Calcium-sensing receptors signal constitutive macropinocytosis and facilitate the uptake of NOD2 ligands in macrophages

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Macropinocytosis can be induced in several cell types by stimulation with growth factors. In selected cell types, notably macrophages and dendritic cells, macropinocytosis occurs constitutively, supporting the uptake of antigens for subsequent presentation. Despite their different mode of initiation and contrasting physiological roles, it is tacitly assumed that both types of macropinocytosis are mechanistically identical. We report that constitutive macropinocytosis is stringently calcium dependent, while stimulus-induced macropinocytosis is not. Extracellular calcium is sensed by G-protein-coupled calcium-sensing receptors (CaSR) that signal macropinocytosis through Gαi, phosphatidylinositol 3-kinase and phospholipase C. These pathways promote the recruitment of exchange factors that stimulate Rac and/or Cdc42, driving actin-dependent formation of ruffles and macropinosomes. In addition, the heterologous expression of CaSR in HEK293 cells confers on them the ability to perform constitutive macropinocytosis. Finally, we show that CaSR-induced constitutive macropinocytosis facilitates the sentinel function of macrophages, promoting the efficient delivery of ligands to cytosolic pattern-recognition receptors.
Macropinocytosis is an actin-driven process whereby cells internalize large volumes of extracellular fluid, generating phase-bright vacuoles (> 250 nm). Many cell types generate such vacuoles, known as macropinosomes, in response to growth factor stimulation. In these instances, macropinocytosis is intended for nutrient acquisition, representing a major amino-acid supply route that enables cell growth. Other proposed functions include recycling of adhesion receptors to the leading edge of migratory cells, bulk membrane retrieval and growth cone collapse in nerve cells.

To trigger macropinocytosis, growth factors remodel the lipid microenvironment. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) is converted to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) by phospholipase C and inositol 3,4,5-phosphatase. Calcium-sensing receptors in constitutive macropinocytosis.

**Results**

Macropinosomes are branched actin-driven structures that control the activity of Rho-family GTPases. One such GEF, T-cell lymphoma invasion and metastasis-inducing protein 1 (TIAM1), has both a PtdIns(3,4,5)P$_3$-binding PH domain and a PtdOH-interacting polybasic domain that mediate its recruitment to nascent ruffles. The subsequent recruitment of Rac1 promotes the formation of branched actin networks that drive membrane extensions and macropinocytic cup formation. Other Rho family GTPases, such as Cdc42 and RhoG, are also required for macropinocytosis, although details of their function remain less clear.

Although macropinocytosis can be induced in a variety of cell types by growth factors and other stimuli, dendritic cells (DCs) and macrophages share a unique behaviour: they perform macropinocytosis constitutively. This intrinsic process is astonishingly active: macrophages were estimated to internalize the equivalent of their entire cell surface every 33 min (ref. 12), while human DCs take up > 1,000 μm$^3$ (ref. 3) of extracellular fluid—roughly 40% of the volume of the cell—every hour. This feature is studied in detail and reveals the involvement of calcium-sensing receptors in constitutive macropinocytosis.

The spatially and temporally restricted changes in lipid composition contribute to the recruitment of several protein effectors, including guanine nucleotide exchange factors (GEFs) that control the activity of Rho-family GTPases. One such GEF, EIPA, is intended for nutrient acquisition, representing a major amino-acid supply route that enables cell growth.

Figure 1 | Two distinct modes of macropinocytosis exist in hMDMs. (a) hMDMs were incubated with fluorescently labelled 70 kDa dextran (0.025 mg ml$^{-1}$) for 15 min at 37°C, in either the presence or absence of M-CSF (200 ng ml$^{-1}$). Cells were then washed and imaged immediately by spinning disc confocal microscopy. (b) Macropinosomes were then counted using ImageJ software and the diameter of each macropinosome was recorded and plotted. A size cutoff (dashed line) was set to distinguish between constitutive and growth factor-induced macropinosomes. The number of macropinosomes under (c) or above (d) 3 μm in diameter per cell was plotted. hMDMs were pretreated with HOE-694 (10 μM), EIPA (10 μM), latrunculin A (2 μM) or LY294002 (10 μM) for 1h and then incubated with fluorescently labelled 70 kDa dextran (0.025 mg ml$^{-1}$) for 15 min at 37°C, in either the presence or absence of M-CSF (200 ng ml$^{-1}$). The number of macropinosomes under (e) or above (f) 3 μm in diameter is plotted. Data represent the means ± s.e.m. of at least three independent experiments using cells from at least 2 separate healthy donors. Scale bar, 10 μm. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, NS, not significantly different.
We proceeded to compare constitutive and growth factor-stimulated macropinocytosis. To analyse constitutive macropinocytosis hMDDMs were bathed in serum-free medium for the duration of the dextran uptake period (15 min; similar results were obtained when the cells were, in addition, preincubated in the absence of serum for up to 16 h). hMDDMs formed numerous cytosolic vacuoles containing 70 kDa dextran, whether incubated in the presence or absence of macrophage colony-stimulating factor (M-CSF; Fig. 1a). The size of the resultant macropinosomes was, however, different under both conditions: vacuoles larger than 3 μm were often formed when M-CSF was present, while smaller macropinosomes were generated in its absence (Fig. 1a). We applied a lower size cutoff of 0.75 μm to exclude endosomes and set an arbitrary size threshold at 3 μm to distinguish between the two forms of macropinocytosis (Fig. 1b). Note that macropinosomes smaller than 3 μm were observed under both conditions and their frequency was unaltered by M-CSF. In contrast, the number of macropinosomes larger than 3 μm was negligible in the absence of M-CSF, but increased on exposure to the growth factor (Fig. 1c,d). These observations suggest that the thresholding approach appropriately distinguished between macropinosomes formed constitutively and those induced by M-CSF. While the 15 min allowed for dextran uptake yielded large and reliable signals, it was conceivable that the occurrence of fusion or fission events during this period made the quantification of macropinosomes inaccurate. However, we found that a much shorter uptake period (3 min) yielded very similar results (Supplementary Fig. 1a–d). Moreover, we monitored macropinosomes loaded by 1-min incubation with 70 kDa dextran for the subsequent 15 min and found that while their size decreased and fluorescence intensity increased—likely as a consequence of fission of small vesicles devoid of dextran—their number was not significantly altered (Supplementary Fig. 1e,f), and that they mix minimally with the late endosomal compartment (Supplementary Movie 1). Altogether, these findings validate the accuracy of the method used to quantify macropinosome formation.

