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Letter

Molecular Force Imaging Reveals That Integrin-Dependent Mechanical Checkpoint Regulates Fcγ-Receptor-Mediated Phagocytosis in Macrophages

Yuru Hu, Hongyun Li, Wenxu Wang, Feng Sun, Chaoyang Wu, Wei Chen,* and Zheng Liu*

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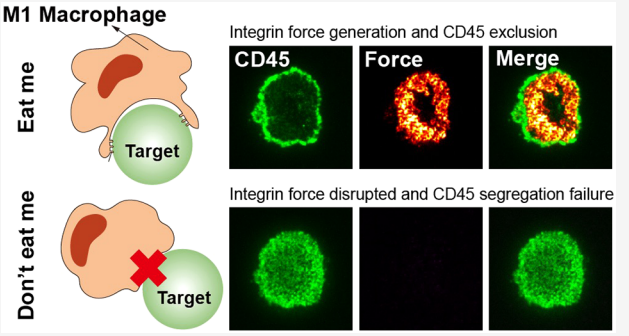
ABSTRACT: Macrophages are a type of immune cell that helps eliminate pathogens and diseased cells. Recent research has shown that macrophages can sense mechanical cues from potential targets to perform effective phagocytosis, but the mechanisms behind it remain unclear. In this study, we used DNA-based tension probes to study the role of integrin-mediated forces in FcγR-mediated phagocytosis. The results showed that when the phagocytic receptor FcγR is activated, the force-bearing integrins create a "mechanical barrier" that physically excludes the phosphatase CD45 and facilitates phagocytosis. However, if the integrin-mediated forces are physically restricted at lower levels or if the macrophage is on a soft matrix, CD45 exclusion is significantly reduced. Moreover, CD47-SIRPα "don't eat me" signaling can reduce CD45 segregation by inhibiting the mechanical stability of the integrin barrier. These findings demonstrate how macrophages use molecular forces to identify physical properties and combine them with biochemical signals from phagocytic receptors to guide phagocytosis.



KEYWORDS: *macrophage, podosome, integrin force, "don't eat me" signal*

Macrophages are known for the clearance of apoptotic cells, pathogens, and tumor cells by phagocytosis.[1] Recently, it has been found that the phagocytic process is regulated by both mechanical and biochemical cues on the target's surface, as the physical properties of phagocytic targets in vivo are widely different.[2−4] Macrophages must integrate the biochemical and mechanical features of targets to perform rapid phagocytosis.[5,6] One prominent example is that macrophages have a strong preference for stiff objects during phagocytosis.[2,7] To engulf a variety of complex objects, macrophages express a series of phagocytic receptors to participate in target recognition and internalization.[8] The Fcγ receptor (FcγR) is a type of phagocytic receptor that initiates phagocytosis by binding selectively to the Fc region of IgG molecules on the target.[9] Macrophages can create an F-actin-rich phagocytic cup around the target, and a phagocytic cup capable of expanding and closing is required for effective phagocytosis.[3,8,10] Accumulating evidence suggests that podosome-like structures are the primary structure of the sites of F-actin polymerization on phagocytic cups in FcγR-mediated phagocytosis.[11] Interestingly, podosome structures exhibit mechanosensitive properties,[12−15] implying that podosomes may act as primary mechanosensory elements responsible for sensing and responding to mechanical cues of targets for more efficient phagocytosis.

An entire podosome structure is approximately 1−2 μm in size and consists of an actin-rich core surrounded by a ring containing integrin receptors.[16,17] Recently, several approaches have been employed to explore the mechanical properties of podosome structures. For example, Labernadie et al. demonstrated a protrusion force microscope (PFM), which is able to measure the protrusion force of individual podosomes of macrophages, and found that the core of the F-actin bundle in a podosome can generate a periodically oscillating, nN-scale protrusion force on the matrix.[18] Glazier et al. then proposed a molecular tension-fluorescence lifetime imaging method for mapping the receptor force on supported lipid bilayers (SLBs) and demonstrated that each αvβ3 integrin molecule in the ring of the podosome in fibroblasts on fluid substrates can generate a pN-level vertical pulling force that balances the protrusion force generated by the core of the podosome.[19] In addition, Pal et al. recently employed single-molecule force imaging to study podosome mechanics,

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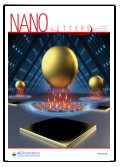

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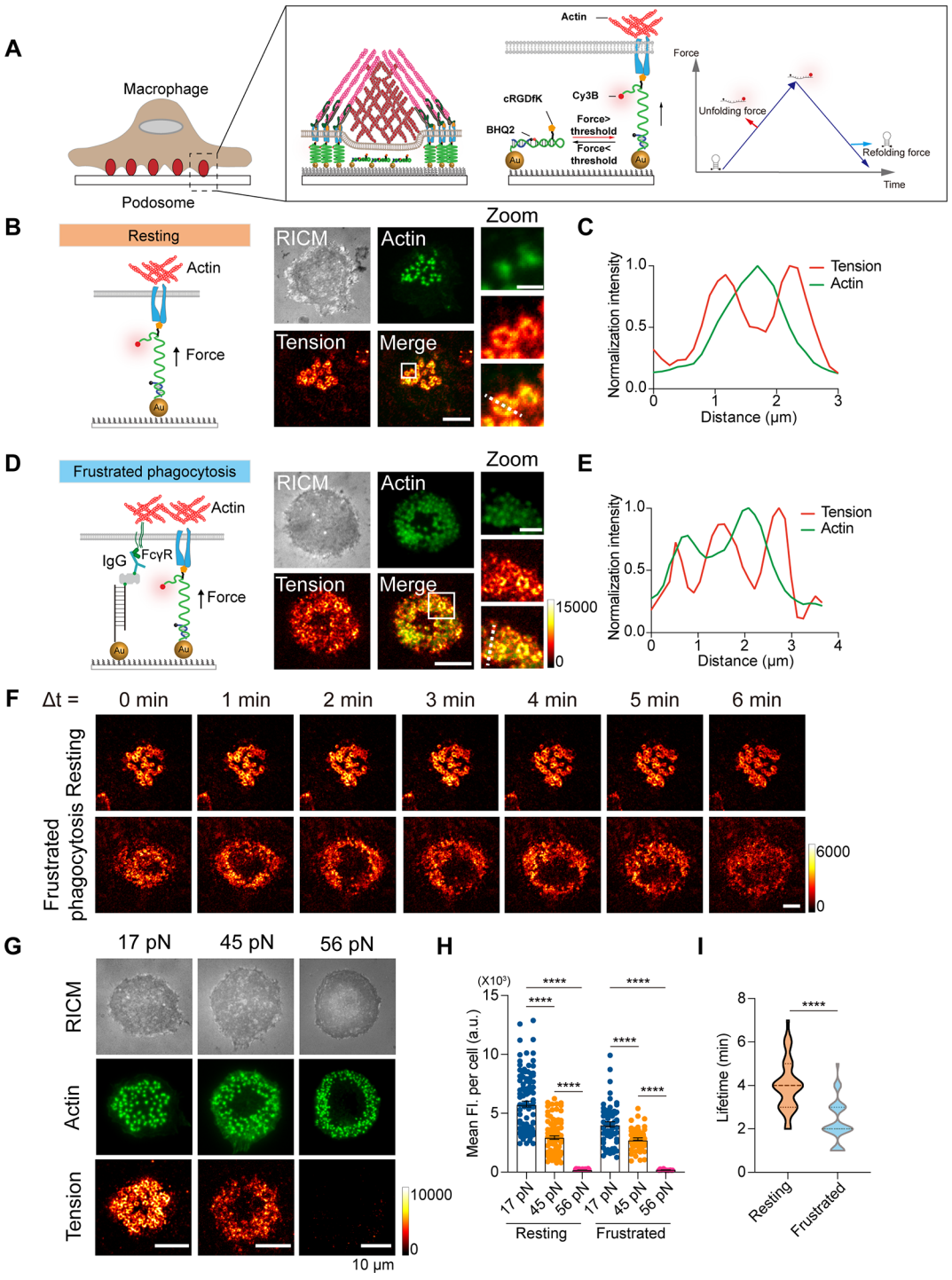

