Bao et al., http://www.jcb.org/cgi/content/full/jcb.201503066/DC1

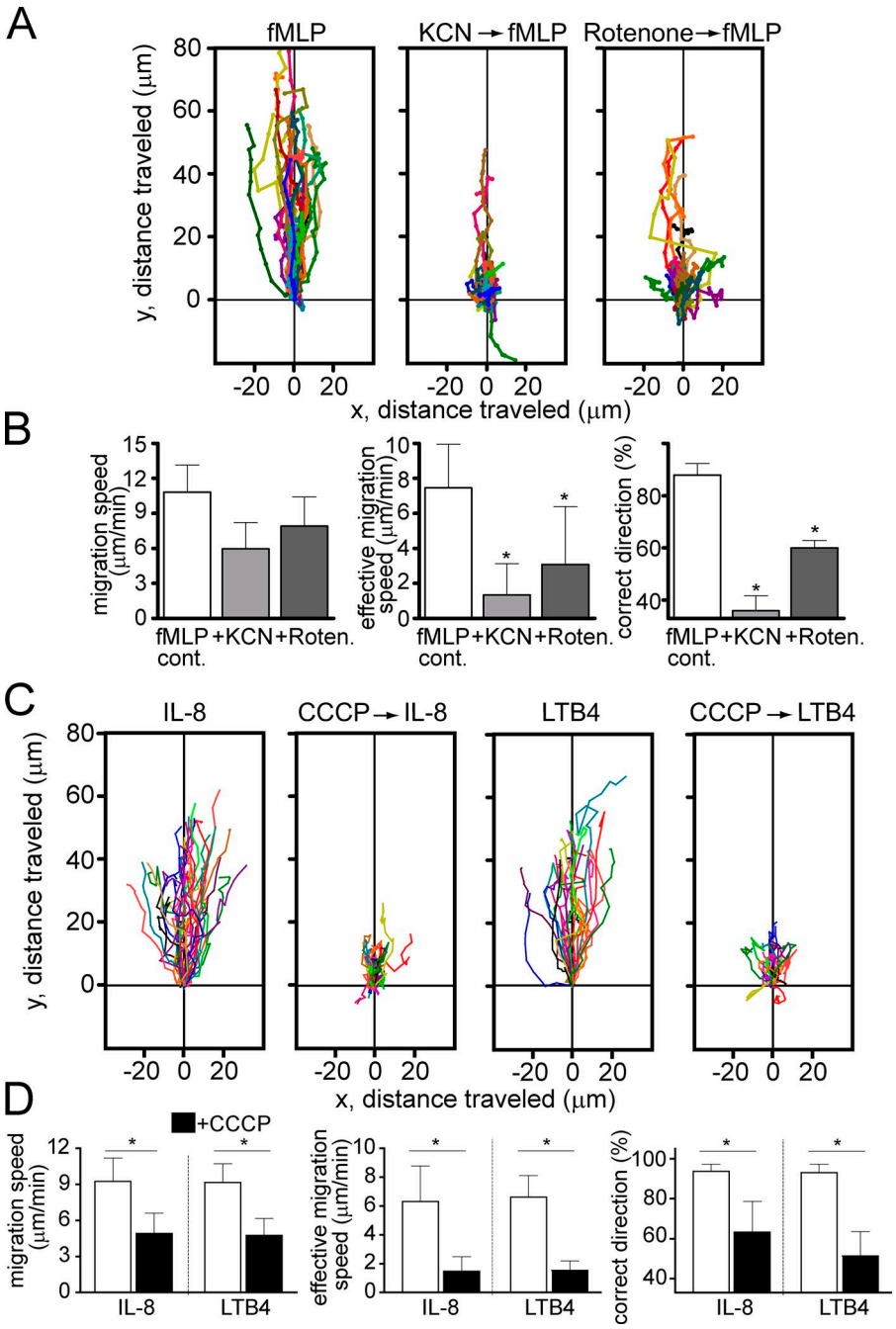


Figure S1. **Inhibition of mitochondria by potassium cyanide (KCN) or rotenone impairs PMN chemotaxis.** (A and B) Freshly isolated primary human PMNs were exposed to a chemotactic gradient using 100 nM fMLP in a micropipette. The chemotactic behavior of untreated cells and of cells treated with 1 mM KCN or 10 µM rotenone for 10 min was recorded and analyzed as shown in Fig. 1 (see also Video 1, bottom). (C and D) PMNs were treated with 1 µM CCCP (10 min) or not and exposed to a chemotactic gradient using a micropipette loaded with 10 µg/ml IL-8 or 1 µM LTB4, and the chemotactic behavior of cells was recorded. Data shown are representative results obtained with cells from at least three different individuals with at least 20 cells analyzed in each individual experiment (data shown are means ± SD [error bars]). Statistical analysis was done with Student's *t* test; *, $P < 0.05$, compared with fMLP control.