We next confirmed that, under the conditions described above, the vacuoles containing dextran were bona fide macropinosomes. Macropinocytosis is typically defined as an amiloride-sensitive, phosphatidylinositol 3-kinase (PtdIns3K)- and actin-dependent process19. Using the amiloride derivatives HOE-694 and 5-(N-ethyl-N-isopropyl)amiloride (EIPA), the PtdIns3K inhibitor LY294002 and the actin-disrupting agent latrunculin A, we assessed whether the vacuoles formed fulfilled the canonical definition of macropinosomes. Both the constitutive and stimulated forms of macropinocytosis were sensitive to the drugs tested, with the exception that the smaller (<3 μm) vacuoles were less sensitive to the amiloride derivatives than anticipated (Fig. 1e,f). The latter observation is reminiscent of earlier reports where the constitutive uptake of fluid by immature DCs was found to be similarly unaffected by amiloride derivatives20. Altogether, these observations establish that there are two morphologically distinct modes of macropinocytosis in hMDDMs and that these can be further distinguished by their sensitivity to amiloride derivatives.

**Constitutive macropinocytosis requires extracellular Ca\(^{2+}\).**

Our results suggest that macropinocytosis is more heterogeneous than previously appreciated. In this regard it resembles phagocytosis, which encompasses a variety of processes that while sharing some gross phenotypic properties, differ in their molecular mechanism. For instance, some forms of phagocytosis require calcium while others do not21. We used this criterion to establish whether the constitutive and M-CSF-induced forms of macropinocytosis are distinct. It is worth noting that calcium is required for optimal M-CSF signalling22. Strikingly, and contrary to our expectation, removal of extracellular calcium abolished the constitutive form of macropinocytosis, but did not affect M-CSF-induced macropinocytosis (Fig. 2a–c; Supplementary Fig. 1a–d). Similar results were obtained when, instead of counting the number of macropinosomes formed, the quantity of 70 kDa dextran taken up was assessed by flow cytometry (Supplementary Fig. 3a–c). The effect was reversible, as the readdition of calcium to cells in calcium-free medium restored constitutive macropinocytosis (Fig. 2d). By contrast, removal of extracellular calcium did not affect the uptake of transferrin, nor the distribution of Rab5 and LAMPI, indicating that other forms of endocytosis remained unaltered (Supplementary Fig. 2a–c).

The preceding results imply that constitutive macropinocytosis is a calcium-dependent event; indeed, the rate of macropinocytosis was directly correlated to the concentration of extracellular calcium (Fig. 2e). To ensure that this feature is common to phagocytes from other species, we tested murine bone marrow-derived macrophages (mBMDMs); these cells proved to be equally sensitive to omission of calcium (Supplementary Fig. 3d; Fig. 2g), as were human monocyte-derived dendritic cells (hMDDCs; Fig. 2f,g), which also perform macropinocytosis constitutively8,20. We also tested A431 cells, an epidermal cell line that expresses high levels of the epidermal growth factor (EGF) receptor. In these cells macropinocytosis is negligible in the absence of growth factors, but it is greatly stimulated by addition of EGF (Fig. 2h,i). Removal of calcium had no effect on the induction of macropinocytosis by EGF (Fig. 2h,i). Altogether these data demonstrate that the constitutive form of macropinocytosis in phagocytes requires extracellular calcium; by contrast, growth factor-induced macropinocytosis is calcium independent.

**Ca\(^{2+}\) does not affect constitutive macropinocytosis.**

Influx of extracellular calcium is an essential component of cytosolic calcium ([Ca\(^{2+}\)]\(_i\)) homeostasis. Removal of external calcium eliminates [Ca\(^{2+}\)]\(_i\), transients that result from influx across the plasma membrane, and causes a progressive decrease in the steady state [Ca\(^{2+}\)]\(_i\). Alterations in [Ca\(^{2+}\)]\(_i\), a well-acknowledged second messenger, can conceivably account for the impairment of macropinocytosis observed when external calcium was omitted. Indeed, a variety of Ca\(^{2+}\)-dependent effectors, including myosin light-chain kinase23,24, protein kinase C25,26 and phospholipase C (PLC)26,27, are involved in both macropinocytosis and phagocytosis. To determine whether the effect of removing extracellular calcium on constitutive macropinocytosis was due to changes in [Ca\(^{2+}\)]\(_i\), we monitored [Ca\(^{2+}\)]\(_i\), and the efficiency of macropinocytosis simultaneously; [Ca\(^{2+}\)]\(_i\), was measured by ratio imaging using Fura2. The removal of extracellular calcium had a minimal effect on [Ca\(^{2+}\)]\(_i\), within a timeframe relevant to the macropinocytosis uptake assays: [Ca\(^{2+}\)]\(_i\), was not significantly different when measured before and shortly (∼30 s) after external calcium removal (Fig. 3a,b). Even when measured after 15 min, the end point of the macropinocytosis determinations, [Ca\(^{2+}\)]\(_i\), had decreased by only 19 nM, from 107 ± 4 nM to 88 ± 3 nM (means ± s.e. of 28 determinations; Supplementary Fig. 4a). Under these conditions, constitutive macropinocytosis was virtually abolished (Fig. 3b). We used thapsigargin to rule out the possibility that the minute change in basal [Ca\(^{2+}\)]\(_i\), that develops during the course of the assay was responsible for the inhibition of macropinocytosis. By inhibiting the Ca\(^{2+}\)-ATPase, thapsigargin causes net release of calcium from the endoplasmic reticulum. In cells bathed in calcium-free medium, this is manifested as an increase in [Ca\(^{2+}\)]\(_i\), (Fig. 3a and Supplementary Fig. 4b). While the increase is transient, it
nevertheless suffices to maintain \([\text{Ca}^{2+}\]) above the baseline level throughout the macropinocytosis assay. This enabled us to test the effects of external calcium removal under conditions where \([\text{Ca}^{2+}\]) did not decrease. As shown in Fig. 3b, macropinocytosis was nevertheless obliterated. These findings imply that the inhibitory effect of calcium removal is not attributable to decreased \([\text{Ca}^{2+}\]), and suggest that \([\text{Ca}^{2+}\]) transients do not suffice to induce macropinocytosis.

