Phagocytosis is one of the earliest cellular functions, developing approximately 2 billion years ago. Although FcR-based phagocytic signaling is well-studied, how it originated from ancient phagocytosis is unknown. Lipid redistribution upregulates a phagocytic program recapitulating FcR-based phagocytosis with complete dependence on Src family kinases, Syk, and phosphoinositide 3-kinases (PI3K). Here we show that in phagocytes, an atypical ITAM sequence in the ancient membrane anchor protein Moesin transduces signal without receptor activation. Plasma membrane deformation created by solid structure binding generates phosphatidylinositol 4,5-bisphosphate (PIP2) accumulation at the contact site, which binds the Moesin FERM domain and relocalizes Syk to the membrane via the ITAM motif. Phylogenetic analysis traces this signaling using PI3K and Syk to 0.8 billion years ago, earlier than immune receptor signaling. The proposed general model of solid structure phagocytosis implies a preexisting lipid redistribution-based activation platform collecting intracellular signaling components for the emergence of immune receptors.
Phagocytosis is the eat to defend functionalization of the original eat to feed feature in the primordial eukaryotic life forms, and the conceptual foundation of cellular immunity. It started at the eukaryogenesis about two billion years ago. In single cell life forms, phagocytosis endows energy/nutrient harvesting. In modern immune cells, phagocytosis has become a functional specialty in a subset of cells. Although phagocytic signaling in immune cells has been mostly delineated, it is unknown how it originated from the ancient architype.

Immune receptors share a common signaling cascade. A receptor ligation leads to the phosphorylation of intracellular ITAM motif. ITAM dual YXXL sequences then bind to SH2 downstream effectors. However, in metazoa, most solid structures can be efficiently recognized. The vast physical and chemical variations of these particles exclude the possibility of preexisting receptors encoded in the host genome. This is particularly evident in the phagocytosis of materials produced after the industrial revolution, as it would not allow the co-evolution of any potential receptor–ligand pairs. Evidence exists that this receptor-independent solid structure uptake is also functional in modern phagocytes.

Binding of a rigid structure creates tension on the plasma membrane. Such a tension is by itself a “sensing” mechanism that regulates coordination of membrane traffic and actin cytoskeleton. Evidence suggests that membrane lipids are actively involved in phagocytosis. In particular, PIP2 tends to aggregate under proper ionic environment and cytoskeletal attachment. In addition, PIP2 is sensitive to the membrane curvature associated with lipid rafts. However, whether PIP2 distribution itself can autonomously induce receptor-independent phagocytosis is not known.

We proposed several years ago that solid particle binding to a phagocyte surface triggers membrane lipid sorting. This lipid redistribution leads to a strong phagocytic programing that resembles FcR-based signaling. In this report, we found that in phagocytes, an atypical ITAM sequence in Moesin, a member of the conserved ERM (Ezrin-Radixin-Moesin) family, transduces such an activation. Particle-induced membrane deformation leads to an autonomous accumulation of PIP2 at the site of contact. Aggregated PIP2 binds to the FERM domain of Moesin, and the latter attracts Syk to the plasma membrane via the ITAM motif. The homologs of Moesin from Caenorhabditis elegans, Drosophila melanogaster, and Danio rerio can also signal in place of mouse Moesin to mediate the uptake. Phylogenetic analysis traces this modality of signaling to about 0.8 billion years ago, earlier than the dawn of immune receptors. This work therefore describes a general model of solid structure phagocytosis and implies a preexisting signaling platform that serves as the basis for the later development of immune tyrosine-based immune receptor signaling.

Results

Moesin mediates receptor-independent phagocytosis. In the absence of receptors, crystalline structure-triggered phagocytosis is dependent on Syk, implicating the involvement of a cryptic ITAM sequence. For FcRs, the ITAM motif is either intrinsic to the receptor (such as FcRIIA) or present in the common γ chain that serves to mediate Syk membrane recruitment. To search for the surrogate ITAM, we used a loosely defined sequence Tyr-X-X-(Leu/Ile)-X(6-12)-Tyr-X-(Leu/Ile) as the probe to search the online database of PROSITE. This search resulted in a total of 1085 independent hits in the mouse genome. All these hits were used to search against several NCBI GEO RNA-seq databases. Among them, a total of seven databases from bone marrow-derived DCs (BMDCs) (three), bone marrow-derived macrophages (BMDMs) (three), and RAW264.7 cells (one) were used to identify the high expressers in these cells. The expression levels were ranked by their abundance. The top 25 high expressers were analyzed further and functions of the corresponding proteins were annotated with UniProt as the reference. From this analysis, 18 of the 25 hits were disqualified for reasons provided in the side notes (Table 1). We produced siRNA (Supplementary Fig. 1a, b) to knock down (KD) the expression of remaining seven genes in DC2.4 cells. Among them, Hmox1 and Csf1r siRNA (three versions each) showed poor knockdown efficiency (Supplementary Fig. 1b), hence not pursued further. In the remaining five genes, Lcp1 and Msn KD showed visible reduction of the uptake of polystyrene (latex) beads (Fig. 1a). Since Lcp1 encodes a cytosolic protein with no known membrane binding ability, we focused our attention on Msn or Moesin, an evolutionarily conserved structural linker protein. The ITAM domain contained in Moesin was previously reported to interact with Syk in signal transduction of adhesion molecule PSGL-1; however, its role in phagocytosis was not studied. To increase the knockdown efficiency, lentivirus-based shRNA with a puromycin selection marker was produced, and the knockeddown cells were cloned. The selected cells showed significantly reduced ability to internalize polystyrene beads (Fig. 1b). This was accompanied by a reduction of phagocytic binding force measured by atomic force microscopy (AFM)-based single cell force spectroscopy (AFM-SCFS) (Supplementary Fig. 1c).

Evolutionarily, Moesin and other two ERM proteins belong to Band 4.1 superfamily and share significant homology with another structural protein Merlin. They are characterized by an N-terminal FERM domain and a C-terminal actin-binding domain flanking a linker sequence. C-terminal is also called ERM-association domain (C-ERMAD), which folds back to cover the FERM sequence, rendering the resting ERM soluble in the cytosol. Binding to the inner plasma membrane leaflet PIP2 displaces this blockage and activates ERM proteins. As Ezrin, Radixin, and Moesin are structurally similar and share an almost identical ITAM-like sequence, the lone involvement of Moesin in macrophage/DC phagocytosis was likely explained by its expression level. Indeed, Moesin expression was the highest among the family members in phagocytes, which was confirmed by online gene expression profiling database search (Supplementary Fig. 2c). In comparison with Moesin, knockdown of Ezrin and Radixin had minimal impact on phagocytic uptake of polystyrene beads (Supplementary Fig. 2d, e). To further confirm the involvement of Moesin, we co-stained actin with the three ERM proteins separately. Actin formed an even sphere around the bound beads. In comparison with Erzin and Radixin, Moesin was strongly accumulated in the phagocytic cup, and was in tight alignment with actin (Fig. 1c). Considering the potential cross-reactivities among these three antibodies, it was possible that Moesin antibody used in Fig. 1c inadvertently stained other ERM members. We performed the Moesin colocalization assay under Erzin and Radixin shRNA knockdown (Supplementary Fig. 3a; the knockdown efficiency and antibody specificity analysis are shown in Supplementary Fig. 3b), and the accumulation of Moesin showed no noticeable difference compared to wild-type cells. Coupled with the phagocytosis data (Supplementary Fig. 2d, e), our results suggested that Moesin was likely involved in the receptor-independent phagocytosis.

