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Specific inhibition of PI3K p110 δ inhibits CSF-1-induced macrophage spreading and invasive capacity

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Abstract

Colony stimulating factor-1 (CSF-1) stimulates mononuclear phagocytic cell survival, growth and differentiation into macrophages through activation and autophosphorylation of the CSF-1 receptor (CSF-1R). We have previously demonstrated that CSF-1-induced phosphorylation of Y721 in the receptor kinase insert triggers its association with the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). Binding of p85 PI3K to the CSF-1R pY721 motif activates the associated p110 PI3K catalytic subunit and stimulates spreading and motility in macrophages and their enhancement of tumor cell invasion. Here we show that pY721-based signaling is necessary for CSF-1-stimulated PtdIns(3,4,5)P (PIP₃) production. While primary bone marrow-derived macrophages (BMM) and the immortalized bone marrow-derived macrophage cell line, M-/-WT, express all three Class IA PI3K isoforms, p110 δ predominates in the cell line. Treatment with p110 δ specific inhibitors demonstrates that the hematopoietically enriched isoform, p110 δ , mediates CSF-1 regulated spreading and invasion in macrophages. Thus GS-1101, a potent and selective p110 δ inhibitor, may have therapeutic potential by targeting the infiltrative capacity of tumor-associated macrophages that is critical for their enhancement of tumor invasion and metastasis.

Keywords

macrophage; CSF-1R; PI3K; p110 δ ; GS-1101; invasion; DMSO

INTRODUCTION

Macrophages are fully differentiated cells of the mononuclear phagocytic lineage and are important for normal development and homeostasis as well as immune surveillance (1, 2). They also promote disease progression in conditions ranging from cancer to atherosclerosis and arthritis (3, 4). Interstitial motility is an essential element of macrophage function and is required for both their beneficial and deleterious activities (5). CSF-1, originally identified as a mononuclear phagocytic cell growth and differentiation factor (6), potently stimulates

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

macrophage motility (5, 7). Furthermore, high circulating levels of CSF-1 are found in advanced breast, endometrial and ovarian cancers (8–10) and breast carcinoma cells express high levels of CSF-1 at invasive fronts where large numbers of tumor-associated macrophages (TAMs) have infiltrated and gathered (11). Indeed, a lack of CSF-1 in the mammary carcinoma-prone MMTV-PyMT mouse significantly inhibits tumour progression, invasion and pulmonary metastasis while CSF-1 overexpression accelerates disease progression (12). An important mechanism underlying this promotion of tumour invasion and metastasis by TAMs is the establishment of a paracrine chemotactic loop between carcinoma cells and TAMs (13). Carcinoma cells secrete CSF-1 and TAMs secrete epidermal growth factor (EGF) to induce co-migration of both cell types (14). This multicellular migratory streaming, which has been imaged *in vivo*, is dependent upon CSF-1 signaling (14, 15).

The pleiotropic effects of CSF-1 are mediated by the CSF-1 receptor (CSF-1R), a receptor tyrosine kinase of the PDGF-R/c-Kit family, via trans-autophosphorylation of a series of intracellular tyrosine residues (2). We have developed a unique macrophage cell line system in which immortalized macrophages derived from the CSF-1R^{-/-} mouse retrovirally express either wild type (WT) or tyrosine mutant (e.g. Y721F) receptors (16). We have recently used this model system to show that signaling triggered by the pY721 motif regulates CSF-1-induced motility in macrophages (17). This motif is necessary and sufficient for association of the receptor with the p85 regulatory subunit of PI3K and subsequent PI3K activation leads to polarized PIP₃ production at the leading edge of the cell (17). The p85 subunit is constitutively bound to one of three Class IA catalytic p110 PI3K subunits, p110 α , p110 β and p110 δ (18). These Class IA PI3K heterodimers signal downstream of receptor tyrosine kinases (RTKs) to mediate a variety of biological effects in different cell types (18, 19). Macrophages express the ubiquitous isoforms, p110 α and p110 β , as well as the hematopoietically enriched p110 δ isoform (20). While p110 δ has been shown to be the main isoform recruited to the CSF-1R in primary macrophages, p110 α associates with the receptor in the immortalized BAC1.2F5 macrophage cell line (20). Nevertheless, p110 δ was demonstrated to regulate migration in both primary and immortalized macrophages (20).

