1   **Cyclic mechanical stresses alter erythrocyte membrane composition and**

2   **microstructure and trigger macrophage phagocytosis**

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18   **Running title:** Red blood cell aging by mechanical stress

19   **Abstract**

20   Red blood cells (RBCs) are cleared from the circulation when they become damaged or display aging

21   signals targeted by macrophages. This process occurs mainly in the spleen, where blood flows through

22   submicrometric constrictions called inter-endothelial slits (IES), subjecting RBCs to large-amplitude

23   deformations. In this work, we circulated RBCs through microfluidic devices containing microchannels that

24   replicate the IES. The cyclic mechanical stresses experienced by the cells affected their biophysical

25   properties and molecular composition, accelerating cell aging. Specifically, RBCs quickly transitioned to a

26   more spherical, less deformable phenotype that hindered microchannel passage, causing hemolysis. This

27   transition was associated with the release of membrane vesicles, which self-extinguished as the spacing

28   between membrane-cytoskeleton linkers became tighter. Proteomics analysis of the mechanically aged

29   RBCs revealed significant losses of essential proteins involved in antioxidant protection, gas transport, and

30   cell metabolism. Finally, we show that these changes made mechanically aged RBCs more susceptible to

31   macrophage phagocytosis. These results provide a comprehensive model to explain how physical stress

32   induces RBC clearance in the spleen.

1

Introduction

34 Circulating red blood cells (RBCs) deliver oxygen from the lungs to all organs in the body. Human RBCs are

35 anucleate and discoidal in shape, with an 8-µm diameter and a thickness ranging between 1 and 2.2 µm[1].

36 Throughout their 120-day lifetime, RBCs undergo large-amplitude deformations in the systemic and

37 pulmonary microcirculations. However, the most severe deformations occur as blood transits the spleen,

38 where RBCs squeeze through 0.5-2.5 µm-gaps between specialized endothelial cell fibers called inter-

39 endothelial slits (IES)[2]. Old and diseased RBCs are retained during splenic transit and eventually removed

40 by red pulp macrophages. The interplay between RBC deformation, RBC aging, and splenic clearance is

41 crucial in hematologic disorders and the preservation of blood stored for transfusion.

42 As RBCs age, their shape transitions from biconcave-discoidal to serrated or spherical, and their

43 intracellular density increases[3, 4]. These changes, often attributed to membrane loss, are associated with

44 decreased deformability, leading to failure to squeeze through the IES[5]. RBC aging hallmarks are

45 accentuated in hemolytic diseases like iron deficiency anemias and malaria[6], which cause life-threatening

46 symptoms and multiple complications. Additionally, aged RBCs exhibit significant biochemical changes

47 affecting cell volume, deformability, and metabolic activity, such as ATP depletion[7] and increased

48 cytoskeleton oxidation[8]. Oxidation, which is also linked to RBC aging during storage[9], promotes the

49 aggregation of band3[10], a membrane protein required for CO_2 transport that regulates RBC stiffness by

50 anchoring the lipid bilayer to the cytoskeleton.

51 How macrophages identify and remove RBCs from the circulation is still poorly understood. This specific

52 macrophage attack is associated with the presence of distinctive age-related signals in the RBC

53 membrane. Previous studies reported an increase in phosphatidylserine (PS) in the outer leaflet of the

54 membrane as RBCs age[11]. PS is a phospholipid usually located in the inner leaflet of the membrane;

55 however, when exposed in the outer leaflet, it acts as a phagocytosis signal[12]. Additionally, the expression

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56   of CD47, a membrane protein that inhibits macrophage attacks, declines as RBCs age[13]. Finally, band3

57   clustering promoted by age-induced oxidation facilitates autoantibody binding and could mediate RBC

58   recognition by macrophages[14].

59   Increased intracellular density is the primary experimental marker of RBC aging because it correlates with

60   other hallmarks of RBC functional decline. Recent data suggest that RBCs exposed to cyclic stretch exhibit

61   significant changes in shape and mechanical properties (i.e., mechanical fatigue)[15,16]. This evidence led us

62   to hypothesize that RBC deformations cause biophysical and biochemical changes equivalent to aging,

63   which can be accelerated significantly in vitro. The main goals of the present study were to test these

64   hypotheses and to investigate the underlying mechanisms for mechanically induced RBC aging. To this

65   end, we fabricated PDMS-based microfluidic devices to model the passage of RBCs through IES in the

66   spleen. We analyzed the biophysical and biochemical changes undergone by RBCs as a function of the

67   number of repetitive passages through these microchannels, finding significant metabolic, proteomic, and

68   biomechanical alterations. Specifically, we demonstrated that RBCs undergo cellular vesiculation and cell

69   rounding accompanied by key biological hallmarks of RBC aging like decreased intracellular ATP and

70   enhanced oxidation, all associated with a higher susceptibility to macrophagic attack. In addition to

71   providing insights into the biomechanical underpinnings of RBC aging, this work offers a proof of principle

72   of using microfluidic devices to systematically and reproducibly accelerate RBC aging *in vitro*.

3

73 **Results**

74 **Microfluidic devices mimic crossing of splenic inter-endothelial slits by RBCs**

75 We developed microfluidic devices to model the crossing of RBCs through splenic inter-endothelial slits

76 (IES) using a two-step photolithography process (see Materials and Methods and Fig 1A-B). The devices

77 test section contains an array with 100 microchannels of tunable width (Fig 1 C). Microchannels with

78 widths < 1 μm were achieved as measured by scanning electron microscopy (Supp Fig 1A).

79 The small height of the device's test section forced the RBCs to orient their discoidal plane parallel to the

80 top and bottom walls, subjecting the cells to large-amplitude deformations when crossing the

81 microchannels. Two types of crossing motion were observed. Most often, the RBCs folded like a "taco

82 tortilla" (Fig 1D and Suppl Video 1), a behavior commonly observed in capillaries[17]. Less frequently, the

83 RBCs twisted to progressively align their discoidal plane parallel to the microchannel sidewalls (Fig 1E and

84 Suppl Video 2). A small percentage of cells failed to cross, got trapped in the microchannel, and eventually

85 burst. By varying microchannel geometry within a range representative of IES *in vivo* observations[18], we

86 found that the frequency of twisting increased with microchannel width (Fig 1F). On the other hand,

87 microchannel length did not affect the type of RBC crossing motion (Fig 1G).

88 We determined the RBC crossing time through microchannels, a parameter strongly related to cell

89 deformability[19], as a function of microchannel width and length (Fig 1H-I). We used this information to

90 choose physiologically realistic microchannel dimensions for our experiments that yielded crossing times

91 consistent with available *in vivo* data[20].

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93 **Successive crossing of microchannels promotes RBC aging**

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94   In humans, RBCs recirculate through the IES in the spleen approximately every 200 minutes leading to 400

95   crossings over a cell's half-life[21]. To decouple the effects of mechanical deformation from other time-

96   dependent processes, RBCs were recirculated through our microfluidic device to cross the microchannel

97   array every 10 minutes (Suppl Fig 1B). Overall, we recirculated the cells up to 480 times over 3 days

98   through 3x0.85-µm channels. In the control experiments, RBCs were recirculated through a sham

99   microfluidic device without microchannels.

100   RBC dynamics were significantly affected as the cumulative number of microchannel crossings (*Nc*)

101   increased. The crossing time grew significantly with *Nc* (Fig. 2A), and so did the percentage of cells

102   undergoing folding vs. twisting (Fig. 2B). The number of cells trapped inside the microchannels (Fig. 2C)

103   and RBC lysis also increased with *Nc* (Fig. 2D). Successive microchannel crossing precipitated RBC aging

104   hallmarks such as oxidation (assessed by RBC methemoglobin content, Fig. 2E) and intracellular ATP

105   decline (Fig. 2F). Altogether, these data demonstrate that cyclic large-amplitude loading promotes an aged

106   phenotype in RBCs.

107

108   **Microchannel crossing results in RBC volume loss and vesiculation**

109   Since RBC aging is associated with changes in cell volume and sphericity[3, 4], we investigated how the

110   cumulative crossing of microchannels affected these parameters. The sphericity index was defined as $SI =$

111   $\pi^{1/3}6^{2/3}V^{2/3}/S$, where V and S are cell volume and surface area measured by fluorescence exclusion[22]

112   (see Materials and Methods, and Suppl Fig. 2A-B). We separated RBCs based on their density by

113   centrifugation to independently study lighter (presumably younger) and denser (older) cells.