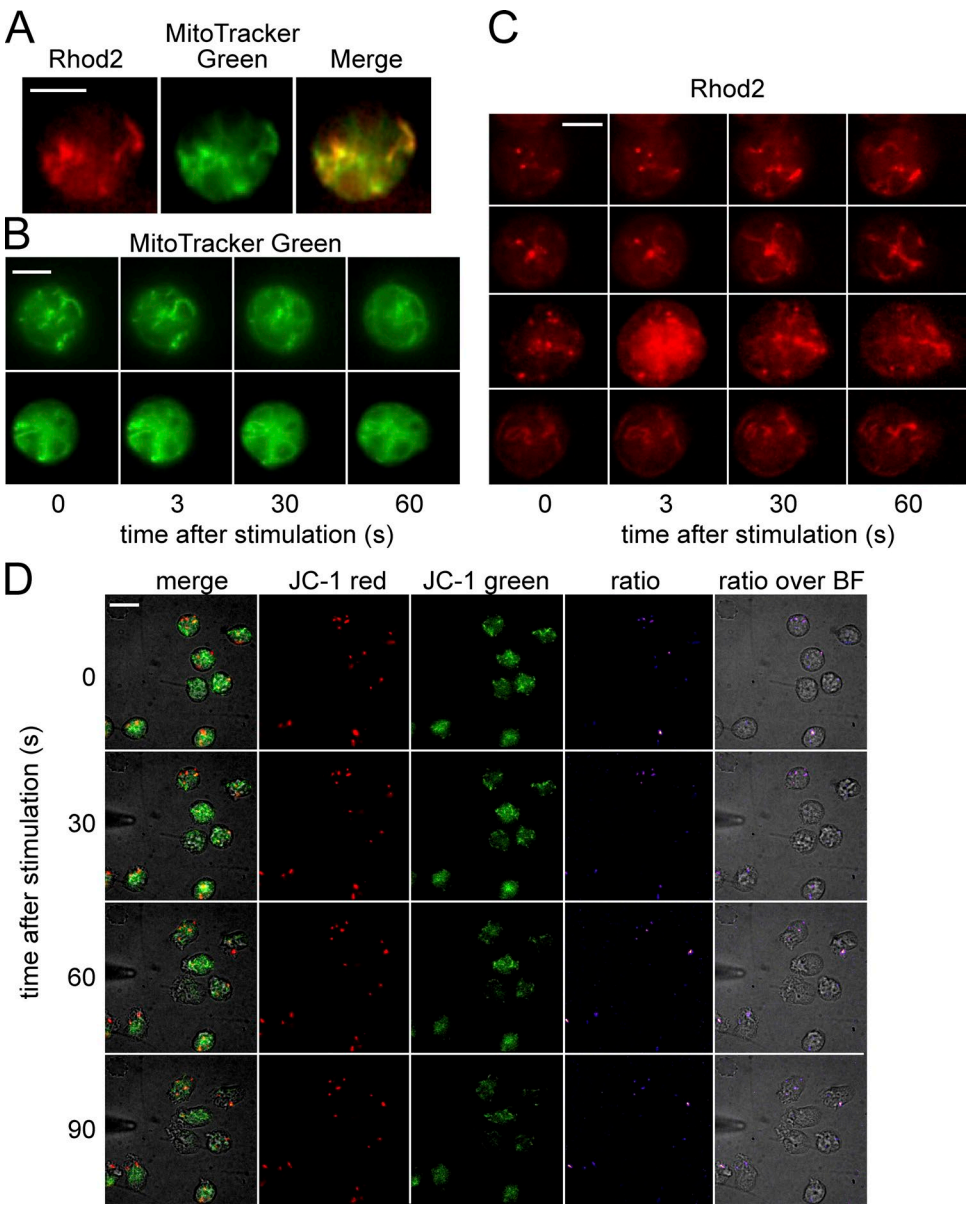


Figure S2. **Differential activation of mitochondria in PMNs.** (A) Costaining of 1 µM Rhod2 (5 min) and 100 nM MitoTracker green (20 min) indicates that Rhod2 staining is associated with mitochondria. (B) MitoTracker green–labeled mitochondria remained globally distributed after stimulation of PMNs in a chemotactic gradient field. Two representative cells are shown. (C) Stimulation of neutrophils loaded with Rhod2 revealed increased mitochondrial Ca^{2+} uptake and differential distribution of activated mitochondria within the cell (four representative cells are shown). Time-lapse images were taken with a fluorescence microscope (DMI6000 B; Leica; objective: 100× oil, NA 1.30; DFC365 FX camera; Leica; bar, 5 µm; see also Video 3). (D) Differential distribution of mitochondria with low and high membrane potential in polarized cells. Additional examples of JC-1–labeled (100 ng/ml, 20 min) PMNs showing