Moesin ITAM signals in receptor-independent phagocytosis. To ascertain the role of FERM domain ITAM as a self-sufficient signaling moiety in phagocytosis, we rescued the Moesin KD
DC2.4 cells with the full length, an ITAM-dead (Y191F + Y205F) full-length Moesin, as well as the FERM domain and ITAM-dead FERM domain constructs (Fig. 1d). Interestingly, the expression of full length or the FERM domain-only construct was sufficient to restore phagocytosis, while ITAM-dead versions failed to rescue (Fig. 1e). This result suggested that the ITAM in FERM is a signaling transducer for non-receptor-mediated phagocytosis while the structural linker function of Moesin is dispensable. Knockdown of Syk via siRNA showed a similar level of reduction in phagocytosis compared to Moesin knockdown DC2.4 cells (Fig. 2a). In the presence of MSU, while the overall Syk and Moesin levels remained the same, there was a strong association between these two molecules as revealed by Moesin co-immunoprecipitation (Fig. 2b). On the other hand, transfected Syk immunoprecipitated via Flag showed increased phosphorylation over time (Fig. 2c), and this was accompanied by increased phosphorylation of Erk1/2 and p38 (Fig. 2d). Following MSU treatment, Syk phosphorylation in Cos-1 was also increased, suggesting Syk was also involved in phagocytosis in these cells (Fig. 2e). To reveal the Moesin domain responsible for Syk binding, we produced a series of Flag-tagged Moesin truncations (Fig. 2f). In Cos-1 cells, each transcript reached similar and reproducible levels of protein expression (in molarity). Here, only the FERM domain of Moesin was able to co-immunoprecipitate Syk, while the linker and C-terminal sequences failed to do so (Fig. 2g). This result indicated that FERM of Moesin could bind to Syk, which was reminiscent of the latter’s involvement in a typical FcR-mediated phagocytosis. Whether this binding correlated with the functional role of Syk in phagocytosis remained a topic for investigation.

Table 1 Top 25 hits of highly expressed ITAM-like sequence-containing proteins in the mouse genome

| #  | Score | Gene Symbol | ITAM (-like sequence) | X(n) | Note |
|----|-------|-------------|-----------------------|------|------|
| 1  | 56.16 | Itm2b       | YTGIVNTRSVGTVYETI     | 7    | ITAM-like sequence in extracellular/luminal domain |
| 2  | 38.70 | Fcgr1g     | YQESFFGQRPEYVSYDL     | 7    | Secretery protein |
| 3  | 32.01 | Tyrobp     | YQGISLVLQFQELK       | 9    | 40S ribosomal protein |
| 4  | 24.71 | Npc2       | YSDQDEALVIFQVEYKI     | 11   | ITAM-like sequence in transmembrane domain |
| 5  | 22.33 | Lcp1       | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 6  | 15.16 | Rps3       | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 7  | 9.63  | Slc8a6     | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 8  | 9.63  | Hmox1      | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 9  | 8.61  | Csf1r      | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 10 | 8.51  | Cd36       | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 11 | 7.96  | Atp1a1     | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 12 | 7.94  | Ndrg1      | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 13 | 6.99  | Slc11a1    | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 14 | 6.10  | Mtn        | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 15 | 5.66  | Myh9       | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 16 | 5.42  | Actr2      | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 17 | 5.42  | Rpl7       | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 18 | 5.15  | Iqgap1     | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 19 | 4.96  | Ctsc       | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 20 | 4.76  | Rps25      | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 21 | 4.39  | Cd81       | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 22 | 4.14  | Dgs1       | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 23 | 4.09  | Glud1      | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 24 | 3.87  | Rpl7a      | YSDQELALVIFQVYK      | 11   | ITAM-like sequence in extracellular domain |
| 25 | 3.86  | Atp6v1e1   | YSDQELALVIFQVYK      | 11   | Subunit of Vacular ATPase |

For those analyzed further or of strong relevance to phagocytosis, their ITAM sequences are listed. The notes explain the likelihood of concerned sequences to serve as membrane proximal signal transducer. The gray font indicates spatial hindrances of their ITAM signaling. The proteins with functional ITAMs have been reported were indicated by "*". Red letters indicate essential amino acid residues for ITAM motif.

Moesin signaling is downstream of PIP2 sorting. ERM can be recruited to the plasma membrane via clustered PIP2 (refs. 28,29). This binding induces the conformational opening of ERM17,24,29. Previous work has revealed that the ITAM motif of Moesin, specifically Y191 and Y205, is localized within the FERM domain of moesin between F2 and F3 subdomains, likely masked by the C-ERMAD in the closed conformation30,31. In contrast, PIP2-binding residues K63 and K278 in the F1 and F2 subdomains, respectively, form a cleft termed the "POCKET" in autoinhibited Moesin. Moreover, K253/K254 and K262/263 residing in the F3 subdomain formed a "PATCH" that can bind to PIP2. This PATCH is more accessible than the POCKET in the autoinhibited Moesin. It was proposed that PIP2 binds and activates Moesin progressively. First, PIP2 transiently binds to the more accessible PATCH and initiates the release to FLAP. Next, the same PIP2 molecule stably binds to the now accessible POCKET to complete conformational activation32. Presumably, this can lead to the

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Fig. 1 Moesin mediates receptor-independent phagocytosis. a Phagocytosis efficiency of DC2.4 cells transfected with non-specific (NS) or indicated siRNA. Data were presented as mean ± s.e.m. (for all following figures, unless noted otherwise), n = 200, collected from a total N = 4 independent experiments. Henceforth, “n” designates number of data points in each group, and “N” independent repeats of experiments. For indicated comparisons, ***p < 0.001, *p < 0.05 and ns p > 0.05 by one-way ANOVA with Scheffé post hoc. b Non-specific (NS) or Moesin shRNA-transfected DC2.4 cell single clones were immunoblotted with antibodies against Moesin and GAPDH (upper). Phagocytosis efficiency of the clones with high KD efficiency (indicated by red dot in the upper panel) is shown (lower). n = 50, N = 3. **p < 0.001 by one-way ANOVA with Scheffé post hoc. c Left: Moesin, Ezrin, and Radixin (green) were visualized with antibodies along with actin cytoskeleton (phalloidin) on RAW264.7 cells incubated with 3 μm polystyrene beads. Structured Illumination Microscopy (SIM) was performed to obtain the fluorescence images. Scale bars, 5 μm. Right: Line profiles corresponding to fluorescence intensities of the respective ERM molecules and actin on an imaginary line across the indicated phagocytic cups. N = 6. d Schematic of Moesin and truncated Moesin fragments containing only FERM domain, or ITAM-YF mutations (Y191F + Y205F). e Moesin KD DC2.4 cells were rescued with the constructs in d by transient overexpression. Actin polymerization inhibitor Cytochalasin D was used as a negative control to establish baseline for phagocytosis. n ≥ 71 for each group. N = 3. For comparison between NS and NS CytoD group, ***p < 0.001 by Student’s t-test. For all other comparisons, ***p < 0.001 and ns p > 0.05 by one-way ANOVA with Scheffé post hoc.