It was conceivable that juxtamembrane rapid \([\text{Ca}^{2+}\]) transients, not detectable by global measurements of \([\text{Ca}^{2+}\]), were responsible for macropinocytosis. We used 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), a fast calcium chelator, to evaluate this possibility. We loaded cells with BAPTA and confirmed that the chelator effectively buffered \([\text{Ca}^{2+}\]) transients. As illustrated in Fig. 3a, the \([\text{Ca}^{2+}\]) elevation normally induced by thapsigargin was eliminated when cells were preloaded with BAPTA. Importantly, buffering \([\text{Ca}^{2+}\]) with BAPTA had no effect on constitutive macropinocytosis (Fig. 3b).

These experiments demonstrate that the inhibition of constitutive macropinocytosis associated with external calcium removal was not caused by elimination of \([\text{Ca}^{2+}\]) transients or by reduction in the resting level of \([\text{Ca}^{2+}\]). Instead, extracellular calcium appears to be exerting its effect on the outer surface of the cell.

**CaSR is necessary for constitutive macropinocytosis.** In specialized cell types, the level of extracellular calcium is monitored by a G-protein-coupled receptor, CaSR. CaSR is expressed in myeloid cells, where it has been implicated in the activation of the inflammasome pathway\(^{28}\). We therefore considered the possibility that CaSR was involved in constitutive macropinocytosis. We initially confirmed that CaSR is expressed in macrophages: unlike HEK293, which were shown earlier to lack CaSR\(^{29}\), transcript encoding the receptor was readily detectable by RT–PCR in hMDMs (Fig. 4a). We next investigated whether activation of CaSR sufficed to elicit macropinocytosis. To this end we used HEK293 cells, which lack the receptor and do not normally perform macropinocytosis (top panel, Fig. 4d). We first confirmed that a fraction of the heterologously expressed CaSR was able to traffic to the plasma membrane in these cells. A CaSR fusion protein containing an extracellular superecliptic phluorin (SeP) tag was readily detected on the surface of non-permeabilized HEK293 cells using an anti-GFP antibody (Fig. 4b). Strikingly, heterologous expression of CaSR not only induced vigorous ruffling in HEK293 cells, as reported\(^{29}\), but sufficed to induce constitutive macropinocytosis (Fig. 4c,d). Using flow cytometry we determined that CaSR-expressing cells accumulated 70 kDa dextran preferentially, compared to untransfected HEK293 cells (Fig. 4e,f). As anticipated, the constitutive macropinocytosis of CaSR-expressing HEK293 cells was reduced by removal of extracellular calcium (Supplementary Fig. 5a).

**Figure 2 | Removal of extracellular calcium abolishes constitutive, but not growth factor-induced macropinocytosis.** (a) hMDMs were incubated with fluorescently labelled 70 kDa dextran (0.025 mg ml\(^{-1}\)) for 15 min at 37 °C, in the presence or absence of M-CSF (200 ng ml\(^{-1}\)), in either calcium-containing or calcium-free medium (see Methods). The number of macropinosomes under (b) or above (c) 3 μm in diameter is plotted. (d) hMDMs were preincubated in either calcium-containing or calcium-free medium and then allowed to undergo macropinocytosis for 15 min at 37 °C. The total number of macropinosomes per cell is plotted. (e) hMDMs were incubated with fluorescently labelled 70 kDa dextran (0.025 mg ml\(^{-1}\)) for 15 min at 37 °C in Na\(^+\)-rich buffer containing the indicated concentrations of CaCl\(_2\). The total number of macropinosomes per cell is plotted. (f) hMDDCs were incubated with fluorescently labelled 70 kDa dextran (0.025 mg ml\(^{-1}\)) for 15 min at 37 °C, in either calcium-containing or calcium-free medium. (g) hMDDCs and murine BMDMs were incubated with fluorescently labelled 70 kDa dextran (0.025 mg ml\(^{-1}\)) for 15 min at 37 °C, in either calcium-containing or calcium-free medium. The total number of macropinosomes per cell is plotted. (h) A431 cells were incubated with fluorescently labelled 70 kDa dextran (0.025 mg ml\(^{-1}\)) for 15 min at 37 °C, in the presence or absence of EGF (200 ng ml\(^{-1}\)), in either calcium-containing or calcium-free medium. The total number of macropinosomes per cell is plotted for each condition, as indicated. Data represent the means ± s.e.m. of at least three independent experiments using cells from at least two separate healthy donors. Scale bar, 10 μm. *P≤0.05, ***P≤0.001, ****P≤0.0001, NS, not significantly different.
experiments using cells from at least two separate healthy donors. Data represent the means ± s.e.m. of at least three independent determinations (± s.e.m.). (b) hMDMs were preloaded with Fura2-AM and treated with or without thapsigargin (100 nM), BAPTA-AM (10 μM), and extracellular calcium as indicated. The total number of macropinosomes per cell and the corresponding \([\text{Ca}^{2+}]_i\) were then plotted. Data represent the means ± s.e.m. of at least three independent experiments using cells from at least two separate healthy donors. ***p<0.001, ****p<0.0001, NS, not significantly different.