114   Our measurements revealed that cells lost volume and became more spherical as *Nc* increased (Fig. 3A-

115   B). These changes were more significant in lighter cells than in denser ones. In contrast, control cells

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116   circulated for an equivalent time through the sham circuit were significantly less affected. After passing

117   through $N_c \approx 60$ microchannels, the statistical differences between light and dense cells became non-

118   significant, implying that the initially young RBCs became similar to the aged ones. Of note, the RBCs

119   needed to loop in the microfluidic circuit for only 10 hours to achieve the aged phenotype. Microchannel

120   crossing time directly correlated with cell sphericity (Fig. 3C) and a significant proportion of cells with $SI \gtrsim$

121   0.8 got stuck inside the microchannels. Consequently, the rate of microchannel RBC retention and

122   hemolysis increased with *Nc* (Suppl Fig. 3A-B). We used membrane flickering spectrometry to assess the

123   RBC membrane (lipid bilayer + membrane skeleton) effective tension and bending modulus. We validated

124   this technique using previously published data for control RBCs[23] (Suppl Fig. 2C-D). We observed that the

125   bending modulus increased with *Nc* (Fig 3D) while tension was not altered significantly (Fig 3E).

126   RBC densification and volume loss have been attributed to vesiculation[24]. Therefore, we studied whether

127   cyclic microchannel crossing caused RBC vesiculation. Vesicle generation was assessed by flow cytometry,

128   which was calibrated using microbeads (Suppl Fig 4A-C). Based on this analysis, RBCs generated vesicles

129   with diameters between 0.1 and 0.5 μm, although the release of smaller vesicles undetectable by the flow

130   cytometer cannot be discarded. RBCs subjected to cyclic microchannel crossing released significantly

131   more vesicles than control RBCs kept in stationary conditions (*Nc = 0*) or circulated through the sham

132   device (Fig 3F). The total number of vesicles increased with *Nc* and plateaued around $N_c \approx 60$, which

133   coincides with the value of *Nc* beyond which RBC volume ceased to decrease (see Fig 3A). Thus, we

134   concluded that volume loss was caused by vesiculation.

135

136   **Successive microchannel crossings alter RBC membrane composition and cytoskeletal organization.**

137   Our observation that cyclic mechanical loading triggered an aged RBC phenotype motivated a more

138   comprehensive analysis of RBC composition and structure. We used mass spectrometry to analyze the

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139 protein content of RBC membranes after 200 microchannel crossings (Fig. 4A, complete list in Suppl Table

140 1). RBCs kept in stationary conditions ($Nc = 0$) were used as controls. The volcano plot for these proteomics

141 data was asymmetric, with few proteins substantially decreased and many other increased albeit to a

142 lower, not significant extent (Fig. 4B). The list of proteins experiencing statistically significant losses with

143 p-value < 0.01 was analyzed using Metascape[25] to investigate the most likely affected pathways (Fig. 4C).

144 Protein clustering analysis revealed three affected groups. One group (Log_{10} p-value = -6.2) consisted of

145 proteins involved in cell metabolism like ATP synthase, suggesting a reduction in overall RBC function. This

146 group was tightly linked to a second group (Log_{10} p-value = -5.1) of proteins involved in gas transport and

147 exchange, such as bisphosphoglycerate mutase (BPGM) and carbonic anhydrases. The third group (Log_{10}

148 p-value = -4.6) comprised proteins involved in protection from oxidation such as catalase and

149 peroxiredoxins, congruent with our finding that RBC oxidation increased with Nc (Fig. 2E). Although less

150 significant (Log_{10} p-value = -2.8), we also found losses in structural proteins such F-actin, filamin-B,

151 dynactin, or the ankyrin repeat domain. The quantity of other structural proteins like spectrin, band3,

152 band4.1, or ankyrin1 remained unaltered.

153 The proteins connecting the RBC lipid bilayer and cytoskeleton, e.g., ankyrin1 or band4.1, form a network

154 with a relatively regular hexagonal pattern[1, 19]. Since successive microchannel crossings did not

155 significantly affect the amounts of these proteins, we hypothesized that their inter-protein spacing in the

156 network shortens as RBC vesiculation reduces the available plasma membrane (Fig 5A). Using stimulated

157 emission depletion microscopy (STED) and image correlation analysis (Fig 5B), we quantified the inter-

158 protein distance of ankyrin1 and band4.1 in RBCs that had transited $N_c \approx 200$ microchannels and static

159 controls (Fig 5C). Our control measurements were in agreement with previous data[26]. Moreover, we

160 observed a significant decrease in inter-protein spacing of ankyrin1 and band4.1 for $N_c \approx 200$. Overall,

161 these results suggest that vesiculation depletes specific proteins essential to RBC function, while most

162 other proteins become slightly enriched as cells become denser. In particular, the network of bilayer-

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163   cytoskeleton linkers becomes tighter, consistent with the observed increase in effective bending modulus

164   with N_c (see Fig 3D).

165

Cytoskeletal integrity modulates RBC aging caused by microchannel crossing

167   To investigate the role of cytoskeletal integrity in mechanically induced RBC aging, we treated RBCs with

168   drugs targeting different cytoskeleton components: Blebbistatin (Blebb) and Y27632 to inhibit myosin

169   activity, and Latrunculin A (LatA) and Cytochalasin D (CytoD) to affect actin polymerization. These

170   treatments increased cell deformability leading to shorter crossing times (Fig. 6A) and lower membrane

171   bending modulus (Fig. 6B), with LatA producing the most significant effects. Besides, Y27632 and

172   especially Blebb significantly reduced membrane tension (Suppl Fig. 5A). Given its more potent effects,

173   we analyzed LatA treatment in more detail. As expected, LatA induced marked changes in RBC morphology

174   as determined by electron microscopy (Suppl Fig 5B). Increasing LatA concentration accentuated both the

175   decrease in RBC effective bending modulus (Fig 6B) and the number of released vesicles (Fig 6C).

176   Consequently, cell volume loss with cumulative microchannel crossing was amplified by LatA treatment

177   in a dose-dependent manner (Fig. 6D). Despite being initially more deformable and experiencing shorter

178   crossing times than previously reported[27], LatA-treated cells displayed increased crossing times (Fig 6E)

179   and hemolysis (Fig 6F) for high Nc, likely due to LatA-induced, accelerated mechanical aging.

180   RBC oxidation is associated with changes in cytoskeletal integrity and cell deformability[28]. Accordingly,

181   RBCs treated with H_2O_2 exhibited increased bending effective moduli and longer crossing times (Suppl. Fig

182   6), indicating a rigidized cytoskeleton. These results confirm that actin cytoskeleton integrity controls IES

183   transit via RBC deformability. Moreover, impaired cytoskeletal integrity accelerates vesiculation, cell

184   spherification, and other RBC aging hallmarks like hemolysis.

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RBC aging caused by microchannel crossing facilitates macrophage attack

187   To address whether mechanically induced RBC aging stimulates macrophages to target RBCs, we co-

188   cultured THP-1-derived macrophages with RBCs after *Nc = 200* microchannel crossings. The RBCs were

189   fluorescently labeled to tag macrophages that internalized RBCs (Fig 7A). This experiment revealed that

190   the number of labeled THP-1 cells (Fig. 7B) and their average fluorescent intensity (Fig. 7C) increased

191   significantly when they were co-cultured with mechanically-aged RBCs as compared to static RBCs (*Nc =*

192   *0*). Since RBC stiffness plays an important role in macrophage phagocytosis[29], we treated RBCs with LatA

193   or H_2O_2 to respectively promote or reduce their deformability. RBC treatment with H_2O_2 increased

194   phagocytosis whereas LatA decreased it (Fig 7B-C).

195   Next, we investigated whether cyclic microchannel crossing generated molecular "eat-me" signals in the

196   RBC membrane. We measured phosphatidylserine (PS) exposure in the outer membrane leaflet[12], finding

197   a shallow increase in PS exposure that was statistically significant for *Nc = 512* (Fig 7D) but not for lower

198   values of *Nc*. Thus, since we observed an increased RBC phagocytosis by macrophages for *Nc = 200* (Fig

199   7B-C), PS exposure alone does not explain increased macrophage targeting of mechanically aged RBCs.

200   We also measured the expression of the transmembrane protein CD47, whose decline with RBC age is

201   associated with RBC clearance[13, 30]. However, we did not find any significant decrease in CD47 expression

202   in RBCs that had crossed *Nc* = 40 or 200 microchannels as compared with static controls (Fig 7E), consistent

203   with our proteomic data (see Fig 4).

