Supplemental Materials

Molecular Biology of the Cell

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Table S1. Matrix of probabilities of statistical significance for pair-wise comparisons of cell-surface expression of pro- and anti-inflammatory markers in macrophages cultured under the conditions presented in Figure 1.

	M/IL4	M/IFNγ	M/IFNγ/ LPS	GM	GM/IFNγ	GM/IFNγ/ LPS
CD40						
M	0.79	0.0069	< 0.0001	0.62	0.017	< 0.0001
M/IL4		0.087	< 0.0001	> 0.99	0.19	< 0.0001
M/IFNγ			0.0025	0.14	> 0.99	0.0036
M/IFNγ/LPS				< 0.0001	0.0011	> 0.99
GM					0.30	< 0.0001
GM/IFNγ						0.0015
CD80						
M	0.22	0.21	< 0.0001	0.85	0.99	< 0.0001
M/IL4		> 0.99	< 0.0001	0.024	0.062	< 0.0001
M/IFNγ			< 0.0001	0.022	0.059	< 0.0001
M/IFNγ/LPS				< 0.0001	< 0.0001	0.052
GM					> 0.99	< 0.0001
GM/IFNγ						< 0.0001
CD163						
M	0.53	0.79	0.40	0.0013	0.0012	0.0011
M/IL4		> 0.99	> 0.99	0.038	0.034	0.032
M/IFNγ			0.99	0.017	0.015	0.014
M/IFNγ/LPS				0.057	0.052	0.049
GM					> 0.99	> 0.99
GM/IFNγ						> 0.99
CD200R						
M	< 0.0001	0.43	0.15	0.21	0.061	0.062
M/IL4		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
M/IFNγ			0.99	> 0.99	0.86	0.87
M/IFNγ/LPS				> 0.99	> 0.99	> 0.99
GM					0.99	0.99
GM/IFNγ						> 0.99

Cell-surface expression levels of CD40, CD80, CD163, and CD200R in macrophages cultured as described in Figure 1 were measured by flow cytometry and expressed as percent of maximum values in each experiment. The significance of the differences in the expression of each marker between all pairs of macrophages were analysed by one-way ANOVA using Tukey's post-hoc multiple comparisons test.

Table S2. Matrix of probabilities of statistical significance for pair-wise comparisons of macropinocytic activity of macrophages cultured under the conditions described in Figure 3.

	M/IL4	M/IFNγ	M/IFNγ/ LPS	GM	GM/IFNγ	GM/IFNγ/ LPS
M	0.053	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
M/IL4		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
M/IFNγ			> 0.99	0.78	> 0.99	> 0.99
M/IFNγ/LPS				0.50	0.99	> 0.99
GM					0.87	0.54
GM/IFNγ						> 0.99

Macrophages were cultured under the conditions described in Figure 3 and the number of macropinosomes formed after a 15 min exposure to 70 kDa TMR-dextran was measured. The significance of the differences in the number of formed macropinosomes between all pairs of macrophages were analysed by one-way ANOVA using Tukey's post-hoc multiple comparisons test.