exposure of ITAM for activation and binding. In Fig. 2g, without any membrane event, the full-length Moesin did not co-purify, suggesting a self-inhibitory state. We wondered if the Moesin FERM domain adhesion to PI(3,4,5)P3 was sufficient to trigger its ITAM motif signaling. By immunofluorescence microscopy, line-profile analysis, and three-dimensional (3D) reconstruction, we first confirmed that in RAW264.7 cell (chosen for their better tolerance of lipid dyes) phagocytosing polystyrene beads, phosphor-ERM and PI(3,4,5)P3 (PH-PLCδ-GFP) both showed strong colocalization with the actin-rich phagocytic cup and pERM/actin were in a close proximity (Fig. 3a, b). In Fig. 3b, pERM (T558) antibody was used as a surrogate measure for Moesin activation as it was likely the dominant ERM member in this setting. 3D reconstitution revealed a well-defined cup of Moesin conforming to a bead with actin forming an outer shell (Supplementary Movie 1). This result implies that Moesin was indeed in its extended conformation in the receptor-independent phagocytosis. To ascertain that PI(3,4,5)P3 was uniquely gathered during phagocytosis, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and PI(3,4,5)P3 distribution with reference to polystyrene beads were analyzed. Figure 3c shows that only PI(3,4,5)P3 was preferentially gathered at the interface of bead/membrane interaction, while PE and PC failed to show any increased accumulation. An AFM tip was used to deliver a polystyrene bead to be in contact with the cell (Fig. 3d, scheme). A kymograph analysis showed that the accumulation of Moesin to a large extent traced the intensity change of PI(3,4,5)P3, suggesting that PI(3,4,5)P3 induced membrane recruitment of Moesin (Fig. 3e, and Supplementary Fig. 4a). Indeed, functional blockage of PI(3,4,5)P3 with Geneticin (G418)33 caused diffusion of PI(3,4,5)P3 probe PH-PLCδ-GFP (Supplementary Movie 2 and Supplementary Fig. 4b) and inhibited the bead uptake (Fig. 3f).
**Figure 2** Moesin ITAM signals in receptor-independent phagocytosis. a) Syk or Moesin siRNA-transfected or NS control DC2.4 cells were immunoblotted with antibodies against corresponding proteins, and GAPDH (upper). The phagocytosis efficiency is compared to NS control (lower). n = 50, N = 3. b) Cell lysates from DC2.4 cells were subjected to IP with anti-Moesin. Cells were treated with 1 µM Na2VO4 for 1 h before harvesting, with or without MSU (200 µg ml−1) was added. N = 3. ***p < 0.001 by one-way ANOVA with Scheffé post hoc. c) Cell lysates from DC2.4 cells transfected Flag-Syk-EGFP were subjected to IP with anti-Flag M2 beads. Cells were treated with 200 µg ml−1 MSU crystal for indicated time before harvesting. N = 3. d) DC2.4 cells were treated with 200 µg ml−1 MSU crystal for indicated time before harvesting. Total cell lysates were subjected to immunoblotting with the indicated antibodies. N = 4. e) Cell lysates from Cos-1 cells transfected with Myc-Syk and Flag-Moesin full-length and fragments were then delivered to cover the prefabricated patterns, and the Si micropatterned mold was then fabricated by high-resolution photolithography and dry etching technique. PDMS membrane was coated with NeutrAvidin to create sufficient surface anchoring. The GPMV were then delivered to cover the prefabricated patterns, and the fluorescent intensity changes of lipid in contact with each pattern of physical contact was autonomous in response to particle binding, we generated giant plasma membrane vesicles (GPMV) from surface-biotinylated RAW264.7 cells that were labeled with fluorescent PIP2, PC, or PE. Several geometric patterns made by polydimethylsiloxane (PDMS), such as round, triangular, and square, were designed with a diameter of 3 µm, and spacing of 3–6 µm. The initial fused silica photomask carrying the designed micropatterns was first made through a photolithography process and the Si micropatterned mold was then fabricated by high-resolution photolithography and dry etching technique. PDMS micropattern array roughly 1 µm thick was produced by replica molding and the pattern was peeled off and attached to a glass substrate (Fig. 4a, b). The PDMS membrane was coated with NeutrAvidin to create sufficient surface anchoring. The GPMV were then delivered to cover the prefabricated patterns, and the fluorescent intensity changes of lipid in contact with each pattern

**Figure 3a–f** suggest that PIP2 served as the initial reaction to an external contact by a solid structure. However, if a contact-driven PIP2 accumulation was the true initial response, it should be autonomous in response to membrane ligation in the absence of biological feedback. To confirm that PIP2 distribution was not dependent on phagocytic signaling, a bead was again delivered by an AFM cantilever to the cell surface. PIP2 accumulation at the site of contact was essentially identical between phagocytic and non-phagocytic HEK293T cells (Fig. 3g and Supplementary Fig. 4c, d). This change was again absent for PC and PE. This further confirmed that PIP2 accumulation in response to a solid structure binding was independent of phagocytic signaling apparatus.

**Autonomous PIP2 sorting in response to physical structures.** To critically establish the notion that PIP2 redistribution to a site of physical contact was autonomous in response to particle binding, we generated giant plasma membrane vesicles (GPMV) from surface-biotinylated RAW264.7 cells that were labeled with fluorescent PIP2, PC, or PE. Several geometric patterns made by polydimethylsiloxane (PDMS), such as round, triangular, and square, were designed with a diameter of 3 µm, and spacing of 3–6 µm. The initial fused silica photomask carrying the designed micropatterns was first made through a photolithography process and the Si micropatterned mold was then fabricated by high-resolution photolithography and dry etching technique. PDMS micropattern array roughly 1 µm thick was produced by replica molding and the pattern was peeled off and attached to a glass substrate (Fig. 4a, b). The PDMS membrane was coated with NeutrAvidin to create sufficient surface anchoring. The GPMV were then delivered to cover the prefabricated patterns, and the fluorescent intensity changes of lipid in contact with each pattern...
was recorded by confocal microscopy and compared with surrounding areas. Regardless of the pattern, PIP2 intensity showed ~3-fold increase in the contact regions compared to the rest (Fig. 4c). These results suggest that PIP2 accumulated in response to the contact of a physical shape without involving any cellular activities and was likely the true initiation signal of phagocytosis.

In our previous work\textsuperscript{15,16}, it was found that the MSU and alum crystal phagocytic signaling is mainly the result of ordered lipid domain (lipid rafts) aggregation driven by solid surface binding to lipid species in these domains. Work by others also indicated that PIP2 tends to colocalize with lipid rafts\textsuperscript{24,34}. We therefore employed a ratiometric imaging method termed

**Fig. 3** Moesin signaling is downstream of PIP2 sorting driven by solid structures. a PIP2 (green) was visualized with PH-PLC6-GFP alongside actin cytoskeleton by SIM on RAW264.7 cells incubated with 3 µm naked polystyrene beads. Line profiles corresponding to fluorescence intensities of PIP2 and actin were generated across the indicated phagocytic cups. N = 5. Scale bars, 5 µm. b Identical to a except that pERM (T558) and actin were analyzed. Phospho-ERM were visualized with anti-pERM antibody. N = 4. Scale bars, 5 µm. c PIP2 (green), PC (red), and PE (red) were visualized with PH-PLC-GFP, TopFluor TMR-PC or TopFluor TMR-PE on RAW264.7 cells incubated with 3 µm biotin-BSA-coated polystyrene beads as in a. N = 3. Scale bars, 5 µm. d Schematic of fluorescence imaging of bead/cell contact by a bead delivered with AFM. e PH-PLC6-mCherry and Moesin-EGFP were co-expressed in RAW264.7 cells. A polystyrene bead was used to contact the cell surface. Images were taken at a 6 s interval for 500 s. Localization of PIP2 and Moesin at the site of contact (indicated with "***") was examined with kymographs generated from the indicated line. The normalized fluorescence is defined as the ratio of the fluorescence intensity of PIP2 or Moesin at the site of contact over non-contact regions on the cell membrane (right). N = 3. Scale bar, 5 µm. f Phagocytosis efficiency was examined for both RAW264.7 and DC2.4 cells in the presence of 0.1 and 10 mM Geneticin. n = 50, N = 4. "***"p < 0.0001 by one-way ANOVA with Scheffé post hoc. g PH-PLC6-GFP-expressing RAW264.7 or HEK293T cells were touched with a single polystyrene bead recorded with a 6 s interval for 900 and 1200 s, respectively. Polarized distribution of PIP2 upon touching at the site of contact (indicated with "***") on cell membrane was examined with kymograph. The normalized fluorescence of PIP2, PC, and PE was calculated for RAW264.7 and HEK293T cells (right). n ≥ 10, N = 3. Scale bars, 5 µm. "**"p < 0.001 by one-way ANOVA with Scheffé post hoc.
Polarization with c-Laurdan (a lipid-packing density sensor used to indicate lipid ordered domains) to determine membrane orders as an indirect measurement for lipid rafts and investigate whether PIP2 was truly localized within the highly ordered membrane (Supplementary Fig. 4e). The results showed that, when induced by solid structures, PIP2 preferentially sorted into highly ordered membranes on RAW264.7 cells compared to control lipids (Supplementary Fig. 4e). This suggests a possible internal link from membrane lipid binding to local PIP2 aggregation, bridged by lipid rafts.