204   Macrophage attack has also been associated with the clustering of specific membrane proteins like

205   band3[31], which could be active in mechanically aged RBCs based on our STED data (Fig 5). This targeting

206   modality relies on the binding of autoimmune antibodies and their posterior recognition by macrophages

207   via the Fc receptor[29, 32]. We opsonized static and *Nc = 200*—RBCs with plasma from the same blood

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208   sample, and measured the percentage of RBCs that bound immunoglobulin G (IgG). As shown in Fig 7F,

209   mechanically aged RBCs exhibited a significantly higher level of IgG binding than controls. Thus, we

210   concluded that mechanical RBC aging enhances macrophage attack by a combination of increased cellular

211   rigidity and a higher association of autoantibody opsonization, with a possible contribution from increased

212   PS exposure at later stages of the process.

213

Discussion

214

215   This work demonstrates that the cumulative exposure of red blood cells (RBCs) to large-amplitude

216   deformations promotes a decline in cell function similar to cell aging, and sheds light onto underlying

217   mechanisms. Cyclic loading is known to lower RBC deformability[15, 16], a mechanical function frequently

218   used as an aging hallmark[18, 33]. We show that cyclic passage through IES (loading) also accentuates

219   biological aging hallmarks like oxidation, ATP depletion, or hemolysis and, notably, degrades the cells'

220   oxygen-carrying efficiency. We investigate the molecular and structural origins of these phenomena and

221   provide a nexus between cyclic loading and RBC targeting by macrophages, which is essential to remove

222   aged RBCs from the circulation.

223   The largest deformations experienced by RBCs *in vivo* occur as they are filtered through the inter-

224   endothelial slits (IES) of the spleen. Thus, we circulated RBCs through microfluidic devices that forced the

225   cells to squeeze through narrow (< 1 μm) microchannels, causing large-amplitude deformations. The flow

226   rate and microchannel dimensions were tuned to match *in vivo* measurements of IES morphology and

227   crossing time[18, 20]. To extricate cyclic loading from other senescence-inducing processes like, *e.g.*,

228   continuous exposure to reactive oxygen species (ROS)[28], we recirculated RBCs in our devices every 10 min,

229   an interval 20 times shorter than the period between successive splenic passages *in vivo*. As RBCs were

230   recirculated, their cumulative crossing of microchannels caused a cascade of biomechanical events that

231   accelerated cell aging.

232   The earliest signs of RBC mechanical aging were detected after 15 load cycles and involved alterations in

233   microchannel crossing dynamics (prolonged crossing time and more frequent cell folding vs. twisting

234   motion). These changes were accompanied by a rise in cell oxidation and a decline in intracellular ATP.

235   Concurrently, cell volume decreased, cell sphericity increased, and the bilayer-cytoskeleton complex

236   stiffened, rendering the cells unable to transit the microchannels and causing hemolysis. The alterations

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237   became significant after 60 microchannel crossings, a comparable albeit smaller figure than the number

238   of loading cycles that elicited mechanical RBC fatigue in previous studies[15, 16]. However, those studies had

239   slower loading timescales (~4 s [15] vs. 0.2 s) or wider microchannels (3 μm[16] vs. < 1 μm) than splenic IES

240   filtration[18, 20], the process modeled in our microfluidic devices.

241   The observed changes were associated with the release of sub-micrometric vesicles. Vesiculation is

242   observed in RBCs aging in vivo or stored for transfusion[34]. Mechanical deformation can trigger vesiculation

243   by loading the linkages between the lipid bilayer and the cytoskeleton beyond their rupture strength.

244   Simulations suggest these conditions are met at the trailing edge of RBCs passing through < 1-μm-wide

245   microchannels[35], and vesiculation has been reported in RBCs subjected to cyclic flow shear in vitro[36].

246   Besides, passage through constrictions elicits Ca^{2+} influxes[37], an ion regulating RBC bilayer – cytoskeleton

247   anchorage and implicated in vesiculation[38]. Our proteomics experiment indicates that calmodulin, a

248   protein involved in Ca^{2+} regulation, is lost in mechanically aged RBCs, suggesting these cells have impaired

249   Ca^{2+} activity, which could exacerbate vesiculation.

250   After 60 microchannel crossings, the RBCs stopped vesiculating and reached minimum volume and

251   maximum sphericity, supporting the theory that vesiculation shuts off as the cells exhaust their available

252   plasma membrane[36]. Our data suggest a mechanism for vesiculation tapering based on the density of

253   bilayer-cytoskeleton linkages. We posit that the distance between linkages gradually decreases as vesicles

254   are released, restricting bilayer fluctuations[39]. Consonantly, we observed that the distance between

255   ankyrin1 and band4.1, two key bilayer-cytoskeleton crosslinkers, decreases significantly in mechanically

256   aged RBCs. Moreover, the effective bending modulus of the bilayer-cytoskeleton complex increased with

257   cyclic mechanical loading.

258   RBC aging and vesiculation have been linked to cellular oxidation, which is recognized to impair RBC

259   deformability[40, 41]. Our data suggest that the interplays between these processes are more complex than

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260   previously appreciated because mechanical loading, in turn, also weakens the RBC antioxidant defenses.

261   Circulating RBCs are constantly exposed to ROS released by exogenous (macrophages, endothelial cells)

262   and endogenous sources. When not neutralized by the RBC antioxidant system, these ROS alter the RBC

263   bilayer-cytoskeleton complex to increase cell stiffness. Our proteomic analysis revealed that mechanically

264   aged RBCs lose a significant amount of antioxidant enzymes (e.g., catalase, peroxiredoxin-1, and

265   peroxiredoxin-2). These results imply a novel positive feedback amplification loop between mechanical

266   and oxidative damage.

267   Large-amplitude mechanical deformation promotes alterations that could favor the removal of RBCs from

268   the circulation. First, as aged RBCs become less deformable, their microchannel crossing time increases

269   significantly. *In vivo*, a prolonged IES crossing time would facilitate interactions between RBCs and

270   macrophages in the spleen. Second, membrane composition changes in mechanically aged RBCs,

271   discussed above, could modulate phagocytic signals recognized by macrophages. We found that THP-1-

272   derived M1 macrophages displayed higher phagocytosis of mechanically aged RBCs compared to controls.

273   Traditionally, PS[11, 12, 32], CD47[13, 30], and band3[10, 14, 32] are considered as removal signals in senescent RBCs.

274   However, our proteomics and flow cytometry measurements did not reveal marked changes in CD47 or

275   band3 content in the membranes of RBCs that had squeezed through microchannels. Likewise, we only

276   detected increased PS exposure after an extremely large number of deformation cycles. Thus, we

277   concluded that cyclic loading may elicit non-traditional "eat-me" signals. Since target stiffness is known

278   to affect phagocytosis efficiency[29], we hypothesized that increased oxidation of membrane proteins in

279   mechanically aged RBCs and its associated cell stiffening facilitate macrophage attack. Consistent with this

280   hypothesis, we observed that H_2O_2 treatment increases RBCs stiffness and promotes phagocytosis. In

281   addition, mechanically aged RBCs had higher opsonization efficiency, which can contribute to RBC

282   phagocytosis via the Fc receptor in macrophages. Autoantibody binding has been related to band3

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283   oxidation[32]. Therefore, we surmise that the increased phagocytosis of mechanically aged RBCs arises from

284   the combination of auto-antibody opsonization and increased cell rigidity and oxidation.

285   Vesiculation allows RBCs to shed membrane fragments containing pro-phagocytic molecules like PS or

286   denaturalized band3[42]. In this regard, vesiculation is believed to protect RBCs until it progressively shuts

287   off with cell age. We tested whether treating RBCs with LatA, a drug that drives F-actin depolymerization,

288   would prolong vesiculation, preserve the cells' deformability, and prevent the aging effects induced by

289   cyclic loading. However, while it initially increased cell deformability and protected the cells from

290   macrophage attacks, LatA treatment also accelerated vesiculation shut off and the adoption of a fragile

291   spherical phenotype. Overall, our results imply that vesiculation introduces a clock in RBC functional

292   decline, winding up with impaired cell deformability, reduced antioxidant protection, decreased gas

293   transport and exchange, and increased macrophage phagocytosis. Notably, we have shown that this clock

294   can be hastened by cyclic mechanical loading.