Although the precise signalling pathways by which PI3K mediates CSF-1-induced macrophage motility are not yet clear, more than one downstream pathway is probably involved (17). Targeting upstream PI3K activation to inhibit the motility of infiltrating TAMs is therefore more likely to be effective than targeting several possible downstream effectors. GS-1101, a potent and selective inhibitor of p110 δ (21), is in Phase III clinical trials as a targeted therapy for B-cell lymphoproliferative disorders, including chronic lymphocytic leukemia and indolent non Hodgkin lymphomas (22, 23). In this report we confirm the necessity of pY721-based signaling for CSF-1-induced PIP₃ production in macrophages and demonstrate that p110 α and p110 β are the major Class IA PI3K isoforms expressed in BMM and M^{-/-}.WT macrophages. We also show that specific inhibition of p110 δ by GS-1101 abrogates CSF-1-induced PIP₃ production to block macrophage spreading and infiltrative capacity.

RESULTS

pY721 CSF-1R-based signaling is necessary for CSF-1-induced PIP₃ production in macrophages

We and others have previously shown that phosphorylation of Y721 in the CSF-1R kinase insert mediates a rapid and prolonged association of p85 PI3K with the receptor in macrophages in response to CSF-1 (17, 24). The resultant activation of PI3K produces an ~9-fold increase in total PIP₃ levels (Fig. 1A, M^{-/-}.WT cells). In contrast, macrophages

expressing a mutant Y721F receptor (M^{-/-}.Y721F) fail to show a CSF-1-induced increase in PIP₃ levels, indicating that PI3K requires direct association with the receptor via the pY721 motif for p110 activation and subsequent PIP₃ production (Fig. 1A).

Macrophages express all three Class IA PI3K isoforms with variable levels in cell lines compared to primary macrophages

To identify the Class IA PI3K isoforms activated by CSF-1 in macrophages, we initially examined their relative expression levels in BMM and M^{-/-}.WT cells compared with MCF10A cells, which are a widely used breast epithelial cell line that we use in macrophage/mammary cell co-cultures. MCF10A cells expressed the ubiquitous isoforms, p110 α and p110 β , but not the hematopoietically enriched isoform p110 δ (Fig. 1B). Compared to the epithelial cell line, M^{-/-}.WT cells expressed very high levels of p110 δ with very little p110 α and p110 β , while primary BMM expressed relatively low levels of all three isoforms (Fig. 1B). Thus, while primary and immortalized macrophages express all three Class IA PI3K isoforms, their expression of the ubiquitous isoforms is very low compared to MCF10A cells. Moreover, in contrast to a previous study, which demonstrated an increased role for p110 δ downstream of the CSF-1R in the BAC1.2F5 spleen-derived macrophage cell line compared to BMM (20), expression of the hematopoietic isoform predominates in M^{-/-}.WT cells, which are bone marrow-derived, indicating that isoform expression levels differ between macrophage cell lines as well as when compared to BMM. Thus PI3K isoform signalling studies in macrophages should include reference BMM where possible.