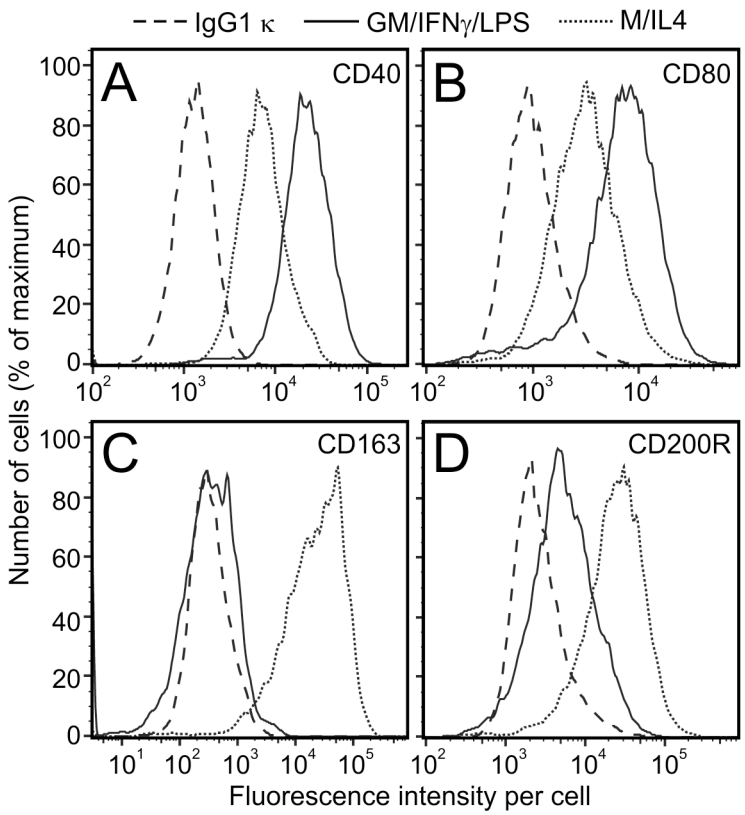

Figure S1. Cell-surface expression of pro- and anti-inflammatory markers in GM/IFNγ/LPS- and M/IL4-cultured macrophages. The histograms represent typical distributions of fluorescence intensities from experiments like those summarized in Figure 1. Only GM/IFNγ/LPS- and M/IL4-cultured macrophages are shown in this figure, but similar experiments were performed for the other five conditions described in Figure 1. GM/IFNγ/LPS- (solid line) and M/IL4-cultured (dotted line) cells were lifted and labeled with either FITC-conjugated anti-human CD40 (a pro-inflammatory marker; A), APC-conjugated anti-human CD80 (a pro-inflammatory marker; B), Alexa-647-conjugated anti-human CD163 (an anti-inflammatory marker; C), or PE-conjugated anti-human CD200R antibody (an anti-inflammatory marker; D). GM/IFNγ/LPS- and M/IL4-cultured cells were also labeled with isotype-matched (IgG1 κ isotype) control antibodies conjugated with either FITC (A), APC (B), Alexa-647 (C) or PE (D). Cell-surface labeling was determined by flow cytometry and the distributions of intensities for each antibody were normalized to the maximum for each experiment. The histograms are representative of 3 independent experiments of each type. Further experimental details, averages for median fluorescence intensities, and probabilities of statistical significance are presented in Figure 1 and Table S1.