Materials and Methods

RBC isolation. Human blood from healthy donors was obtained in Heparin treated BD Vacutainer tubes from the San Diego Blood Bank. Upon reception, red blood cells (RBCs) were isolated using a density gradient technique[43]. First, a 15 ml centrifuge tube (Falcon tube, Fisher Scientific) was filled with 3 ml of Histopaque 1119 (Sigma Aldrich); then, a 3 ml layer of Histopaque 1077 (Sigma Aldrich) was added on top and finally 6 ml of whole blood were carefully added to prevent the three layers from mixing. The tube was centrifuged at 700 g for 30 min at room temperature; the top layers containing plasma, mononuclear cells, and granulocytes were discarded and the RBCs were resuspended in PBS (Dulbecco's Phosphate Buffered Saline, Sigma Aldrich). The tube was centrifuged again at 200 g for 10 min at room temperature and the supernatant cells were removed to discard platelets and damaged RBCs. Finally, RBCs were resuspended in AS-3 media[44] (dextrose, sodium chloride, adenine, citric acid, sodium citrate and sodium phosphate were all bought from FisherScientific) in a 5% hematocrit concentration and stored at 4° C until used. All the experiments were conducted within one week of RBC isolation. Separation of denser and lighter RBCs was performed by discontinuous gradient of Histopaque. A layer of RBCs was carefully added on top and centrifuged for 15 minutes at 700 g. Layers were aspirated carefully and only bottom(denser) and top (lighter) cells were kept.

Microfluidic device fabrication. A series of polydimethylsiloxane (PDMS) microfluidic devices with multiple constrictions of customizable geometry were designed and manufactured in the San Diego Nanotechnology Facility of UC San Diego. A two-step photolithography process combining both positive and negative photoresist was used to fabricate the mold of the device, resulting in a two-layer device. The thicker layer, used for the inlet and outlet regions, was made with negative photoresist to allow for minimized flow resistance and RBC deformation outside of the constrictions. The central part of the

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317   device containing the constrictions required higher resolution which was obtained leveraging the

318   thinner layer of positive photoresist.

319   The process started by cleaning a 4-inch silicon wafer (University Wafer, South Boston, MA) in a sonic bath

320   of acetone for 5 min; then it was rinsed in methanol, isopropanol and finally water. Subsequently, the

321   wafer was dehydrated at 180° C for 10 min and its surface activated using a plasma treatment (PVA TePla

322   PS100) for 5 min. A coat of MCC Primer 80/20 (Microchemicals, Westborough, MA) was applied to

323   improve the attachment of the photoresist to the wafer. After a short (2 min) bake at 110° C, the first

324   layer of SU8 2050 negative photoresist (Microchemicals, Westborough, MA) was applied by spin coating

325   at 3000 rpm for 30 sec. After 6 min of soft bake at 95°C, the inlet and outlet regions of the device were

326   exposed using a MLA150 (Heidelberg Instruments Mikrotechnik GmbH, Germany) under a laser with 375

327   nm wavelength and a dose of 3000 mJ/cm^2. The post exposure bake was carried at 95° C for 5 min and

328   then the wafer was developed using SU8 developer (Microchemicals, Westborough, MA). The second

329   part of the process started by applying again a coat of MCC Primer and then spin coating AZ12XT-20PL-05

330   (Microchemicals, Westborough, MA) for 30 sec at 800 rpm. After soft baking the sample for 2 min at 110°

331   C, the whole device, including inlet/outlet and central region was exposed using a dose of 200 mJ/cm^2.

332   Afterwards, the post exposure bake was carried on for 1 min at 90° C and the wafer was developed using

333   AZ300 MIF (Microchemicals, Westborough, MA). The heights of the channel were verified using a Dektak

334   150 surface profilometer (Veeco Instruments Inc. Plainview, NY) and the wafer was passivated with

335   tridecafluoro-1,1,2,2-tetra-hydrooctyl-1–trichlorosilane e (Gelest, Morrisville, PA) for 15 min inside a

336   vacuum chamber to prevent PDMS adhesion to the wafer.

337   PDMS replicas of the device were made by casting a previously degassed mixture of the PDMS oligomer

338   and crosslinking agent (Sylgard®184, Dow Corning Inc, Midland, MI) in a 10:1 (w/w) proportion on the

339   passivated silicon wafer. The sample was then cured at 65° C overnight. The next day the master was

340   peeled off from the wafer, cut into several single devices and the inlet and outlet holes were punched (2.5

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341   mm) with a biopsy puncher (Miltex, Integra Lifesciences, Plainsboro Township, NJ). Finally, we activated

342   the surface of both coverslip (Corning, 24x60 mm and thickness 1.5 mm) and PDMS chip under a UV ozone

343   lamp (Model 30, Jelight Co. Irvine, CA) for 4 min with an oxygen inflow of 0.2 l/min and bonded them

344   together at 65° C for a minimum of 4 h before they were ready to be used.

345   **RBC morphological determinations**. To calculate the volume and the surface of RBCs we used a method

346   based on Fluorescence exclusion as previously described[45, 46]. Briefly, RBCs were suspended at a

347   concentration of 10^4 cells/ml in the AS-3 media supplemented with 1 mg/ml of FITC-Dextran (Sigma-

348   Aldrich). The solution containing the cells was introduced into one of the microfluidic devices described

349   previously and imaged using a Leica DMI 6000B inverted phase contrast microscope (Leica Camera,

350   Wetzlar, Germany). Chambers' heights [4.5-5 µm] were rigorously characterized using profilometer

351   (Dektak 150 Surface Profiler, Veeco). In the fluorescent field, RBCs and PDMS pillars appeared dark

352   whereas the rest of the chamber, where the Dextran was present, resulted in brighter regions. Image

353   analysis was performed using a in house MATLAB (The Math Works Inc., Natick, MA, USA) scripts. For each

354   image, a calibration using PDMS pillars was used to obtain the relationship between fluorescence intensity

355   and chamber height as described previously[45]. The linear relationship between the height of the object

356   and the intensity is described by: $IB = \alpha \cdot h + IP$, where IB and IP are the background and PDMS pillar

357   fluorescence respectively and h the height of the chamber. To measure single cell volume, a region of

358   interest was automatically defined around each cell where the background was calculated; then α was

359   obtained and finally the heights of the pixels conforming the cell were obtained. To calculate the surface,

360   an equatorial symmetry for the cells was assumed, and an enclosed area was generated using half of the

361   pixel heights. The cell was considered valid if the difference between the volume computed from the

362   enclosed area and the volume calculated from the pixel height was smaller than a threshold (10%).

363   Echinocytes were discarded from surface calculation due to the limitations of the equatorial symmetry

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364   assumption. Our *V* and *S* measurements produced results in agreement with previously obtained data by

365   other techniques[47, 48] (Suppl Fig. 2A-B).

366   **Determination of RBCs 'effective' bending modulus and membrane tension**. To measure the

367   mechanical properties of RBCs we used thermal flickering spectrometry as described in [23]. Using this

368   technique we captured the contributions from both the lipid bilayer and the cytoskeleton. RBCs were

369   diluted into culture medium at 10^4 cells/ml and seeded into a thin coverslip (Corning). Leica DMI 6000B

370   inverted phase contrast microscope (Leica Camera, Wetzlar,Germany) controlled by a dedicated

371   workstation connected to a Zyla3-Tap Enclosed C-mount 16 bit camera (Andor Technology, Belfast, UK)

372   was used for high speed (300 fps) image acquisition. Imaging was performed in a controlled

373   environment at 37 °C by using the setup described above. A MATLAB (The Math Works Inc., Natick, MA,

374   USA) code was written to analyze the images and obtain the mechanical properties of the cells. First, the

375   RBC contour at the cell equator was detected for each frame using the method described in [49]. Then, the

376   power spectrum of mean square mode amplitudes was obtained by applying the Fourier transform and,

377   from these data, the 'effective' bending modulus (κ) and tension (σ) were fitted using the following

378   equation:

379    $$\langle |h(q_x, y = 0)|^2 \rangle = \frac{1}{P} \frac{k_B T}{2\sigma} \left(\frac{1}{q_x} - \frac{1}{\sqrt{\frac{\sigma}{\kappa} + q_x^2}} \right),$$

380   where k_B is the Boltzmann constant, *T* is the absolute temperature, and *P* is the mean perimeter of the

381   RBC contour. This equation was derived from the energy of deforming a flat sheet[50] and describes shape

382   fluctuations of the cell's equator in only a limited range of modes. On one hand, smaller modes are

383   excluded because they are affected by the geometry of the surface and an important deviation from the

384   spherical harmonics expression. On the other hand, high modes, with smaller wavelengths, are affected

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385   by noise and their fluctuations lie outside the spatial and temporal resolution of the experiment. For this

386   reason, we use modes 6 to 18 for fitting the equation above.