p110 δ is the primary mediator of CSF-1-induced PI3K signaling in macrophages

Class IA PI3K isoforms exert distinct actions downstream of RTKs that are not necessarily correlated with their relative expression levels. Studies with microinjected inhibitory antibodies to specific PI3K isoforms indicated that the CSF-1R signals through p110 δ to mitogenesis while it regulates actin cytoskeletal remodeling and motility through p110 α and p110 β in BAC1.2F5 cells (25). Subsequent studies with p110 δ or p110 α PI3K-deficient macrophages and with specific small molecule inhibitors demonstrated that p110 δ was the primary mediator of CSF-1-induced chemotaxis (20). To more closely examine the role of p110 δ in macrophage motility, GS-1101, a highly selective and potent p110 δ small molecule inhibitor (IC₅₀ = 2.5nM) (21), and IC488743, a p110 δ inhibitor with an IC₅₀ of 13nM (26) from Gilead Sciences (Seattle, WA) were used in a series of signaling and single cell assays in both BMM and M^{-/-}.WT macrophages.

CSF-1-induced PI3K activation in M^{-/-}.WT macrophages results in Akt phosphorylation in the activation loop (T308) and the C-terminus (S473) that peaks at 2.5 minutes (17). CSF-1-stimulated Akt phosphorylation was abrogated by pretreatment with the pan-PI3K inhibitor, wortmannin (WM) (Fig. 2A). Akt phosphorylation was significantly inhibited by 1 μ M of GS-1101 (pS473 53%, pT308 62%) and IC488743 (pS473 68%, pT308 72%) with almost complete inhibition at greater inhibitor concentrations (Fig. 2A). Since p110 isoform levels may vary between macrophage cell lines and primary macrophages (Fig. 1B), the effect of GS-1101 was examined in BMM and the extent of p110 δ inhibition was comparable to that seen in the macrophage cell line (Fig. 2B). PI3K inhibition did not affect CSF-1-induced tyrosine phosphorylation in macrophages (data not shown).

To confirm that inhibition of p110 δ alone prevents Akt activation, a PIP₃ ELISA of M^{-/-}.WT cells in the presence or absence of the isoform-specific inhibitors was carried out. Consistent with the phospho-Akt levels, blockade of p110 δ alone by GS-1101 prevented a CSF-1-induced rise in PIP₃ production similar to that observed with macrophages expressing the Y721F CSF-1R mutant (Fig. 2C). Taken together these results indicate that

p110 is the primary PI3K isoform activating the PI3K/Akt pathway downstream of the CSF-1R in macrophages.

PI3K p110 δ mediates CSF-1-induced spreading and invasion

CSF-1 stimulates rapid and circumferential spreading in macrophages prior to elongation and commencement of migration (5). The spreading response, which increases macrophage footprint area by ~40% at 5 minutes, is dependent upon pY721-based CSF-1R signaling (17). To determine whether PI3K p110 regulates CSF-1-stimulated spreading, we plated M^{-/-}.WT cells on fibronectin-coated coverslips for 2 days prior to removing CSF-1 overnight. The cells were then pretreated with GS-1101 (5 μ M) or vehicle control (DMSO) and stimulated with CSF-1 for 0 or 5 minutes before fixation and staining for F-actin. Stained cells were individually traced to determine mean footprint area before and after CSF-1 stimulation. DMSO is a widely used solvent for cell based assays and is required to solubilize GS-1101 and IC488743. Although it is widely considered to be non toxic to cells in culture at concentrations as high as 1%, macrophages in 0.1% DMSO failed to show the expected spreading response upon CSF-1 treatment (data not shown). To assess whether DMSO inhibits CSF-1-induced spreading, we exposed M^{-/-}.WT cells to either 0%, 0.04% or 0.1% DMSO and measured mean footprint area 0 and 5 minutes after CSF-1 stimulation. While cells treated with 0.04% DMSO increased their footprint by 30% in response to CSF-1 (n > 30 cells), which was not significantly different to control cells (42%), there was a significant decrease in the spreading response of cells in 0.1% DMSO (16%, p < 0.01)(Fig. 3A). To reduce the DMSO volume to a final concentration of 0.04%, the concentration of isoform specific inhibitors had to be reduced commensurately. Thus, we compared CSF-1-induced Akt phosphorylation in cells pretreated with 2 μ M or 5 μ M of either GS-1101 or IC488743 to confirm that reduced inhibitor concentrations significantly inhibited CSF-1-stimulated PI3K activation in M^{-/-}.WT macrophages. Both inhibitors were as effective at 2 μ M as at 5 μ M (Fig. 3B).

