Supplemental material

JCB

Yoshida et al., http://www.jcb.org/cgi/content/full/jcb.201504097/DC1

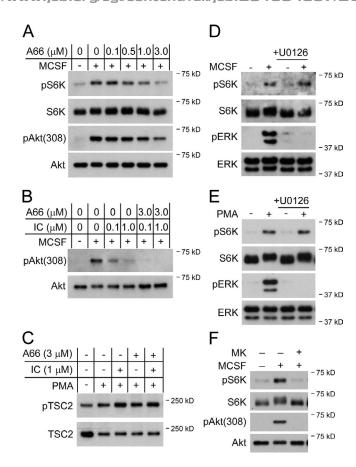


Figure S1. Inhibitors of mTORC1 signaling in BMM. (A) The PI3K type 1a p110α inhibitor A66 decreased the levels of pAkt-308, and inhibited mTORC1 activity (pS6K) in a dose-dependent manner in response to M-CSF (5-min stimulation). (B) Effects of IC87114 and A66 on PI3K, as indicated by Akt phosphorylation. (C) PI3K inhibitors did not decrease phosphorylation of TSC2 in response to PMA. (D and E) Effects of MEK inhibitor U0126 on mTORC1 activation by M-CSF and PMA in BMM. U0126 (10 μM for 30 min) inhibited phosphorylation of ERK but did not inhibit activation of mTORC1 (pS6K) in response to M-CSF (5-min stimulation; D), or PMA (30-min stimulation; E). (F) The Akt inhibitor MK2206 (MK) inhibited activation of mTORC1 by M-CSF.

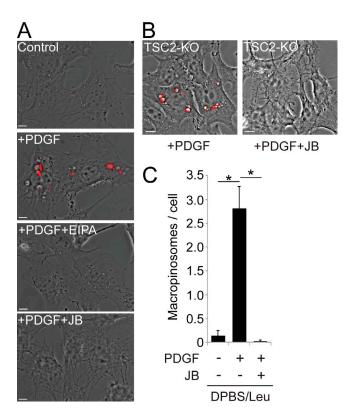


Figure S2. **PDGF-stimulated macropinocytosis in MEFs.** (A) MEFs were incubated 30 min in EIPA, JB, or DPBS, then 15 min in DPBS containing FDx and no stimulus (control), PDGF (+PDGF), PDGF plus EIPA (+PDGF+EIPA), or PDGF plus JB (+PDGF+JB). Bars, 10 μ m. (B) Macropinosome formation in TSC2-knockout (KO) MEFs stimulated by PDGF in the presence of FDx with (right) or without (left) JB. FDx-labeled macropinosomes are indicated by the red overlay. Bars, 10 μ m. (C) Quantification of macropinocytosis in TSC2KO MEFs stimulated with PDGF in the presence or absence of JB. *, P < 0.05.

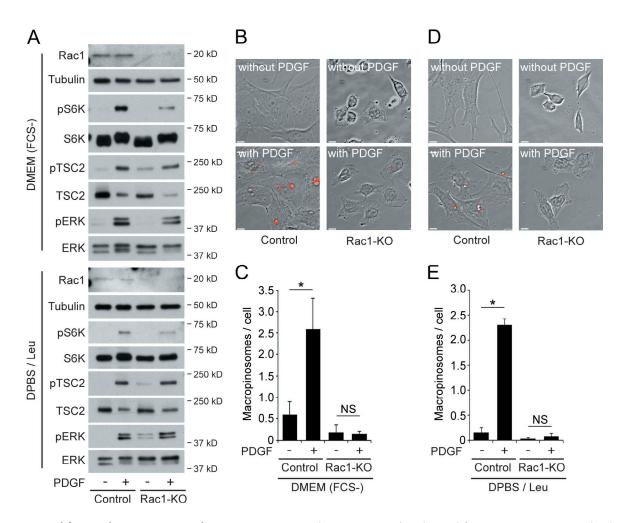


Figure S3. Rac deficiency abrogates PDGF signaling to mTORC1. (A) Signaling in WT (control) and Rac1-deficient (Rac1-KO) MEFs stimulated with or without PDGF. Rac1 deficiency reduced mTORC1 activity (pS6K), but not the phosphorylation of TSC2 or ERK in response to PDGF. (B and D) Macropinosome formation in WT (control) and Rac1-deficient (Rac1-KO) MEFs stimulated with or without PDGF in DMEM (FCS-; B) or DPBS/leucine (D). FDx-labeled macropinosomes are indicated by the red overlay. Bars, 10 µm. (C and E) Quantification of macropinocytosis in control and Rac1-KO MEFs with or without PDGF in DMEM (FCS-; C) or DPBS/leucine (E). *, P < 0.05.