That CaSR is in fact responsible for constitutive macropinocytosis in myeloid cells was verified using NPS2143, a selective antagonist of the receptor. Exposure of hMDMs to NPS2143 resulted in a dose-dependent inhibition of constitutive macropinocytosis (Fig. 4f,h), accompanied by elimination of the spontaneous ruffling (Supplementary Movie 2 and 3). NPS2143 also potently inhibited constitutive macropinocytosis, indicating a requirement for Gz-dependent signalling (Fig. 5a,b). PTX also inhibited macropinocytosis, albeit to a lesser extent, suggesting that Gz-dependent signalling is at least partially involved (Fig. 5b). The observed inhibition could have resulted from suppression of a stimulatory signal, or from the generation of an inhibitory signal. Cytosolic 3’-5’-cyclic AMP (cAMP) levels are controlled by both stimulatory (Gz) and inhibitory (Gs) G proteins, and inhibition of the latter is anticipated to elevate cAMP. Increased cAMP could conceivably account for the observations, because it controls the phosphorylation and activity of vasodilator-stimulated phosphoprotein (VASP), a regulator of actin filament elongation; of note, VASP has been implicated in the reorganization of the actin cytoskeleton that underlies macropinocytosis. Indeed, we found that removal of calcium or inhibition of CaSR by NPS2143 increased the phosphorylation of VASP, likely due to stimulation of protein kinase A activity in response to elevated cAMP.

Although suggestive, this pharmacological evidence is not conclusive, due to possible off-target effects of these drugs. More definitive evidence of the involvement of CaSR in constitutive macropinocytosis was obtained by silencing the expression of the receptor with siRNA. Using electroporation, we achieved a ≈70% reduction in the levels of CaSR transcript, as determined by quantitative PCR, in hMDMs (Fig. 4j). This was accompanied by a commensurate reduction in the macropinocytosis efficiency (Fig. 4j). Collectively, these experiments demonstrate that CaSR is required for constitutive macropinocytosis in macrophages.

CaSR signals constitutive macropinocytosis via Gz. Like other serpentine receptors, CaSR couples to heterotrimeric G proteins. To assess the involvement of G proteins in macropinocytosis we used the recently described pan-Gz inhibitor, BIM46187 (refs 31,32), and the Gz, inhibitor, pertussis toxin (PTX). BIM46187 potently inhibited constitutive macropinocytosis, indicating a requirement for Gz-dependent signalling (Fig. 5a,b). PTX also inhibited macropinocytosis, albeit to a lesser extent, suggesting that Gz-dependent signalling is at least partially involved (Fig. 5b). The observed inhibition could have resulted from suppression of a stimulatory signal, or from the generation of an inhibitory signal. Cytosolic 3’-5’-cyclic AMP (cAMP) levels are controlled by both stimulatory (Gz) and inhibitory (Gs) G proteins, and inhibition of the latter is anticipated to elevate cAMP. Increased cAMP could conceivably account for the observations, because it controls the phosphorylation and activity of vasodilator-stimulated phosphoprotein (VASP), a regulator of actin filament elongation; of note, VASP has been implicated in the reorganization of the actin cytoskeleton that underlies macropinocytosis. Indeed, we found that removal of calcium or inhibition of CaSR by NPS2143 increased the phosphorylation of VASP, likely due to stimulation of protein kinase A activity in response to elevated cAMP.

However, an even greater increase in VASP phosphorylation, induced by direct stimulation of adenyl cyclase with forskolin, had no effect on constitutive macropinocytosis (Fig. 5d). These observations imply that the inhibitory effects of BIM46187 and PTX on macropinocytosis are not a consequence of the increase in cAMP.

CaSR signals macropinocytosis via PtdIns3Kγ and PLCγ. Generation of PtdIns(3,4,5)P3 is required for the completion of macropinocytosis. We explored whether PtdIns3Kγ is involved in the activation of constitutive macropinocytosis by CaSR. It is noteworthy that the Gz subunit of heterotrimeric G proteins, which was shown above to be engaged by CaSR, can activate PtdIns3Kγ (refs 37,38). By transfecting into hMDMs a biosensor consisting of the PH domain of Akt fused to green fluorescent protein (GFP) (PH(Akt)-GFP), we detected the presence of PtdIns(3,4,5)P3 at the plasma membrane, with noticeable enrichment at sites of ruffling (Fig. 6a; Supplementary Movie 4). Strikingly, removal of extracellular calcium or pharmacological inhibition of CaSR resulted in depletion of PtdIns(3,4,5)P3 from the membrane and loss of membrane ruffles (Fig. 6a; Supplementary Movie 5). The accumulation of PtdIns(3,4,5)P3 in ruffles required Gz-dependent signalling, as BIM46187 caused dissociation of PH(Akt)-GFP from the plasma membrane and loss of ruffles (Fig. 6b). These data suggest that tonic stimulation of CaSR is required to maintain PtdIns3Kγ activity in resting hMDMs. To determine whether PtdIns3Kγ was responsible for the generation of PtdIns(3,4,5)P3 and the macropinocytosis induced by CaSR, we treated hMDMs with AS605240, a PtdIns3Kγ-specific inhibitor. AS605240 caused a dose-dependent decrease in constitutive...
macropinocytosis (Fig. 6c). We also generated mBMDMs from PtdIns3Kγ knockout mice and compared the efficiency of macropinocytosis to mBMDMs isolated from wild-type mice. Constitutive macropinocytosis was markedly reduced in mBMDMs lacking PtdIns3Kγ (Fig. 6d).

