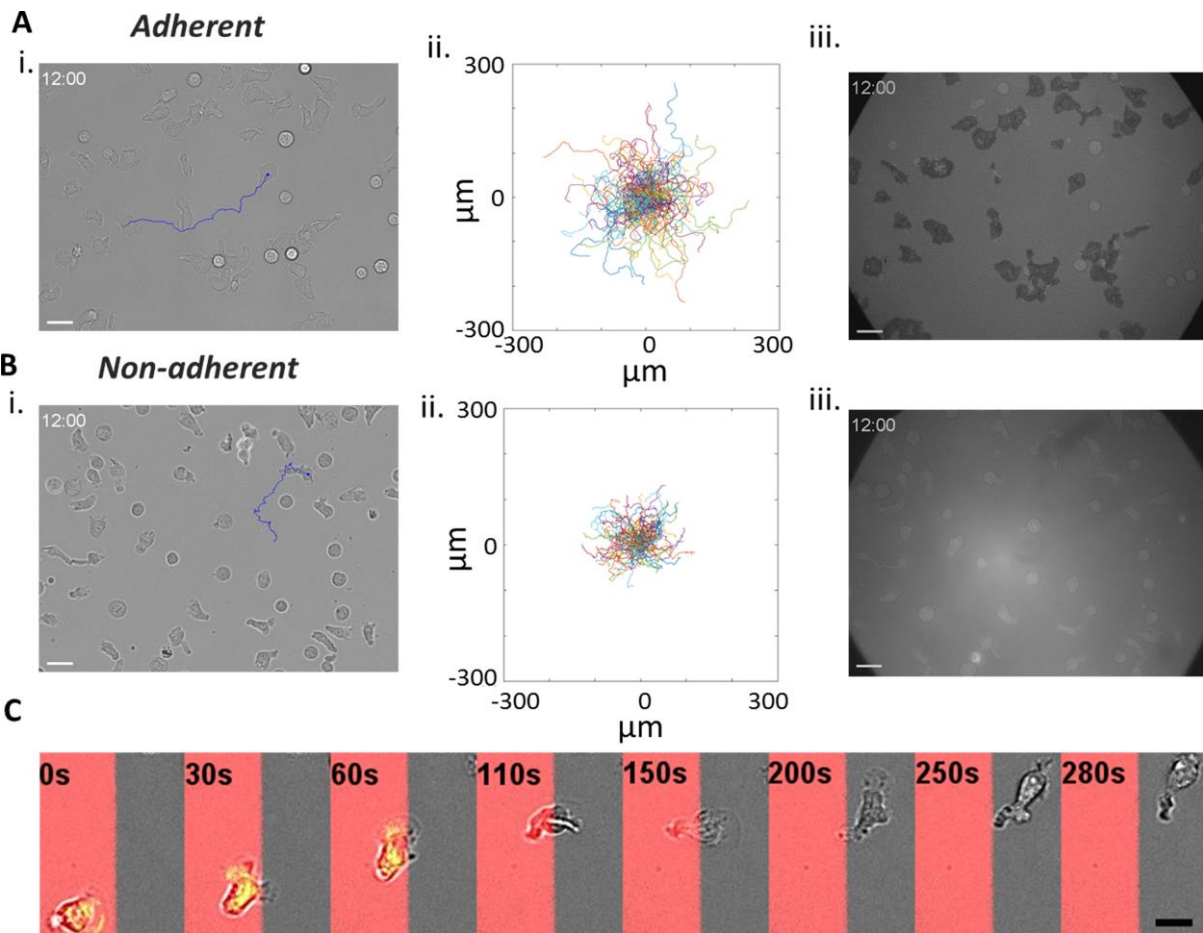
552 Importantly, we also showed that treadmilling of actin and transmembrane proteins, whose essential
553 role has been widely inferred in cell motility, was associated with sophisticated mechanisms of
554 internal vesicular transport to sort proteins that support continuous swimming. Vesicular transport is
555 indeed implemental to complete the cycle of paddling proteins and promote sustained propulsion,
556 and sorting of proteins transported by vesicles is also necessary to explain heterogeneity of proteins
557 dynamics at cell surface. Internal transport of a treadmilling transmembrane protein and absence of
558 internal frontward recycling of a diffusion transmembrane protein was evidenced here by FRAP with
559 respectively integrin LFA-1 and protein MHC. Mechanisms of internal integrins recycling by
560 anterograde vesicular transport have also been documented in the literature⁵⁷⁻⁶¹. Moreover, the
561 cycle of extrusion/treadmilling of integrins in our experiments occurred within a minute, which is in
562 line with the treadmilling and swimming time scales (in contrast to literature data that reported
563 timescales around 15-30 min^{58,59}).

564 Our swimming model sheds also new light on the ubiquitous motility of amoeboid cells. The
565 adaptation of mammalian amoeboid cells to various environments has repeatedly been attributed to
566 their capability to switch between different migration modes versus the environment^{10,14,15,19,33,62}.
567 The mode of adherent crawling is generally attributed to a sequence of cell front
568 protrusion/attachment and cell rear pulling /detachment, whereas the more intriguing mode of non-
569 adherent migration in 3D has been explained by various mechanisms depending on cell types^{9,19}:
570 blebbing in the cell front and transfer of actin cytoskeleton into the novel bleb^{51,63,64}, intercalation of
571 protrusion into gaps and discontinuities of the matrix to advance like on a ladder⁶⁵⁻⁶⁷, chimneying via
572 active gel pushing-off the wall¹¹, cell rear contractility stabilizing a single bleb conformation¹⁸, water
573 permeation throughout the cell body⁶⁸ or treadmilling coupled to friction with the substrate^{9,8,12}. In
574 our case, there was no evidence of a mode switching between crawling and swimming sequences on
575 patterned substrates, and treadmilling of a heterogeneous membrane can propel ubiquitous
576 migration of lymphocytes versus solid^{8,12} or liquid environments. In this scheme, speed depends on
577 cell/environment coupling conditions. An adhesive solid environment provides optimal coupling and
578 cells speed is close to actin treadmilling speed (unless detachment issues slow down motion); a liquid
579 environment provides a minimal coupling due to the molecular heterogeneity of cell membrane
580 treadmilling (the environment fluidity is not directly responsible for weak coupling, as attested by our
581 model or by the squirmer model⁴³); finally, a non-adherent confined environment provides an
582 intermediate coupling, where friction relies on surface forces rather than on hydrodynamic
583 lubrication³⁵. The regime of hydrodynamic friction (or lubrication) appears indeed only for objects
584 much faster than crawling cells⁶⁹. Interestingly, the propulsion by heterogeneous membrane
585 treadmilling supports systematic migration in non-adherent confined environments because a
586 solid/cell interface with null friction is not physically relevant. Furthermore, non-adherent confined
587 propulsion is expected to be at least as fast as swimming because coupling of the membrane with a
588 solid can only be more efficient than with a liquid. Altogether, the mechanism of heterogeneous
589 membrane treadmilling is operational in polarized cells displaying backwards actin treadmilling. This
590 mechanism can allow ubiquitous adaptation of lymphocytes to various microenvironments via a
591 single mode of migration¹⁰ associated to different friction/adhesion coupling conditions. It may exist
592 for other cell types and coexist with other propulsion mechanisms.

593 In the end, heterogeneous membrane treadmilling can drive ubiquitous amoeboid migration within
594 fluid and solid, and with or without adhesion, however the physiological role of swimming remains
595 enigmatic for leukocytes. Since the traffic of immune cells (or invasivity of cancer cells) relies mostly
596 on matrix-associated migration and since swimming is clearly relevant to planktonic eukaryotic cells
597 (like amoeba), it is possible that the basic machinery for swimming is an evolutionary conserved

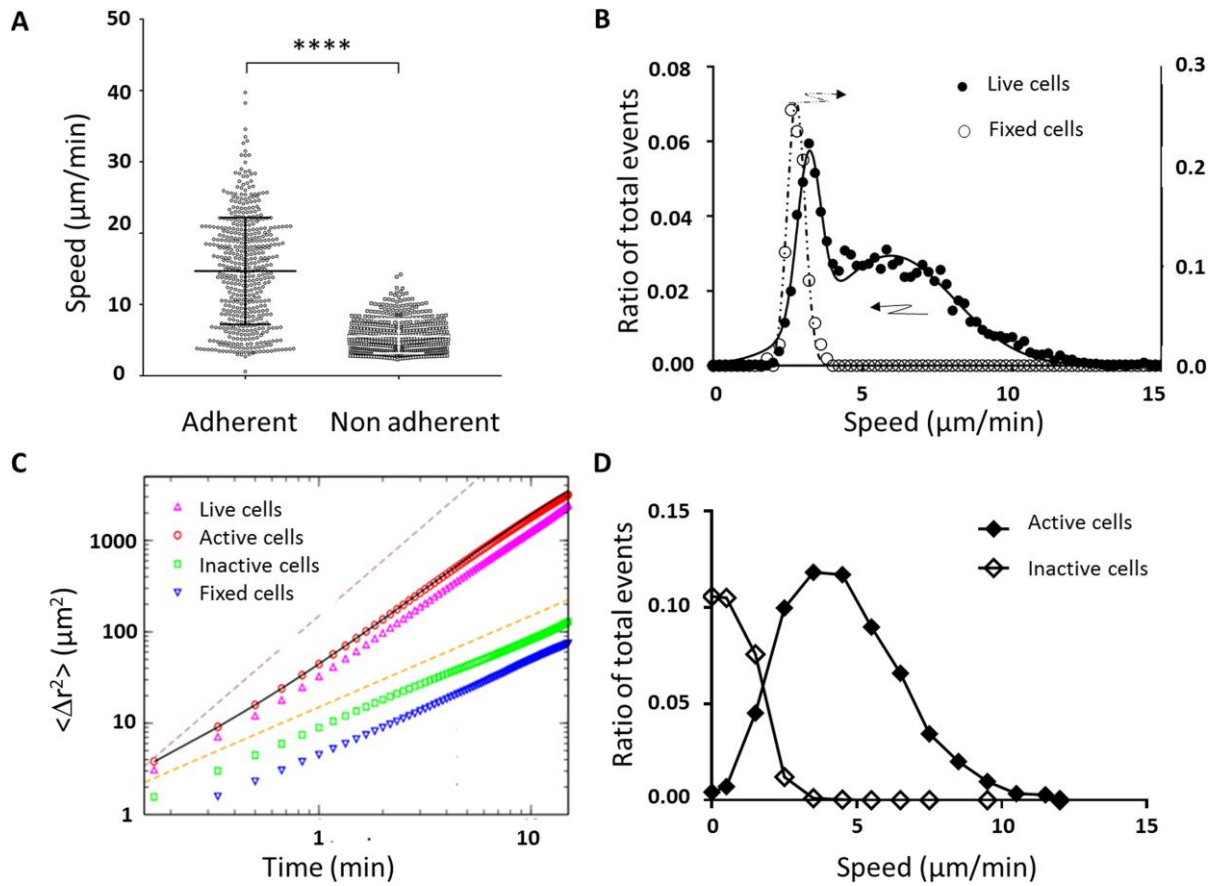
598 ability that has found novel functions for ubiquitous crawling in mammalian cells. Nevertheless,
599 swimming might be instrumental to extend the ubiquitous migration capabilities of immune cells to
600 non-confining conjunctive tissues, or liquid-rich infected niches like post-wound oedema zones.