Although two previous reports suggest that geometric shapes of phagocytic target with identical size affected the uptake, we failed to detect any difference in distinct geometry to attract PIP2. To attribute the ability of phagocytosis to a physical property of the target, we wondered if the “stiffness”, a surface property extensively implicated in cellular sensing, was involved. A series

**Fig. 4** Autonomous PIP2 sorting in response to physical structures. **a** Schematic of the procedure of making PMSD pattern. **b** SEM images of PDMS micropatterns. **c** Biotinylated-GPMVs were labeled with BODIPY FL-PIP2, TopFluor-TMR PC, or PE and incubated with PDMS triangular and rectangular patterns coated with NeutrAvidin. Images were taken when GPMV were settled onto a specific pattern. Fold changes, defined as the ratio of fluorescence intensity of a given lipid at the pattern contacting regions over non-contacting regions, were measured for all shapes with or without 10 mM NaN3. n ≥ 30, N = 5. Scale bars, 5 μm. ***p < 0.001 by one-way ANOVA with Scheffé post hoc. **d** Polystyrene beads with different degrees of crosslinking on glass surfaces were subjected to force-indentation displacement probing with a stiff probe. The values of stiffness (Young’s modulus) were obtained via indentation analysis with Veeco software. n ≥ 12, N = 3. ***p < 0.001 and *p < 0.05 by one-way ANOVA with Scheffé post hoc. **e** RAW264.7 cells were incubated with 2 μm, biotin-BSA-labeled polystyrene beads with varied degrees of crosslinking for 90 min at 37 °C. The efficiency of phagocytosis was analyzed as per phagocytosis assay. n = 50, N = 4. ***p < 0.001 and **p < 0.01 by one-way ANOVA with Scheffé post hoc. **f** Correlation analysis of stiffness as shown in **d** and phagocytosis efficiency as shown in **e** by non-parametric method. Spearman’s rank correlation coefficient r = 1. p = 0.0083
of polystyrene beads with variable stiffness were produced through manipulating the degree of crosslinking (from 10 to 90%), and their stiffness was determined individually by AFM based-nanoindentation (Fig. 4d). As a trend, the degree of crosslinking was as expected associated with the stiffness, which was echoed by the degree of phagocytosis (Fig. 4e). Non-parametric correlation analysis resulted in a Spearman coefficient \( r = 1 \) (\( p = 0.0083 \)), indicating that stiffness of phagocytic target is positively correlated with phagocytic efficiency (Fig. 2f). This analysis suggests that in addition to the contour of a solid structure, there may be a cut off in “hardness”; objects with a surface value below this limit are inefficient in triggering phagocytosis.

**PIPP-moesin-syk axis vs. fcr signaling.** Since particle binding is sufficient to drive PIP2 sorting, we wondered if any membrane ligation with sufficient force can trigger Moesin-based phagocytosis. Complete CD4 or chimeric extracellular/transmembrane CD4/41 intracellular Moesin ITAM construct was transfected into Cos-1, Cos-7, HEK293T, and NIH3T3 cells, along with an ITAM-dead control (Fig. 5a). As expected, in mildly phagocytosing Cos-1 cells, polystyrene beads coated with anti-CD4 IgG were efficiently phagocytosed, similar to FcRIIA-transfected cells in phagocytosing antibody-opsonized beads (Fig. 5b). Minimally phagocytosing Cos-7 cells showed relatively lower phagocytosis; however, the “mock” CD4 receptor and FcRIIA demonstrated similar efficiencies (Fig. 5b). NIH3T3 (fibroblast) and HKE293T (embryonic kidney cells), however, were mostly unresponsive. Notably, in Cos-1 and Cos-7 cells, all three versions of CD4 phagocytosed anti-CD4 antibody-coated beads, suggesting that in the presence of endogenous Moesin, surface ligation itself was sufficient. In contrast, in Moesin KD cells, only CD4 with an intact Moesin ITAM mediated phagocytosis with high efficiency, in comparison with CD4 alone or with the ITAM-dead mutant (Fig. 5c). This result suggests a scenario that sufficient binding force exerted on the membrane from a solid particle can induce phagocytosis via Moesin; in the absence of Moesin, the ITAM built into the “pseudo” receptor can provide similar stimulatory signal for phagocytosis, reminiscent of a phagocytic receptor. These results collectively indicate that Moesin ITAM domain can signal following surface ligation or behave as a signaling motif in a “receptor”-like structure. Importantly, this “pseudo” receptor signaling was similar to FcR-based phagocytosis in its sensitivity to PI3K, Syk, Cdc42, and Src inhibitors (Supplementary Fig. 5a), suggesting a signaling cascade similar to the typical FcR-mediated phagocytosis. Of note, the “pseudo” receptor signaling on DC2.4 cells displayed the same sensitivity (Supplementary Fig. 5a), suggesting again a shared signaling cascade.

In the “pseudo” receptor analysis, there was a potential confounding factor in its signaling cascade. Src family kinases are important for ITAM phosphorylation. In line with this notion, Src inhibitors AZD0530 and PP2 reduced the phagocytosis (Supplementary Fig. 5a). As CD4 is known to recruit Lck39, whether the latter, although typically restricted to T cells40, was involved was not clear. To this end, we expanded the analysis. We expressed mouse ICAM-1 and CD8a on Cos-1 cells and successfully induced significant levels of phagocytosis of anti-ICAM-1 and anti-CD8a-coated latex beads (Supplementary Fig. 5b, c). Similarly, whether these molecules carried Moesin ITAM did not significantly impact the uptake efficiency. Under Moesin knockdown, however, the chimeric ITAM sequence became critical. (Supplementary Fig. 5b, c). These additional analyses have two implications. First, it is likely that other Src family members may work in place of Lck as ICAM-1 was not known to engage this particular kinase. Second, Moesin ITAM’s signal capacity to induce phagocytosis in “pseudo” receptors is likely a generalizable effect, not limited to CD4 molecule.