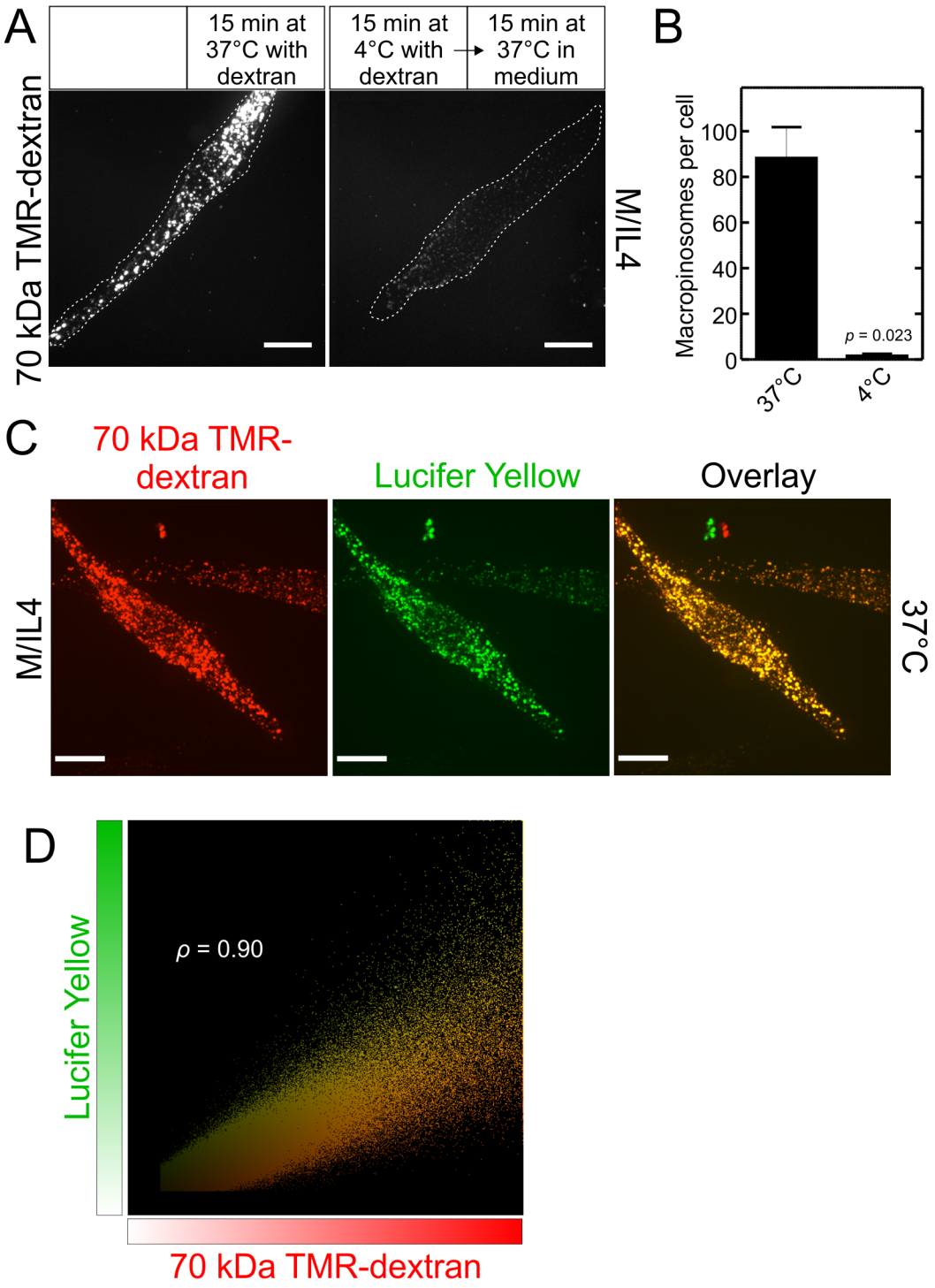

Figure S2. Validation of fluid-phase uptake of 70 kDa TMR-dextran. (A) M/IL4-cultured macrophages were incubated with fluorescently labelled 70 kDa dextran (TMR-dextran, 125 µg/ml) for 15 min either at 37ºC (left) or 4ºC (right) in calcium-containing medium. Cells were then washed and either imaged live immediately (left) or after a further 15 min incubation at

37ºC in dextran-free medium (right). (B) The number of macropinosomes (*i.e.*, TMR-positive vacuoles) per cell then was determined as described for Figure 2C. Data are means ± SEM of \ 19–21 cells from 3 independent experiments from at least 2 different donors. (C) M/IL4-cultured macrophages were incubated with both 70 kDa TMR-dextran (125 μg/ml) and Lucifer Yellow (125 μg/ml) for 15 min at 37ºC in calcium-containing medium. Cells were then washed and imaged live immediately by spinning disc confocal microscopy. Channels for both TMR-dextran and Lucifer Yellow were recorded and their overlay is shown in the right panel. (D) Correlation of the red and the green fluorescence intensity for individual pixels in the images shown in C. The Pearson correlation coefficient (ρ) of the image shown was 0.90 and the average Pearson coefficient from 10 additional cells was 0.83 ± 0.02. Scale bars, 15 μm.