601 **FIGURES**



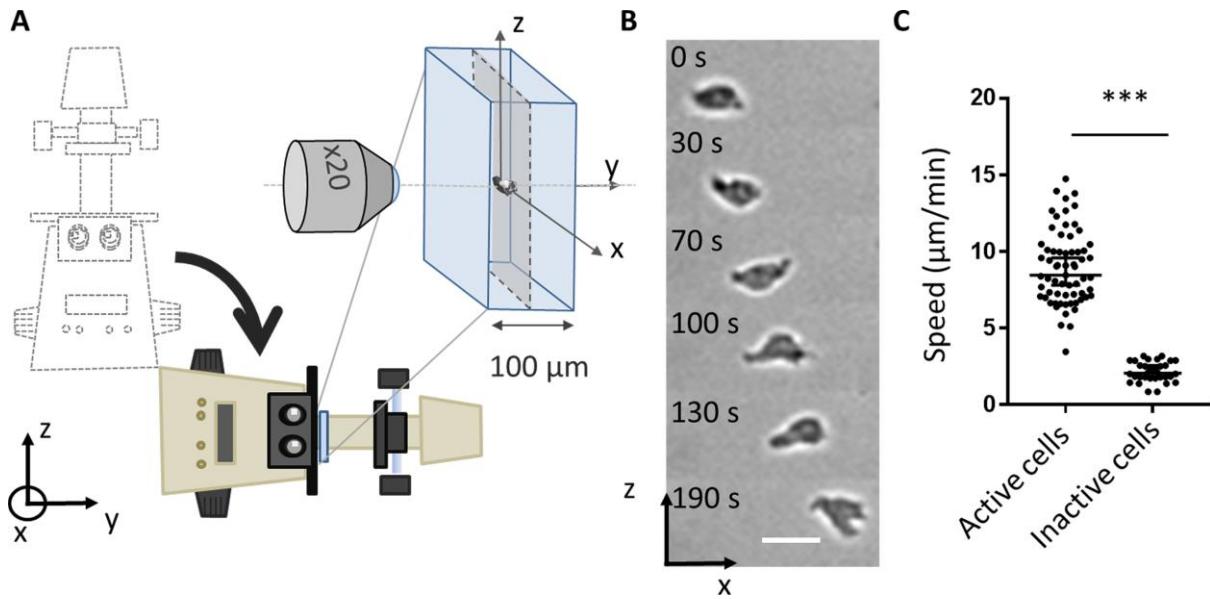
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603 **Figure 1 : From adherent to non-adherent 2D migration.** Primary human effector T lymphocytes **(A)**
 604 Adhesive crawling on ICAM-1- treated substrate, and **(B)** non-adherent migration on Pluronic F127-
 605 treated substrate; **i.** Bright field images. Blue lines correspond to the track of a particular cell for 12
 606 min. Scale bar is 20 μm . See also Movie 1. **ii.** Representative tracks of motile cells in a single
 607 experiment (time lag = 20 s, duration 16 min, n > 100 cells). **iii.** Reflection Interference Contrast
 608 Microscopy (RICM) images. Cell contact zone is dark for cells crawling on ICAM treated surfaces,
 609 revealing an adhesion phenotype, and white for cells on non-adhesive surface attesting the absence
 610 of adhesion to the surface (see also Suppl. Mat **Erreur ! Source du renvoi introuvable.**). Scale bar is
 611 20 μm . **(C).** Image sequence of a cell migrating over adjacent stripes of adhesive ICAM-1 (red) and
 612 anti-adhesive Polyethelene glycol (grey) prepared by LIMAP⁷⁰ with a width of 40 μm . The sequence is
 613 a merge of fluorescent images (ICAM-1, red), bright field images (cell morphology, grey) and
 614 reflection interference contrast microscopy images (adhesion zone, green). Scale bar is 20 μm . See
 615 also Movie 3.



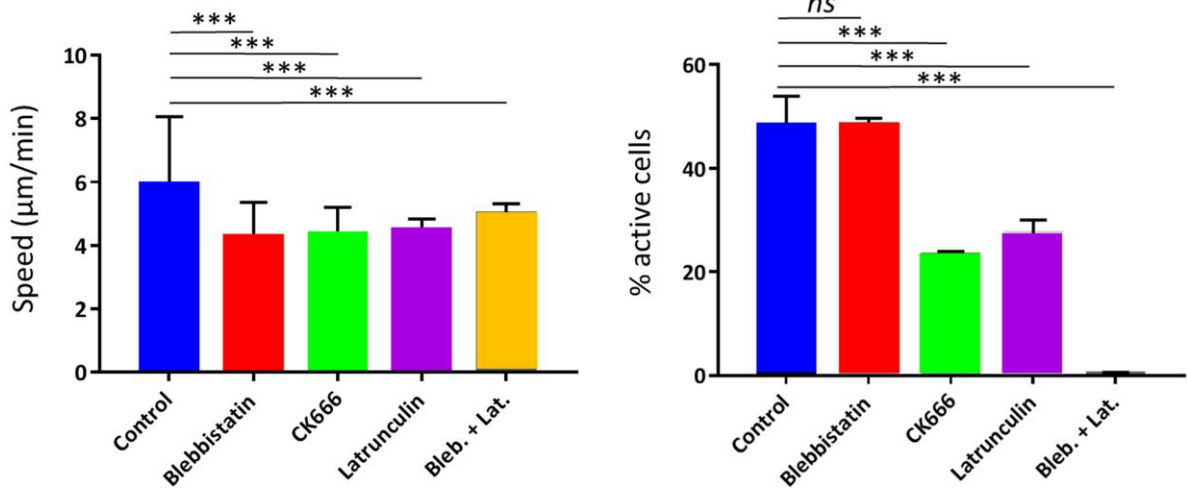
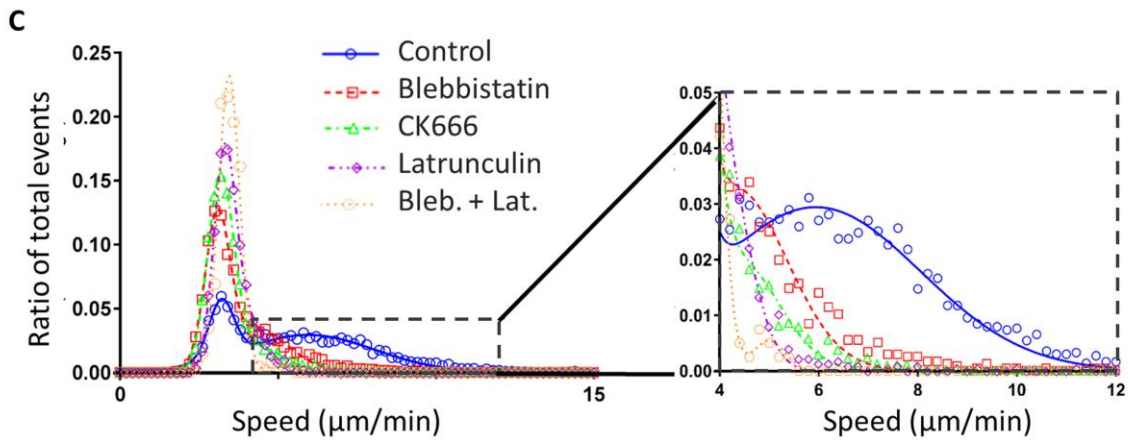
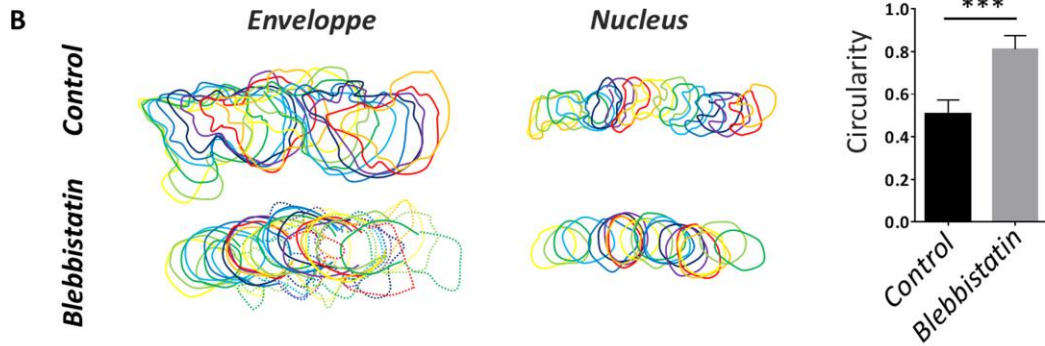
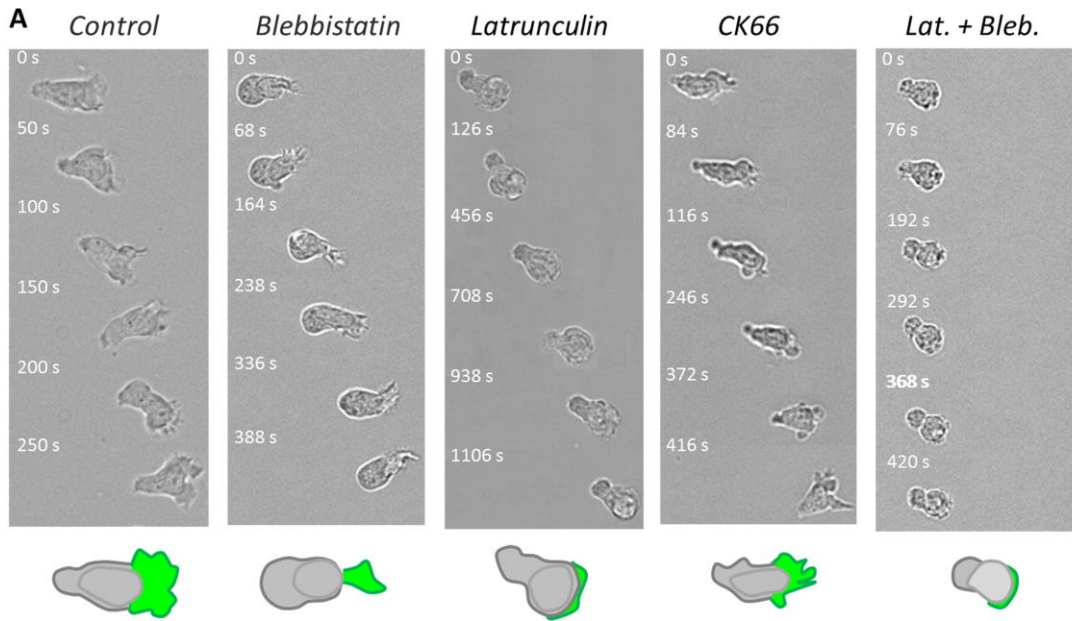
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617 **Figure 2 : Active polarized cells migrate without adhesion in 2D at $5 \mu\text{m}\cdot\text{min}^{-1}$.** (A) Raw curvilinear
 618 speed of cells crawling on adherent ICAM-1-treated substrates and migrating on anti-adherent
 619 Pluronic F127-treated substrates, estimated by averaging the displacements of cell mass centres over
 620 intervals of 30 s. $N = 500$ cells, p value of t test. (B) Histogram of raw curvilinear speed of live non-
 621 adherent migrating cells (filled dots, left y-axis) and fixed cells (hollow dots, right y-axis). Data are
 622 fitted with a single Gaussian for fixed cells (dotted line) and a double Gaussian for live cells (dark
 623 line). Live cells are composed of one population of diffusing cells and one of migrating cells with an
 624 average speed of $5.9 \mu\text{m}\cdot\text{min}^{-1}$. (C) Mean square displacement $\langle [r(t) - r(0)]^2 \rangle$ as a function of
 625 time for all cells and all steps combined in the case of live cells (upward pointed triangles) and fixed
 626 cells (downward pointed triangles). Fixed cells have purely diffusive behaviour corresponding to $D_t =$
 627 $2.34 \mu\text{m}^2\cdot\text{min}^{-1}$. Circles and squares show the mean square displacement for active and inactive cells,
 628 respectively. Black line is a fit of active cells using **Erreur ! Source du renvoi introuvable.** (Suppl.
 629 Mat.) with $v_s = 4.3 \mu\text{m}\cdot\text{min}^{-1}$, $D_r = 0.19 \text{ min}^{-1}$, and $D_t = 7.28 \mu\text{m}^2\cdot\text{min}^{-1}$. Dashed lines show the limit
 630 cases of purely ballistic and diffusive behavior with slopes of respectively 2 and 1. (D) Histogram of
 631 average speed per cell determined using Equation 1 as in (C) for active cells (filled diamonds) and
 632 inactive cells (hollow diamonds). Root mean square speed of active cells is equal to $4.9 \mu\text{m}\cdot\text{min}^{-1}$.
 633 Lines are guides for the eye.