Although Syk binding to FcR-associated ITAM is similar to its binding to Moesin, we wondered if the former represents an evolutionarily more advanced/efficient form of phagocytic signaling. First, in comparison with the standard FcR signaling, we noticed that Moesin-mediated Akt and Erk activation was nearly identical (Supplementary Fig. 6a), suggesting both mechanisms were activation-wise similar. In Moesin KD cells, IgG-coated beads were phagocytosed as efficiently as WT cells, suggesting that FcRs no longer require Moesin (Fig. 5d). Without IgG coating, the phagocytic efficiency was reduced in the absence of Moesin (Fig. 5d). In the phagocytosis of 1, 3, and 6 \( \mu \)m beads, IgG-coated ones in comparison showed high efficiency in taking up smaller beads, and had better capacity to internalize the larger ones (Fig. 5e). A similar analysis indicated that IgG coating promoted small bead internalization in terms of the number of uptakes per cell (Fig. 5f). With large sizes, the overall volume and surface area internalized were increased in the presence of IgG coating (Fig. 5g). Therefore, FcRs appear to have evolved to become independent of Moesin and demonstrate a much better efficiency in solid particle phagocytosis.

In contrast to FcR-based phagocytosis, there are several phagocytic mechanisms that do not seem to involve an ITAM-like structure. Scavenger receptors are known as non-opsonic phagocytic receptors for the lack of any apparent signaling motif in their intracellular domains41. They are believed to engage their targets via polycationic and polyanionic interactions (the electrostatic patch model)42. To test whether these receptors can also use Moesin as the conduit to relay the extracellular binding across the membrane, polystyrene beads were coated with MalBSA (maleylated-BSA, a ligand for SR-A6/SR-A1, previously known as MARCO/SR-A)21. This treatment increased the phagocytosis in comparison to BSA alone-coated beads (Supplementary Fig. 6b); the effect was mediated via SR-A6/SR-A1 as macrophages from mice with deficiencies in these two receptors did not show enhanced phagocytosis of MalBSA-coated targets, in comparison with similar macrophages from WT mice. However, in the absence of Moesin, MalBSA did not show any enhanced phagocytosis over BSA alone (Supplementary Fig. 6c), suggesting that Moesin was involved in the SR-A6/SR-A1-mediated phagocytosis.

**Evolutionary implications of PIP2 signaling in immune signaling.** Considering the possibility that Moesin-based signaling was evolutionarily conserved, we cloned the ERM protein from Caenorhabditis elegans, Drosophila melanogaster, and Danio rerio (Fig. 6a). Upon transflecting these sequences into Moesin KD DC2.4 cells, phagocytosis was restored, suggesting ERM proteins are conserved through the evolution as a phagocytic signaling mechanism in the absence of FcRs (Fig. 6b).

To identify the origin of ERM signaling, phylogenetic analyses based on model organisms were performed (see Methods for technical details). For Moesin, the evolutionary lineages furthest to H. sapiens found by BLAST were D. melanogaster and C. elegans, which diverged from the vertebrates at about 797 million years ago, indicating the latest time of hypothetic origin of Moesin (Fig. 7; Table 2 as the amino acid substitution table; Supplementary Figs. 7, 8a). The search for Syk traced back to a common origin of 435 million years ago. However, the times of common ancestors of the PI3K catalytic subunit, and of Syk and its analogous ZAP70 were found as early as 1.496 and 1.032–0.757 billion years ago, respectively (Fig. 7; Supplementary Figs. 7, 8b, c). The earliest identifiable origin of ITAM-containing surface molecules such as Fc common y chain, CD3 \( \zeta \) chain, and
Fig. 5 PIP2-Moesin-Syk axis is qualitatively similar to but quantitatively less robust than FcR signaling. a Schematic of a series of CD4-EGFP chimeric sequences, with or without a WT or mutated ITAM. b Cos-1, Cos-7, HEK293T, and NIH3T3 cells were transfected with the chimeric molecules by transient overexpression. Empty vector control and FcRIIA for phagocytosis efficiency control were also shown. ***p < 0.001 and ns p > 0.05 by one-way ANOVA with Scheffé post hoc. Phagocytosis efficiency was measured with anti-CD4-coated or IgG-coated 3 μm beads. n = 50, N = 3. c Identical to b except that Moesin KD DC2.4 cells were rescued with the constructs as shown in b. NS was used as a positive control to establish the full capacity of phagocytosis in DC2.4 cells without Moesin knockdown. n = 50, N = 3. ***p < 0.001 and ns p > 0.05 by one-way ANOVA with Scheffé post hoc. d Efficiency of Moesin-dependent (top) and FcR-dependent (bottom) phagocytosis was measured with biotin-BSA-coated beads or IgG-coated beads in shMoesin or shNS treated DC2.4 cells. n = 50, N = 3. *p < 0.5, ***p < 0.0011 and ns p > 0.05 by one-way ANOVA with Scheffé post hoc. e Impact of particle size on the efficiency of Moesin-dependent and FcR-dependent phagocytosis was measured as per phagocytosis assay with 1, 3, or 6 μm biotin-BSA-coated beads (upper) or IgG-coated beads (lower) in RAW264.7 cells. n = 50, N = 4. f To investigate the effects of particle sizes on phagocytosis, phagocytosis assays were performed with 1, 3, 4.5, and 6 μm biotin-BSA-coated (red line) or IgG-coated (black line) polystyrene beads on RAW264.7 cells. The numbers of beads internalized per cell on average were counted for each condition. n = 50, N = 4. *p < 0.05, ***p < 0.0001 and ns p > 0.05 by one-way ANOVA with Scheffé post hoc. g Volume and surface area internalized by RAW264.7 cells in f were calculated from the following formula. Volume = 4/3πr³, and surface area = 4πr². *p < 0.5, ****p < 0.0011, and ns p > 0.05 by one-way ANOVA with Scheffé post hoc.
DAP12 by BLAST was also around 435 million years ago, although its function is not clear (Fig. 7; Supplementary Figs. 7, 9a–c). The RAG transposon invasion into the immunoglobulin superfamily of the vertebrate genome took place around 600 million years ago43, and the vertebrate genome duplications were estimated to be around 600–700 million years ago (Fig. 7; Supplementary Fig. 7). Those events provided the "tools" and the "materials" for the emergence of modern immune receptors44,45. The high diversity of immune receptors (FcRs, BCR, and TCR)-based signaling cascade (ITAM-Syk/ZAP70-P13K) could undergo the diversifications in specific determinations to give rise to the modern immune receptors only after those two events. As expected, the coalescent time of the FcR II family members was only 90 million years ago, followed by a rapid radiation (Fig. 7; Supplementary Figs. 7, 9d). Therefore, modern FcR-based phagocytosis that relies on the Fc portion of antibodies cannot account for the phagocytosis beyond this time point. ERM-based phagocytosis is a likely candidate for an evolutionarily conserved form of solid particle uptake, already in the full complement of immune tyrosine-based apparatus.

Discussion

Figure 8 and Supplementary Movie 3 suggest a model on how the primordial Moesin-based signaling was gradually adopted by the modern FcR-based phagocytosis, and how the intracellular ITAM-Syk signaling cascade is shared by two different phagocytic mechanisms.