387   **Flow Cytometry**. Flow Cytometry was used to measure the vesicles released by RBCs. Briefly, after

388   finishing every experiment, RBC samples were resuspended at 10^6 cells/ml in PBS and analyzed using an

389   Accury C6 (BD Biosciences, USA). Blank samples, containing only PBS, were used to set the forward and

390   side scattering thresholds to limit noise measurements, that would reflect in an incorrect reading in the

391   number of vesicles. Manual gating for the RBCs and samples was performed using a user made MATLAB

392   (The Math Works Inc., Natick, MA, USA) code.

393   Phosphatildylserine exposure on the outer leaflet of RBCs was measured using the Annexin V Alexa Fluor™

394   kit (V13241, ThermoFIsher). RBCs were resuspended at a concentration of 10^6 cells/ml in the Annexin V

395   binding buffer. Then, 5 µL of Alexa Fluor® 488 annexin V and 1 µL of Propidium Iodide were added into

396   100 µL of cell suspension and incubated for 30 min. Finally, cells were centrifuged and resuspended in the

397   binding buffer for analysis using the flow cytometer. Negative controls were used to set up the threshold

398   for the detection of positive cells.

399   To measure the expression levels of CD47 we incubated the cells with anti-CD47 (Clone B6H12,

400   BDbiosciences) and 2% BSA for 1 h at room temperature. We then washed thrice in PBS and incubated

401   the cells with the secondary antibody (Goat anti-Mouse IgG Alexa Fluor 488, ThermoFisher). Shortly after,

402   the samples were analyzed using the flow cytometer. Appropriate negative controls were prepared to

403   differentiate positive cells.

404   To measure the percentage of opsonized RBCs we first treated cells with 2% BSA for 1 h, washed with PBS

405   multiple times and incubated with plasma from the same blood sample for 1 h. Finally, RBCs were

406   incubated with anti-Human IgG Alexa Fluor 488 (Thermo Fisher) for 2 h, fixed with 4% PFA and analyzed

407   using the flow cytometer. Negative controls were used to establish a fluorescent threshold.

408 **Stimulated emission depletion (STED) microscopy.** RBCs samples were bonded to Nº 1.5 glass coverslips

409 treated with 100 ng/ml Poly-L-Lysine (Sigma-Aldrich) for 1 h. Cells were fixed using 4% PFA for 15 min,

410 washed multiple times in PBS and permeabilized with 0.1% Triton-X (Sigma-Aldrich) for 10 min. Non-

411 specific antibody binding was prevented by incubating with 2% BSA (Thermo Fisher) for 2 h. Cells were

412 incubated with the respective primary antibody (rabbit anti human, anti-ANK1 or anti-EPB41, Sigma-

413 Aldrich) overnight. Finally, a secondary antibody (goat anti-rabbit IgG, Thermo Fisher) was added for 1h

414 and samples were fixed using ProLong Diamond (Thermo Fisher) for at least 24 h. Samples were imaged

415 in the Microscope Core Facility at UCSD (NS047101) using a Leica SP8 confocal with STED and lightning

416 deconvolution. Post processing of the images and data analysis was performed with an in-house designed

417 MATLAB script. Briefly, the autocorrelation of the images was obtained and the distance between the two

418 higher peaks was used to calculate the distance in between protein complexes.

419 **Hemolysis**. The content of hemoglobin in the supernatant was used to measure the percentage of RBCs

420 lysis. Hemoglobin concentration was measured analyzing absorbance at 415 nm in a UV-Visible

421 spectrophotometer (Biomate 3s, ThermoFisher). Before starting the different experiments, a sample

422 containing the same concentration of RBCs was used to later calibrate the absolute hemolysis lysing the

423 cells with 1% SDS. After finishing every experiment RBCs were pelleted down by centrifuging at 1000

424 rpm for 2 min (Eppendorf, 5415C) and the supernatant absorbance was assessed and normalized using

425 the maximum hemolysis value.

426 **RBC oxidation**. RBCs oxidation was assessed by measuring the intracellular content of Methemoglobin

427 (MetHb) as previously described[51]. RBCs obtained from the different samples were washed multiple

428 times with PBS to remove any extracellular hemoglobin from lysed cells. Then, RBCs were resuspended

429 in PBS at a final concentration of 10^7 cells/ml, lysed with 1% SDS and the absorbance at 645 nm was

430 measured using a UV-Visible spectrophotometer (Biomate 3s, ThermoFisher). The solution was then

431 treated with potassium hexacyanoferrate ($K_3[Fe(CN)_6]$, ThermoFisher) to completely oxidized Hb into

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432   MetHb and the absorbance was measured again. Finally, the percentage of MetHb was obtained by

433   normalizing the two measurements at 645 nm.

434   **ATP concentration.** The intracellular ATP concentration was measured using the Luciferin technique

435   (ATP Determination Kit; ThermoFisher). Briefly, 10^5 cells were extracted from each sample and washed

436   with PBS multiple times to remove any cell remaining in the supernatant. The standard reaction solution

437   containing luciferase and luciferin was prepared according to the manufacturer guidelines and

438   introduced in a black with clear bottom 96-well plate (Corning). To prevent any possible ATP

439   degradation, RBCs samples were lysed with DI water and quickly added to each well. When ATP reacted

440   with the standard solution, light was emitted with a wavelength of 560 nm and captured with a

441   luminometer (Tecan infinite M1000 Pro). A standard reference curve was made from different ATP

442   concentration from an ATP vial provided with the kit. This curve was used to measure the ATP

443   concentration for each sample. Finally, to obtain the intracellular ATP concentration, the average

444   measured concentration was normalized by the total number of cells lysed and the mean volume

445   obtained in previous experiments. ATP measured concentrations were similar to previous studies[52].

446   **RBCs recirculation and crossing time determination.** Flow was driven through the device at a fix flow

447   rate (100µl/h) using a peristaltic pump (IPC, ISMATEC) (Suppl Fig 1F); wider channels (5 µm) were added

448   at both sides of the microchannel array to ensure that microchannel clogging by stuck RBCs did not

449   significantly affect the pressure difference across the array. The maximum number of constrictions

450   crossed by the RBCs was estimated based on the flowrate and the volume of fluid inside the circuit.

451   Therefore, *Nc* represents the maximum number of constrictions that RBCs could have undergone for a

452   period of time. RBCs were recirculated at room temperature.

453   To measure the crossing time RBCs were washed multiple times in PBS and resuspended at 1% HC in AS-

454   3. The device was left running for 15 min before the image acquisition started to allow the flow to

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455 stabilize. The passage of RBCs through misconstructions was imaged using a DMI 6000B inverted phase

456 contrast microscope (Leica Camera, Wetzlar, Germany) equipped with ahigh frame rate (100 fps) camera

457 (Zyla3-Tap Enclosed C-mount 16 bit, Andor Technology, Belfast, UK). The crossing time was manually

458 calculated as the difference between the first and last frame where the RBCs contacted the constriction.

459 **Proteomics analysis**. Control RBCs and cells transited through 200 constrictions were collected and

460 washed multiple times with PBS. Then, cells were lysed with DI water and centrifuged at 14000 g for 10

461 min to collect the cell membranes. The pellet was resuspended in DI water and washed multiple times to

462 remove as much Hemoglobin as possible. The total protein concentration was measured using a Micro

463 BCA Protein Assay Kit (Thermo Fisher) and resuspended at a final concentration of 120 µg/ml for all

464 samples. Samples were submitted to the Biomolecular and Proteomics Mass Spectrometry Facility

465 (BPMSF, S10 OD016234) at UCSD and analyzed using a label free quantification. The Benjamini-Hochberg

466 correction method[53] was used to adjust the p-values. Protein set enrichment analysis was performed using

467 Metascape on the KEGG, Canonical pathways, Gene Ontology, Reactome, and CORUM databases[25].

468 **Phagocytosis assay.** Macrophages were differentiated from THP-1 monocytic cells based on[54]. Briefly,

469 $5x10^5$ THP-1 cells/ml were treated with 25 ng/ml phorbol myristate acetate (PMA, ThermoFisher) in

470 RPMI 1640 with 10% FBS. After 72 h, the media was renewed and a rest period of 72 h was performed

471 before cytokine activation. To polarize macrophages into M1 phenotype, cells were treated with 50

472 ng/ml of IFNγ (eBiosciences) and 200 ng/ml of LPS (eBiosciences) for 72 h. At this point cells were

473 already activated and ready to interact with RBCs.