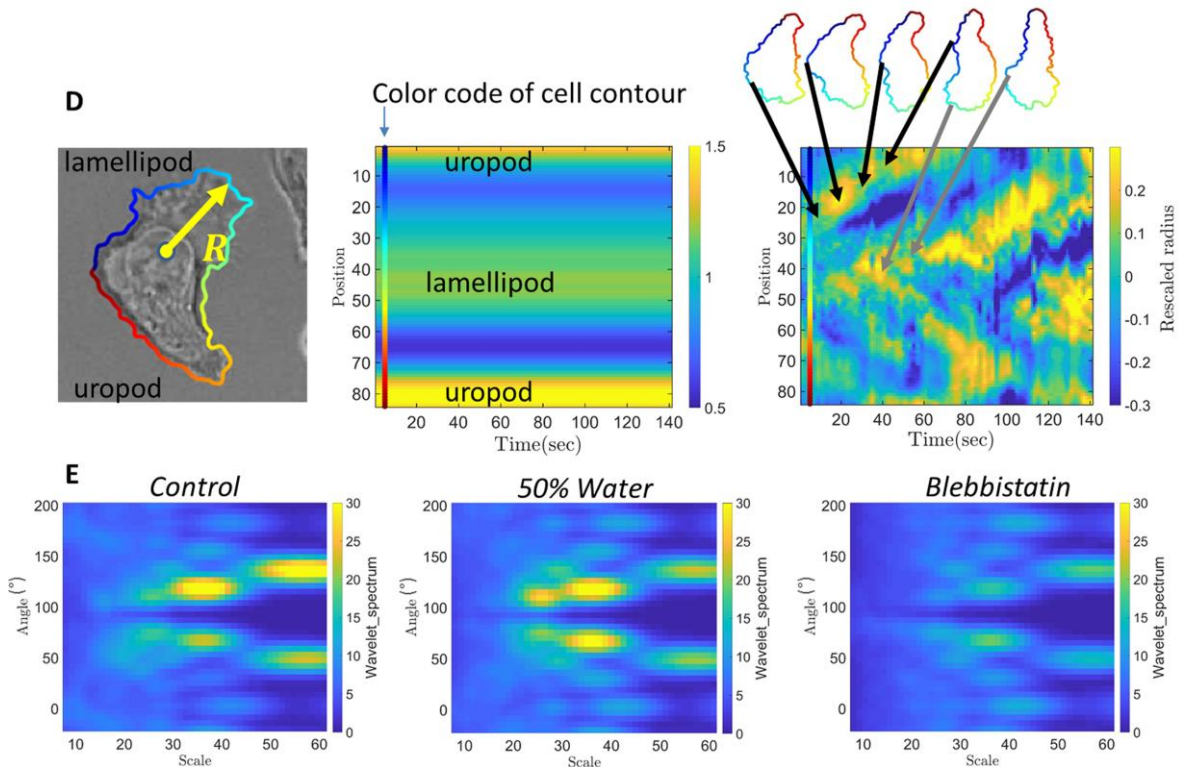
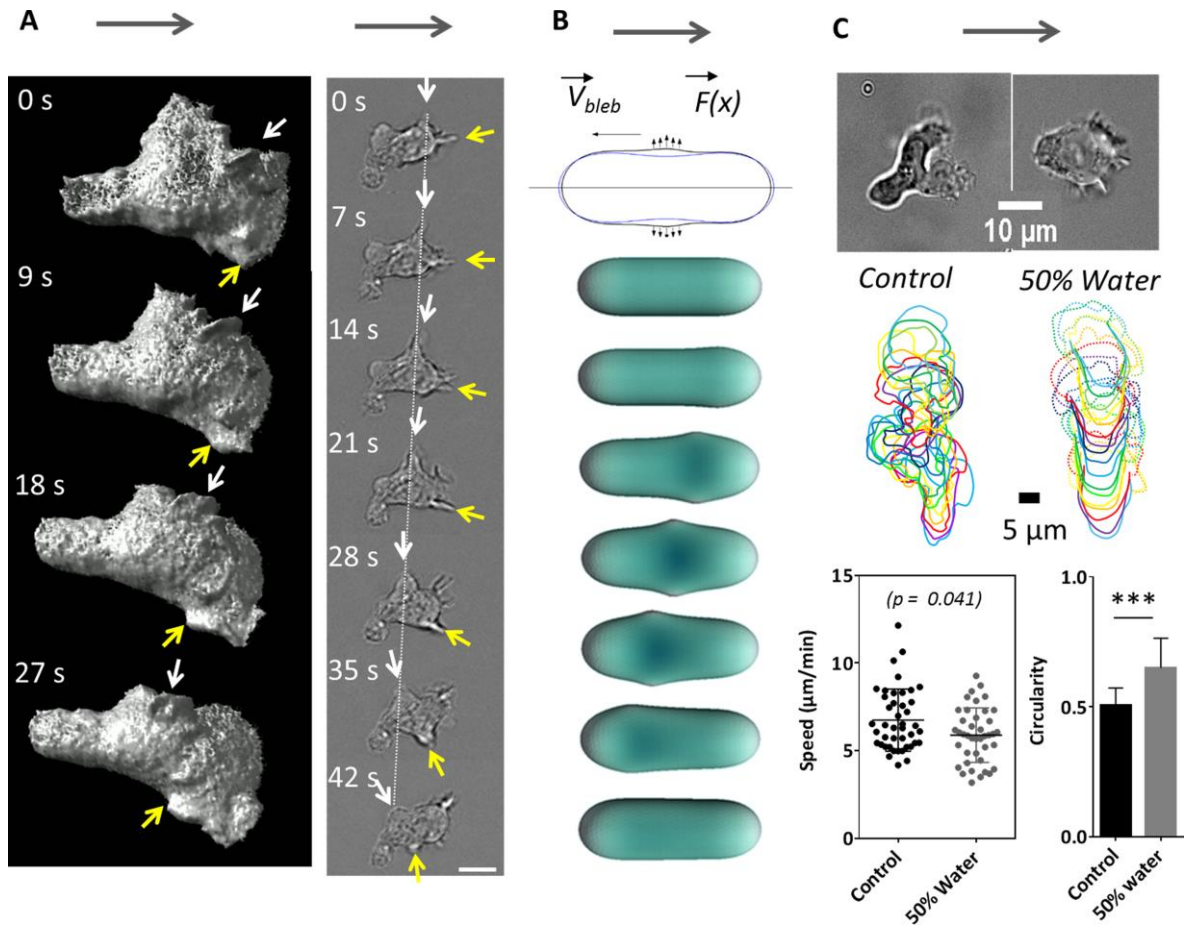


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635 **Figure 3 : Lymphocytes swim in free suspension.** (A) Schematic of the set-up used to image
 636 swimming in suspension with a microscope tilted by 90° and a flow channel oriented vertically. (B)
 637 Sequence of images of a cell swimming in the centre of the channel in the direction of the x axis.
 638 Scale bar is 20 μm . See also Movie 4. (C) Speed of active and inactive cells for a distance to wall larger
 639 than 40 μm . Nexperiments = 6 , Ncells >16, Nsteps >80. *P < 0.05, **P < 0.001, ***P < 0.001 (two-
 640 tailed Student's t test).