Thus, we examined the effect of inhibition of p110 on the CSF-1-induced cell spreading response using GS-1101 at 2 μ M. CSF-1-starved M^{-/-}.WT cells were pre-treated with DMSO or GS-1101 prior to CSF-1 stimulation and their footprint area measured at 0 and 5 minutes. While control macrophages demonstrated the expected increase in footprint area (39%), spreading failed to occur following inhibition of p110 (Fig. 3C). Representative images of M^{-/-}.WT cells pretreated with DMSO or GS-1101 then stimulated with CSF-1 are shown in figure 3D. Thus p110 PI3K mediates CSF-1-stimulated spreading, which is underpinned by actin polymerization and adhesion structure formation (5, 17), in macrophages.

To determine whether p110 inhibition similarly affects macrophage motility and infiltrative capacity, a matrigel invasion assay was carried out. Compared to control cells, M^{-/-}.WT macrophages pretreated with GS-1101 or IC488743 showed a 65–70% reduction in their ability to infiltrate through matrigel in a modified Boyden chamber assay (Fig. 4A). To further examine the role of p110 in the regulation of macrophage invasive capacity, a matrix degradation assay was carried out. BMM, which degrade matrix more efficiently than M^{-/-}.WT cells (data not shown), were plated on Cy3-labeled gelatin in the presence or absence of GS-1101 for 24 hours before fixation, F-actin staining and immunofluorescent microscopy. Inhibition of p110 produced a significant reduction in both the number of macrophages demonstrated to be actively degrading Cy3-labeled gelatin (Fig. 4B) and the area of digestion (Fig. 4C). Importantly, inhibition of p110 for 24 hours did not affect cell proliferation as the average number of cells per field was equivalent in the DMSO and GS-1101 treated samples (data not shown). Taken together, the results indicate a profound reduction in total matrix area degraded in the presence of GS-1101. Representative images for BMM treated with either DMSO or GS-1101 illustrate the profound reduction in

extracellular matrix digestion upon inhibition of p110 (Fig. 4D). Thus PI3K signaling regulates the infiltrative and degradative capacity of macrophages, largely through the activity of p110 .

DISCUSSION

Most epithelially-derived tumors contain large numbers of TAMs, which are the dominant innate immune cell in mammary cancers of women and mice (3). There is a strong correlation between poor prognosis and TAM numbers in many solid tumors and high circulating levels of CSF-1 are found in advanced breast, endometrial and ovarian cancers (8–10, 27). CSF-1 stimulates TAMs to secrete a variety of factors to promote tumor growth and progression via several mechanisms, including stimulation of angiogenesis and lymphangiogenesis (28–30) and suppression of the immune response (31). TAMs also promote tumor invasion and metastasis by secreting EGF to establish an EGF/CSF-1 paracrine dialogue with CSF-1-secreting carcinoma cells (13, 14). CSF-1 and EGF are both potent chemokines for their respective target cells (5, 32), and alternating tumor cells and TAMs can be seen migrating out of primary tumors along collagen fibers (15).

If a CSF-1/EGF chemotactic circuit between TAMs and tumor cells is an underlying driver of invasion and metastasis, inhibition of macrophage migration should break the circuit and prevent carcinoma cell egress to inhibit tumor invasion and metastasis. To investigate this possibility, we initially used our novel cell line system (16) to identify the CSF-1R tyrosine residue(s) responsible for transducing the CSF-1 signal to motility in macrophages. Phosphorylation of a single receptor tyrosine, Y721, mediates association of PI3K with the receptor to trigger both macrophage migration and enhancement of tumor cell invasion *in vitro* (17). Here we have extended these findings to confirm that CSF-1-stimulated PIP₃ production requires CSF-1R Y721 and show that the predominant PI3K isoform activated by association with the receptor is p110 . To determine whether specific inhibition of this hematopoietically enriched PI3K isoform is sufficient to prevent macrophage migration, we examined the effects of GS-1101, a potent and highly selective p110 inhibitor, on macrophage motility signaling. Confirming our initial findings, p110 inhibition not only completely blocks CSF-1-induced PIP₃ production but GS-1101 also prevents macrophage spreading, invasive capacity and degradation of extracellular matrix.