The generation of PtdIns(3,4,5)P3, fosters the recruitment of PLCγ1 through its PH domain. PLCγ1, along with PtdIns3K activity, serves to deplete PtdIns(4,5)P2 from sites of macropinocytosis, allowing for the release of PtdIns(4,5)P2-binding proteins and actin remodelling39. Indeed, blocking PLC with U73122 causes inhibition of growth factor-induced macropinocytosis in epithelial cells40. Inhibition of PLC also inhibited constitutive macropinocytosis in hMDMs (Fig. 6e). PLC catalyses the hydrolysis of PtdIns(4,5)P2, releasing DAG that can be converted by diacylglycerol kinases into PtdOH; the latter is required for constitutive macropinocytosis in dendritic cells8.

Using a biosensor consisting of the Spo20p domain fused to GFP (GFP-2PABD)8, we confirmed the presence of PtdOH at the plasma membrane and in membrane ruffles of hMDMs (Fig. 6f). The expression of CaSR mRNA, normalized to actin mRNA, was determined by quantitative PCR (qPCR); the total number of macropinosomes per cell is also plotted. Scale bar, 10 μm. *P ≤ 0.05, **P ≤ 0.01, NS, not significantly different.

**Figure 4 | CaSR is necessary for constitutive macropinocytosis in hMDMs and is sufficient to induce macropinocytosis when heterologously expressed in HEK293 cells.** (a) The expression of CaSR mRNA in HEK293 cells and hMDMs was determined by RT–PCR. The expression of GAPDH mRNA was determined as a control. (b) HEK293 cells were transfected with SeP-CaSR and, 24 h after transfection, were incubated on ice with an anti-GFP monoclonal antibody for 15 min to label SeP-CaSR expressed at the cell surface. Cells were then washed, fixed and labelled with a fluorescein secondary antibody (red). Cells nuclei were labelled with DAPI (cyan). (c) HEK293 cells were transfected with SeP-CaSR and, 24 h after transfection, were labelled with fluorescent phalloidin (red). (d) Untransfected (upper panel) and HEK293 cells transfected with SeP-CaSR (lower panel) were incubated with labelled 70 kDa dextran (0.025 mg/ml) and M-CSF (200 ng/mL) for 15 min at 37°C. Cells were then gently lifted and dextran uptake assessed by flow cytometry. Gates were drawn on GFP(+) (SeP-positive) and GFP(-) (SeP-negative) populations as shown in (e) and the relative dextran uptake in the respective populations in shown in (f). (g) hMDMs were pretreated with the indicated dose of the specific CaSR antagonist NPS2143 and then incubated with labelled 70 kDa dextran (0.025 mg/ml) for 15 min at 37°C. (h) The total number of macropinosomes per cell is plotted at the indicated concentration of NPS2143 for hMDMs (main panel) and hMDDCs (inset, where 10 μM was used). (i) hMDMs were pretreated with NPS2143 (10 μM), and then incubated with 70 kDa dextran (0.025 mg/ml) and M-CSF (200 ng/mL) for 15 min at 37°C. (j) CaSR expression was knocked down in hMDMs using CaSR-specific siRNA oligonucleotides. The cells were then incubated with labelled 70 kDa dextran (0.025 mg/ml) for 15 min at 37°C. The expression level of CaSR mRNA, normalized to actin mRNA, was determined by quantitative PCR (qPCR); the total number of macropinosomes per cell is also plotted. Scale bar, 10 μm. *P ≤ 0.05, **P ≤ 0.01, NS, not significantly different.
incubated with labelled 70 kDa dextran (0.025 mg ml⁻¹) containing or calcium-free medium for 30 min, then lysed, fractionated by 12% SDS–PAGE and subjected to immunoblotting with antibodies to phosphorylated VASP (pVASP) and tubulin.

A representative immunoblot is shown on top and quantification of the total number of macropinosomes per cell is plotted. (c) hMDMs were incubated with forskolin (10 μM) or NPS2143 (10 μM) in either calcium-containing or calcium-free medium for 30 min, then lysed, fractionated by 12% SDS-PAGE and subjected to immunoblotting with antibodies to phosphorylated VASP (pVASP) and α-tubulin (as loading control). A representative immunoblot is shown on top and quantification of the pVASP/α-tubulin ratio from three independent experiments is shown below. (d) hMDMs were pretreated with forskolin (10 μM) for 30 min and then incubated with labelled 70 kDa dextran (0.025 mg ml⁻¹) for 15 min at 37°C. The total number of macropinosomes per cell was then plotted. Scale bar, 10 μm. **p ≤ 0.01, NS, not significantly different.

**Figure 5 | Gαq-dependent signalling is required for constitutive macropinocytosis.** (a) hMDMs were pretreated in the absence or presence of BIM46187 (10 μM) for 1 h and then incubated with fluorescent 70 kDa dextran (0.025 mg ml⁻¹) for 15 min at 37°C. (b) hMDMs were pretreated with BIM46187 (10 μM) or PTX (0.1 μg ml⁻¹) for 1 h and then incubated with fluorescent 70 kDa dextran (0.025 mg ml⁻¹) for 15 min at 37°C. The total number of macropinosomes per cell is plotted. (c) hMDMs were incubated with forskolin (10 μM) or NPS2143 (10 μM) in either calcium-containing or calcium-free medium for 30 min, then lysed, fractionated by 12% SDS-PAGE and subjected to immunoblotting with antibodies to phosphorylated VASP (pVASP) and α-tubulin (as loading control). A representative immunoblot is shown on top and quantification of the pVASP/α-tubulin ratio from three independent experiments is shown below. (d) hMDMs were pretreated with forskolin (10 μM) for 30 min and then incubated with labelled 70 kDa dextran (0.025 mg ml⁻¹) for 15 min at 37°C. The total number of macropinosomes per cell was then plotted. Scale bar, 10 μm. **p ≤ 0.01, NS, not significantly different.