642 **Figure 4 : Actin propels swimming more by polymerization than by contractility. (A)** Bright field
643 image sequences showing the shape and dynamics of cells swimming on an antiadhesive substrate
644 versus addition of actomyosin inhibitors. From left to right: wild type cells, cells treated with 50 μ M
645 Blebbistatin, 0.05 μ M Latrunculin, 100 μ M CK666, and combined Latrunculin and Blebbistatin. See
646 also Movie 6. Cartoons at the bottom reproduce the cell in the first image to illustrate in each case
647 the shape of the cell body (rear and nucleus) in grey and of the cell front or lamellipod in green.
648 Blebbistatin-treated cells have a roundish cell body without travelling protrusion, and a reduced but
649 active lamellipod; Latrunculin-treated cells have almost no lamellipod; CK666-treated cells have a
650 perturbed lamellipod forming blebs and spikes. Cells treated with Blebbistatine and Latruculin have a
651 roundish non contractile cell body and no lamellipod (scale bar is 10 μ m). **(B) Left-** Representative
652 sequences of envelope and nucleus contours of a control cell (top) and of a cell treated with 50 μ M
653 Blebbistatin (bottom) suggesting a difference of contractile activity. Time lag between each contour
654 is 10 s. **Right-** Quantification of the circularity of cells envelopes for control cells and 50 μ M
655 Blebbistatin treatment on image sequence taken at magnification x60 and cell contour determined
656 using Ilastik. Ncell > 10, Nexp = 3. ***P < 0.001 (two-tailed Student's t test). **(C) Top** - Histogram of
657 raw curvilinear speeds for swimming cells in response to above mentioned actin inhibitors
658 treatments. The lines correspond to a fit by a double Gaussian function. Insert presents a zoom of
659 the histogram for the active cells corresponding to the Gaussian of high mean speed. **Bottom Left** -
660 Average speed and SD corresponding to the Gaussian of high mean speed. **Bottom right** -
661 Percentage of active motile cells, error bars correspond to SEM. Ncells = 4342 (HBSS), 2353
662 (Blebbistatin), 5582 (CK666), 2255 (Latrunculin), 403 (Blebbistatine + Latrunculin); Nexperiments > 5.
663 ***P < 0.0001 with respect to homogeneous substrate, one-way ANOVA followed by the Tukey
664 multiple comparison test.

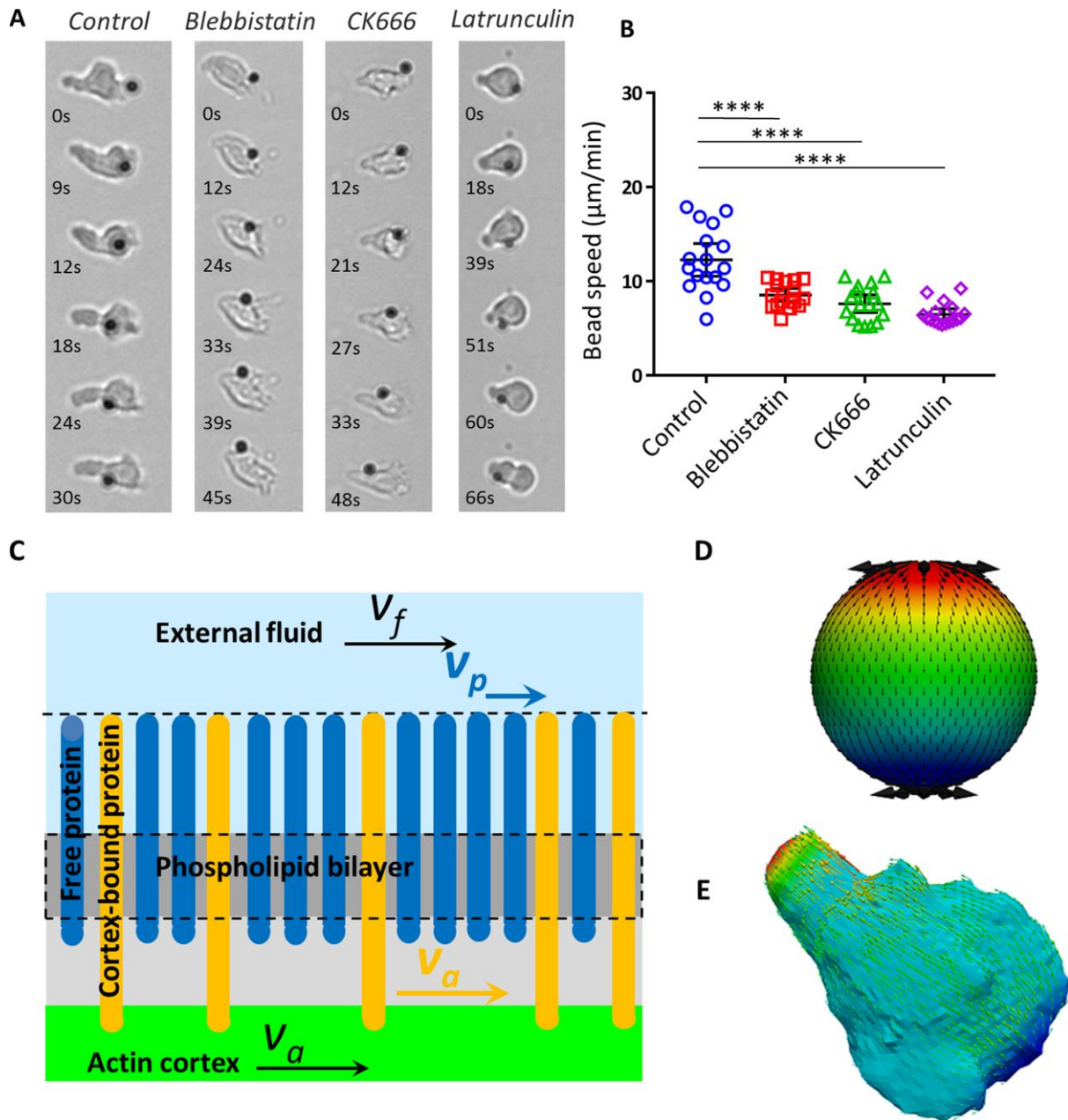


665

666

667 **Figure 5 : Protrusion paddling alone seems not efficient enough to propel swimming. (A)** Images
 668 sequence of swimming cell with micron scale protrusions travelling along cell body. **Left-** SoSPIM