redistribution of mitochondrial structures with different membrane potential levels (high: red, JC-1; low: green, JC-1; black, no potential) during cell polarization. Time-lapse images were taken with a fluorescence microscope (DMI6000 B; Leica; objective: 100× oil, NA 1.30; Spot Boost EMCCD camera, EM 150; bar, 10 µm; see also Video 4).

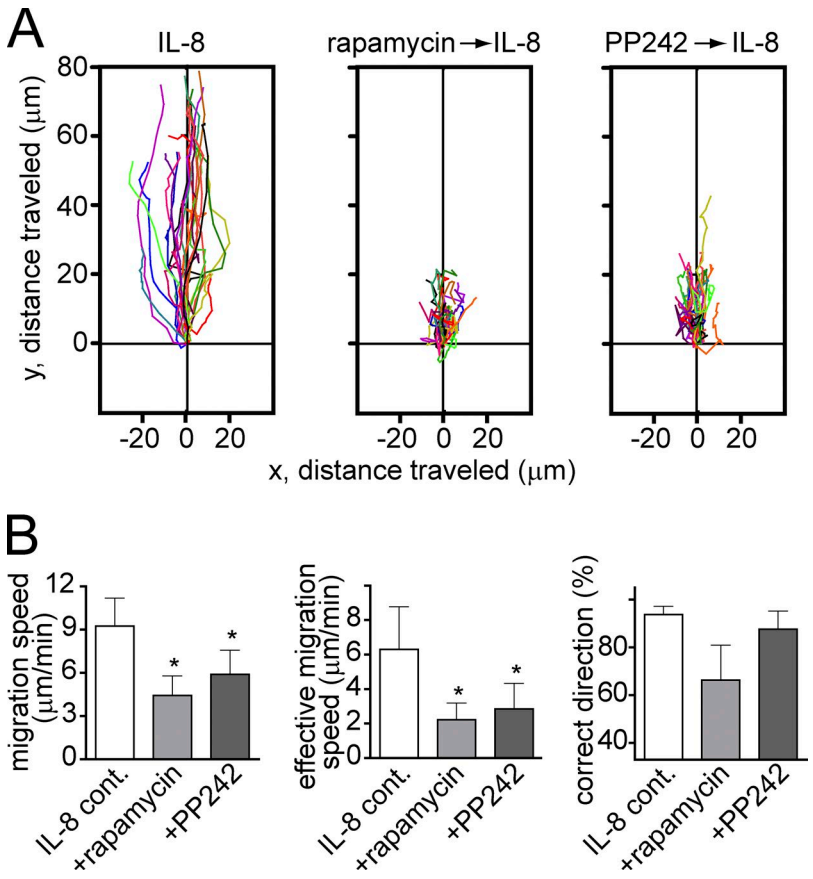


Figure S3. **Inhibition of mTOR by rapamycin or PP242 impairs IL-8–induced PMN chemotaxis.** (A) Freshly isolated primary human PMNs were exposed to a chemotactic gradient using 10 μg/ml IL-8 in a micropipette, and cell migration was monitored. The data shown are from a single representative experiment out of four experiments done with cells from two different donors. (B) The chemotactic behavior of untreated cells and of cells pretreated for 30 min with 1 μM rapamycin or 1 μM PP242 was recorded (data shown are means ± SD [error bars]; see also Video 5, bottom).