Figure 1. Mapping the integrin-mediated forces of macrophages during FcγR-mediated frustrated phagocytosis using RSDTPs. (A) Schematic of measuring the integrin-ligand tensions of podosomes in macrophages using RSDTPs. RSDTPs convert the integrin tension to fluorescent signals in a digital and reversible manner. (B, D) Left: schematic illustrating induction of resting (B) or frustrated phagocytosis states (D) of macrophages using distinctly functionalized coverslips. Right: representative RICM and TIRF images of F-actin stained macrophages and associated tension signals reported by 17 pN-RSDTPs. (C, E) Intensity profiles of tension signals and actins along marked lines in (B) and (D). (F) Time-lapse images of tension map of macrophages in different states. (G) Representative RICM images of macrophages in a frustrated state and corresponding TIRF images of their actin and tension signals measured by RSDTPs with different unfolding forces. (H) Average intensities of mean tension signals of macrophages in two states (resting and frustrated phagocytosis) measured using different RSDTPs (n, number of cells: for resting, blue = 102, orange = 88, magenta = 20; for frustrated phagocytosis, blue = 62, orange = 70, magenta = 13). Data are presented as mean values ± sd from three independent experiments. (I) Violin plots of macrophage podosome lifetimes in different states from three independent experiments. (n = 39 podosomes for resting state and 71 for frustrated state). Unpaired two-tailed Student's t-tests are used to assess statistical significance (****P < 0.0001). The scale bar is 10 μm (enlargement, 2 μm).

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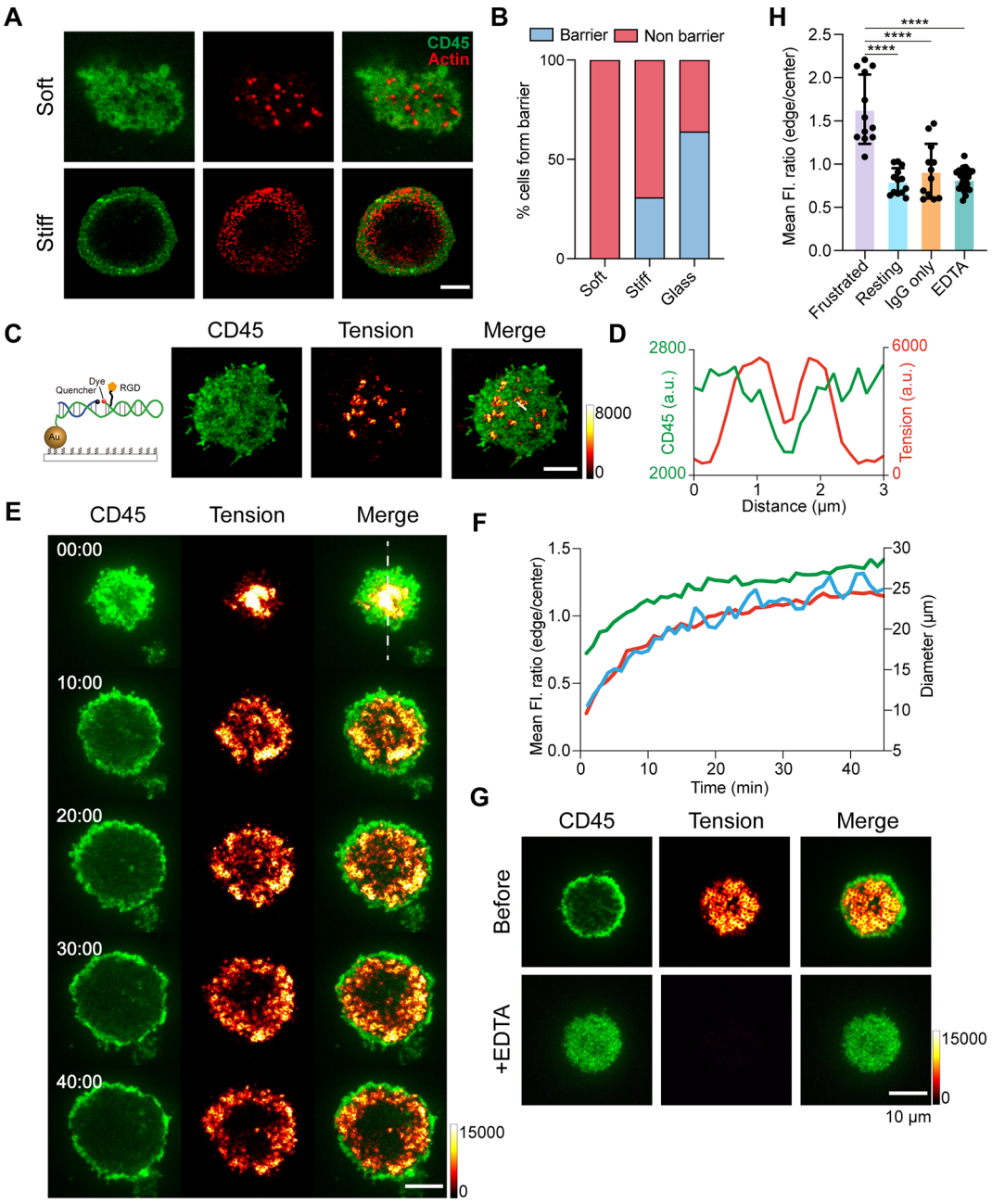