474 RBCs from control or cells transited through 200 constrictions were collected washed three times with

475 PBS and resuspended at a concentration of 10^6 cells/ml. Following, RBCs were stained with CellTracker

476 Deep Red CMFDA Dye (Invitrogen) for 30 min at 37ºC and washed multiples times with PBS. Finally,

477 RBCs were opsonized with plasma from the same blood samples for 30 min, cocultured with

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478   macrophages in RPMI +10%FBS for 48 h and fixed afterwards for imaging using 4% paraformaldehyde

479   (PFA, ThermoFisher). Cells were imaged using a DMI 6000B inverted phase contrast microscope (Leica

480   Camera, Wetzlar, Germany) and active macrophages were assessed by measuring its fluorescence. To

481   measure the mean fluorescence of each macrophage, cells were manually gated and the mean intensity

482   of inner pixels was calculated.

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498   **Figure legends**

499   ***Figure 1. Design of a microfluidic device to replicate the passage of RBCs through splenic inter***

500   ***endothelial slits.*** **A,** Scheme of the key steps in the fabrication process. See details in the Materials and

501   Methods section. **B,** Actual device next to 1-cent coin for scale. **C,** Scanning electron microscopy image

502   showing a close-up of the micro-constriction array. **D-E,** Time-lapse sequences of RBC passage reveal two

503   characteristic types of motion: folding (D) and twisting (E). See Suppl. Videos 1 and 2 for a high-time-

504   resolution depiction of these two motions. Bars correspond to 15 μm (C) and 5 μm (D and E). Type of RBCs

505   motion was measured as a function of slit width (**F**) or length (**G**). RBCs crossing times were measured

506   with respect to slit width (**H**) and length (**I**) and compared with in vivo data previously calculated by other

507   authors[20]. Each cell is represented as a circle; error bars represent median and interquartile range.

508   Statistically significant differences, as determined using an unpaired t test with Welch's correction, are

509   indicated (*, p< 0.05; **, p<0.01, ***, p< 0.001).

510

511   ***Figure 2. Successive microchannel crossing promotes RBC aging.*** **A,** Crossing time was measured as

512   indicated in methods in RBCs that had previously gone through 0, 15, or 75 microchannels (0.85μm width

513   and 3μm length) by recirculating in the microfluidic circuit. Each cell is represented as a triangle or circle

514   depending on whether it underwent folding or twisting, respectively. The control corresponds to cells that

515   have gone to the indicated number of previous passages through a sham microfluidic device containing

516   no constrictions.   **B,** Percentage of cells displaying folding vs. twisting motion after previous passage

517   through 0, 15, or 75 microchannels. **C,** Percentage of RBCs that failed to pass through a micro-constriction

518   vs. number of previous microchannels they have passed through (—●—,dots, continuous line) and vs.

519   number of previous passages through a mock microfluidic device containing no constrictions (--■--,

520   squares, discontinuous line). Data was modeled using the Hill dose-response equation [55, 56]; **D,** percentage

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521   of RBC lysis vs. number of previous microchannels RBCs have passed through (—●—,dots, continuous line)

522   and vs. number of previous passages through a mock microfluidic device containing no constrictions (--■--

523   squares, discontinuous line). Data was modeled using a double exponential decay equation. **E,** RBC

524   oxidation vs. number of constrictions the cells have previously passed. **F,** Intracellular ATP concentration

525   vs. number of previous microchannels and vs. number of previous passages through a mock microfluidic

526   device containing no constrictions. Data was modeled using the Hill dose-response equation. In panels A-

527   D, the data come from at least 20 determinations of 3 independent experiments, in E and F, from the

528   evaluation of 5 independent experiments. All datapoints are represented as mean ± SD. In all the graphs

529   the blue dot (●) represents the point with the 50% change. Statistically significant differences, as

530   determined using an unpaired t test with Welch's correction, are indicated (*, p< 0.05; **, p< 0.01; ***,

531   p< 0.001).

532

533   ***Figure 3. Microchannel crossings result in RBC volume loss associated with vesiculation.*** **A** and **B,** Two

534   subpopulations of RBCs were separated based on density and circulated multiple times through our

535   microfluidic circuit (constrictions' dimensions: 0.85µm width and 3µm length). Cell volume (A) and

536   sphericity index (B) were plotted vs. number of microchannels previously passed by RBCs of low-density

537   (grey dots, —●—) or high-density (black squares, —■—) and compared with RBCs recirculated for an

538   equivalent time through a mock microfluidic device containing no constrictions (control, discontinuous

539   line; grey diamonds, --◆--, low-density cells; black diamonds, --◆--, high-density cells). Data was modeled

540   using the Hill dose-response equation. The data are represented as mean and 95% confidence interval of

541   at least 20 determinations of 3 independent experiments. Statistically significant differences, as

542   determined using an unpaired t test with Welch's correction, are indicated (*, p< 0.05; **, p<0.01; ***,

543   p< 0.001). **C,** Scatter plot of micro-constriction crossing time vs. sphericity index where each point

544   represents a single cell. **D-E,** 'effective' bending elastic modulus (D) and cortical tension (E) were assessed

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545   in at least 20 RBCs after they passed through the indicated number of constrictions. Each point represents

546   a cell and the error bars indicate mean ± SD (**, p<0.01; Wilcoxon test). **F,** Vesicle release was determined

547   by flow cytometry and represented as fold change relative to static controls. Dotted line corresponds to

548   cells were recirculated for an equivalent time without constrictions. The means ± SEM of 5 independent

549   experiments are shown. Data was modeled using the Hill dose-response equation. In all graphs the blue

550   dot (●) represents the point with the 50% change.

551

552   *Figure 4.* **Repetitive microchannel crossings modify RBC protein composition.** RBCs were subjected to

553   200 microchannel crossings (0.85µm width and 3µm length), membranes were isolated, and proteins

554   were extracted and analyzed by mass spectrometry. The differences in protein content between *Nc=200*

555   RBCs and static controls are presented. Values correspond to mean of three independent analysis. **A,** a

556   list of proteins significantly modified (log fold change < -2 or > 2 and a P-value >0.05) and proteins

557   important in membrane cytoskeleton connectivity were classified by its main function. Proteins were

558   classified based on its function, gas transport (blue), metabolism (yellow), structural (orange) and

559   protection from oxidation (green). B, Volcano plot of all the proteins measured. Statistically significant

560   changes were obtained by performing the Benjamini-Hochberg correction method. **C,** Protein clustering

561   analysis revealed that deminished proteins were mostly localized in two clusters: oxidation, gas transport

562   and metabolism. Statistical differences in **C** are indicated as [Log_{10} p-value].

563

564   *Figure 5.* **Passage through micro-constrictions increases the density of cytoskeleton-membrane**

565   **anchoring complexes. (A),** A scheme explaining how vesiculation and membrane loss decreases the

566   distance between the anchoring complexes that connect the membrane and the cytoskeleton . **(B, C)** STED

567   microscopy was used to assess the distance between proteins that connect the membrane and the

568 cytoskeleton (Ankyrin-1 and Band4.1) as indicated in Materials and Methods in static vs RBCs that crossed

569 200 microchannels (0.85µm width and 3µm length). In B, a representative image of a RBC labeled with an

570 anti-Band4.1 antibody (scale bare 1 µm). The auto correlation matrix used to calculate the distance

571 between two proteins is showed at the bottom. Red dashed line used to emphasize the hexagonal

572 structure of the protein network observed in the autocorrelation image. Circles in C represent single cell

573 measurements and bars indicate mean ± SD of at least 25 determinations. Statistically significant

574 differences were measured using an unpaired t test with Welch's correction and are indicated (*, p<0.05).

575

576 *Figure 6.* **Cytoskeleton targeting drugs increase RBC deformability and demonstrate a relationship**

577 **between vesiculation and cytoskeletal integrity. A,** Microchannel (0.85µm width and 3µm length)

578 crossing time in RBCs treated with Y27632, Blebbistatin, Cytochalasin D or Latrunculin A (in all cases 2 µM

579 for 30 min), and control vehicle-treated cells. Circles represent single cell measurements and bars indicate

580 mean ± SD of at least 25 determinations in 3 independent experiments. Statistically significant differences,

581 as determined using an unpaired t test with Welch's correction, are indicated (*, p< 0.05; ***, p< 0.001).