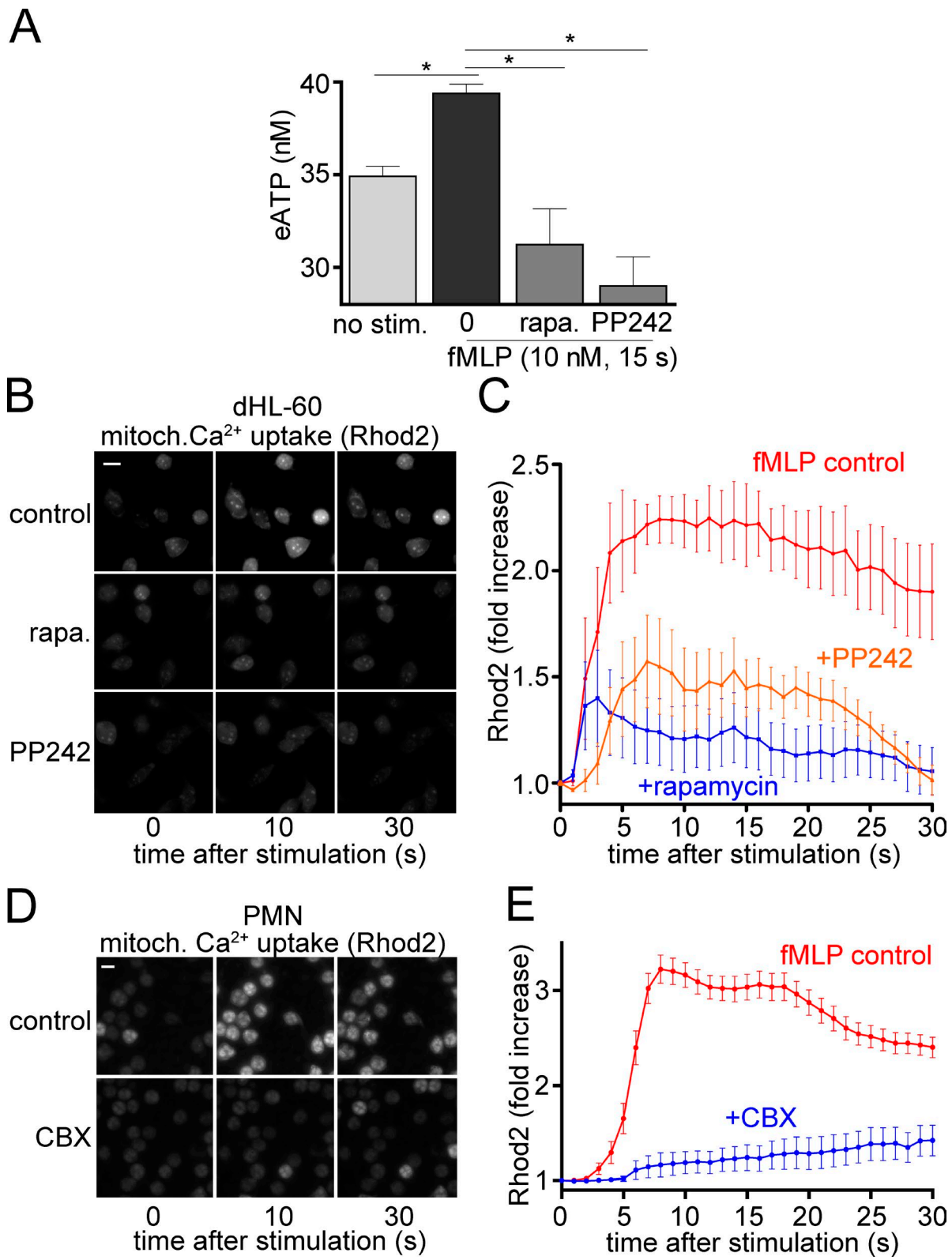


Figure S4. **Inhibition of mTOR impairs FPR-induced ATP release and mitochondrial activation.** (A) Purified human PMNs (10^6/ml) were incubated with or without 1 µM rapamycin or 1 µM PP242 for 30 min and stimulated with 10 nM fMLP for 15 s, and ATP concentrations in supernatants were measured with a luciferin/luciferase bioluminescence assay kit. (B and C) Differentiated HL-60 cells were loaded with Rhod2 and incubated with or without 1 µM rapamycin or 1 µM PP242 for 30 min, and mitochondrial Ca^{2+} uptake in response to 1 nM fMLP was assed as described in Fig. 2 using microscopy (bar, 