Figure 2. Integrin tension cooperates with the FcγR to induce CD45 efflux. (A) Representative confocal microscopy images of CD45 and F-actin of macrophages on soft (1 kPa) and stiff (80 kPa) hydrogel substrates. (B) Proportional representation of macrophages succeeded or failed to form integrin barriers on different substrates (number of cells that were included from randomly selected at least 10 fields of view: n = 68 for soft hydrogel, 78 for stiff hydrogel, and 61 for glass). (C) Representative TIRF images of CD45 and associated tension signals of integrins reported by 17 pN RSDTP. (D) Intensity profiles of CD45 and integrin signals in (C). (E) Representative time-lapse images of CD45 and tension maps of macrophages during the expansion process after seeding on a coverslip modified with 17 pN RSDTP and IgG. (F) Temporal changes of the diameters of CD45 (the green line) and the tension signal (the magenta line). The spatial distribution of CD45 is measured as the ratio of its mean fluorescence intensity in the periphery to that in the center (the blue line). (G) Representative TIRF images of CD45-FITC and associated tension signals before and after treatment with EDTA. (H) Mean ratio of edge/center CD45 for macrophages placed on different surfaces (n = 12 for Frustrated, 13 for Resting, 12 for IgG only, and 24 for EDTA treatment). Data are mean values ± sd from three independent experiments. Ordinary one-way ANOVA tests were used to assess statistical significance (****P < 0.0001). The scale bar is 10 μm.

demonstrating that integrin-mediated forces are dispensable for podosome formation in macrophages on solid substrates.[20] Although these results reveal that podosome structures are highly structurally and mechanically complex, how the mechanobiology of podosome formation integrates with phagocytic receptors to promote effective phagocytosis in macrophages remains largely unknown.

In this study, we used our lab's recently developed reversible shearing DNA-based tension probes (RSDTPs),[21,22] which combine the technical advantages of reversibility of traditional DNA hairpin probes[23,24] with the wide force range of the tension gauge tether (TGT) approach,[25] to image integrin forces of podosome structures at the macrophage−target interface during frustrated phagocytosis. Briefly, RSDTP is

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designed to activate when the integrin force increases above its unfolding force (critical force) and deactivate when the integrin force drops below its refolding force.[21] Since RSDTP has different unfolding and refolding forces, a low level of force is sufficient to keep the probe in the open state after mechanical force activation.[21] Thus, the critical force value reported by the RSDTP represents the minimum force threshold required to activate the probe quickly rather than an instantaneous force value. In addition to characterizing integrin forces of macrophages, we further investigate how the force-bearing integrins coordinate with the FcγR to facilitate phagocytosis.

MAPPING THE DYNAMICS OF INTEGRIN FORCES DURING FcγR-MEDIATED FRUSTRATED PHAGOCYTOSIS

To investigate how integrin forces are involved in FcγR-mediated macrophage phagocytosis, we first attempted to map and quantify integrin forces in real time while macrophages were in a resting state or in a state of frustrated phagocytosis[11,26] using RSDTPs with different force thresholds (Figure 1A). We prepared two types of coverslips: one was modified only with RSDTP (conjugated by cRGDfK peptide) for resting state experiments, and the other was comodified with immobilized biotinylated IgG molecules and RSDTP for probing integrin forces during frustrated phagocytosis (Figure S1). When macrophages were in a resting state (Figure 1B,C), we observed tiny ring-like tension signals reported by 17 pN RSDTP generated by integrins in podosome structures mainly located in the center region of cells; immunofluorescence data showed that F-actin-rich bundles were packed in the center of the ring, which is similar to previous reports.[19,20] Dual-channel time-lapse TIRF imaging of integrin tension and actin immunofluorescence suggests that integrin ring tension signals and actin signals are generated almost simultaneously (Figure S2).

In contrast, frustrated phagocytosis occurred when IgG and cRGDfK molecules on the coverslips were simultaneously identified by the FcγRs and integrin receptors on the macrophage membrane (Figure 1D,E). Remarkably, more podosomes were produced during frustrated phagocytosis, and while the integrin tension signals were still located on the podosomes, the signals did not form perfect small-ring structures. Moreover, these force-bearing integrins formed a larger ring-like barrier at the edge of macrophages, which has recently been described as a diffusion barrier because it can exclude the transmembrane protein tyrosine phosphatase CD45 from the phagocytic cup.[11,27] Additionally, we observed that the integrin force barrier can dynamically expand and close during frustrated phagocytosis as if the cells were trying to engulf an infinitely large target, which is not found in the resting state (Figure 1F and Movies S1 and S2). Next, we attempted to quantify the magnitudes of integrin tension during frustrated phagocytosis using three different force probes (17 pN, 45 pN, or 56 pN). Approximately 30 min after macrophages were seeded on substrates, strong circular fluorescent signals were only observed on the substrates modified with either 17 pN or 45 pN RSDTPs but not 56 pN RSDTPs (Figure 1G,H). A statistical analysis showed that integrin tensions in resting macrophages followed a distribution similar to that of macrophages during frustrated phagocytosis (Figure 1H) and were consistent with the previously reported tension range of podosomes in fibroblasts seeded on SLBs.[19] In addition, the average lifetime of ring-like tension signals in the resting state was approximately 4 min (Figure 1I), which is consistent with previous reports.[18] In comparison, the lifetime of tension signals in podosome structures during frustrated phagocytosis was approximately 2 min, which is much shorter than that in resting macrophages.

Overall, we show that the integrin tensions of podosomes are less than 56 pN in macrophages and that the magnitude of force distribution is not significantly altered when the FcγR is activated, but the force-bearing integrins in the podosomes reposition into phagocytic cups and form a unique dynamic mechanical barrier.

