**Figure 1.**

**Amino acids increase activation of mTORC1 by M-CSF and PMA in macrophages.** (A) Time course of signaling in BMMs after stimulation with M-CSF. mTORC1 activity, as indicated by phosphorylation of S6K (pS6K), increased within 5 min and remained elevated. PI3K (pAkt-308), mTORC2 (pAkt-473), and MEK/ERK (pERK) were activated transiently. (B) Time course of signaling in response to PMA. MEK/ERK activity increased quickly, whereas mTORC1 was activated after a delay. PI3K and mTORC2 were not activated. (C and D) Relative to amino acid–depleted medium (HBSS, −), amino acid–rich medium (DMEM, +) increased mTORC1 activation in response to M-CSF (5-min stimulation; C) and PMA (30-min stimulation; D), but did not affect stimulation of MEK/ERK in response to either stimulus. (E and F) The addition of leucine to DPBS was sufficient to augment activation of mTORC1 by M-CSF (E) or PMA (F), as indicated by phosphorylation of S6K and 4EBP1, but did not alter stimulation of PI3K by M-CSF or of MEK/ERK by PMA.

**Figure 2.**

**Macropinocytosis is required for activation of mTORC1 by M-CSF and PMA in macrophages.** (A) Macropinosome formation in response to M-CSF and PMA. BMMs were incubated for 5 min in buffer containing fluorescein dextran with M-CSF or PMA, and then were washed, fixed, and observed by phase-contrast and fluorescence microscopy (red overlay; Bars, 10 µm). (B) Time course of macropinosome formation after stimulation with M-CSF (diamonds), PMA (squares), or with buffer only (triangle). *n* ≥ 25 cells per time point. (C–F) Effects of PI3K inhibitors on macropinocytosis (top) and mTORC1 activity (bottom). M-CSF–stimulated macropinocytosis and mTORC1 activity were inhibited partially by A66 (C) and were inhibited more completely by IC87114 (IC) or the combination of IC87114 and A66 (D). PMA-stimulated macropinocytosis and mTORC1 activity were unaltered by either A66 (E) or IC (F). B–F show the means ± SEM from three independent experiments, with >25 cells scored per condition. \*, P < 0.05, one-tailed *t* test.

**Figure 3.**

**Macropinocytosis is required for activation of mTORC1 in BMM.** (A–D) Effects of JB on macropinocytosis (top) and mTORC1 activity (bottom). JB treatment blocked both macropinocytosis and mTORC1 activity in response to M-CSF in DMEM (A), PMA in DMEM (B), or M-CSF in DPBS containing 0.4 mM leucine (C). (D) JB did not inhibit activation of mTORC1 in response to M-CSF in DPBS containing 5.6 mM glucose. (E) JB inhibited 4EBP1 phosphorylation in response to PMA in BMM. (F and G) EIPA inhibited activation of mTORC1 by M-CSF (5-min stimulation; F) and PMA (30-min stimulation; G). (H) Macropinosome formation in response to M-CSF was inhibited by EIPA (+E), but not by U0126 (+U). Macropinocytosis measurements of A–D and H show the means ± SEM from three independent experiments, with >25 cells scored per condition. \*, P < 0.05, one-tailed *t* test.

**Figure 4.**

**Macropinocytosis is required for leucine-dependent activation of mTORC1 by PDGF in MEFs.** (A) Amino acid–dependent activation of mTORC1 by PDGF. MEFs were incubated 30 min in DPBS and then stimulated for 15 min with PDGF (2 nM) in DPBS containing glucose (Glu; 5.6 mM) and leucine (Leu; 0.4 mM) and scored for macropinosome formation (top) and mTORC1 activity (bottom). (B) Effects of leucine concentration on macropinocytosis (top) and mTORC1 activity (bottom). (C and D) Stimulation of macropinocytosis and mTORC1 by PDGF and 0.4 mM leucine were inhibited by EIPA (C) and JB (D). (E) Stimulation of mTORC1 by PDGF and glucose (5.6 mM) was not inhibited by EIPA (EI) or JB. (F) Activation of mTORC1 after 30 min in PDGF and Ala-Leu. MEFs were incubated in DPBS for 30 min, followed by 30 min in DPBS, with or without PDGF, with or without 4 mM Ala-Leu. (G) Effects of Akt inhibitor MK2206 (MK; 2 µM for 30 min) on macropinocytosis (top), Akt activity, and mTORC1 (bottom). Macropinocytosis measurements of A–G show the means ± SEM from three independent experiments, with >25 cells scored per condition. \*, P < 0.05, one-tailed *t* test. (H) Stimulation of mTORC1 in TSC2-deficient (TSC2KO) and WT MEFs. mTORC1 activity in TSC2KO MEFs was increased by PDGF and inhibited by JB.

**Figure 5.**

**PKC inhibition blocks PDGF-induced macropinocytosis and mTORC1 activation independent of TSC function.** (A) Macropinosome formation in TSC2-WT (left) and TSC2-knockout (KO) (right) MEFs stimulated by PDGF in the presence of FDx with (bottom) or without (top) calphostin C (cal C). FDx-labeled macropinosomes are indicated by red overlay. Bar, 10 µm. (B) Macropinocytosis and activation of mTORC1 in TSC2-WT and TSC2-KO MEFs was increased by PDGF and inhibited by calphostin C. Bars indicate the means ± SEM of three trials. \*, P < 0.05.