10 µm; see also Video 7). (D and E) Freshly isolated PMNs were loaded with Rhod2 as described in Fig. 2 and incubated for 5 min with or without the ATP release blocker carbenoxolone (CBX; 100 µM), and mitochondrial Ca^{2+} uptake after fMLP (1 nM) stimulation was monitored in real time using fluorescence microscopy (DMI6000 B; Leica; objective: 100× oil, NA 1.30; DFC365 FX camera; Leica; bar, 10 µm; see also Video 8). Data are shown as means ± SD (error bars) of normalized gray values from 15–25 cells.

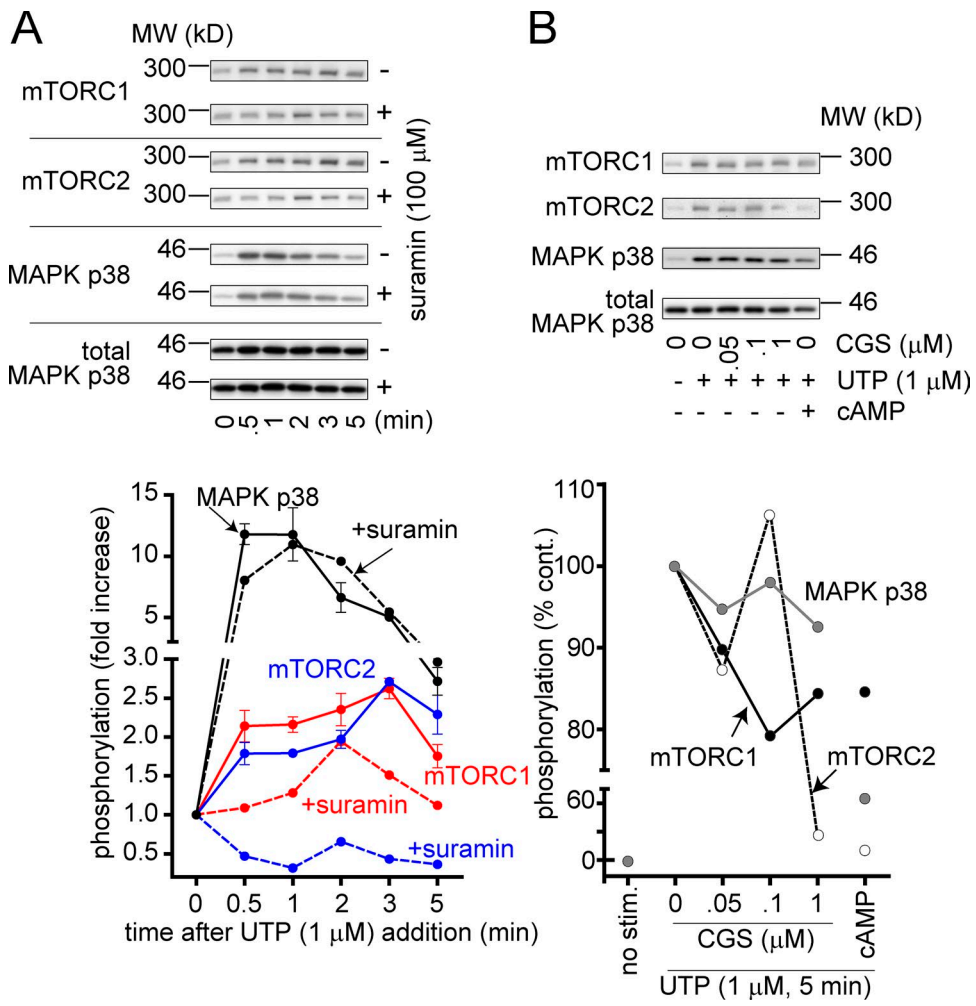
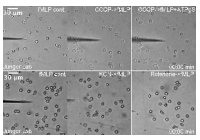
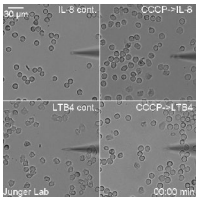