ERM protein emerged from the founder Band 4.1 at the transition towards multicellularity46. The role of Moesin has been mostly recognized as a structural linker, similar to other ERM members47. It is believed that PIP2/ERM interaction mainly serves as an initial membrane recruitment and ERM in turn stabilizes cortical actin48,49. In recent years, the extended functions of ERM are increasingly appreciated50–53. Yet, the involvement of its ITAM is poorly understood. In one case, it was reported to signal downstream of PSGL-1 (ref. 21). Structurally, the ITAM motif is well maintained across FcR y chain, CD3 chains including ζ, ιγ/β, and DAP12, with exceptions of Dectin 1 (YXXXL) and FcRII (11 AA spacer). We found that in addition to the 10 AA spacer, all ERM ITAM have a lysine following the first tyrosine, which in the case of most immune ITAM is an acidic residue. Despite these differences, our results show that Moesin ITAM contributes to an FcR-like phagocytic event. There remains the important question whether the linker and actin-binding domains of Moesin are also involved in the overall phagocytosis. In FcR-based phagocytosis, ITAM-containing transducers (such as FcγRIIA and the common γ chain) are not known to have actin-binding capacity54. As membrane anchoring effects of the linkers are important, the latter is likely provided in trans. Our data seem to suggest that ERM domain, once attached to PIP2, can function in a manner similar to the canonical ITAMs. As in our experiment Moesin knockdown is not complete, the residual Moesin may again play the role of membrane anchoring along with other linkers, such as other ERM and spectrin55. Whether an actin-binding domain cis to the ITAM would render the phagocytosis more efficient is an interesting question for the future.

Our current understanding of PIP2 functions focuses on several fronts: precursor of messengers, attachment anchors, and membrane organization4. It contributes to the negative membrane potential in the inner leafllet that forms the "electrostatic switch" to help organize signaling molecules via their cationic charges56. PIP2 is mostly localized at and constitutes 1–2% of lipids to the inner leafllet19. Its distribution is a balanced outcome of its synthesis and metabolism20,28. However, it is important to note that PIP2 is regulated by lipid rafts and membrane curvature24,34. Cholesterol has an "innate" ability to regulate the richness of PIP2 (refs. 13,34,57). Previously we reported that lipid raft aggregation is the initiation mechanism of receptor-independent phagocytosis. We speculated that since solid structures attract lipid rafts, an ITAM sequence contained in one or more raft-associated proteins may serve as the signal transmitter across the plasma membrane16. However, deletion of individual ITAM-containing receptors such as DAP12 and Fcγ chain did not produce an anticipated effect in reducing solid particle phagocytosis16. In light of this report, it is likely that aggregation of lipid rafts triggers the accumulation of PIP2, which in turn serves as the docking site of Moesin in its extended configuration, exposing the ITAM domain. In other words, Moesin ITAM and PIP2 appear to bridge the missing link for the phagocytic cup insertion58. This regulation may serve the purpose of

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**Fig. 6** Moesin function is conserved in solid structure phagocytosis. **a** Schematic of Moesin proteins in *Mus musculus* (Mm), *Danio rerio* (Dr), *Drosophila melanogaster* (Dm), and *Caenorhabditis elegans* (Ce), identified by NCBI Blast search. Functional domains and sequences of ITAM motif are shown. Two-letter abbreviations of all the listed species presented in this figure are shown on the left. **b** Moesin KD DC2.4 cells were rescued with the molecules indicated in a by transient overexpression. Vector transfection of the KD and WT cells were also shown. n = 50, N = 3. *p < 0.05, **p < 0.01, and ***p < 0.001 by one-way ANOVA with Scheffe post hoc
secondary lipid messenger formation as well as precise recruitment and expulsion of other signaling molecules. To study its involvement in our system, several assays in this report were designed to identify the original "innate" response to a solid particle contact. The motion patterns of PIP2 in non-phagocytes and in GPMV’s response to fabricated micropatterns imply that the local distribution of this lipid may be the first available intracellular beacon to herald an extracellular contact. This proposal is in line with PIP2’s propensity to be organized by membrane curvature and lipid domains.

Our results suggest that the PIP2/Moesin-mediated signal transduction was likely operational prior to the RAG transposon
invasion and the 2R genome duplication, which certainly predated the FcR diversification. This seems to suggest that the ERM/Syk signaling platform had been in existence before the FcR, and the immune receptor signaling “hijacked” this platform. This report provides evidence that phagocytosis can be induced by surface ligation-mediated lipid sorting, which fulfills the conceptual void how phagocytes can recognize almost inexhaustible variations of solid structures. In addition, this report proposes a potential link of how ancient, relatively inexhaustible variations of solid structures.

Table 2 Amino acid substitution models selected by SMS

| Protein          | Model    |
|------------------|----------|
| Moesin           | LG + G+1+F |
| Phosphatidylinositol 3-kinase catalytic subunit type 3 | LG + G+1+F |
| Tyrosine-protein kinase SYK/ZAP70 | JTT + G+1 |
| High-affinity immunoglobulin epsilon receptor subunit gamma | WAG + G |
| Low-affinity immunoglobulin gamma Fc region receptor II-a | JTT + G |
| TYRO protein tyrosine kinase-binding protein | JTT |
| T cell surface glycoprotein CD3 zeta chain | VT |

Phagocytosis assay. Cells were seeded into 24-well plates at 10^5 cells per well the night before the experiment. Biotin-BSA-coated beads resuspended in Opti-MEM at 1:1000 were centrifuged to the bottom of the dish at 930 g for 3 min at 4 °C. Cells were chilled on ice for 5 min before being moved into a 37 °C, 5% CO2 incubator. Cells were incubated with beads during the first 3 min. After incubation, the plate was washed with phosphate-buffered saline (PBS) buffer before 4% PFA fixation for 10 min. After fixation, cells were directly labeled with 1:200

Plasmin. PH-PiC6-GFP (Addgene #35142), PH-PiC2-mCherry (Addgene # 36073), and Lifeact-TdTomato (Addgene # 54528) vectors are purchased from Addgene. Lifeact-GFP (60113) for purchased from ThermoFisher. C57BL/6 female ZP70^−/^ and Syk^−/^ double knockout bone marrow from C57BL/6 mice and wild-type bone marrow from C57BL/6 female were purchased from EASYBIO. Anti-Myc (M20002) (1:1000), anti-Flag (M20008) (1:1000), anti-Zap70 (2705) (1:1000), anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST. Anti-phospho-tyrosine (9102) (1:1000), anti-phospho-Akt (9271) (1:800), anti-Akt (9272) (1:1000), anti-Syk (29177) (1:1000), anti-phospho-ERM (ab76247) (1:1000), and anti-mouse-IgG (7076) were purchased from CST.
Atomic force microscopy-based single cell force spectroscopy. Experiments were performed as previously described using a JPK CellHesion unit. In brief, a clean cantilever was placed on top of a glass block holder. Using a video camera attached to AFM, a laser was focused on the cantilever and properly aligned with respect to the position detector. Suitable silica crystals or polystyrene beads were placed directly onto the surface of a glass coverslip (diameter 2.4 cm); equal parts of the AB epoxy were mixed together and spread thinly at the side of the same glass coverslip. The clean cantilever gradually was moved to approach the glue so that only the very edge of the tip was over the glue. It was then lowered until a change in vertical deflection was observed and the tip had visible glue drawn up. Then the cantilever was pulled away from the glue until an observable snapping motion of the tip was observed, and was moved to individual silica crystals or polystyrene beads. The cantilever with glue was slowly lowered until the tip was in focus, and another deflection of the laser would be observed once the tip was in contact with the target. When raising the cantilever, the silica crystal or polystyrene bead on the glass coverslip should disappear if there was a successful attachment. To be certain that the target was glued to the tip, it was also examined under a compound light microscope. Functionalized cantilever tips were left to harden in 37°C for 30 min or room temperature overnight for further curing.

All instruments of AFM were turned on 30 min before single cell force spectroscopy (SCFS) experiments to ensure thermal equilibration and reduce drift during experiments. The coverslip seeded with DC2.4 cells was mounted onto a customized holder for AFM. The laser was focused on the back of the cantilever during experiments. The coverslip seeded with DC2.4 cells was mounted onto a customized holder for AFM. The laser was focused on the back of the cantilever during experiments.