Inhibition of CaSR impairs constitutive ruffling. PtdIns(3,4,5)P3 and PtdOH at the membrane serve to recruit GEFs that activate Rho-family GTPases such as Rac1 (ref. 8); this in turn can initiate membrane ruffling and macropinocytosis. To establish whether CaSR utilizes this pathway we compared the actin cytoskeleton and assessed the activation of GTPases in control and NPS2143-treated hMDMs. Phalloidin staining revealed clearly defined cortical F-actin and F-actin-rich membrane ruffles extending from the surface of untreated hMDMs (Fig. 7a). In NPS2143-treated cells the band of cortical F-actin was thinner, abnormal F-actin puncta appeared in the cytoplasm, and virtually no actin-containing ruffles were observed (Fig. 7a). Differential interference contrast microscopy of live cells confirmed that dynamic membrane ruffling was arrested by NPS2143 (Supplementary Movies 2 and 3).

Rac1 and Cdc42 are thought to drive the actin remodelling required for macropinocytosis17. To monitor the activation state of these GTPases we employed a construct encoding PAK(PBD), a biosensor for GTP-bound Rac1 and Cdc42. hMDMs electroporated with PAK(PBD) conjugated to YFP were imaged under control and calcium-free conditions, as well as immediately after treatment with NPS2143. As expected, PAK(PBD) accumulated robustly and in a highly localized fashion in the membranes subtending macropinocytic ruffles in control cells (Fig. 7b; Supplementary Movie 6). In sharp contrast, the biosensor dissociated from the plasmalemma and became exclusively cytosolic when calcium was removed or when CaSR was inhibited (Fig. 7b; Supplementary Movie 7). These results strongly suggest that CaSR-driven signals promote activation of Rac1 and/or Cdc42. Consistent with this, we found that macropinosome formation by hMDMs was profoundly depressed by C. difficile toxin B which is known to inhibit Rac1 and Cdc42 (as well as other members of the Rho-GTPase family)41, but not by the C3 toxin of Clostridium botulinum that specifically inhibits RhoA (Fig. 7c). These data imply that CaSR-dependent activation of Rac and/or Cdc42 is implicated in constitutive membrane ruffling and macropinocytosis in hMDMs.

Macropinocytosis facilitates the uptake of NOD2 ligands. Extracellular danger signals sensed by pattern-recognition receptors such as nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and 2 (NOD2) initially enter the endocytic pathway before reaching the cytosolic compartment42. NOD ligands are transported in a pH-dependent manner across the membrane of endosomes/lysosomes by members of the solute carrier family of peptide transporters—SLC15A3 and SLC15A4 (ref. 42). Because large volumes of extracellular fluid are internalized in macrophages and DCs via macropinocytosis, we analysed the possible role of CaSR and of constitutive macropinocytosis in the uptake of ligands that activate pattern-recognition receptors. Fifteen minutes after being added to the medium bathing hMDMs, the fluorescently labelled NOD2 ligand muramyl dipeptide (MDP) was taken up into endomembrane vesicles; MDP was also detectable in the cytosol (Fig. 8a), presumably as a result of transport across endomembranes by peptide transporters42. That the ligand was being effectively sensed by NODs was indicated by the robust phosphorylation of the p65 subunit of NFκB induced by MDP (Fig. 8b,c). Strikingly, inhibition of CaSR by the removal of external calcium or with NPS2143 drastically reduced the phosphorylation of p65 elicited by MDP (Fig. 8b,c). This suggested that inhibition of CaSR-dependent constitutive macropinocytosis reduces the amount of MDP taken up and subsequently translocated to the cytosol for sensing.

To ensure that the reduction in p65 phosphorylation caused by NPS2143 was due to impaired macropinocytosis, as opposed to non-specific effects of the drug on the NFκB signalling cascade, we incubated hMDMs with lipopolysaccharide (LPS) that stimulates NFκB through Toll-like receptor 4 (TLR4). TLR4 senses LPS at the cell surface and does not require internalization. As shown in Fig. 8d, LPS (0.5 μg ml⁻¹) induced robust phosphorylation of p65. Importantly, treatment with NPS2143 had no discernible effect on LPS-induced p65 phosphorylation (Fig. 8d). Comparable results were obtained using lower
To ensure that the stimulation was not caused by LPS internalization due to macropinocytosis, we also performed similar experiments in cells treated with either latrunculin A or NPS2143. The inhibition of CaSR-dependent macropinocytosis or of macropinocytosis in general had no significant effect on LPS-induced phosphorylation of p65 (Supplementary Fig. 6). Altogether, these data demonstrate that constitutive macropinocytosis, which is initiated and maintained by CaSR, aids in the sentinel function of phagocytes by facilitating the uptake of pattern-recognition receptor ligands.

**Discussion**

Unlike most cell types, DCs and macrophages display constitutive macropinocytosis, a unique behaviour that appears well suited for their sentinel function. This distinguishing feature has been proposed to serve as a mechanism for the acquisition of antigen for presentation on class I and II major histocompatibility molecules. Indeed, inhibition of macropinocytosis markedly reduces the efficiency of antigen presentation by DCs both in vitro and in vivo. The signals that initiate and maintain this constitutive process, however, have remained largely unknown. Here we show that extracellular calcium is a key stimulant of the signalling pathways that trigger constitutive macropinocytosis in macrophages and DCs. Further, we identify CaSR as the receptor that senses extracellular calcium and initiates the aforementioned signalling pathways.