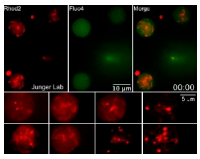
Figure S5. **Blocking P2 receptors with suramin or activating A2a receptors with CGS21680 reduces P2Y2-induced mTOR and MAPK p38 phosphorylation.** (A) Differentiated HL-60 cells were pretreated with or without 100 μM suramin for 5 min and stimulated with 1 μM UTP for the indicated times. (B) Cells were treated for 5 min with the indicated concentrations of the A2a agonist CGS21680 and the P2Y2 receptor agonist UTP (1 μM). Activation of MAPK p38, mTORC1, and mTORC2 signaling was determined by immunoblotting as described in Fig. 5. Total MAPK p38 antibodies were used as a protein-loading control. Data are expressed as means ± SD (error bars) of at least three independent experiments; Student's *t* test; *, $P < 0.05$, compared with corresponding controls.



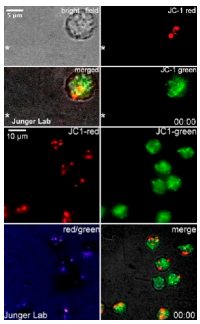
Video 1. **Inhibition of mitochondria abolishes FPR-induced PMN chemotaxis.** Freshly isolated human PMNs were plated onto glass coverslips coated with 40 μg/ml human fibronectin and placed into a temperature-controlled stage incubator at 37°C. Cells were incubated for 10 min with or without 1 μM CCCP ± 100 μM ATPγS (top) or with or without 1 mM potassium cyanide (KCN) or 10 μM rotenone (bottom) and exposed to a chemotactic gradient using a micropipette loaded with 100 nM fMLP, and bright field images of cells migrating to the tip of the micropipette were recorded in real time using video microscopy (DMI6000 B; Leica; objective: 20×, air, NA 0.40; Spot Boost EMCCD camera, EM 50). Frames were taken every 20 s for 7 min.



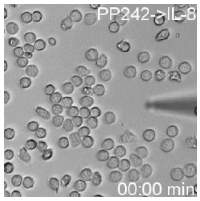
Video 2. **Inhibition of mitochondria by CCCP reduces PMN chemotaxis in response to IL-8 or LTB4.** PMNs were incubated for 10 min with or without 1 μM CCCP, and bright field images of chemotaxis toward a micropipette loaded with 10 μg/ml IL-8 or 1 μM LTB4 were recorded over time as described in the legend for Video 1 using video microscopy (DMI6000 B; Leica; objective: 20×, air, NA 0.40; DFC365 FX camera; Leica). Frames were taken every 20 s for 7 min.



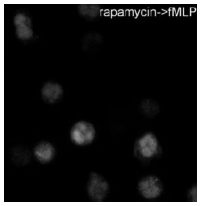
Video 3. **FPR stimulation triggers mitochondrial Ca^{2+} uptake.** PMNs plated onto glass coverslips were loaded with 5 µM Fluo4 (20 min; green) and 1 µM Rhod2 (5 min; red), washed with HBSS, and exposed to a chemotactic gradient using a micropipette as described above. The micropipette was loaded with 100 nM fMLP and fluorescein (green) to allow recording of the fMLP gradient and of Ca^{2+} signaling (top). In addition, several examples of cells with Rhod2 (red) were included (bottom). The bright field, Rhod2 (red), and Fluo-4/fluorescein (green) channels were recorded in real time with a fluorescence microscope (DMI6000 B; Leica; objective: 100× oil, NA 1.30; DFC365 FX camera; Leica). Frames were taken every 1 s for 1 min.