**Figure 6.**

**Stimulation of macropinocytosis in BMMs increased solute uptake and amino acid–dependent recruitment of mTOR to macropinosomes and endolysosomes.** (A and B) Immunofluorescence localization of mTOR and LAMP-1 in BMMs stimulated 5 min with M-CSF. Insets show mTOR association with macropinosome-associated endolysosomes (A) and with tubular endolysosomes (B). (C) BMMs fixed and imaged after 1, 5, or 10 min with LY and M-CSF. LY was initially distributed in macropinosomes but also localized to tubular compartments by 10 min. (D) Total LY fluorescence per cell, from image analysis of preparations shown in C (*n* > 10 cells per point; \*, P < 0.05). (E and F) Immunofluorescence localization of LAMP-1 after incubation of BMMs with LY and M-CSF for 5 min (E) or 10 min (F). Insets shows a macropinosome (E) and tubular endolysosomes (F) labeled with both LAMP-1 (top right) and LY (bottom left); top left: phase-contrast, bottom right: overlay. (G) Immunofluorescence localization of mTOR after 10-min incubation in LY with (bottom; insets a and b) or without (top) M-CSF. (H) Quantitation of mTOR colocalization with LY (\*, P < 0.05). (I–K) Macropinocytosis and colocalization of mTOR and LY in BMMs expressing pRac1WT-IRES2-mCFP (RacWT) or pRac1(N17)-IRES2-mCFP (RacDN), fixed after stimulation for 5 min with FDx (I) or 10 min with LY (J and K) with or without M-CSF. CFP-positive BMMs were scored for macropinosome labeling with FDx (I), integrated intensity of LY per cell (J), and colocalization of mTOR with LY (K). RacDN significantly decreased macropinocytosis, LY accumulation, and mTOR colocalization with LY-positive organelles (\*, P < 0.05). (L and M) Effects of amino acids on LY accumulation and colocalization of mTOR and LY. BMMs were incubated 10 min in DMEM (+AA) or HBSS (−AA) containing LY, with or without M-CSF. Amino acids did not affect the integrated cellular accumulation of LY (L), but increased the association of mTOR with LY-positive endocytic compartments (M). \*, P < 0.05. Bars, 10 µm.

**Figure 7.**

**Rapid delivery of extracellular small solutes into endolysosomes by M-CSF–induced macropinosomes in macrophages.** (A) BMMs with TRDx-labeled endolysosomes were stimulated with M-CSF and then imaged by time-lapse phase contrast (PC) and fluorescence (TRDx) microscopy. Corresponding distributions of macropinosomes and lysosomes are indicated in overlay images (middle row) and by the lines that track macropinosomes between time points (top and bottom rows). Time after the addition of M-CSF is indicated in the bottom row (minutes). Macropinosomes were repeatedly engaged by endolysosomes and shrank gradually. (B) PC images show phase-bright macropinosomes and the time course of maturation for one LY-labeled macropinosome. Inverted contrast images of macropinosomes (LY) and endolysosomes (TRDx) show dye exchange between the compartments. Time after addition of M-CSF is indicated in the bottom row (min:sec). LY/TRDx fluorescence ratio images (Ratio) show the relative distributions of macropinosomes (white) and endolysosomes (black). Tubular endolysosomes containing TRDx elongated toward the phase-bright macropinosome containing LY (*t* = 7:00–7:20), and wrapped around it (*t* = 7:40). The two dyes mixed in the macropinosome (*t* = 8:00), which then disappeared quickly (*t* = 8:00–9:00). (C) Size-selective solute exchange between tubular endolysosomes and macropinosomes. Tubular endolysosomes prelabeled with LY and TRDx contacted phase-bright macropinosomes and delivered LY before delivery of the larger TRDx. Asterisks indicate the position of a macropinosome in corresponding PC, LY, and TRDx fluorescence images. LY/TRDx ratio images show the early entry of LY into the macropinosome (26:20), followed by entry of the TRDx and rapid shrinkage of the macropinosome (26:40). Bars, 5 µm.

**Figure 8.**

**Signaling for mTORC1 activation is localized to macropinocytic cups.** (A) BMMs expressing CFP and YFP-BtkPH, a probe for PIP3, were imaged during M-CSF–stimulated macropinocytosis. The image series are aligned such that circular ruffle closure occurs at *t* = 80 s (top row). Pseudocolor ratio images (YFP-BtkPH/CFP) show strong YFP-BtkPH recruitment to the macropinocytic cup at *t* = 120 s (bottom row). (B) Ratiometric imaging of the DAG probe C1δ-YFP in BMMs, as described in (A). *t* = 80 s marks the end of ruffle closure. Maximal C1δ-YFP recruitment occurs at *t* = 140 and 160 s (C) Ratiometric imaging of the PIP3 probe YFP-BtkPH during PMA-stimulated macropinocytosis. YFP-BtkPH was not recruited to the macropinocytic cup. Bars, 5 µm. (D) Two pathways of growth factor receptor (GFR) signaling to mTORC1. GFR signaling activates PI3K, which activates mTORC1 by a cytosolic pathway, involving Akt, TSC2, and Rheb, and a vesicular pathway, involving PKC-dependent, macropinosome-mediated delivery of leucine to endolysosomes. PMA activates both pathways independent of PI3K. Stimuli are indicated in blue type; inhibitors are indicated in gray type. (E) The macropinosome as a discrete unit of GFR signaling. PI3K-generated PIP3 accumulates in macropinocytic cups (red line), activating Akt (cytosolic pathway) and PLCγ. PLCγ generates DAG in the cup, leading to PKC-dependent macropinocytosis (vesicular pathway). Extracellular solutes internalized by macropinocytosis are delivered rapidly into LAMP-1 (blue lines)–enriched endolysosomes by piranhalysis or after tubular endolysosomes wrap around macropinosomes. Small solutes exchange more rapidly between macropinosomes and endolysosomes than large solutes, providing a rapid mechanism for activation of mTORC1 by amino acids inside macropinosomes and endolysosomes.