582 **B,** 'Effective' bending elastic modulus of the cells treated in the same conditions as panel A or with 20 µM

583 LatA. Circles represent single cell measurements and bars indicate mean ± SD of at least 10 determinations

584 of 3 independent experiments. Statistically significant differences, as determined using a paired Wilcoxon

585 test, are indicated (*, p< 0.05; **, p<0.01; ***, p< 0.001). **C,** Vesiculation caused by passing through a

586 single micro-constriction by RBC treated with different LatA concentrations. Bars indicate mean ± SD of 4

587 independent experiments, p values were calculated using an unpaired t test with Welch's correction and

588 are indicated as (*, p< 0.05). **D,** Normalized cell volume vs. number of microchannels previously passed

589 by RBCs after different doses of LatA treatment. Bars indicate mean ± SD of at least 20 determinations in

590 3 independent experiments. **E,** Micro-constriction crossing time after previous passage through 0, 15, or

591 75 microchannels in RBCs treated with 2 µM Latrunculin A and vehicle-control-treated RBCs. Circles

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592   represent single cell measurements and bars indicate mean ± SD of at least 20 determinations in 3

593   independent experiments. **F,** Hemolysis vs. number of microchannels previously passed by RBCs treated

594   with different doses of LatA. Bars indicate mean ± SD of 6 independent experiments. For **D-F** statistically

595   significant differences were measured between control and 2 µM LatA using an unpaired t test with

596   Welch's correction and are indicated (*, p< 0.05; **, p<0.01; ***, p< 0.001).

597

598   *Figure 7*. **RBC passage through microchannels facilitates macrophage attack. A,** THP-1 derived

599   macrophages (blue arrow) were cultured with fluorescently-labeled RBCs (red arrow); an example of a

600   macrophage that have incorporated fluorescence is shown (yellow arrow). **B,** Percentage of labelled

601   macrophages detected after co-culture with fluorescent RBCs either static, after 200 microchannel

602   crossings (0.85µm width and 3µm length) or treated with H_2O_2 (0.3 mM) or LatA (2 µM). **C,** Average

603   fluorescence intensity of macrophages cultured with labelled RBCs treated as in panel B. In B and C, the

604   bars represent mean ± SD of 4 independent experiments (*, p<0.05, using an unpaired t test with Welch's

605   correction). **D,** Exposure of phosphatidylserine (PS) on the outer plasma membrane of RBCs as

606   determined by flow cytometry. A statistically significant difference was found between *Nc = 0* and *Nc =*

607   *480* as determined using an unpaired t test with Welch's correction, and is indicated by (***, p< 0.001).

608   Note also that the Y-axis only reaches 2%. **E,** Expression of transmembrane protein CD47 as determined

609   by flow cytometry. No statistically significant differences were found as determined using an unpaired t

610   test with Welch's correction. **F,** The percentage of opsonized RBCs, static vs *Nc= 200*, that bound

611   autoantibodies was assessed by flow cytometry. A statistically significant difference was found between

612   *Nc = 0* and *Nc = 200* as determined using an unpaired t test with Welch's correction, and is indicated by

613   (**, p< 0.01). Means ± SD of 5 independent experiments are shown in **D**-**F.**