MECHANICAL−CHEMICAL CROSSTALK BETWEEN Fcγ AND INTEGRIN RECEPTORS DETERMINES THE ESTABLISHMENT OF MECHANICAL BARRIERS

CD45 is a tyrosine phosphatase widely present on the surface of macrophages.[28] Recent findings indicate that physically excluding CD45 from the phagocytic cup is required to initiate FcγR-mediated phagocytosis.[27,29] The fundamental exclusion mechanism of the barriers created by IgG or integrin receptors is considered to be steric exclusion or electrostatic repulsion.[30] Although these receptors may transmit mechanical signals, the role of mechanotransduction in the process of CD45 exclusion and phagocytosis is unknown.

To test whether mechanotransduction of these receptors affects force-bearing integrin barrier formation and drives CD45 exclusion, we labeled CD45 in cells with FITC-modified antibodies and analyzed its distribution in macrophages under various stimulation conditions. We first seeded cells separately on soft and rigid hydrogel substrates, where both surfaces were modified with IgG and RGD molecules (Figure 2A,B). Immunofluorescence results showed that the cells could generate podosomes on a soft hydrogel surface but could not form a phagocytic cup and CD45 was almost uniformly distributed in the cells. In contrast, after analyzing cells from randomly selected at least 10 fields of view, we found approximately 30% of the cells on the rigid hydrogel surface (80 kPa) and 64% of cells on glass could generate a phagocytic cup in podosome structures (Figure 2B and Figure S4C). Furthermore, macrophages activated only by RGD failed to form a phagocytosis cup on either the soft or rigid hydrogel (Figure S4D). These findings imply that the IgG-FcγR signaling pathway can also be modulated by ECM stiffness and then coupled with integrin signaling to induce the formation of a diffusion barrier in the phagocytosis cup.

Simultaneous force measurements and CD45 diffusion imaging showed that CD45 homogeneously diffused on IgG-stimulated cells, while IgG molecules exerted a sparse and dynamic force on 4.2 pN probes (Figure S5A). Furthermore, except for the 1−2 μm region around each podosome structure, CD45 was also fairly homogeneously distributed on the resting macrophage (Figure 2C,D and Figure S5B). We recorded the time−space distribution of CD45 in association with integrin force signals on the interface during frustrated phagocytosis (Figure 2E,F and Movie S3). During the initial spreading of cells, the integrin force signal was localized in the central region of the cell adhesion site and CD45 molecules were also slightly extruded there. Then, the cells spread rapidly on the surface, and we could clearly observe the formation of a large ring-like integrin force barrier at the 10 min time point, which progressively expanded until it stabilized at 40 min. More importantly, signals of CD45 were kept outside the

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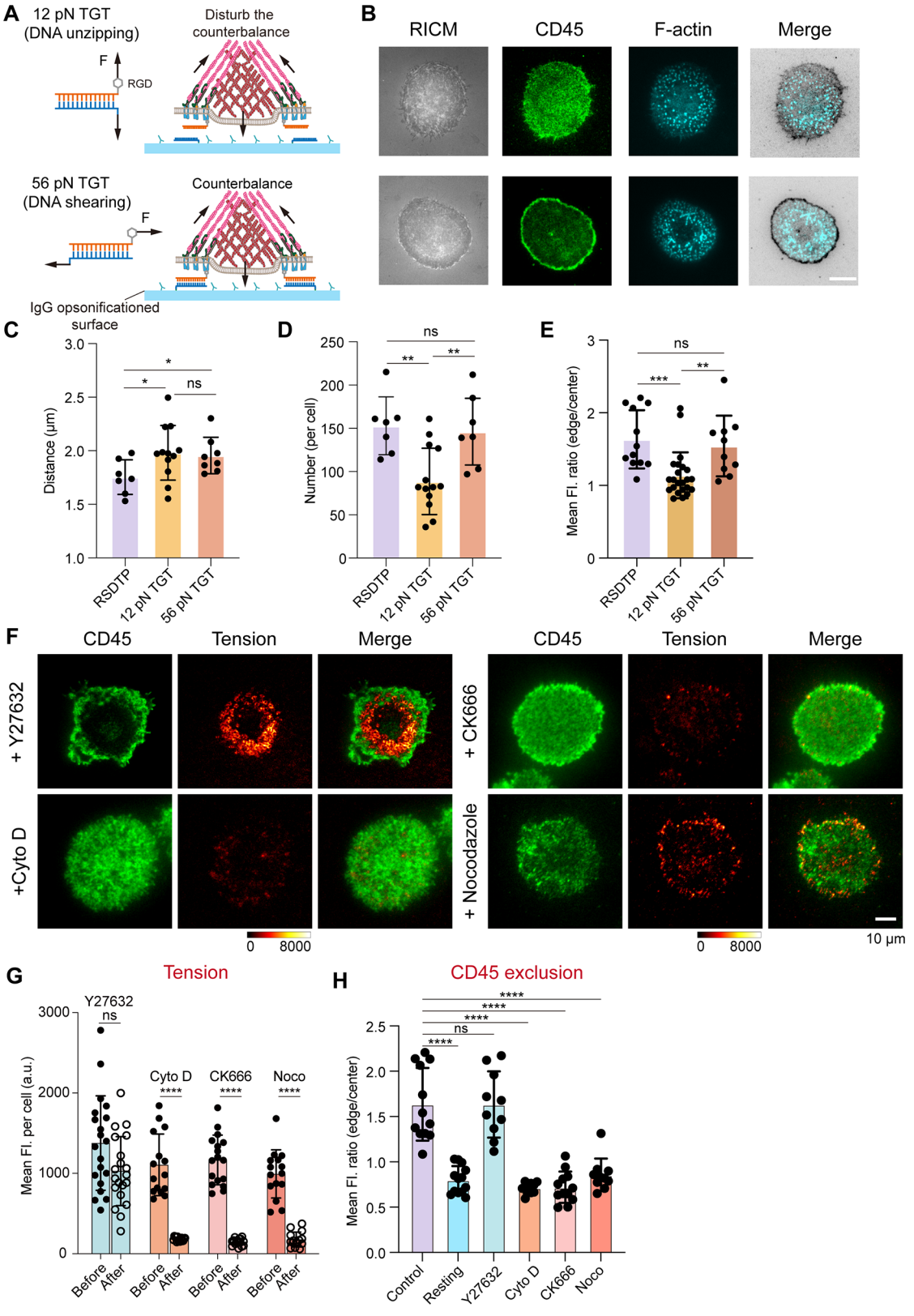