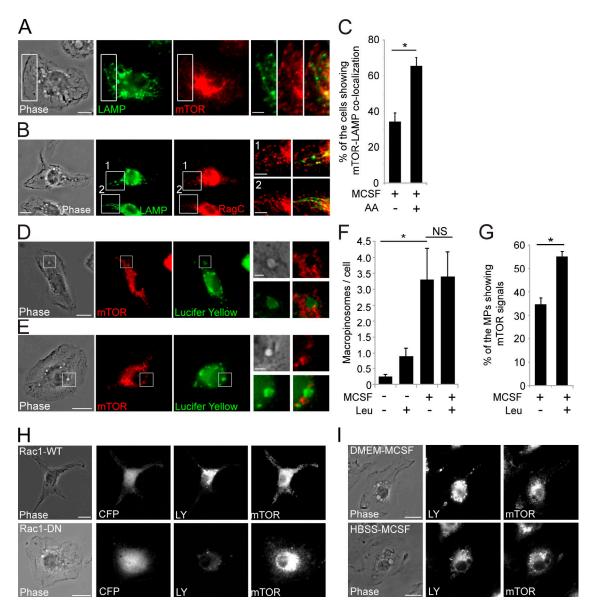


Figure S4. Macropinocytosis and mTOR localization in BMM. (A and B) Immunofluorescence localization of LAMP-1 and mTOR (A) or RagC (B) on macropinosomes and endolysosomes in BMMs stimulated 5 min with M-CSF. Insets show regions indicated by boxes, enlarged to show colocalization. (C) Amino acids increased the fraction of cells with mTOR localized to LAMP-1-positive organelles. Analysis of colocalization shows the means ± SEM from >10 cells per condition. *, P < 0.05, two-tailed t test with unequal variance. (D and E) Immunofluorescence localization of mTOR relative to LY. BMMs were stimulated 5 min with M-CSF and LY in DPBS with (D) or without (E) 0.4 mM leucine, and then cells were fixed and prepared for immunofluorescence localization of mTOR. Insets show phase contrast (top left), mTOR (top right), LY (bottom left), and LY/mTOR overlay (bottom right). mTOR localized near the LY-positive macropinosome in D, but there was no colocalization in E. (F and G) Quantitation of the immunofluorescence of cells prepared as in D and E. (F) 0.4 mM leucine did not alter the number of LY-positive macropinosomes per cell after stimulation with M-CSF. (G) Leucine increased the percentage of LY-positive macropinosomes (MPs) with associated mTOR fluorescence. (H and I) Effects of Rac1-DN on mTOR/LY colocalization. (H) BMMs expressing pRac1-IRES2-mCFP (top) or pRac1(N17)-IRES2-mCFP (bottom) were stimulated 10 min with M-CSF and LY and were then fixed and prepared for immunolocalization of mTOR. mTOR colocalization with LY was decreased in BMMs expressing pRac1(N17)-IRES2-mCFP. (I) Stimulation of BMMs with M-CSF in amino acid—rich medium (DMEM-M-CSF) resulted in greater colocalization of LY and mTOR than in BMMs stimulated in amino acid—poor medium (HBSS-M-CSF). Bars, 10 µm.

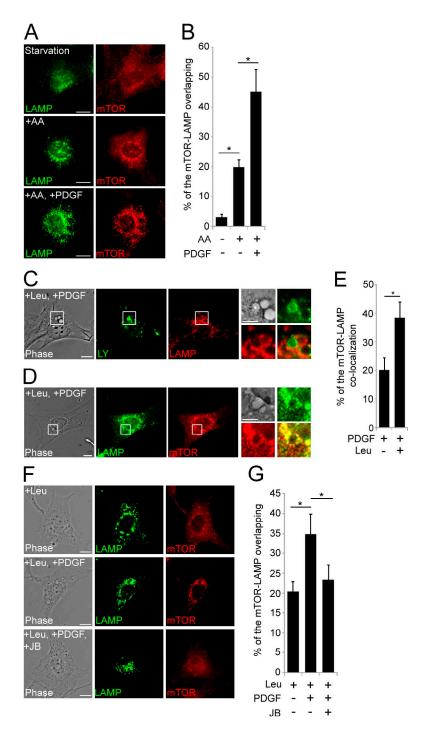
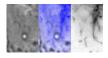
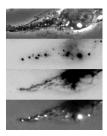