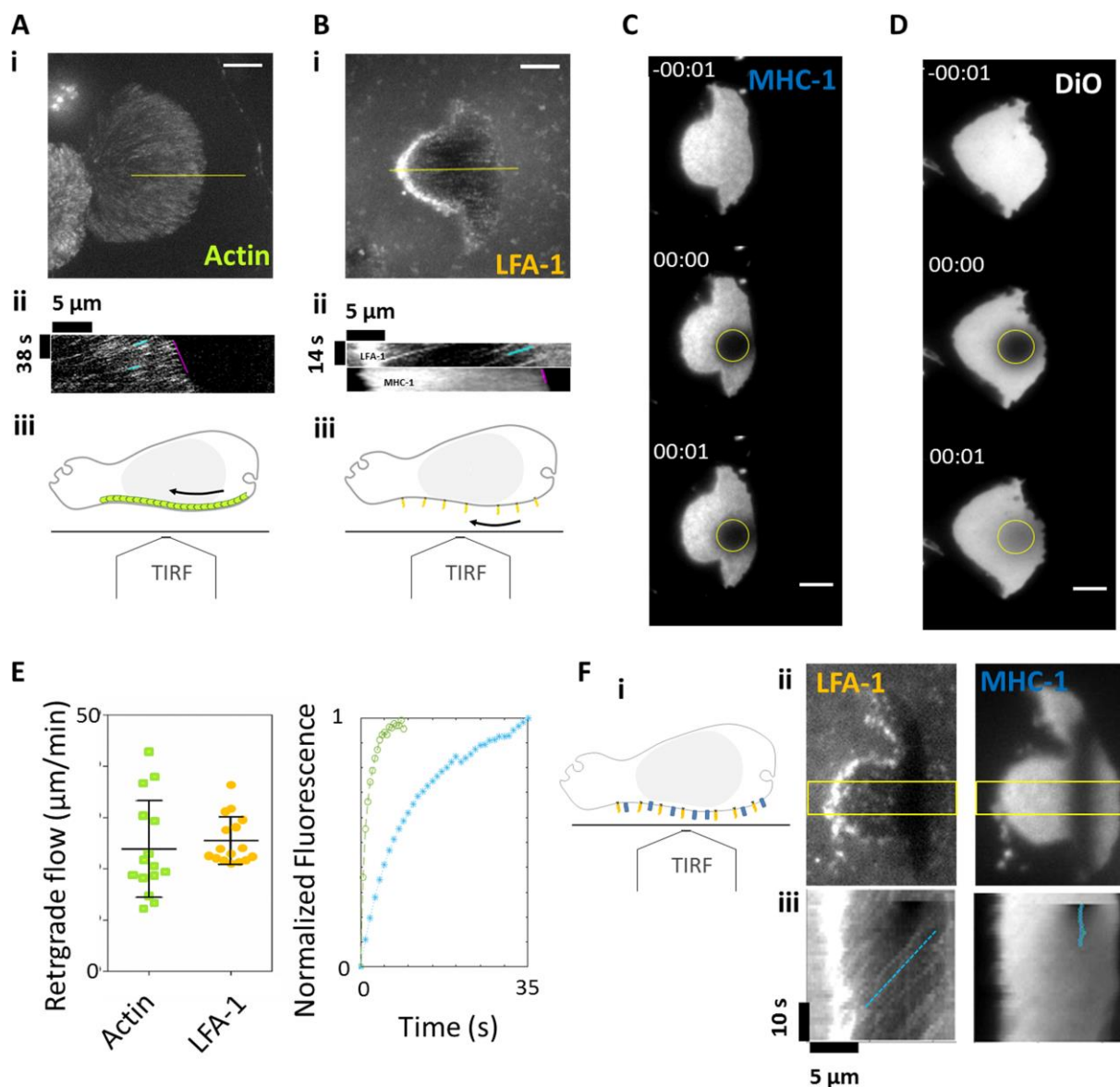
669 images of a cell transfected with RFP-Lifeact reveals the shape and motion of waves of actin
670 protrusion in three dimensions. White and yellow arrows point to particular protrusions. (See also
671 Suppl. Mat. Movie 7). **Right-** Bright field images of a swimming cell showing dynamics of protrusions.
672 (See also Suppl. Mat. Movie 8). Protrusions (white and yellow arrows) travel backwards in the frame
673 of the cell and of the lab (white dashed line). Grey arrows indicate swimming direction. Scale bar is
674 10 μm . **(B)** Schematic illustrating the model of cell swimming by protrusive blebs. **Top-** Blue and black
675 contours are the initial and deformed configurations of the cell in the model. **Bottom-** Sequence of
676 cell shapes obtained by the numerical simulation. Simulation yield that a cell propelled by shape
677 waves that is 1000 times slower than the protrusion wave (details in Suppl. Mat.). **(C)** Osmotic
678 swelling affects cell shape but not swimming speed. **Top-** Representative images of a cell in medium
679 and in water dilution at 50%. **Middle-** Representative cell contour sequences (Time lag is 10s
680 between each contour). The cell swollen by osmotic stress displays less deformations of cell body
681 (full lines) but lamellipod protrusions are still distinguishable (dotted line). **Bottom-** Swimming speed
682 in medium and with water dilution at 50%. (Ncells > 30, p value of t test); and circularity of cells on
683 image taken at magnification x60, with cells contours determined using Ilastik. Ncell > 10, Nexp = 3.
684 ***P < 0.001 (two-tailed Student's t test). (See also Suppl. Mat. Movie 10). **(D)** Measurement of cell
685 protrusion dynamics. **Left-** Representative example of an elliptic Fourier contour reconstruction.
686 Radius R Definition of edge distance to the centre of mass. **Middle, Right-** Kymographs of Rescaled R
687 versus position along the cell perimeter, either averaged in time (middle) or with time-average
688 contribution subtracted (Right). Color scale on the left corresponds to the color code used in contour
689 reconstruction, allowing one to identify the position in kymographs of lamellipod, uropod, and
690 propagating protrusions. (see also Suppl. Mat. **Erreur ! Source du renvoi introuvable.**). **(E)**
691 Spatiotemporal spectrum of protrusion dynamics by wavelet transform. Average wavelet spectra of
692 rescaled R kymographs without time-averaged contribution (see also Suppl mat. **Erreur ! Source du**
693 **renvoi introuvable.**).



694

695

696 **Figure 6 : External membrane retrograde flow can propel swimming efficiently.** (A) Bright field
 697 images of ICAM-coated beads travelling from front to back on the cell membrane of swimming
 698 primary human effector T-cells in HBSS control media, and with 50 μM Blebbistatin, 100 μM CK666
 699 and 0.05 μM Latrunculin (left to right). Scale bar is 10 μm . See also Movie 11. (B) Travelling speed of
 700 ICAM-coated beads versus inhibitors type ($n=17$ cells for each case, error bar is SD, ****: $p<0.0001$,
 701 Dunnet's multiple comparison test vs control condition). (C) Cartoon of the external and internal
 702 structure of the cell membrane considered in the modelling of membrane dynamics. (D-E)
 703 Retrograde flow pattern on a model spherical cell (D) and a cell with an experimental shape (E)
 704 extracted from soSPIM images of (Figure 5-A). Swimming speed is found in both cases equal to the
 705 speed of the membrane at equator. See also Movie 12.

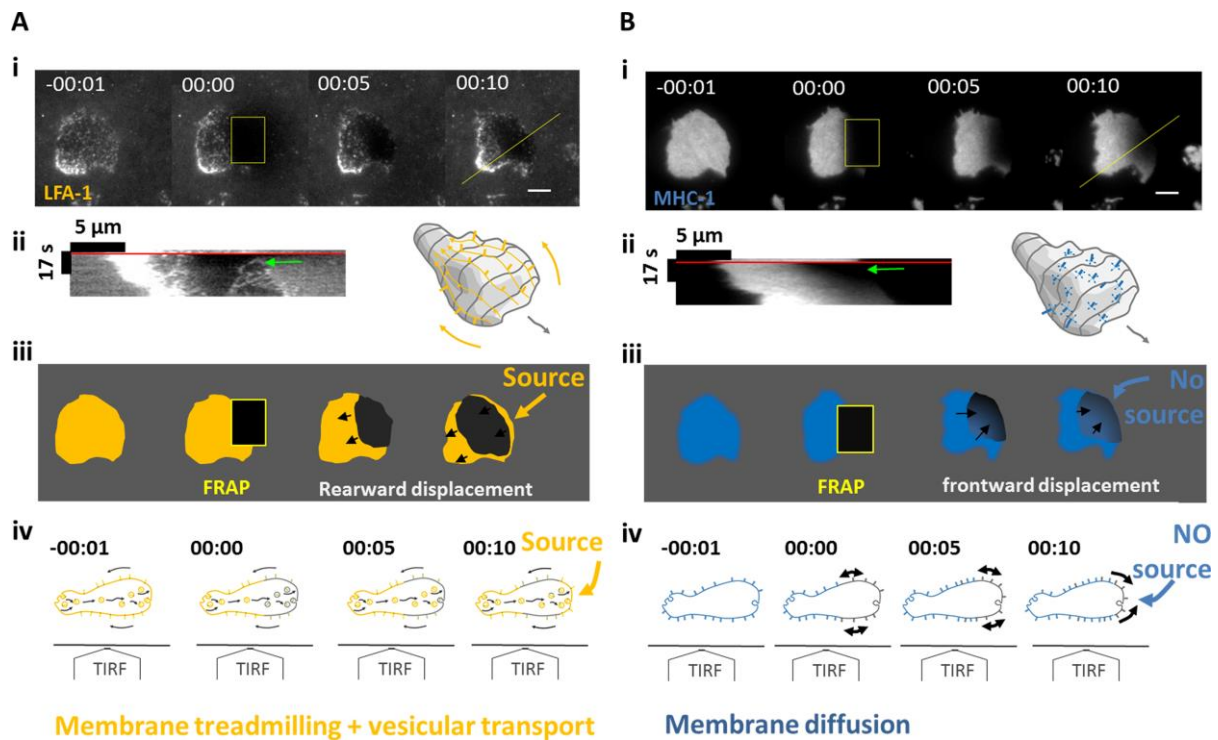


706

707 **Figure 7 : Actin-bound proteins LFA-1 treadmill backwards at the cytoplasmic membrane whereas**
 708 **non-actin-bound proteins MHC-1 diffuse without net transport. (A,B)** Backwards transports of actin
 709 and actin-bound LFA-1 transmembrane protein. (i) Maximum intensity projection of TIRF sequences
 710 on adhesion free cells transduced with RFP-actin (A) and stained with antibody M24 that binds the
 711 actin-bound integrin LFA-1 in its high affinity state (B). Scale bars are 5 μm. (ii) Representative
 712 kymographs along the yellow line in figures (i) with the front advance highlighted in magenta and
 713 clusters retrograde flow highlighted in blue. See also Movie 13 and Movie 14 (iii) Schematics
 714 representing a side view of a non-adherent cell observed with a TIRF objective and highlighting the
 715 retrograde flow of internal actin (Green units) and external LFA-1 integrins (orange bars). (C,D)
 716 Diffusive dynamics of non-actin bound transmembrane protein MHC-1 and lipidic DiO marker. TIRF-
 717 FRAP experiments on adhesion free cells, (C) stained with anti-HLA-ABC that binds the non-actin-
 718 bound MHC-1 type I proteins, and (D) with membrane lipidic marker DiO. Yellow circles indicate
 719 frapped regions used to calculate fluorescence recovery. Scale bars are 5 μm. (E) Quantification of
 720 transmembrane proteins advection and diffusion. **Left-** Speed values for retrograding actin
 721 measured by cluster tracking as in (D) (n=7 cells, 51 clusters tracked) and by FRAP as in Suppl. Mat.
 722 (n=8 cells), and for LFA-1 clusters (n=16 cells, 106 clusters tracked), all normalized to the front of the
 723 cell. **Right-** Averaged FRAP curves for DiO (blue, n=10 cells) and MHC-1 (green, n=14 cells). All values
 724 were normalized and corrected by a non-bleached cell. Diffusion coefficient is of $3.1 \pm 1.8 \mu\text{m}^2/\text{s}$ for