Figure 3. CD45 efflux is regulated by the mechanical properties of the barrier. (A) Schematic showing the different podosome stabilities of macrophages placed on 12 pN TGT (unzipping mode) or 56 pN TGT (shearing mode) modified and IgG opsonized coverslips. (B) Representative RICM and TIRF images of CD45, F-actin, and merged images of frustrated macrophages seeded on different surfaces. (C) Average distance of podosomes to their nearest neighbors per macrophage on different surfaces (n = 7 for RSDTP, 12 for 12 pN TGTs, and 8 for 56 pN TGTs). (D) Total number of podosomes per macrophages on different surfaces (n = 7 for RSDTP, 12 for 12 pN TGTs, and 8 for 56 pN TGTs). (E) Mean ratio of edge/center CD45 for macrophages placed on different surfaces (n = 12 for RSDTP, 23 for 12 pN TGTs and 11 for 56 pN TGTs). (F) Representative TIRF images of CD45 and 17 pN RSDTP signals of macrophages after treatment with Y27632, Cyto D, CK666, or nocodazole. (G) Mean intensities of tension signals per cell before and after drug treatment in (F) (n = 20 for Y27632 treatment, 15 for Cyto D

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Figure 3. continued

treatment, 17 for CK666 treatment, and 15 for nocodazole treatment). (H) Quantification of effects of different drug treatments on the average ratio of mean fluorescence intensity of CD45 in peripheral region to that in central region of macrophages (n = 12 for Control, 13 for Resting, 10 for Y27632 treatment, 10 for Cyto D treatment, 12 for CK666 treatment, and 10 for nocodazole treatment). Data are mean values ± sd from three independent experiments. Statistical comparisons in (C−E) were tested using an unpaired two-tailed Student's t-test and in (G) using a paired, two-tailed Student's t-test. Ordinary one-way ANOVA tests were used to assess statistical significance in (H). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, ns, not significant P > 0.05. The scale bar is 10 μm.

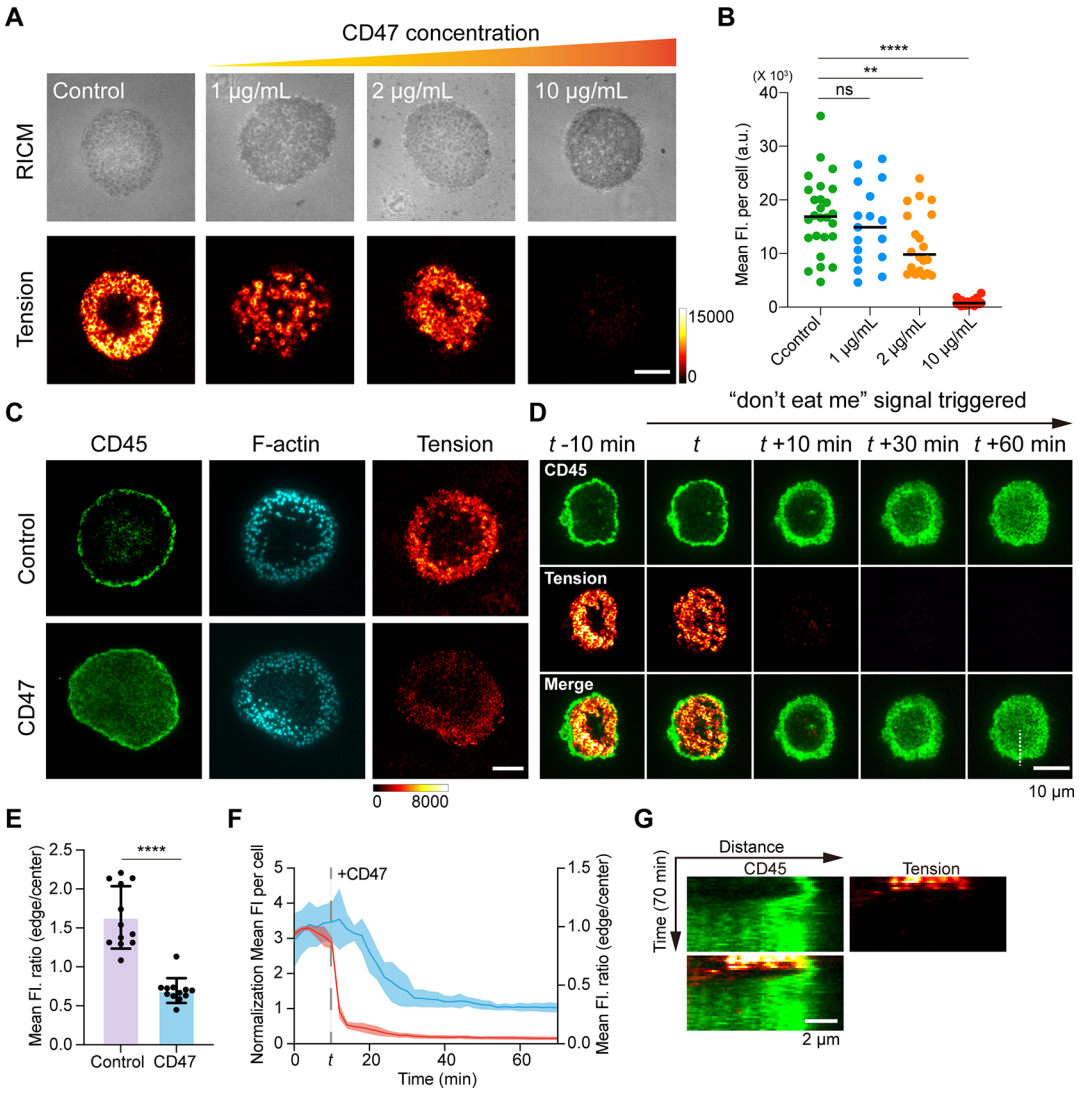

Figure 4. CD47 attenuates the integrin force of the barrier and affects the exclusion of CD45. (A) Representative RICM and 17 pN tension maps of macrophages in frustrated state incubated with 1, 2, or 10 μg/mL or without CD47. (B) Corresponding scatter plots of mean intensities of tension signal in (A) (n = 26 for control, 17 for 1 μg/mL, 20 for 2 μg/mL, and 25 for 10 μg/mL). Data are mean values from three independent experiments. Ordinary one-way ANOVA tests were used to assess statistical significance (****P < 0.0001, **P < 0.01, ns, not significant P > 0.05). (C) Representative TIRF images of CD45, actin, and 17 pN RSDTP signals of control and CD47-treated macrophages. (D) Representative time-lapse images of CD45 and tension maps of macrophages before and after CD47 treatment. (E) Mean ratio of edge/center CD45 of macrophages incubated with or without CD47 (n = 12 for Control and CD47 treatment). Data are mean values ± sd from three independent experiments. Statistical comparisons were tested using an unpaired two-tailed Student's t-test (****P < 0.0001). (F) Plots of the mean normalization force signal fluorescence intensity (left, magenta line) and the mean ratio of edge/center CD45 fluorescence intensity (right, blue line) as functions of time (error bars, sd). Data were obtained from four independent replicates. (G) Kymograph of the macrophage representing the CD45 and tension signal dynamics before and after CD47 treatment. The scale bar is 10 μm (kymograph, 2 μm).