Western blotting and co-immunoprecipitation assays. For western blot analysis, in most experiments, 10^7 cells were lysed with RIPA buffer. Total proteins were subjected to SDS-PAGE and immunoblotted with indicated antibodies. For co-immunoprecipitation, appropriate antibodies were incubated with protein A-Sepharose beads for 1 h at room temperature, followed by further incubation with 10^7 cell lysates for 4–5 h at 4°C. Immunocomplexes were washed four times with RIPA buffer and resolved by western blot analysis.

Immunofluorescence. Cells were fixed with 4% PFA for 10 min at room temperature followed by permeabilization with 0.1% Triton X-100 for 3 min at room temperature. After being washed three times, cells were incubated with primary antibodies at 4°C overnight. Cells were washed again three times and incubated.
with secondary antibodies or Alexa568-Phallolidin at 37 °C for 30 min. Cells were mounted with ProlongGold (Thermo P36975) mounting media and cured overnight before imaged by a microscope.

Fluorescence microscopy. Structured illumination microscopy (SIM) was performed on Carl Zeiss LSM780, an ELTRA super resolution microscope. Confocal microscopy was carried out on Nikon A1+, a resonant scanning confocal system. Widefield microscopy was done on an Olympus IX-73 microscope or a home-built TIRF microscope.

SIM images were processed first with Zen software for calculation and channel alignment. Confocal images were processed and deconvolved with Nikon NIS Elements software. Images from an Olympus IX-73 microscope were deconvolved with CellSens software. Images from the home-built TIRFM were collected with MicroManager and processed in FIJI and deconvoluted with Autoquant X. Line-profile analysis was performed with Autoquant X. Kymograph analysis and all fluorescence intensities were measured with FIJI. 3D reconstruction of fluorescence images was done with Imaris 7.2.3.

GPMV generation, modification, labeling, and observation. GPMV were made according to Sezgin et al.61, with modifications. In short, cells were first cultured in a 35-mm culture dish overnight to reach 70% confluence. Before vesiculation, cell surface was biotinylated with an EZ-Link Sulfo-NHS-BCN-Biotin kit (Thermo 21327). This step was added to provide extra adhesion of GPMV to PDMS patterns coated with NeutrAvidin. Biotinylated cells were washed twice with 1 mL and then incubated with 1 mL freshly prepared 25 mM PFA/2 mM DTT at 37 °C for 60 min. GPMV were concentrated by leaving the suspension in a 1.5 mL Eppendorf tube for 30 min. GPMV were labeled with Bodipy FL-PF2, TopFlour TMR-PC, and TopFlour TMR-PE at 1:50 dilution on ice for 40 min. Labelled and biotinylated GPMV were then incubated with NeutrAvidin-coated PDMS patterns for 30 min at room temperature. Samples were observed with a Nikon A1R+ confocal microscope.

Polystyrene microparticles with tunable rigidity. Styrene (ST) and divinylbenzene (DVB, 55 wt%) were obtained from Dongda Chemical Engineering Group Co. Poly(vinyl alcohol) (PVA-2 17, degree of polymerization 1700, degree of hydrolysis 88%) was ordered from Kuraray (Japan). Benzoyl peroxide (BPO, with 25 wt% moisture content, reagent grade) was bought from Beijing Chemical Reagents Company. Fast Membrane Emulsifier (FM0210/500 M) and microporous membrane were provided by Senhui Microsphere Tech (Suzhou) Co., Ltd.

The premix membrane emulsification technique and the FMEM-500M equipment were used to prepare O/W (oil in water) emulsion. Different percentages of the crosslinking agent were used to tune the PST microparticle rigidity. In a typical process, 9 g ST as monomers, 0.43 g BPO as initiator (4.3 wt% of total monomers), and 1 g DVB and other dosages (10–90 wt% of total monomers) were commingled together and used as the oil phase. Deionized water (150 mL) containing 1.0 wt% PVA was used as the water phase. The oil phase and the water phase were mixed by low-speed stirring and poured into the premix silos. Then this coarse emulsion was extruded through the microporous membrane under a nitrogen pressure. The preliminary emulsified emulsion was extruded through emulsified emulsion with the same membrane as coarse emulsion in the next pass. The final emulsion was obtained after repeating the above process for three times. The obtained emulsion was transferred to a four-neck glass separator flask equipped with a semicircular anchor type blade, a condenser, and a nitrogen inlet nozzle. The emulsion was bubbled with nitrogen gas for 1 h. Then, the nozzle was lifted and the temperature was increased to 70 °C gradually. The polymerization was carried out for 20 h under nitrogen atmosphere. The prepared uniform microparticles were washed with hot water and then ethanol for four times each.

Nanoindentation. The rigidity of single polystyrene microparticles with different degrees of crosslinking was probed by an atomic force microscope (Veeco BioScope Catalyst, USA). Briefly, the microparticles or bead solution diluted with ethanol was dipped on the surface of a clean glass substrate. The beads adhered to glass surface primarily due to the capillary force. The topography of the surface was first scanned in tapping mode with a stiff probe (Veeco TESP, spring constant 20–80 N m⁻¹, resonant frequency 230–320 kHz) and the location of individual beads was then identified for force-indentation displacement probing. The force applied for indentation ranged from 100 to 1500 nN. The observed indentation depth was less than 10 nm, about 0.3% of the size of beads. The same beads were probed three times and at least 12 beads were investigated for each sample. The rigidity (Young’s modulus) of these beads was obtained through indentation analysis based on the Hertz model with a Poisson ratio of 0.33 for bulk polystyrene using the native Veeco analyzing software.

PDMS patterns. Micropatterned array was fabricated with conventional semiconductor microfabrication and soft lithography. Briefly, a fused silica photomask carrying the designed micropatterns was first made through a photolithography process (Beijing Zhongke Shengzhe Technology Development Co., Ltd.). The Si micropatterned mold was then fabricated by high-resolution photolithography and dry etching techniques (Department of Microelectronics, Peking University).

PDMS micropatterned array was generated by replica molding. Briefly, PDMS and the curing agent (SYL Gardner™) were combined in a 3:1 ratio and mixed thoroughly using a pipette for 15 min. PDMS mixture was degassed for 1 h under vacuum. A thin layer of degassed PDMS mixture was cast onto the chlororotimiylsilane (MACRILIC)-coated resist master by spinning at 1000 rpm for 1 min. After overnight incubation at 60 °C, individual hardened patterns (1 mm × 1 mm) were peeled off and stored at room temperature. To prepare for GPMV/pattern contact experiment, patterns were pre-coated with NeutrAvidin (A2666, Invitrogen) and stored at −20 °C.

Phylogenetics. The amino acid sequences (RefSeq) of each protein in Homo sapiens (NP_001129691.1, NP_004097.1, NP_0024351.1) and other dosages (10–90 wt% of total monomers) were commingled together and used as the oil phase. Deionized water (150 mL) containing 1.0 wt% PVA was used as the water phase. The oil phase and the water phase were mixed by low-speed stirring and poured into the premix silos. Then this coarse emulsion was extruded through the microporous membrane under a nitrogen pressure. The preliminary emulsified emulsion was extruded through emulsified emulsion with the same membrane as coarse emulsion in the next pass. The final emulsion was obtained after repeating the above process for three times. The obtained emulsion was transferred to a four-neck glass separator flask equipped with a semicircular anchor type blade, a condenser, and a nitrogen inlet nozzle. The emulsion was bubbled with nitrogen gas for 1 h. Then, the nozzle was lifted and the temperature was increased to 70 °C gradually. The polymerization was carried out for 20 h under nitrogen atmosphere. The prepared uniform microparticles were washed with hot water and then ethanol for four times each.