CaSR is ubiquitous, but is expressed at particularly high levels in the parathyroid gland and kidney, where it plays a critical role in extracellular calcium homeostasis. In myeloid osteoclast precursors CaSR directs migration towards bones, allowing for the initiation of bone remodelling. CaSR, however, is also expressed in a number of tissues that are seemingly not involved...
in calcium homeostasis, including monocytes, macrophages and DCs, where it has been speculated to regulate immune function by controlling the activation of the inflammasome. Our findings extend the immune function of CaSR in myeloid cells. It is relevant that extracellular calcium is elevated at sites of inflammation and injury. CaSR may function in the migration towards, and acquisition of antigen at sites of injury and inflammation, thereby assisting the immune surveillance function of antigen-presenting cells.

Although previous work identified various signalling intermediates required for constitutive macropinocytosis, such as PtdIns3K, PLC and Rac1, efforts to identify the initiating event had failed. We show here that CaSR can trigger the pathways that underlie constitutive macropinocytosis. We demonstrate that removal of extracellular calcium or pharmacological inhibition of CaSR block the steady state formation of PtdIns(3,4,5)P3 as well as the constitutive activation of Rac1/Cdc42 (Figs 6 and 7). The activation of PtdIns3K facilitates the recruitment of PLC, which, in turn, catalyses the formation of DAG at the plasma membrane. DAG not only activates protein kinase C, but can be converted to PtdOH by diacylglycerol kinases. The generation of PtdOH—DAG not only activates protein kinase C, but can be converted to PtdOH by diacylglycerol kinases. The generation of PtdOH—

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**Methods**

**Reagents.** Tetramethyl rhodamine-conjugated 70,000 MW dextran, Alexa Fluor 488 conjugated transferrin, EGF, Superscript VILO cDNA synthesis kit, and the anti-GFP antibody (catalogue number, A11120; dilution, 1:1,000) and Fura2-AM were acquired from Life Technologies (Carlsbad, CA, USA). 5-(N-ethyl-N-isopropyl)amiloride, LY294002, thapsigargin, NPS2143, Calnex 231, PTX, LPS, DAPI, latrunculin A, the anti-actin monoclonal antibody (catalogue number, A5414; dilution, 1:1,000) and the anti-tubulin monoclonal antibody (catalogue number, T5168; dilution, 1:1,000) were purchased from Sigma-Aldrich (St Louis, MO, USA). Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human and murine recombinant M-CSF, IL-4 and GM-CSF were from Human 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Confocal microscopy. Scale bar, 10 μm. (Walsh, MA, USA). The TaqMan gene expression mastermix and the CaSR TaqMan gene expression assay were from Applied Biosystems (Walsh, MA, USA).

Solutions. The calcium-free medium contained 1 mM KH2PO4, 154 mM NaCl, 5.6 mM NaHCO3, 2 mM MgCl2, 2 mM EGTA and 10 mM glucose, pH 7.2 at 37°C. The calcium-containing medium contained 1 mM KH2PO4, 154 mM NaCl, 5.6 mM NaHCO3, 2 mM MgCl2, 2 mM CaCl2 and 10 mM glucose, pH 7.2 at 37°C. The Na+ rich buffer contained 140 mM NaCl, 3 mM KCl, 1 mM MgCl2, 5 mM glucose, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) adjusted to pH 7.2 at 37°C.

Macrophage and dendritic cell isolation and cell culture. For hMDMs and hMDCs, peripheral blood mononuclear cells were isolated from the blood of healthy donors by density-gradient separation with Lymphoprep (Cedarlane, Burlington, ON, Canada). Monocytes were separated from other mononuclear cells in the washed buffy coat by adherence to glass coverslips in 12-well plates at a density of 3.0 × 105 mononuclear cells per 10-cm dish. Monocytes were then cultured for 7 days in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, antibiotic/antimycotic (MultiCell, Wisent, St Bruno, Canada) and either 25 ng ml–1 GM-CSF + 20 ng ml–1 IL-4 (for hMDCs) or 25 ng ml–1 M-CSF (for hMDMs). The medium and cytokines on the cells were replaced every 2–3 days.

For murine BMDMs, bone marrow was collected from the long bones of healthy wild-type or Pldln3KO knockout mice. The Pldln3KO knockout mice were generated26 and kindly provided by Dr J. M. Penninger (Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria). The precursor cells (plated at a density of 4.5 × 105 cells per 10-cm dish) were cultured in the presence of 10 ng ml–1 M-CSF in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotic/antimycotic for 7 days. The medium and cytokines on the plate were aspirated and replaced every 2–3 days.

A431 and HEK293 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in DMEM (Life Technologies, Wisent, St Bruno, Canada) supplemented with 10% heat-inactivated fetal bovine serum. When cultures reached confluence, they were detached using 0.05% trypsin-EDTA and replated on either coverslips for macroinocytosis assays or in flasks for maintenance.

Transfections. For hMDMs, DNA transfections were performed using the Neon transfection system (Life Technologies, Carlsbad, CA, USA). hMDMs were suspended using Accutase (Gibco, Life Technologies, Carlsbad, CA, USA) and the cells were washed 3 ×. Transfections were performed according to the manufacturer’s protocol. For siRNA transfections of hMDMs, the HiPerFect transfection reagent (Qiagen, Hilden, Germany) was used according to the manufacturer’s protocol. For HEK cells, transfections were performed with the FuGENE 6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

PCR. To detect the expression of CaSR messenger RNA (mRNA) in hMDMs and HEK cells, one step RT–PCR was performed. RNA was purified from hMDMs and HEK cells using the GeneJet RNA purification kit (Thermo Fisher Scientific) and used as a template for the generation of complementary DNA (cDNA) and subsequent PCR amplification using the OneStep RT–PCR kit from Life Sciences according to the manufacturer’s instructions. The primary sequences of the forward and reverse primers are listed in the ‘Reagents’ section. The amplification products were visualized by electrophoresis on agarose gels prestained with ethidium bromide. Uncropped gels are presented in Supplementary Fig. 7a.