Thus, we have identified p110 as a primary drug target to inhibit macrophage infiltration into disease sites, including tumors and next we aim to examine whether GS-1101 will prevent macrophage infiltration of mammary spheroids and their stimulation of spheroid invasion into the surrounding matrix. Notably, GS-1101 is currently in Phase III clinical trials for hematopoietic malignancies. GS-1101 has been shown to promote apoptosis in B-cell lines and primary cells from patients with different B-cell malignancies, including chronic lymphocytic leukemia, mantle cell lymphoma, multiple myeloma, and Hodgkin lymphoma (21, 22, 26, 33). Several lines of evidence demonstrate that GS-1101 interferes with the crosstalk between malignant B cells and their microenvironment, suggesting that disruption of intrinsic and extrinsic survival signals could be a critical mechanism for the clinical activity of GS-1101 (22, 34). We believe that the clinical usefulness of this drug could extend to the treatment of invasive solid tumors.

MATERIALS AND METHODS

Cell culture

M^{-/-}.WT and M^{-/-}.Y721F macrophages were cultured in supplemented alpha modified minimal essential medium (+MEM) containing 10% newborn calf serum (NBCS; Life Technologies, Mulgrave, VIC), as described previously (16). Primary BMM were extracted

from C57BL/6 mouse femurs and tibias, differentiated and maintained in +MEM containing 10% fetal calf serum as described previously (35). All macrophages were grown in 120ng/ml human recombinant CSF-1 (a gift from Chiron Corporation, Emeryville, CA) for at least 1 week when thawed from GM-CSF-maintained stocks. MCF10A cells (a gift from Dr E. Thomas) were maintained in HuMEC Basal Serum-Free Medium supplemented with HuMEC Supplement Kit (Life Technologies).

Antibodies and other reagents

GS-1101 and IC488743 were supplied by Gilead Sciences. Wortmannin was purchased from Sigma-Aldrich (W1628, Castle Hill, NSW). Antibodies used included anti-phosphotyrosine (4G10, Millipore, Kilsyth VIC), anti-GAPDH (Abcam, Cambridge MA), anti-Akt (BD Transduction Laboratories, North Ryde NSW) and anti-PI3K p110 (Y387, GeneTex, Irvine CA). Cell Signaling Technology (Danvers MA) supplied anti-PI3K p110 (polyclonal), anti-PI3K p110 (C33D4), anti-PI3K p110 (D55D5), anti-phospho-Akt Ser473 (193H12), and anti-phospho-Akt Thr308 (polyclonal). HRP-conjugated secondary antibodies were from Cell Signaling Technologies and Alexa-Fluor-488-conjugated phalloidin and Prolong Gold antifade reagent with DAPI were from Molecular Probes (Life Technologies).

PIP₃ ELISA

PIP₃ production was detected with PI-3 Kinase Activity ELISA: Pico? Kit (Echelon Biosciences, Logan UT). Briefly, cells were starved of CSF-1 for 16 hours, treated with 5μM GS-1101, IC488743 or DMSO for 1 hour and stimulated with CSF-1 for 0 or 30 seconds. PIP₃ was extracted by sequential centrifugation in methanol:chloroform:HCl buffer and measured using the PIP₃ Mass ELISA Kit (K-2500s; Echelon) according to manufacturer's instructions.