The data that support the findings of this study are available from the corresponding author on request.

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References
1. Tauber, A. I. Metchnikoff and the phagocytosis theory. Nat. Rev. Mol. Cell Biol. 4, 897–901 (2003).
2. Kaufmann, S. H. Immunology’s foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. Nat. Immunol. 9, 705–712 (2008).
3. Yutin, N., Wolf, M. Y., Wolf, Y. I. & Koonin, E. V. The origins of phagocytosis and eukaryogenesis. Biol. Direct. 4, 9–9 (2009).
4. Freeman, S. A. & Grinstein, S. Phagocytosis: receptors, signal integration, and the cytoskeleton. Immunol. Rev. 262, 193–215 (2014).
5. Jaunouille, V. et al. Actin cytoskeleton reorganization by Syk regulates Fcgamma receptor responsiveness by increasing its lateral mobility and clustering. Dev. Cell 29, 534–546 (2014).
6. Kubo, Y. et al. Ezrin, Radixin, and Moesin (ERM) proteins function as pleiotropic regulators of human immunodeficiency virus type 1 infection. Virology 375, 130–140 (2008).
7. Chen, T. et al. Interaction of phosphorylated Fc epsilon RI gamma and the cytoskeleton. J. Biol. Chem. 275, 25308–25315 (1996).
8. Gauthier, N. C., Masters, T. A. & Sheetz, M. P. Mechanical feedback between membrane tension and dynamics. Trends Cell Biol. 22, 527–535 (2012).
9. Bohdanowicz, M. & Grinstein, S. Role of phospholipids in endocytosis, phagocytosis, and macropinocytosis. Physiol. Rev. 93, 69–106 (2013).
10. Salvemini, I. L. et al. Low PIP(2) molar fractions induce nanometer size clustering in giant unilamellar vesicles. Chem. Phys. Lipids 177, 51–63 (2014).
11. Salvemini, I. L. et al. Low PIP(2) molar fractions induce nanometer size clustering in giant unilamellar vesicles. Chem. Phys. Lipids 177, 51–63 (2014).
12. Carvalho, K., Ramos, L., Roy, C. & Picart, C. Giant unilamellar vesicles containing phosphatidylinositol(4,5)bisphosphate: characterization and functionality. Biophys. J. 95, 4348–4360 (2008).
13. Johnson, C. M. & Rodgers, W. Spatial segregation of phosphatidylinositol 4,5-bisphosphate (PIP2) signaling in immune cell functions. Immunol. Endocr. Agents Med. Chem. 8, 349–357 (2014).
14. Chierico, L., Joseph, A. S., Lewis, A. L. & Battaglia, G. Live cell imaging of membrane/cytoskeleton interactions and membrane topology. Sci. Rep. 4, 6056 (2014).
15. Flach, T. L. et al. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nat. Med. 17, 479–487 (2011).
16. N. G. et al. Remodeling independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. Immunity 29, 807–818 (2008).
17. Fehon, R. G., McClatchey, A. I. & Bretscher, A. Organizing the cell cortex: the role of ERM proteins. Nat. Rev. Mol. Cell Biol. 10, 387–402 (2010).
18. Zu, Y. et al. Characterization of interleukin 2 stimulated 65-kilodalton phosphoprotein in human T cells. Biochemistry 29, 1055–1060 (1992).
19. Takata, K., Horiuchi, S. & Morino, Y. Scavenger receptor-mediated recognition of maleylated albumin and its relation to subsequent endocytic degradation. Biochem. Biophys. Acta 984, 273–280 (1989).
20. Bretscher, A., Edwards, K., & Fehon, R. G. ERM proteins and myelin: integrators at the cell cortex. Nat. Rev. Mol. Cell Biol. 3, 586–599 (2002).
21. Golovina, K., Blinov, A., Akhmeteyeva, E. M., Omelyanchuk, L. V. & Chang, L. S. Evolution and origin of merlin, the product of the neurofibromatosis type 2 (NF2) tumor-suppressor gene. BMC Evol. Biol. 5, 69 (2005).
22. Pouwee, G. A. A glimpse of the ERM proteins. J. Biomed. Sci. 23, 35 (2016).
23. Louvet-Vallee, S. ERM proteins: from cellular architecture to cell signaling. Biol. Cell 92, 305–316 (2000).
24. Dupuy, A. G. & Caron, E. Integrin-dependent phagocytosis: spreading from microadhesin to new concepts. J. Cell Sci. 121, 1773–1783 (2008).
25. Dustin M. Complement receptors in cell adhesion and migration. Biochim. Biophys. Acta 1839, 620–634 (2014).
26. Yeung, T. et al. Receptor activation alters inner surface potential during phagocytosis. Science 313, 347–351 (2006).
27. Hong, Z. K., Staiculescu, M. C., Hampel, P., Levitan, I. & Forcags, G. How cholesterol regulates endothelial biomechanics. Front. Physiol. 3, 426 (2012).
28. Flannagan, R. S., Jaoumaille, V., Grinstein, S., Jaoumaillé, V. & Grinstein, S. The cell biology of phagocytosis. Annu. Rev. Pathol. 7, 61–98 (2012).
29. Trinkle, W. S. & Grinstein, S. Barriers to the free diffusion of proteins and lipids in the plasma membranes. J. Cell Biol. 208, 259–271 (2015).
30. Tokuzawa, Y. et al. Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. Mol. Cell. Biol. 23, 2699–2708 (2003).
31. Szegi, E. et al. Elucidating membrane structure and protein behavior using giant plasma membrane vesicles. Nat. Protoc. 7, 1042–1051 (2012).
32. Guindon, S. et al. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321 (2010).
33. Lefort, V., Longueville, J. E. & Gascuel, O. SMS: Smart Model Selection in PhyML. Mol. Biol. Evol. 34, 2422–2424 (2017).
34. Kumar, S., Stecher, G., Suleski, M. & Hedges, S. B. TimeTree: a resource for timelines, timetrees, and divergence times. Mol. Biol. Evol. 34, 1812–1819 (2017).
35. Hedges, S. B., Marin, J., Suleski, M., Paymer, M. & Kumar, S. Tree of life reveals clock-like speciation and diversification. Mol. Biol. Evol. 32, 835–845 (2015).
36. Kumar, S. & Hedges, S. B. TimeTree2: species divergence times on the iPhone. Bioinformatics 27, 2023–2024 (2011).
37. Hedges, S. B., Dudley, J. & Kumar, S. TimeTree: a public knowledge-base of divergence times among organisms. Bioinformatics 22, 2971–2972 (2006).
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Author Contributions
Libin M. and Z.T. performed all the experiments unless noted otherwise. Lin M. advised on phytogenic analysis. Y.H. performed siRNA screening of knockdown cells. N.K. provided technical assistance on AFM-related experiments. J.C. performed AFM-SCFS. H.R. assisted in imaging analysis. W.W. and F.G. provided technical assistance on polystyrene microparticles synthesis with guidance from G.M. B.W. coordinated the manufacture of silicon wafer with guidance from Y.D. Y.S., Libing M. and Z.T. designed all experiments and analyzed data. Lin M., Z.T. and T.X. contributed to manuscript preparation. T.X. performed nanoindentation work and supervised the project. Y.S. wrote the manuscript with inputs from T.X and M.W.A.

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