integrin belt throughout the expansion, further confirming the functionality of the dense podosome belts as diffusion barrier. After we added 2.5 mM EDTA to the solution to inhibit integrin activation, the integrin tension was immediately diminished, followed by CD45 flowing back from the cell edge until it was evenly distributed throughout the cells (Figure 2G and Movie S4). Immunofluorescent staining also showed that the actin core of podosomes entirely disappeared after the addition of EDTA (Figure S5C). These results further suggest that the mechanical force of integrin is involved in the

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efflux of CD45, which may be achieved by regulating the diffusion efficiency of the podosomes as well as the podosome-dense barrier (Figure 2H).

THE MECHANICAL PROPERTIES OF INTEGRIN BARRIERS REGULATED CD45 SEGREGATION

Next, to determine the causal relationship between the mechanical properties of the integrin barrier and CD45 segregation, we used TGT probes to limit the integrin force on integrins. TGT probes are irreversible DNA duplexes that rupture once transmitted tension exceeds a defined threshold[25] (Figure 3A and Figure S6), thus disrupting the transmission of mechanical forces and reducing the mechanical stability of integrin−ligand interaction. Since we have demonstrated that the integrin force of most podosomes was less than 56 pN (Figure 1G, H), TGT probes with thresholds of 12 pN and 56 pN were used to induce macrophages to form integrin barriers with different mechanical stabilities. Sixty minutes after seeding macrophages on coverslips comodified with TGT probes and IgG molecules, actin immunofluorescence staining results revealed that a phagocytic cup consisting of podosomes could be generated on surfaces treated with either of the TGT probes (Figure 3B). Furthermore, the mean distance between each podosome in the phagocytic cups did not alter substantially when macrophages were cultured on different TGT probes, and the number of podosomes on low-threshold TGT probes decreased dramatically (Figure 3C,D). Interestingly, we found that CD45 molecules can be well extruded from the phagocytic cup on the 56 pN TGT probes but homogeneously dispersed in the cells on the 12 pN TGT probe-treated surface (Figure 3B,E). This result clearly demonstrates that reducing the mechanical stability of the integrin barrier leads to the failure of CD45 segregation.

Next, we attempted to identify which proteins maintain the mechanical stability of the integrin barrier (Figure 3F−H). We subjected cells to F-actin inhibitor Cytochalasin D (Cyto D) or Arp2/3 inhibitor CK666, which rapidly extinguished the integrin force signal and caused a uniform CD45 distribution in the cell. Moreover, treatment with the microtubule-disruptor nocodazole transformed larger ring-like diffusion barriers into smaller spot-like integrin plaques, reminiscent of focal adhesion (FA) rather than original integrin rings.[31] Notably, CD45 segregation was minimal, suggesting that dense integrin force barriers formed by the podosome structures are necessary for effective CD45 segregation.

Furthermore, the Rho-associated protein kinase (ROCK) inhibitor (Y27632) only slightly decreased the integrin force barrier intensity. This observation is consistent with a previous study on single-podosome mechanics,[19] which showed that the integrin force in podosome structures highly depends on actin rather than myosin II activation. As expected, CD45 segregation was also nearly unaffected in Y27632-treated cells since the mechanical stability of the integrin barrier was not disrupted (Figure 3G,H). However, the dynamics of the integrin force barrier were significantly slower than those of the control, which echoes a previous report that myosin II activity is required for phagocytic cup closure[10] (Figure S7 and Movie S5).

"DON'T EAT ME" SIGNALING MEDIATES PHAGOCYTOSIS BY REDUCING INTEGRIN FORCE

CD47 is a classic "don't eat me" signal that suppresses phagocytosis by binding to the immune receptor SIRPα on macrophage membranes.[32−34] However, the molecular mechanism by which the CD47-SIRPα signaling pathway inhibits macrophage phagocytosis remains unclear. For example, myosin IIA was thought to be a potential primary target of SIRPα.[35] However, Morrissey et al. recently proposed that integrin molecules are targets of CD47-SIRPα signaling and demonstrated that the CD47-SIRPα signaling axis could limit phagocytosis by inhibiting integrin activity from the inside out.[36] These results inspired us to explore the effect of "don't eat me" signaling on phagocytosis from a mechanical perspective. We first asked whether the activation of CD47-SIRPα signaling inhibits integrin-mediated force transmission, and we tested this possibility by exposing macrophages to different amounts of the extracellular domain of CD47 (Figure 4A,B). The results showed that CD47 decreased the integrin force signals of podosomes in a dose-dependent manner and that 10 μg/mL of CD47 dramatically attenuated the integrin force on the diffusion barriers.

Since we observed that CD45 exclusion is an integrin force-dependent process (Figure 3A,B), we hypothesized that activation of CD47-SIRPα signaling would eventually result in the failure of CD45 segregation in cells. To test this hypothesis, we compared the changes in CD45 distribution, podosome structures, and integrin force in macrophages before and after the activation of CD47-SIRPα signaling (Figure 4C−G and Movie S6). As we expected, within 10 min after the addition of CD47 to the solution, CD45 gradually refluxed from the cell periphery after a dramatic decrease in the integrin tension signal. Interestingly, immunofluorescence staining of actin showed that the phagocytosis cup consisting of podosomes was still present and that the actin bundles in each podosome were not significantly affected, suggesting that SIRPα activation leads to a decrease in integrin force by directly affecting integrin activity rather than by disrupting the structure of the actin bundle in the podosome structure (Figure 4C). Furthermore, when nocodazole or the PAK (p21-Activated kinase) inhibitor PF-3758309 was used to convert the adhesion structure of the podosome into an FA-like structure[37] before the addition of CD47, we observed that the force signals also disappeared in the FA-like structure (Figure S9), confirming that the observed reduction in the integrin force signal due to CD47 is not a consequence of inhibited podosome formation.