Immunoblotting

Subconfluent (~70–80%) 100-mm dish cultures of cells were starved of CSF-1 for 16 hours to upregulate CSF-1R expression then incubated with 240 ng/ml CSF-1 at 37°C for the indicated times. Following incubation, cells were rinsed in ice-cold PBS, scraped into 200μl of lysis buffer (1% NP-40, 10mM Tris-HCl, 50mM NaCl, 30mM Na₄P₂O₇, 50mM NaF, 500μM Na₃VO₄, 5μM ZnCl₂, 1mM benzamidine, 10μg/ml leupeptin and 10μg/ml aprotinin, pH 7.2) at 4°C, vortexed and centrifuged at 13,000g for 30 minutes. 35–50μg of protein was loaded for SDS-PAGE. SDS-PAGE and western blots were performed using the Criterion Bis-Tris XT gel electrophoresis system (Bio-Rad, Gladesville NSW). Blotted membranes were incubated with HRP substrate (Millipore) and the chemiluminescent signal detected by the ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare, Rydalmere NSW).

F-actin staining and footprint area quantification

Cells were seeded onto fibronectin-coated coverslips (BD BioCoat) in six-well tissue culture dishes and grown for a minimum of 2 days. When 60–70% confluent, cells were starved of CSF-1 for 16 hours. Upregulated cells were treated with either DMSO or GS-1101 (2μM) for 1 hour then stimulated with 120 ng/ml CSF-1 for indicated times then fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and quenched with 0.1% glycine in Fix buffer as previously described (35). Coverslips were blocked with 10% goat serum in 1% BSA/TBS with Alexa-Fluor-488-conjugated phalloidin for F-actin staining. Coverslips were mounted on slides with Prolong Gold antifade reagent with DAPI. Samples were imaged using an Olympus IX-81 inverted fluorescence microscope and images analysed by ImageJ to calculate footprint area from cell outline tracing (35).

Matrigel invasion assay

Cells were seeded at 1×10^5 cells/insert on BD BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences) in 24-well tissue culture dishes in the presence of 5μM GS-1101, 5μM IC488743 or 0.1% DMSO for 16 hours in a CSF-1 gradient with no CSF-1 in the chamber insert and 120ng/ml CSF-1 below. Inserts were fixed in 4% paraformaldehyde and cells remaining on the chamber were removed. The membranes were cut and mounted then examined by phase contrast with an Olympus IX-81 microscope. Ten representative fields at 20x magnification were captured per sample to calculate the mean number of cells that had traversed through the matrigel.

Matrix degradation assay

Glass-bottomed 35 mm dishes (MatTek, Ashland MA) were coated with a thin layer of Cy3-labeled gelatin using the QCM™ Gelatin Invadopodia Assay (Red) kit (Millipore) as per the manufacturers instructions. BMM were seeded at a density of 2×10^5 cells per dish in media containing 10% FCS, 120ng/ml CSF-1, 20ng/ml recombinant murine IL-4 (PeproTech, Rocky Hill NJ) and either 2μM GS-1101 or DMSO for 24 hours. Dishes were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-10, quenched with 0.1% glycine, blocked with 10% goat serum in 1% BSA/TBS with Alexa-Fluor-488-conjugated phalloidin for F-actin staining and mounted in 1% N-Propyl Gallate in 1xPBS. Samples were imaged with an Olympus IX-81 inverted fluorescence microscope and representative images analysed by ImageJ for cell number, matrix degradation site numbers and area normalized to cell number for each field.

Significance testing

Student's t test was used for all comparisons.

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Abbreviations

BMM	bone marrow-derived macrophages
CSF-1	colony stimulating factor-1
CSF-1R	colony stimulating factor-1 receptor
PI3K	phosphoinositide 3 -kinase
PIP₃	PtdIns(3,4,5)P
RTK	receptor tyrosine kinase
TAM	tumor-associated macrophage
WM	wortmannin
WT	wild type

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Figure 1.