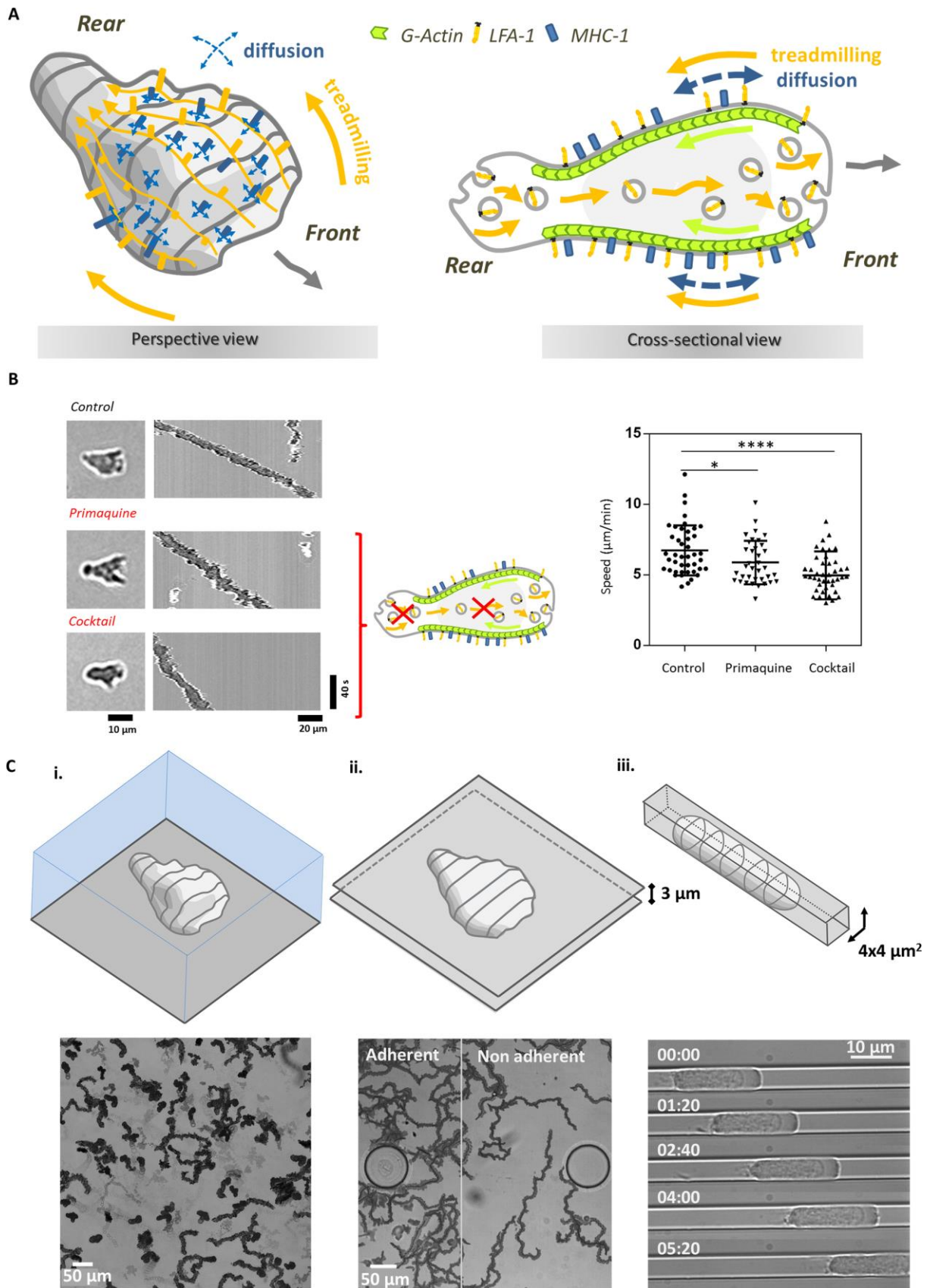
725 DiO and $0.26 \pm 0.22 \mu\text{m}^2/\text{s}$ for MHC-1. **(F)** Single cell evidence of average backward advection for
726 actin-bound LFA-1 and average absence of motion for non-actin-bound MHC-1. **(i)** Schematics
727 representing a side view of a non-adherent cell double-stained for LFA-1 (orange) and MHC-(blue).
728 **(ii)** TIRF images after FRAP bleaching of a line pattern revealing actin-bound activated LFA-1 in its
729 high affinity state (left) and non-actin-bound MHC-1 (right). **(iii)** Kymographs in the yellow rectangle
730 in figures (ii) with the motion of clusters and the centre of mass of the FRAP region highlighted in
731 blue for respectively LFA-1 and MHC-1. See also Movie 15.

732



734

735 Figure 8 : **Treadmilling actin-bound transmembrane protein LFA-1 is recycled by internal vesicular**
 736 **transport whereas diffusive transmembrane protein MHC-1 is not.** (A) Staining of high affinity LFA-
 737 1 and (B) of MHC-1. (i) Sequences of TIRF images before FRAP, after FRAP of cell leading edge, then 5
 738 and 10s after FRAP. Scale bar is 5 μm. See also Movie 16. (ii) Kymographs along yellow lines of figures
 739 A and B, with red lines indicating FRAP time and green arrows pointing at the cell front shortly after
 740 FRAP. 3D cartoon illustrates backward treadmilling of LFA-1 and 2D diffusion of MHC-1. (iii)
 741 Schematics of experimental results in (i) illustrating that fluorescence recovers from the cell leading
 742 edge for LFA-1, revealing a source at cell front, and from the back for MHC-1 revealing the absence of
 743 source at cell front. (iv) Side view schematics of non-adherent cells observed with a TIRF objective
 744 illustrating the dynamics of transmembrane proteins evidenced in TIRF experiments. For actin-
 745 binding LFA-1, source at cell front reveals internal frontward transport of fresh material exocytosed
 746 at cell front, whereas for non-actin binding MHC-1, purely diffusive transport dominates.



747

748 **Figure 9 : Treadmilling of transmembrane proteins recycled from rear to front by internal transport**
 749 **provides ubiquitous propulsion.** (A) Schematics of heterogeneous membrane treadmilling
 750 mechanism. The perspective view displays a swimming cell on which actin-bound proteins (orange)
 751 are advected backwards and entangled with non-actin bound proteins. Swimming is promoted by

752 advected proteins and hindered by diffusing proteins, which results in swimming significantly slower
753 than treadmilling. The cross sectional view displays the complete cycling of actin-binding proteins
754 (orange), which comprises endocytosis at cell rear, internal advection by vesicles forwards,
755 exocytosis at cell front and advection at cell membrane backwards by linkage to retrograde actin flow
756 (green). **(B)** Specific perturbation of paddling cycle by inhibitors of endocytosis (cartoon). **Left-**
757 Representative images of a cell, **middle-** representative kymographs of the swimming motion of the
758 corresponding cells and **right-** plot of the raw curvilinear swimming speeds for control conditions,
759 with Primaquine at 100 μM and with a cocktail of inhibitors (pitstop2 at 50 μM ; Dynasore at 200 μM ;
760 and secinH3 at 20 μM). $N = 30$ cells, p value of t test <0.01 (*), and < 0.0001 (****). **(C)**
761 Heterogeneous treadmilling fosters ubiquitous motility without adhesion. **(i)** Swimming on a non-
762 adherent surface, **(ii)** crawling between two surfaces separated by 3 μm , with adherent and non-
763 adherent zones (see also Movie 17), and **(iii)** in a non-adherent tube of cross-section $4 \times 4 \mu\text{m}^2$ (see
764 also Movie 18). Data for cases (i) and (ii) correspond to projected images taken every 10 s for 3 min
765 of a given area with several cells. The round shapes in (ii) correspond to 100 μm diameter glass pillars
766 that sustain the structure. Data for case (iii) show a sequence of images of a single cell for 5 min.

767 **Movies**

768 **Movie 1 : From Crawling to Swimming in the vicinity of a substrate.** (Left) Crawling on adhering
769 ICAM-1-treated substrate. (Right) Swimming on Pluronic[®] F127 treated surface. First sequence, 20x
770 bright field transmission microscopy, then 63x bright field transmission microscopy and finally x63
771 reflected interference contrast microscopy.

772 **Movie 2 : 3D imaging of primary human effector T lymphocyte swimming in spinning disk**
773 **microscopy.** Videomicroscopy sequence of swimming T lymphocytes stained with CMFDA (5-
774 chloromethylfluorescein diacetate) on Pluronic[®] F127 treated surface for 14 min 40 sec with a time
775 lapse every 20 s and stack of 10 slices taken every 1 μm . Some unpolarized cells do not swim. The
776 arrow points a polarized and swimming cell that crosses the whole field of view. Scale bars in μm
777 indicated on axis, magnification: 63X.

778 **Movie 3 : Immediate transition between crawling and swimming.** Migration on alternative 40 μm
779 wide stripes of adherent ICAM-1 and non-adherent Pluronic[®] F127 prepared by LIMAP³¹.
780 Superimposition of fluorescent image (ICAM-1, red), bright filed transmission image (greyscale) and
781 reflection interference contrast microscopy image (bright green corresponds to cells adhesion
782 fingerprints) taken at 63x.

783 **Movie 4 : Swimming in bulk suspension.** Movie of two cells suspended in a medium of matched
784 density using a microscope tilted by 90° and a flow chamber oriented vertically for sideway
785 observations.

786 **Movie 5 : Correlation of swimming direction with cell polarization axis.** T lymphocytes swimming
787 on Pluronic[®] F127 treated surface imaged in bright field at 10x of primary human effector. The red
788 bars direction represents the instant tangent to trajectory, and their norm is proportional to the ratio
789 between instant speed and maximal speed reached by the cells. The yellow bars direction represents
790 the orientation of the ellipse having the same second-moment as the segmented cell, and their norm
791 scales with the value of the ellipse eccentricity, as $(\exp(\text{ecc})-1)/(\exp(1)-1)$, where ecc is the
792 eccentricity of the ellipse which varies between 0 and 1. (For more details see Suppl. Mat. **Erreur !**
793 **Source du renvoi introuvable.** and corresponding paragraph).

794 **Movie 6 : Role of actomyosin network in swimming motion.** Movies in bright field at 63x of primary
795 human effector T lymphocytes swimming on Pluronic[®] F127 treated surface in the presence of actin
796 inhibitors: control, 50 μM Blebbistatin, 0.05 μM Latrunculin or 100 μM CK666. Scale bar 20 μm .