In addition, we also found that the activation of CD24-Siglec-10 signaling, described as another "don't eat me" pathway,[38] can also cause CD45 segregation failure by reducing the mechanical stability of the integrin barrier (Figure S10A,B). Uniquely, the integrin barrier was disrupted and returned to a resting like state after the activation of Siglec-10 (Figure S10C). This indicates that the activation of CD24-Siglec-10 signaling may have a slightly different mechanical effect on the integrin barrier compared to CD47, which requires further investigation in the future. Additionally, when MEFs (nonphagocytic cells lacking SIRPα or Siglec-10) were cocultured with CD47 or CD24, the force signal remained unchanged (Figure S11). This finding suggests that the effects of these two "don't eat me" signaling pathways are specific and do not interfere with the DNA probes.

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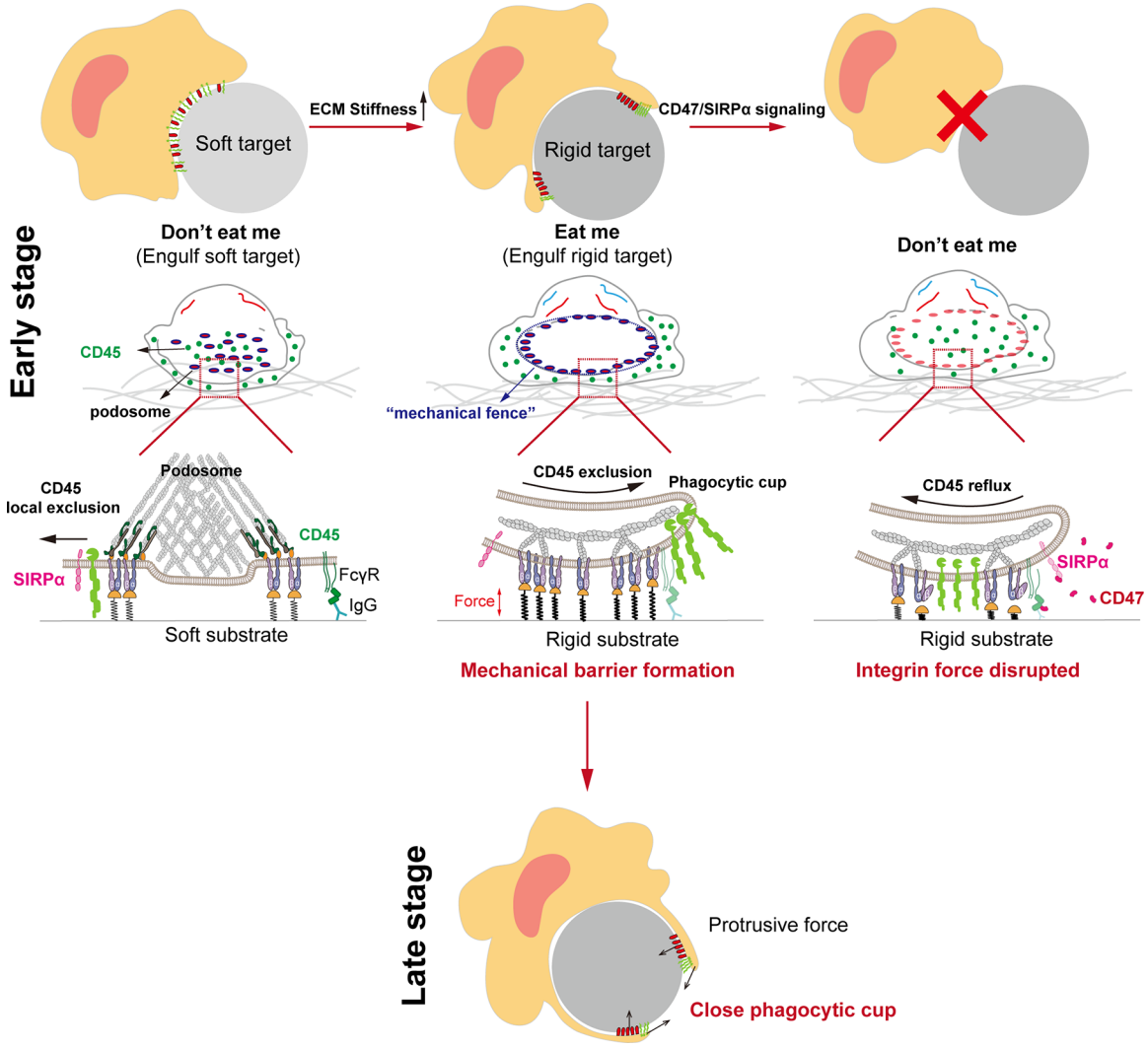

Figure 5. Integrin barrier acts as a mechanical checkpoint in macrophages to regulate FcγR-mediated phagocytosis. During the early stage of phagocytosis, macrophages contact and recognize targets to collect mechanical and chemical cues. When the target is soft, the macrophage is unable to rearrange its podosomes into an effective belt-shaped CD45 filter, despite individual podosomes still locally excluding CD45 (early stage, left). In contrast, when a macrophage attempts to engulf a rigid target, podosomes are relocalized into the phagocytic cup to assemble a "mechanical fence" for CD45 segregation (early stage, middle). On the other hand, once the "don't eat me" signal is activated, both the integrin force of podosomes and the mechanical stability of the diffusion barrier are significantly reduced, resulting in CD45 segregation failure (early stage, right). In the late stage, macrophages orchestrate the pulling force of integrin and the protrusive force of the F-actin bundle to close the phagocytic cup and complete the phagocytosis.