CSF-1R pY721-based signaling is required for CSF-1-induced PIP₃ production. (A) PIP₃ levels in CSF-1-starved M-/- WT and M-/- Y721F cells stimulated with CSF-1 for 0 or 30 seconds were measured by PIP₃ ELISA; n=4, *** denotes p<0.001. (B) Primary and M-/- WT macrophages grown in the continuous presence of CSF-1 were lysed and the lysates examined for expression of PI3K Class IA isoforms by SDS-PAGE and immunoblotting. MCF10A mammary epithelial cell lysates were included for comparison and GAPDH immunoblotting was carried out as a loading control. A representative blot (n=4) is shown.

Figure 2.

Inhibition of PI3K p110 by GS-1101 abrogates PIP₃ production and Akt activation in macrophages. (A) CSF-1-starved M^{-/-}.WT cells were pretreated with the indicated doses of GS-1101, IC488743 or wortmannin for 1 hour prior to CSF-1 stimulation for 0 or 2.5 minutes. Akt activation was measured by densitometry of pS473 and pT308 Akt bands and compared as % reduction in signal compared to DMSO-treated control cells. (B) CSF-1-starved BMM were pretreated with the indicated doses of GS-1101 prior to CSF-1 stimulation for 0 or 2.5 minutes and Akt activation measured by SDS-PAGE and immunoblotting as before. (C) PIP₃ levels in CSF-1-starved M^{-/-}.WT cells pretreated with GS-1101 (5μM), IC488743 (5μM) or DMSO (0.1%) then stimulated with CSF-1 for 0 or 30 seconds were measured by PIP₃ ELISA; n=3, *** denotes p<0.001.

Figure 3.

GS-1101 inhibits macrophage spreading. (A) To examine the effect of DMSO on CSF-1-induced macrophage spreading, CSF-1-starved M^{-/-}.WT cells were pretreated with the indicated concentrations of DMSO then stimulated with CSF-1 for 0 or 5 minutes, fixed and stained for F-actin. Cell outlines of at least 30 cells/sample were traced in ImageJ to determine footprint area and a representative experiment (n=3) is shown; * denotes p<0.5, ** p<0.01 and *** p<0.001. (B) CSF-1-starved M^{-/-}.WT cells were pretreated with the indicated doses of GS-1101 or IC488743 prior to CSF-1 stimulation for 0 or 2.5 minutes and Akt activation measured by SDS-PAGE and immunoblotting as before. (C) CSF-1-starved M^{-/-}.WT cells were pretreated with 2μM GS-1101 or 0.04% DMSO then stimulated with CSF-1 for 0 or 5 minutes, fixed and stained for F-actin and cell outlines traced as before; *** denotes p<0.001 (D) M^{-/-}.WT macrophages treated with DMSO or GS-1101 before CSF-1 stimulation for the indicated times were fixed and stained for F-actin. Scale bar represents 20 μM.

Figure 4.

GS-1101 inhibits macrophage invasion and matrix degradation. (A) CSF-1-starved M-/-WT cells pretreated with GS-1101 (5 μ M), IC488743 (5 μ M) or DMSO (0.1%) added to matrigel inserts and their capacity to migrate towards CSF-1 in the bottom chamber over 16 hours measured; n=4, *** denotes $p < 0.001$. For the matrix degradation assay, BMM were seeded onto Cy3-labeled gelatin with GS-1101 (2 μ M) or DMSO (0.04%) for 24 hours prior to fixation and staining for F-actin. At 20x magnification, 5 fields were captured per sample and areas of degraded matrix were matched to overlying F-actin stained cells and traced in ImageJ to determine: (B) the percentage of BMM actively degrading matrix and (C) the percentage area of matrix degraded per field. (D) Representative images of BMM treated with DMSO or GS-1101 taken show F-actin-stained BMM (green) and Cy3-labeled gelatin (red) at 60X magnification. Scale bar represents 20 μ M.