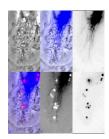
Figure S5. Amino acid-dependent colocalization of mTOR with LAMP-1 and endocytosed LY. (A) Colocalization of mTOR with LAMP-1 in MEFs stimulated 10 min with PDGF in DMEM (+AA) or HBSS (starvation). Bars, 10 µm. (B) Quantitative analysis of MEFs stained for LAMP-1 and mTOR. (C) Colocalization of LY and LAMP-1 in MEFs after 15 min stimulation by PDGF. Insets show phase contrast (top left) and the association of endolysosomes (LAMP, bottom left) with macropinosomes (LY, top right). Bar, 10 µm. (D) Colocalization of mTOR and LAMP-1 in MEFs. Insets show phase contrast (top left), mTOR (bottom left), LAMP (top right), and overlay (bottom right). Bar, 10 µm. (E) Quantification of leucine-dependent colocalization of mTOR and LAMP. Means ± SEM from >10 cells per condition. *, P < 0.05, one-tailed, paired t test with unequal variance. (F) mTOR/LAMP-1 colocalization after 15-min stimulation of MEFs with PDGF in DPBS/Leu, with or without PDGF, with or without JB. Bars, 10 µm. (G) Quantification of mTOR/LAMP-1 colocalization in MEFs under the conditions shown in F. (B and G) Means ± SEM from >10 cells per condition. *, P < 0.05, one-tailed, two-sample t test with unequal variance.



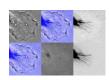
Video 1. **Piranhalysis after macropinosome formation.** (right) Time-lapse video of a macrophage stimulated with M-CSF after preloading endolysosomes with TRDx (inverted contrast, right). (left) Phase contrast. (middle) Phase contrast with blue overlay of TRDx fluorescence. TRDx-labeled tubular and vesicular endolysosomes surrounded macropinosomes immediately after macropinosome closure and repeatedly contacted and separated from it as the organelle shrank. Frame intervals = 20 s (data of Fig. 7 A).



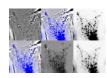
Video 2. **Fusion of macropinosomes and tubular endolysosomes.** Time-lapse video of a macrophage stimulated with M-CSF and LY after preloading endolysosomes with TRDx. (top) Phase contrast. (second Panel) LY fluorescence (inverted contrast). (third panel) TRDx fluorescence (inverted contrast). (bottom) LY/TRDx ratio image. LY-labeled macropinosomes are bright and TRDx-labeled endolysosomes are black. Macropinosomes were wrapped by tubular endolysosomes before disappearing into the endolysosomal compartment. A large macropinosome was wrapped by tubular endolysosomes, exchanged dyes, and then was resorbed into the tubular endolysosomal network. Frame intervals = 20 s (data of Fig. 7 B).



Video 3. Fusion of macropinosomes and tubular endolysosomes. (bottom and top right) Time-lapse video of macrophage stimulated with M-CSF and LY (bottom right, inverted contrast) after preloading endolysosomes with TRDx (top right, inverted contrast). (top left) Phase contrast. (top middle) Phase contrast with blue overlay of TRDx fluorescence. (bottom middle) LY/TRDx fluorescence ratio image. Bright spots indicate LY-labeled macropinosomes. Black indicates relative distribution of TRDx-labeled endolysosomes. (bottom left) Phase contrast with blue overlay indicating TRDx fluorescence and red overlay indicating LY fluorescence. Macropinosomes were wrapped by tubular endolysosomes before disappearing into the endolysosomal compartment. Frame intervals = 20 s.



Video 4. Solute size—dependent delivery of dye from endolysosomes into macropinosomes. (bottom and top right) A macrophage stimulated with M-CSF after preloading endolysosomes with LY (bottom right, inverted contrast) and TRDx (top right, inverted contrast). (top left) Phase contrast. (top middle) Phase contrast with blue overlay of TRDx fluorescence. (bottom middle) LY/TRDx fluorescence ratio image. Bright spots indicate compartments with elevated concentrations of LY, relative to TRDx. (bottom left) Phase contrast with blue overlay indicating TRDx fluorescence and red overlay indicating increased LY/TRDx ratio. Macropinosomes labeled transiently with LY before shrinking and merging with the endolysosomes. Frame intervals = 20 s (data of Fig. 7 C).



Video 5. Solute size-dependent delivery of dye from endolysosomes into macropinosomes. (bottom and top right) Time-lapse video of macrophage stimulated with M-CSF after preloading endolysosomes with LY (bottom right) and TRDx (top right). (top left) Phase contrast. (top middle) Phase contrast with blue overlay of TRDx fluorescence. (bottom middle) LY/TRDx fluorescence ratio image. Bright spots indicate compartments with elevated concentrations of LY, relative to TRDx. (bottom left) Phase contrast with blue overlay indicating TRDx fluorescence and red overlay indicating increased concentrations of LY. LY entered macropinosomes from endolysosomes earlier than did TRDx. Frame intervals = 20 s.