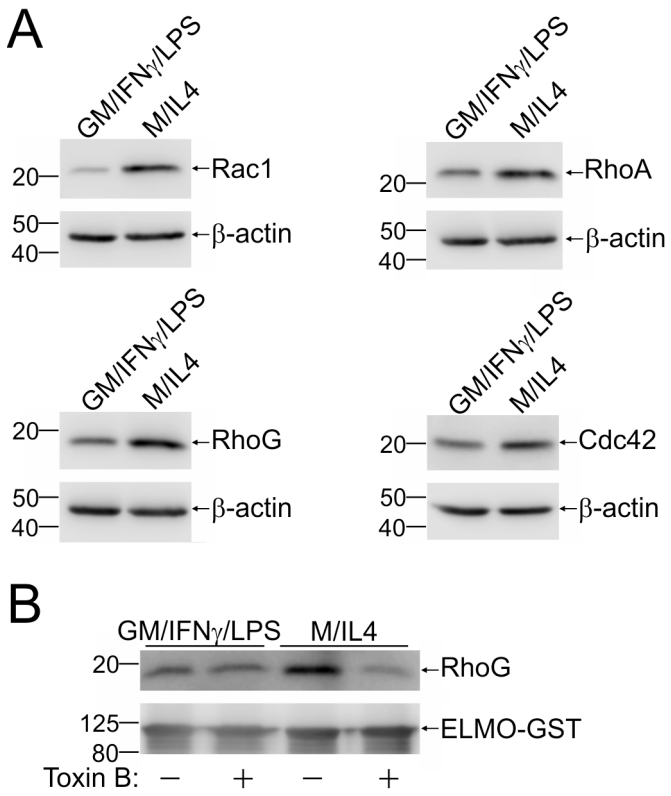

Figure S3. Typical immunoblots of Rac1, RhoA, RhoG, and Cdc42, and of RhoG pull-downs. (A) GM/IFNγ/LPS- and M/IL4-cultured macrophages were lysed, separated by 10% SDS-PAGE, and subjected to immunoblotting with anti-Rac1, anti-RhoA, anti-RhoG, or anti-Cdc42 antibodies. The blots were stripped and re-blotted with anti-β-actin antibody, as a loading control. The immunoblots are representative of 3–4 independent experiments. (B) GM/IFNγ/LPS- and M/IL4-cultured macrophages were lysed, cleared by centrifugation at 14,000 g, followed by immediate mixing and incubation with recombinant ELMO-GST loaded onto glutathione-Sepharose beads, followed by washing and centrifugation at 500 g. Pulled down fractions were separated by 10% SDS-PAGE and immunoblotted with an anti-RhoG antibody. The blots were stripped and re-blotted with anti-GST antibody, as a loading control. Treatment with *C. difficile* toxin B (3 h in serum-free medium) was used as a negative control to inhibit RhoG. The immunoblot is representative of 4 independent experiments.