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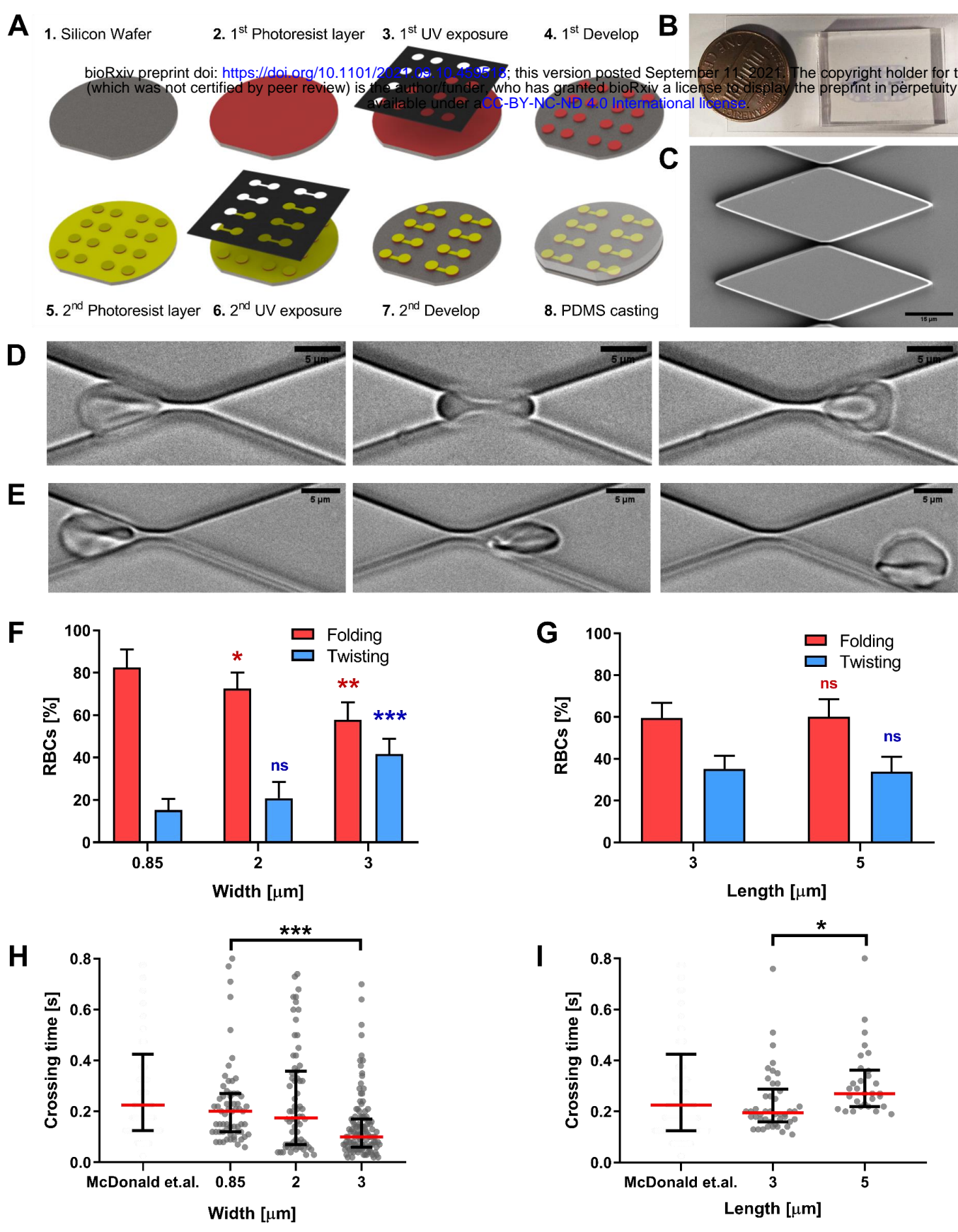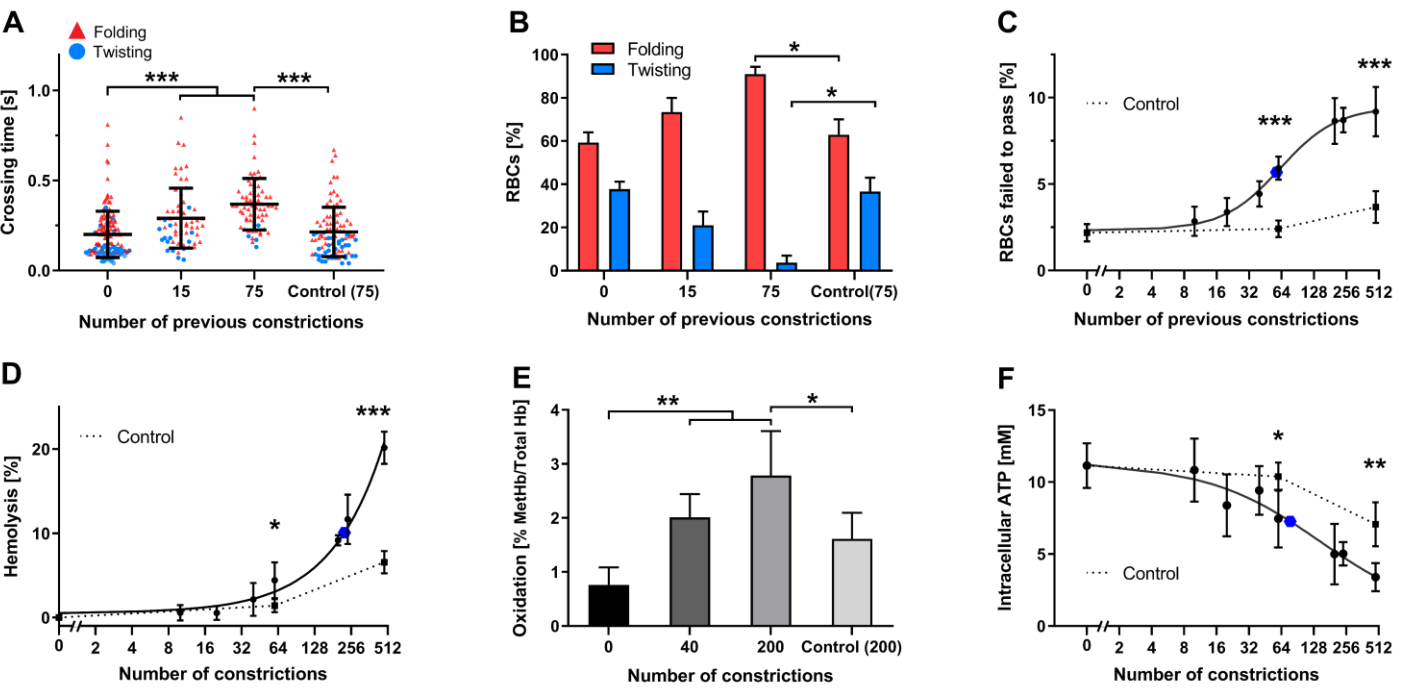735   **235**, E97-102 (1978).
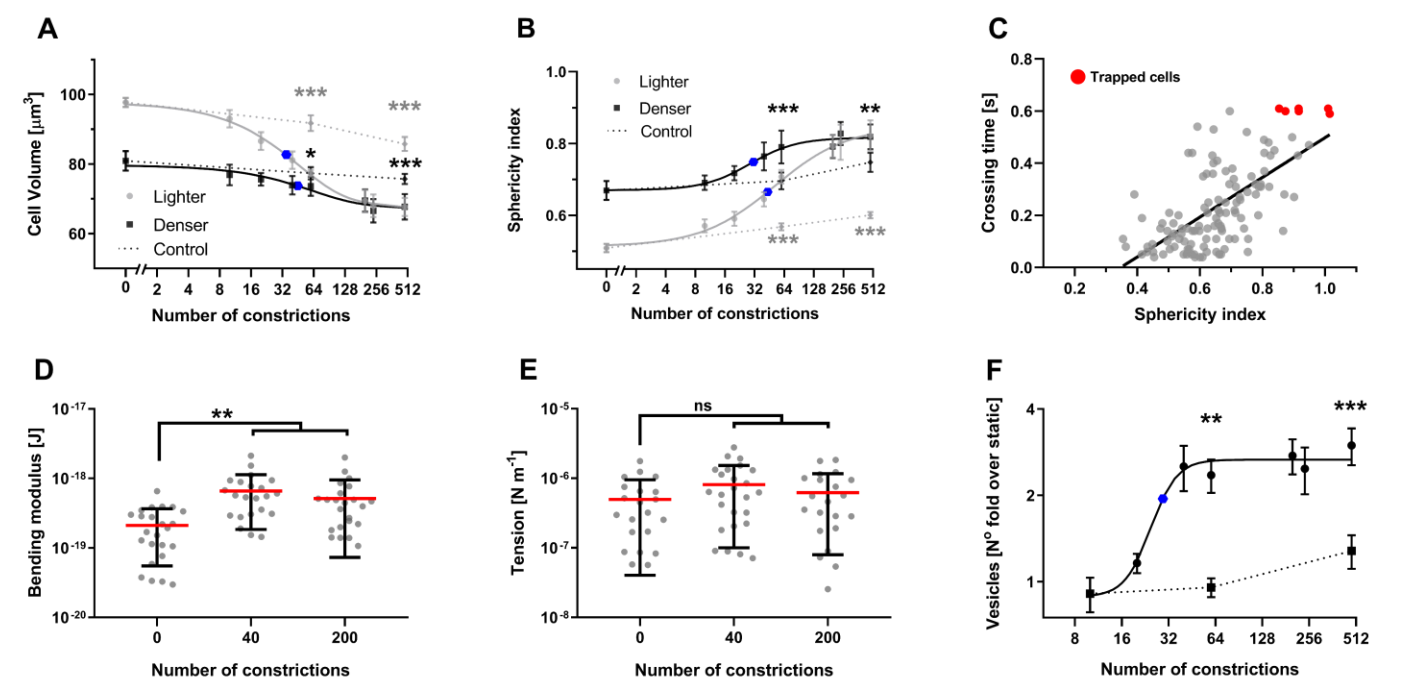
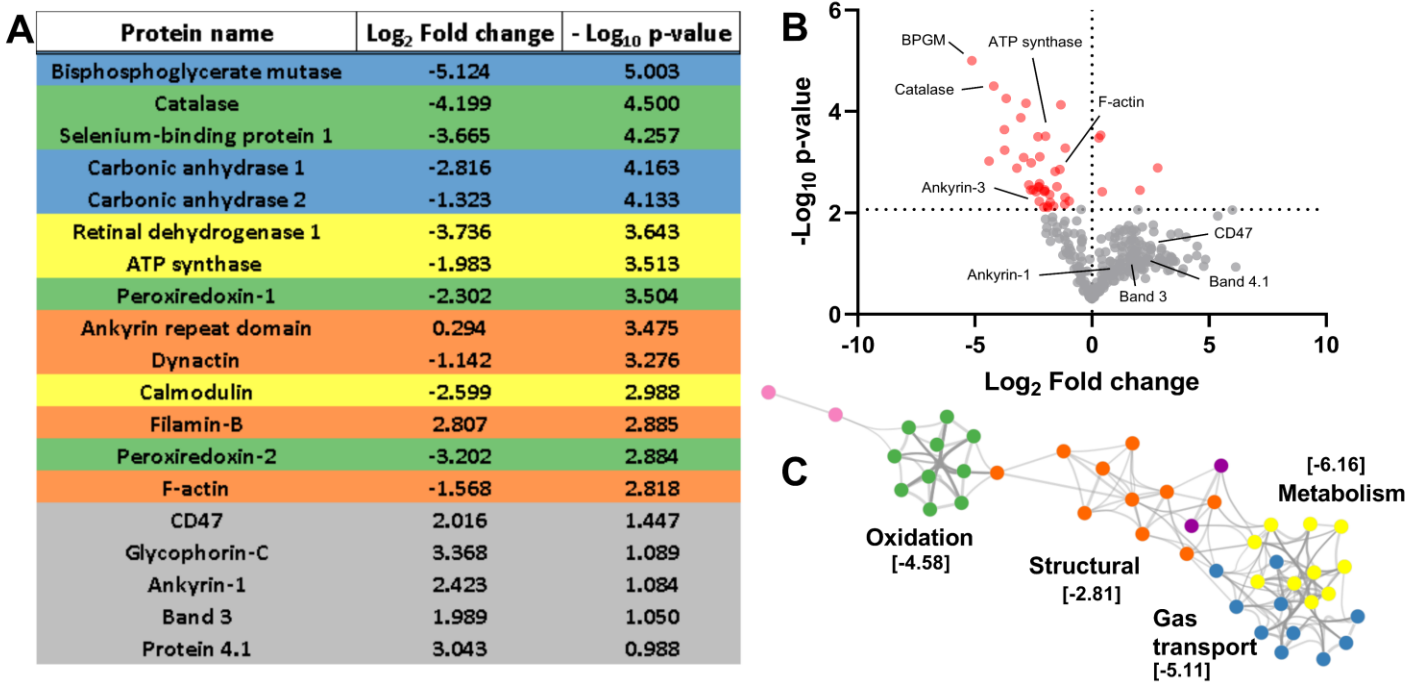# Figure 1

Figure 2

Figure 3

Figure 4

A

Protein name	Log$_2$ Fold change	- Log$_{10}$ p-value
Bisphosphoglycerate mutase	-5.124	5.003
Catalase	-4.199	4.500
Selenium-binding protein 1	-3.665	4.257
Carbonic anhydrase 1	-2.816	4.163
Carbonic anhydrase 2	-1.323	4.133
Retinal dehydrogenase 1	-3.736	3.643
ATP synthase	-1.983	3.513
Peroxiredoxin-1	-2.302	3.504
Ankyrin repeat domain	0.294	3.475
Dynactin	-1.142	3.276
Calmodulin	-2.599	2.988
Filamin-B	2.807	2.885
Peroxiredoxin-2	-3.202	2.884
F-actin	-1.568	2.818
CD47	2.016	1.447
Glycophorin-C	3.368	1.089
Ankyrin-1	2.423	1.084
Band 3	1.989	1.050
Protein 4.1	3.043	0.988

Figure 5

Figure 6

Figure 7