To quantify the expression of CaSR mRNA after siRNA knockdown, RNA was purified from hMDMs using the GeneJet RNA purification kit (Thermo Fisher Scientific) and used as a template for the generation of cDNA using the SuperScript VILO cDNA synthesis kit (Life Technologies). The CaSR-specific Taqman gene expression assay (Life Technologies) was then used for real-time quantitative PCR to quantify the expression of CaSR, which was normalized relative to actin mRNA.

Macroinocytosis assay. Cells were plated on 18 mm coverslips 24 h before performing the macroinocytosis assay. In the case of drug treatments, cells were pretreated with their respective drugs for 1 h before the assay, except for PTX, Tat-C3 and C. difficile toxin B, which required 24, 4 and 4 h pretreatments, respectively. The concentrations for the drugs used are as follows: EIPA (10 μM), HOE-694 (10 μM), latrunculin A (2 μM), LY294002 (10 μM), PTX (0.1 μg ml–1), BFA (0.05% trypsin-EDTA, 1 μg ml–1), AS605240 (10 μM), MDP (1 μg ml–1), MDP-rhodamine (1 μg ml–1), Mıl, (1 μg ml–1) and 100 μg ml–1 of C. difficile toxin B, which required 24, 4 and 4 h pretreatments, respectively. After pretreatment, the medium was aspirated from the cells and replaced with either calcium-containing or calcium-free medium with rhodamine-conjugated 70 kDa dextran (25 μg ml–1) and either M-CSF (200 ng ml–1) or EGF (200 ng ml–1) where indicated. Cells were incubated at 37°C for 15 min and then washed and imaged immediately. Imaging was done by spinning disk confocal microscopy on an Axiovert 200 M equipped with a 63 × objective and a separate 10× objective. The Axiovert 200 M was used.
1.5 × magnifying lens (Carl Zeiss, Oberkochen, Germany). The microscope was fitted with a piezo focus drive and diode-pumped solid-state lasers (440, 491, 561, 638 and 655 nm; Spectral Applied Research, Richmond Hill, ON, Canada). Micrographs were acquired using a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) under the control of Volocity software. Macropinosomes were then counted from the acquired images using the measurement tool in the Image software.

### Intracellular calcium measurements
MDMs were loaded with Fura2-AM at a concentration of 3 μM in HBSS (MultiCell, Wisent, St Bruno, Canada) for 20 min at 37°C. They were then washed with fresh HBSS 3 × and incubated at 37°C for an additional 10 min. hMDMs were then placed into a magnetic Chemidive cover slip holder, covered in HBSS and placed onto the temperature controlled (37°C) microscope stage. The hMDMs were imaged on a Leica DM IRB microscope (Leica, Wetzlar, Germany). Calcium measurements were done by fluorescence ratio imaging using a filter wheel (Sutter Instruments, Novato, CA, USA) to rapidly switch between excitation filters. Fura2 was excited by light from an X-Cite 120 lamp (EXFO Photonic Solutions, Quebec, Canada) using dual excitation (340 and 380 nm) and single emission (510 nm). The emitted light was captured by a CCD camera (Cascade II; Photometrics, Tucson, AZ, USA). The filter wheels, shutters and camera were controlled by the MetaFluor software (MDM Analytical Technologies, Sunnyvale, CA, USA).

After experimental measurements were taken, ionomycin was added at a concentration of 6 μM to acquire an Rmax (340/380) value for approximation of absolute [Ca2+]i values. The Grynkiewicz formula was used to convert the fluorescence ratios to [Ca2+]i values.

### Immunoblotting
Cells were washed 3 × with ice-cold PBS and were subsequently lysed using a small volume of ice-cold RIPA buffer. Total protein concentration was calculated using the Bradford assay and 50 μg of protein of each sample was loaded and separated by 12% SDS-PAGE. The protein was transferred to a polyvinylidene difluoride (PVDF) membrane and blocked in Tris-buffered saline containing 0.05% Tween-20 (TBST) and 10% fat-free milk for 30 min. The primary antibody was added to the membrane in 2.5% milk in TBST for 1 h at room temperature and washed 3 × with TBST for 10 min each. The membrane was reblocked with 10% milk in TBST for 15 min and the HRP-conjugated secondary antibodies were added in 2.5% milk in TBST for 45 min. The membrane was washed 3 × with TBST for 10 min each and blot was visualized on the Odyssey FC (LI-COR, Lincoln, NE, USA). Band intensity was quantified using ImageStudio Lite software. Uncropped versions of all the gels presented can be seen in Supplementary Fig. 7.

### Data presentation and statistics
Unless otherwise indicated, images are representative of ≥50 cells from at least three separate experiments. Data presented in text and graphs are the means plus standard error of at least three independent experiments. Unpaired t-tests were used to establish the significance of experimentally observed differences.

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Author contributions
J.C. conceived the project, designed and performed the experiments, analysed the data and wrote the manuscript. D.S. designed and performed experiments and analysed the data. C.B. and M. Gütschow synthesized BIM46187. M. Glogauer helped design experiments and provided the PtdIns3Kγ knockout mice. S.G. conceived the project, designed the experiments, analysed the data and wrote the manuscript.

Additional information
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