797 **Movie 7 : 3D live imaging by so-SPIM of the actin cytoskeleton of swimming primary human**
798 **effector T lymphocyte.** Video microscopy movie of RFP-Lifeact transfected cell, showing lamellar-
799 protrusion forming with random orientation in cell front and travelling backwards at around 10
800 $\mu\text{m}\cdot\text{min}^{-1}$.

801 **Movie 8 : Live bright field imaging of swimming primary human effector T lymphocytes showing**
802 **protrusion retrograde motion and nucleus forward squeezing through constricted rings.** Bright field
803 videomicroscopy at 63x of primary human effector T lymphocytes swimming on Pluronic[®] F127
804 treated surface. Scale bars 10 μm .

805 **Movie 9 : Swimming of a cell by the motion of two blebs on the cell surface.** The color here
806 represents the mean curvature of the cell surface.

807 **Movie 10 : Lymphocytes swimming with perturbation of cell body deformation.** Bright field
808 videomicroscopy at 63x of primary human effector T lymphocytes swimming on Pluronic[®] F127
809 treated surface in pure medium (left) and in a medium diluted with water at 50% (right). Osmotic

810 stress cancels the deformations of cell body by swelling but cell speed is $8 \mu\text{m}\cdot\text{min}^{-1}$ like for control
811 cells.

812 **Movie 11 : Cell membrane retrograde flow revealed by attached beads.** Bright field
813 videomicroscopy of ICAM-coated beads travelling from front to back on the cell membrane of
814 swimming T-cells in HBSS control media, 50 μM Blebbistatin, 0.05 μM Latrunculin, or 100 μM CK666.

815 **Movie 12 : Numerical simulation of swimming by retrograde flow.** Cell shape is extracted from
816 experiments. The swimming is shown in the laboratory frame. Color code on the surface represents
817 the production/consumption of the cortex material. Small spheres are fictitious tracers moving with
818 the cortex velocity. Transmission coefficient $\beta = 1$.

819 **Movie 13 : Evidence that actin bound proteins LFA-1 are advected backwards at cell membrane**
820 **whereas non actin bound proteins MHC-1 are diffusive.** TIRF-FRAP experiments on primary human
821 effector T-cell (Top left) transfected with a GFP-Actin by lentiviral infection, (Top right) stained with
822 membrane lipidic marker DiO, (Bottom left) stained with antibody Mab24 that binds an actin-bound
823 protein, the integrin LFA-1 in its high affinity state, and (Bottom right) stained with anti-HLA-ABC that
824 binds the non-actin-bound MHC-1 type I proteins. Scale bars 5 μm .

825 **Movie 14 : Cytoskeleton retrograde flow.** TIRF imaging of a primary human effector T lymphocyte
826 transfected with GFP-Actin and displaying backward travelling of clusters. Scale bar 5 μm .

827 **Movie 15 : Single cell evidence of average backward advection for actin-bound LFA-1 and average**
828 **absence of motion for non-actin-bound MHC-1.** TIRF images after FRAP bleaching of a line pattern
829 revealing actin-bound activated LFA-1 in its high affinity state (left) and non-actin-bound MHC-1
830 (right). Scale bar 5 μm .

831 **Movie 16 : Evidence that advected proteins LFA-1 are recycled at the cell front by internal vesicular**
832 **transport whereas diffusive proteins MHC-1 are not.** TIRF-FRAP experiments on primary human
833 effector T-cell (Right) stained with antibody Mab24 that binds the actin-bound proteins LFA-1 in high
834 affinity state, and (Left) stained with anti-HLA-ABC that binds the non-actin-bound proteins MHC-1.
835 The cell front is frapped and fluorescence recovers only from the front for LFA-1, in agreement with
836 an internal vesicular recycling of integrins from back to front, and only from the rear for HLA, in
837 accord with a surface diffusion mechanism.

838 **Movie 17 : Lymphocytes are motile when confined between non-adherent plates.** Movies in bright
839 field at 20x of primary human effector T lymphocytes moving between two glass substrates
840 patterned with an adherent zone covered with ICAM-1 (left) and an anti-adhesive zone (right). The
841 round shapes correspond to 100 μm diameter glass pillars that sustain the structure.

842 **Movie 18 : Lymphocytes are motile when confined in tubes.** Movies in bright field at 63x of a
843 primary human effector T lymphocytes moving in a non-adherent microfluidic channel of cross-
844 section $4 \times 4 \mu\text{m}^2$.

845 **Material and methods**

846 ***Cells***

847 Whole blood from healthy adult donors was obtained from the “Établissement Français du Sang”.
848 Peripheral blood mononuclear cells (PBMCs) were recovered from the interface of a Ficoll gradient /
849 “Milieu de separation des lymphocytes” (eurobio, les Ulis, France). T lymphocytes were isolated from
850 PBMCs with Pan T cell isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), then were
851 stimulated for 48 h with anti-CD3/anti-CD28 Dynabeads (Gibco by Thermo Fischer Scientific,
852 Waltham, MA) according to the manufacturer’s instructions. T lymphocytes were subsequently
853 cultivated in Roswell Park Memorial Institute Medium (RPMI) 1640 (Gibco by Thermo Fischer
854 Scientific, Waltham, MA) supplemented with 25 mM GlutaMax (Gibco by Thermo Fischer Scientific,
855 Waltham, MA), 10% fetal bovine serum (FBS; Gibco by Thermo Fischer Scientific, Waltham, MA) at
856 37°C, 5% CO₂ in the presence of IL-2 (50 ng/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) and
857 used 6 to 10 days after stimulation. At the time of use, the cells were >99% positive for pan-T
858 lymphocyte marker CD3 and assessed for activation and proliferation with CD25, CD45RO, CD45RA
859 and CD69 markers as judged by flow cytometry.

860 ***Quantitative cytometry for integrin expression level***

861 For the quantification, we used the CellQuant calibrator kit (ref 7208, Biocytex). T lymphocytes were
862 stained by indirect immunofluorescence with specific monoclonal antibodies, CD49d (HP2/1) for VLA-
863 4 and CD11a (Hi111) for LFA-1, then analyzed by quantitative flow cytometry. The expression level of
864 the tested antigen was determined using the kit calibration beads.

865

866 ***Transfection of cells***

867 For soSPIM experiments with LifeAct transduced cells, virus was produced in HEK 293T cells by co-
868 transfecting the lentiviral plasmids pLenti.PGK.LifeAct-Ruby.W (a gift from Rusty Lansford - Addgene
869 plasmid #51009) with psPAX2 and pMD2.G (a gift from Didier Trono - Addgene plasmid #12260 and
870 #12259). PBMC were transduced by spinoculation of virus using polybrene, after 48h activation with
871 CD3-CD28 Dynabeads. The cells were then cultured with IL-2, and used 8 days after activation. The
872 expression of LifeAct-RFP was controlled by flow cytometry. For TIRF-FRAP experiments cells, RFP-
873 Lentivirus for RFP-actin transduction were bought from Merck (Lentibrite™ RFP-β-actin lentiviral
874 biosensor) and cells were transduced 48h after activation with an MOI of 10. For GFP-actin
875 transfection, plasmid EGFP-Actin-7 from Addgene (ref 56421) was used with the electroporation
876 program Amaxa T20.

877 ***Microfluidic channels and surface treatments***

878 PDMS microchannels were fabricated using standard soft lithography. A positive mould was created
879 with a negative photoresist SU-8 3000 (Microchem) on silicon wafers (Siltronix), then replicas were
880 moulded in polydimethylsiloxane (PDMS) elastomer (Sylgard 184, Dow Corning) and sealed on glass
881 cover slides via plasma activation (Harricks Plasma). The device is composed of one channel with one
882 inlet and one outlet punched with a 2.4 mm puncher (Harris Uni-Core). For adherent crawling
883 experiments, Ibidi channels IV^{0.4} (Clinisciences) were coated overnight at 4°C with 10µg/ml human
884 ICAM-1-Fc (R&D Systems) in Phosphate Buffer Solution (PBS) (Gibco). Channels were subsequently
885 blocked with a solution containing 2.5% bovine serum album (BSA) (w/v; Axday, France) and 2.5%
886 Pluronic acid F-108 (w/v; BASF, Germany) in PBS for 30 min at room temperature, then rinsed three
887 times with PBS and finally with HBSS. Cells were injected at densities around 1.5x10⁶/ml and allowed
888 to equilibrate for 10 min at 37°C before image acquisition. For non-adherent migration or swimming
889 experiments, Ibidi channels IV^{0.4} and PDMS microchannels were incubated with Pluronic F-127
890 (Sigma-Aldrich) for 30 min at room temperature, then rinsed three times with PBS and finally with
891 HBSS. Cells were injected at densities around 0.75x10⁶/ml in Ibidi channels and 6x10⁶/ml in PDMS
892 microchannels of 40 µm height. Cells were allowed to equilibrate for 10 min at 37°C before image
893 acquisition. For migration in non-adherent confined environment, microfluidic tubes and channels of
894 height 2-4 µm were treated with Pluronic F-127 as described above. The adhesive/antiadhesive
895 patterns were prepared by optical patterning. We used an inverted microscope (TI Eclipse, Nikon,
896 France) coupled to a UV laser source and a Digital Micromirror Device (Primo™, ALVEOLE, Paris,
897 France)⁷⁶. UV was projected on ICAM-1 substrates in presence of a soluble photo-activator (PLPP™,
898 ALVEOLE, Paris, France) to degrade the proteins⁷⁷. Samples were then rinsed with PBS solution and
899 passivated with BSA and Pluronic F-127 for 15mins at room temperature.

900

901

902 ***Cell treatments***

903 Stock solutions of Blebbistatin (Fischer Bioblock Scientific), CK666 (Sigma-Aldrich), Latrunculin
904 (L12370; Molecular Probes), Primaquin (Primaquine biphosphate, Sigma-Aldrich), Pitstop2 (Sigma-
905 Aldrich), Dynasore (Dynasore monohydrate, Sigma-Aldrich) and cytohesin (secinH3, S Sigma-Aldrich)
906 were prepared in DMSO following manufacturer's specification, stored at -20°C and then diluted in
907 culture medium for use in experiments. Cells were resuspended in solutions of 50 µM Blebbistatin,
908 100 µM CK666, 50 nM Latrunculin, 100 µM Primaquin, or a cocktail of 50 µM pitstop2, 200 µM
909 dynasore, and 20 µM secinH3, and then injected in the microchannels and allowed to settle in the
910 channels for a period of 30 min at 37 °C before image acquisition.