In summary, we have used DNA-based tension probes for real-time imaging of integrin forces in FcγR-mediated phagocytosis, and we found that when the IgG-FcγR signaling pathway is activated, the magnitude of the force on the integrins remains the same (17−45 pN), but the integrins relocalize into the phagocytic cup and expand outward to exclude the phosphatase CD45, which supports the idea that integrins can function as a diffusion barrier to coordinate phagocytosis,[27] and the results with TGT probes further suggest that the mechanical stability of integrin barriers is a critical factor for determining the successful separation of CD45 from the phagocytic cup. In addition, macrophages do not generate a considerable diffusion barrier by integrins on soft ECM matrices, suggesting that the mechanical−chemical crosstalk between FcγR and integrin receptors regulates the formation of diffusion barriers, and that likely explains why macrophages have a higher uptake efficiency for rigid targets.[7] We also discovered that activation of "don't eat me" signaling can reduce the mechanical stability of the diffusion barrier, resulting in CD45 segregation failure. While the precise impact of the CD47-SIRPα signaling axis on integrin mechanical properties remains to be elucidated, our study reveals that the CD24-Siglec-10 pathway, as another "don't eat me" signal pathway described by Barkal et al.,[38] also exerts a similar inhibitory effect on integrin mechanics. This suggests that the two independent signaling axes, CD47-SIRPα and CD24-Siglec-10, may have a similar signaling target, which does not affect podosome formation but can alter its mechanical properties. Further research is needed to investigate this.

It should be noted that the ring-like integrin-dependent mechanical barrier formed by macrophages during target phagocytosis is only the first step in initiating phagocytosis. Recently, Vorselen et al. found that the protrusive force of F-actin in podosomes may play a "tooth" function during the phagocytosis process,[4] to achieve subsequent chewing and swallowing of particles. This indicates that the pulling force of

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integrin and the protrusive force of F-actin bundles in the podosome structures will play different roles in different stages of phagocytosis. Combining our research data and literature results,[3,4,11,27,36] we have drawn a schematic diagram of the FcγR-mediated phagocytosis process, as shown in Figure 5. When macrophages contact a target in the early stages, the integrin barrier likely serves as a mechanical checkpoint in macrophages to determine whether they initiate phagocytosis. Macrophages cannot form an effective mechanical barrier to exclude CD45 on soft phagocytic targets, making it difficult to continue the phagocytosis process. However, on a rigid target, a CD45-isolating mechanical barrier formed by integrins in the phagocytic cup can be well established, which will initiate the phagocytosis process, and then the protrusive force generated by F-actin bundles will dominate the chewing process and close the entire phagocytic cup in a "zipper-like" manner.[4] If the macrophage's "don't eat me" signal is activated during the phagocytosis process, then the mechanical stability of the isolation barrier will be disturbed, leading to phagocytic failure.

Overall, our results and model suggested that the first step for promoting efficient phagocytosis in FcγR-mediated phagocytosis is the establishment of a mechanically stable integrin barrier at the phagocytic cup by podosomes. Unlike the function of a "molecular clutch" in complement-receptor (CR)-mediated phagocytosis,[6] integrins on podosomes utilize a mechanical fence-like structure to interpret the mechanical properties of the target and drive FcγR-mediated phagocytosis. This may provide a new strategy for immune therapy, such as increasing the mechanical force of podosomes through small-molecule agonists to enhance pulling force or protrusive force of phagosome and enhancing the efflux of CD45 at the phagocytic cup, which may promote the phagocytic efficiency of phagocytic cells toward tumors.

Our study's limitations include a 2D experimental design for easier imaging, and the study was in vitro, not considering the complex tumor microenvironment. Future directions should involve 3D molecular force imaging to explore mechanical forces' functions in actual engulfment processes and dynamic in vivo force imaging of macrophages.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.3c00957.

Additional experimental details, materials, methods, surface and hydrogel preparation, DNA sequence of probes, synthesis of DNA probes, and immunofluorescence staining or force signal data (PDF)

Movie S1: Time-lapse imaging of the RICM and tension map for a THP-1 M1 macrophage in the resting state (AVI)

Movie S2: Time-lapse imaging of the RICM and tension map for a macrophage in the frustrated state (AVI)

Movie S3: Time-lapse imaging of the interaction between CD45, labeled with FITC, and the integrin force reported by 17 pN RSDTP at the phagocytic cups during phagocytosis (AVI)

Movie S4: Time-lapse imaging of the interaction between CD45 and the 17 pN integrin force before and after EDTA treatment (AVI)

Movie S5: Time-lapse imaging of integrin force dynamics at phagocytic cups during phagocytosis after treatment of cells with Y27632 (AVI)

Movie S6: Time-lapse imaging of the interaction between CD45 and the 17 pN integrin force before and after activation of the "don't eat me" signal (AVI)

■ AUTHOR INFORMATION

Corresponding Authors

Wei Chen − *The Institute for Advanced Studies, TaiKang Center for Life and Medical Sciences, Hubei-MOST KLOS & KLOBM, School & Hospital of Stomatology, Wuhan University, Wuhan 430072, People's Republic of China*; Email: w.chen@whu.edu.cn

Zheng Liu − *The Institute for Advanced Studies, TaiKang Center for Life and Medical Sciences, Hubei-MOST KLOS & KLOBM, School & Hospital of Stomatology, Wuhan University, Wuhan 430072, People's Republic of China*; orcid.org/0000-0002-4252-4617; Email: zheng.liu@whu.edu.cn

Authors

Yuru Hu − *The Institute for Advanced Studies, TaiKang Center for Life and Medical Sciences, Hubei-MOST KLOS & KLOBM, School & Hospital of Stomatology, Wuhan University, Wuhan 430072, People's Republic of China*

Hongyun Li − *The Institute for Advanced Studies, TaiKang Center for Life and Medical Sciences, Hubei-MOST KLOS & KLOBM, School & Hospital of Stomatology, Wuhan University, Wuhan 430072, People's Republic of China*

Wenxu Wang − *The Institute for Advanced Studies, TaiKang Center for Life and Medical Sciences, Hubei-MOST KLOS & KLOBM, School & Hospital of Stomatology, Wuhan University, Wuhan 430072, People's Republic of China*

Feng Sun − *The Institute for Advanced Studies, TaiKang Center for Life and Medical Sciences, Hubei-MOST KLOS & KLOBM, School & Hospital of Stomatology, Wuhan University, Wuhan 430072, People's Republic of China*

Chaoyang Wu − *The Institute for Advanced Studies, TaiKang Center for Life and Medical Sciences, Hubei-MOST KLOS & KLOBM, School & Hospital of Stomatology, Wuhan University, Wuhan 430072, People's Republic of China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.nanolett.3c00957

Author Contributions

Z.L., Y.H., and W.C. conceived the project and directed the research. Y.H. designed and performed the overall experiments and analyzed the data with the help of H.L., W.C., F.S., W.X., and C.W. Z.L., Y.H., and W.C. wrote the paper.

Notes

The authors declare no competing financial interest.

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