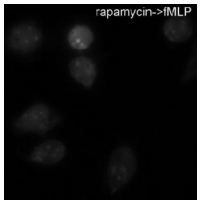
Video 4. **Mitochondria with higher membrane potential accumulate at the front of cells.** PMNs were loaded with 100 ng/ml JC-1 (20 min), washed with HBSS, and exposed to a chemotactic gradient using a micropipette containing 100 nM fMLP (tip indicated by asterisk), and red and green JC-1 fluorescence signals as well as bright field images were acquired over time (top) by fluorescence microscopy (DMI6000 B; Leica; objective: 100× oil, NA 1.30; Spot Boost EMCCD camera, EM 150). More examples of cells stained with JC-1 were added, and red/green ratio data were included (bottom). Frames were taken every 1 s for 1.5 min.



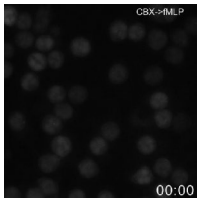
Video 5. **Inhibition of mTOR impairs PMN chemotaxis.** PMNs were incubated for 30 min with or without 1 µM rapamycin or 1 µM PP242, and bright field images of chemotaxis to a micropipette loaded with 100 nM fMLP (top) or 10 µg/ml IL-8 (bottom) were recorded by time-lapse video microscopy (DMI6000 B; Leica; objective: 20×, air, NA 0.40; DFC365 FX camera; Leica). Frames were taken every 20 s for 7 min.



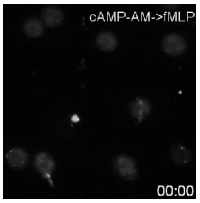
Video 6. **Blocking mTOR impairs FPR-induced mitochondrial activation in PMNs.** PMNs were loaded with 1 µM Rhod2 (5 min), washed, and incubated for 30 min with or without 1 µM rapamycin or 1 µM PP242, and real-time images of mitochondrial Ca^{2+} uptake in response to 1 nM fMLP were recorded by fluorescence microscopy (DMI6000 B; Leica; objective: 100× oil, NA 1.30; DFC365 FX camera; Leica). Frames were taken every 1 s for 30 s.



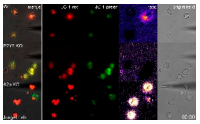
Video 7. **Blocking mTOR impairs mitochondrial activation in HL-60 cells.** Differentiated HL-60 cells were loaded with 1 µM Rhod2 (5 min) and treated with rapamycin or PP242 and stimulated with fMLP, and movies of mitochondrial Ca^{2+} uptake were recorded in real time by fluorescence microscopy (DMI6000 B; Leica; objective: 100× oil, NA 1.30; DFC365 FX camera; Leica). Frames were taken every 1 s for 30 s.



Video 8. **CBX abolishes FPR-induced mitochondrial activation.** PMNs were loaded with Rhod2 and incubated for 5 min with or without 100 µM CBX, and images of fMLP-induced mitochondrial Ca^{2+} uptake were recorded by time-lapse video microscopy (DMI6000 B; Leica; objective: 100× oil, NA 1.30; DFC365 FX camera; Leica). Frames were taken every 1 s for 30 s.



Video 9. **cAMP impairs FPR-induced mitochondrial activation.** PMNs were loaded with Rhod2 and incubated for 20 min with or without 1 µM cAMP-AM, and fMLP-induced mitochondrial Ca^{2+} uptake was recorded by fluorescence microscopy (DMI6000 B; Leica; objective: 100× oil, NA 1.30; DFC365 FX camera; Leica). Frames were taken every 1 s for 30 s.



Video 10. **Defective mitochondrial activation and chemotaxis of neutrophils from P2Y2 and A2a receptor knockout (KO) mice.** Neutrophils were isolated from the peritoneal cavities of wild-type (WT) mice or P2Y2 and A2a receptor KO mice. Cells were loaded with JC-1 and FPR-induced polarization (bright field), and mitochondrial membrane potential changes (red and green for JC-1 red and JC-1 green, respectively) were recorded by fluorescence microscopy (DMI6000 B; Leica; objective: 100× oil, NA 1.30; DFC365 FX camera; Leica). Frames were taken every 5 s for 2 min.