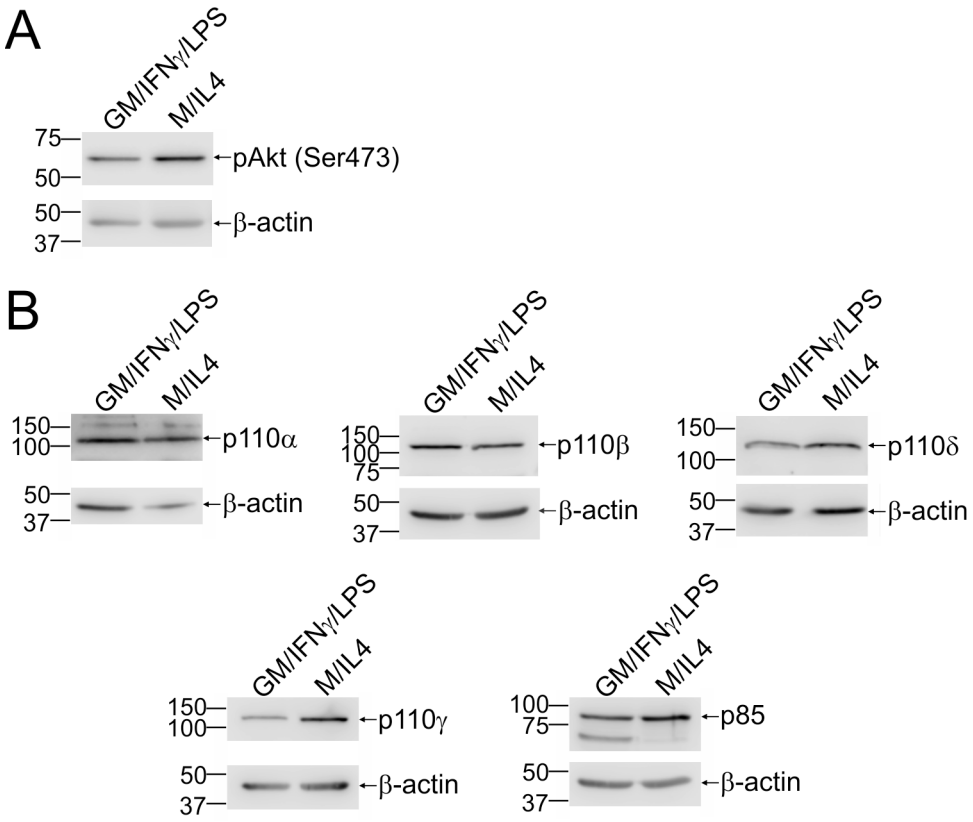

Figure S4. Typical immunoblots of phospho-Akt and different subunits of PtdIns3K. (A) GM/IFNγ/LPS- and M/IL4-cultured macrophages were lysed, separated by 10% SDS-PAGE, and subjected to immunoblotting with anti-phospho-Akt antibody. Then blots were stripped and re-blotted with anti-β-actin antibody, as a loading control. The immunoblot is representative of 4 independent experiments. (B) GM/IFNγ/LPS- and M/IL4-cultured macrophages were lysed, separated by 10% SDS-PAGE, and subjected to immunoblotting with anti-p110α, anti-p110β, anti-p110δ, anti-p110γ, or anti-p85 antibodies. The blots were stripped and re-blotted with anti-β-actin antibody, as a loading control. The immunoblots are representative of 3–6 independent experiments.

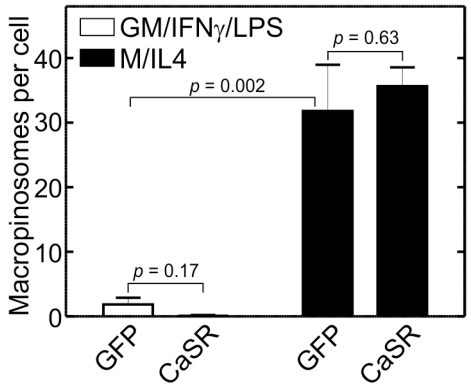

Figure S5. Overexpression of CaSR does not increase macropinocytic activity of GM/IFNγ/LPS-cultured macrophages. (A) GM/IFNγ/LPS- and M/IL4-cultured macrophages were transfected with fluorescently-tagged CaSR (SeP-CaSR) or GFP, as a control. After 24 h the cells were incubated with fluorescently labelled 70 kDa dextran (TMR-dextran, 125 μg/ml) for 15 min at 37ºC. Cells were then washed, fixed and imaged immediately; only transfected cells (SeP- or GFP-positive) were selected for measurements of macropinocytosis, which was quantified as the number of macropinosomes (*i.e.*, TMR-positive vacuoles) per cell, as described for Figure 2C. Data are means ± SEM of at 15–30 cells from 2–3 independent experiments.