911

912 ***Viscosity and osmolarity measurements***

913 Viscosity changes were performed using Dextran of average molecular weight of 1500-2800 KDa
914 (Sigma-Aldrich) at concentrations of 50 and 150 g/L. HBSS alone has a viscosity value of 0.001 Pa.s
915 while the viscosity for HBSS+50g/L Dextran is 0.01 and for HBSS+150g/L Dextran it is 0.1 Pa.s. Adding
916 Dextran to the media increased the viscosity as well as the osmolarity up to 355 mosm/kg for the
917 solution HBSS+150g/L Dextran. Dextrose (Sigma-Aldrich) was then used as an osmolarity control in
918 HBSS media supplemented with 25mM HEPES. Osmolarity measurements for the different media
919 were performed using the automatic Micro-Osmometer Type 15 (Loser Messtechnik), calibration was
920 done using standard solutions of 300 and 900 mosm/kg H₂O according to the manufacturer's
921 instructions.

922

923

924 ***Experimental fluidic setup***

925 All experiments were performed in a home-made chamber precisely thermostated at 37°C to limit
926 temperature instability potentially inducing flow drifts within fluidic devices. For swimming close to a
927 surface, we used Ibidi channels for experiments in HBSS, Dextrose and 50g/L Dextran solutions, and
928 40 µm high PDMS microchannels for experiments in 150g/L Dextran to limit the observation range in
929 the z axis because cells did not sediment. To minimize flow, channels were sealed with a plastic cap
930 for Ibidi channels, or with a 250µm thickness PDMS film for the PDMS microchannels. On the
931 microscope stage, the devices were surrounded by a 100% humidity chamber to minimize
932 evaporation through PDMS. For experiments of swimming in suspension, cells were resuspended in
933 66% Ficoll to limit sedimentation effects, and injected in 100 µm high channels. Minimization of drifts
934 for swimming in suspension was more challenging than for the swimming close to a substrate. The
935 microfluidic channel was set vertical (along the gravity axis) and the whole microscope was tilted by
936 90° to get side-observation view. The channel was connected to a microfluidic flow control system
937 (Fluigent MFCS-EZ) to control the unidirectional flow towards the bottom, and we used 2 meter long
938 tubes of 0.5 µm internal diameter to further limit drift by hydraulic resistance. Cell motion were
939 recorded for at least 100 frames every 10 seconds.

940

941 ***Cell motion imaging***

942 Experiments were performed with an inverted Zeiss Z1 automated microscope (Carl Zeiss, Germany)
943 equipped with a CoolSnap HQ CCD camera (Photometrics) and piloted by µManager^{1,4}. Different
944 objectives were used for bright-field mode (Plan-Apochromat 20x/0.8, 63x/1.4 objectives) and for
945 reflection interference contrast microscopy (RICM) mode (Neofluar 63/1.25 antireflex). A narrow band-
946 pass filter ($\lambda=546 \text{ nm} \pm 12 \text{ nm}$) was used for RICM. Three dimensional imaging was performed on

947 cells stained with a lipophilic tracer DiO (Invitrogen) and cells transfected with lifeAct-RFP cells. The
948 imaging was done using a Spinning disk (Inverted Nikon Eclipse TI) equipped with two cameras
949 (Photometrics EMCCD evolve) and controlled by Metamorph, and a home-made single-objective
950 selective plane illumination microscopy (soSPIM) set-up.

951

952 ***soSPIM imaging and analysis***

953 The soSPIM system, for single-objective Selective Plane Illumination Microscope, is a recently
954 developed architecture which enables to combine the advantages of low photo-toxicity and high
955 optical sectioning of light-sheet microscopy techniques with the high sensitivity provided by high
956 numerical aperture objectives⁷⁸. The set-up is composed of a high numerical aperture objective (CFI
957 Plan Apochromat VC 60x WI 1.27NA), a beam steering unit and dedicated micro-fabricated devices
958 containing mirrors angled at 45° alongside micro-wells. The soSPIM components are mounted on a
959 conventional inverted microscope (Nikon Ti-E). The micro-fabricated chambers (see ⁷⁸ for detailed
960 descriptions of the chambers) are placed on an axial translation piezo stage (Mad City Lab) within a
961 controlled environment chambers (Tokai Hit) for live cell imaging. Fluorescence emission is collected
962 through the same objective used for excitation and is captured on a sCMOS camera (ORCA-Flash 4.0
963 V2, Hamamatsu). The whole acquisition process is steered under MetaMorph environment
964 (Molecular Device) using a home-made designed plugin which synchronize the excitation and
965 acquisition processes. Further details of soSPIM setup, calibration, and synchronization are described
966 in⁷⁸. The 3-dimensional time series data sets acquired with the soSPIM set-up were analysed using
967 the freely available software UCSF Chimera⁷⁹ (developed by the Resource for Biocomputing,
968 Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-
969 GM103311)). This software enables to render surfaces of equal fluorescence intensity as well as
970 normalization and alignment of whole 3D time series which enhances the possibility to visualize cell
971 membrane movement in our case.

972 ***Molecular motility imaging***

973 For TIRF-FRAP experiments, cells were resuspended in HBSS-Dextran 150g/L solution at a
974 concentration of 4.5×10^6 cells/ml, in the presence of CD11a/CD18 (Biolegend, clone M24) and HLA-
975 A,B,C (Biolegend, clone W6/32) primarily conjugated antibodies. Alternatively, cells were labelled
976 with Vybrant® DiO by 10 min incubation at 37°C in the presence of 5 µl of dye per 1.5×10^6 cells, then
977 washed twice with HBSS and resuspended in HBSS-Dextran. For LifeAct-RFP cells, no further staining
978 was required. Cell suspensions were loaded into the devices and centrifuged for 3 min at 200 RCF.
979 Cells were allowed to equilibrate for at least 10 min at 37°C before image acquisition. Movies were

980 recorded on a Nikon Eclipse Ti microscope, equipped with iLas2 system and controlled by
981 Metamorph software. For DiO and MHC-1 staining, diffusion coefficients were calculated using the
982 SimFRAP ImageJ plugin*. LFA-1 cluster speed values were calculated from kymographs performed
983 along the cell axis, while Actin flow was calculated measuring the displacement of the frapped
984 region. All values were corrected by the advance of the front edge, measured by a kymograph along
985 the cell axis, to obtain a value relative to the cell front.

986 ***Cell tracking***

987 For swimming experiment in the vicinity of a substrate (in 2D), cells were tracked with a home-made
988 program (MATLAB software, The MathWorks, Natick, MA, USA) and raw curvilinear speeds of
989 swimming cells were calculated using trajectory time points every 30s. Residual flow drift was
990 corrected on each cell trajectory using the mean x- and y-movements values of all cells between 2
991 pictures. For high viscosity experiments, the fraction of cells squeezed toward the substrate by
992 depletion force were discarded from the analysis. For swimming experiment in suspension (in 3D), a
993 stack of bright-field images was taken every 10 s across the 100 μm height of the channel with a
994 spatial pitch of 5 μm . To determine the position a particular cell on the x-axis at a given time, we
995 analyzed the intensity distribution of the image of this cell on all images of the x-axis stacks. The best
996 focus corresponded to the image with the minimum standard deviation of the intensity, which
997 yielded an x-position with a precision of 2.5 μm . Each cell trajectory was fragmented in 30s steps and
998 cell-step speed was calculated using coordinates along x- and y-axis. The speed component along y
999 was considered negligible because we selected cells with an orientation perpendicular to Y-axis. Total
1000 cell speed was calculated as the mean of all the 30s steps-speed for each cell.

1001 The circularity of cells was determined on bright field images at magnification x63. Images were first
1002 binarised using the Pixel classification workflow of the Ilastik software⁸⁰. The binaries images were
1003 then analyzed in Matlab and the circularity was calculated using the image processing toolbox,
1004 $Circularity = Perimeter^2 / (4\pi Area)$.

1005

1006 ***Beads advection experiments***

1007 Streptavidin-coupled beads with a diameter of 2.7 μm (Dynabeads[®] M-270 Streptavidin, Invitrogen)
1008 were washed three times with 0.1% BSA (w/v), then incubated with 0.5 $\mu\text{g}/\text{ml}$ biotin-coupled Protein-
1009 A (Sigma-Aldrich) for 1 hour under stirring at room temperature and rinsed with 0.1% BSA. The beads
1010 were then incubated with 500 $\mu\text{g}/\text{ml}$ ICAM for 2 hours at room temperature and rinsed with 0.1%
1011 BSA. A final concentration of 0.125 mg/ml Dynabeads was added to the cell suspension. Bright-field

1012 images (Plan-Apochromat $\times 20/0.8$) were taken every 3s. Beads were tracked manually from the
1013 moment the bead attached to the cell front until it reached the cell rear. Cell are moving in the frame
1014 of the laboratory and cell rear was taken as a reference of bead position. All experiments were
1015 performed at least in triplicate for each substrate and/or drug.

1016

1017 **Contributions**

1018 LA and PN worked on all experiments and analysis except TIRF-FRAP section; AF developed all
1019 modelling and simulations on treadmilling mechanism, and contributed to swimming speed analysis;
1020 NGS performed experiments of beads tracking and all FRAP-TIRF assays; MSR performed modelling of
1021 shape deformation mechanism; XL performed migration assays on patterned substrates; ST
1022 performed elliptic Fourier reconstruction of contours and wavelet transforms ; MB managed cell
1023 culture, performed transfection with RFP-Lifeact, and made quantitative cytometry, MPV
1024 participated to experiments and analysis, RG and JBS performed 3D live imaging by soSPIM, CH and
1025 SD prepared GFP-actin cells; SR performed viscosity measurements and participated to discussions;
1026 CM supervised and participated to all modelling developments, designed the study and wrote the
1027 paper; OT supervised and participated to all experiments and analysis, designed the study and wrote
1028 the paper.

1029

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1046

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