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A ERK/RSK-Mediated Negative Feedback Loop Regulates M-CSF-Evoked PI-3 Kinase/AKT Activation in Macrophages

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Abstract

Activation of the RAS/ERK and its downstream signaling components is essential for growth factor-induced cell survival, proliferation and differentiation. SHP2, encoded by Ptpn11, is a positive mediator required for most, if not all, receptor tyrosine kinase (RTK)-evoked RAS/ERK activation, but differentially regulates the PI3 kinase/AKT (PI-3K) signaling cascade in different cellular contexts. The precise mechanisms underlying differential effects of SHP2 deficiency on the PI-3K pathway remain unclear. We found that mice with myelomonocytic cell-specific (Tg(LysM-Cre);Ptpn11fl/fl mice) Ptpn11 deficiency exhibit mild osteopetrosis. Ptpn11-/− bone marrow macrophages (BMMs) showed decreased proliferation in response to macrophage colony stimulating factor (M-CSF) and decreased osteoclast generation. M-CSF-evoked ERK1/2 activation was decreased, while AKT activation was enhanced, in SHP2-deficient BMMs. ERK1/2, via its downstream target RSK2, mediates this negative feedback by negatively regulating phosphorylation of M-CSF receptor (M-CSFR) at Tyr721 and, consequently, its binding to p85 subunit of PI3K and PI3 kinase activation. Pharmacologic inhibition of RSK or ERK phenotypically mimics the signaling defects observed in SHP2-deficient BMM. Furthermore, this increase in PI3K/AKT activation enables BMM survival in the setting of SHP2 deficiency.

Key words: Macrophages, SHP2, M-CSF, MAPK, PI3 kinase, c-Fms, and AKT
Introduction

Receptor tyrosine kinases (RTKs) regulate the growth, proliferation, differentiation and survival of multiple cell types during development and in adulthood. Despite their pleiotropic actions, RTKs typically initiate a limited and common set of downstream signaling events. Most RTKs activate RAS, which in turn triggers a kinase cascade consisting of one or more RAF family members, MEK1/2, ERK1/2, and ERK-dependent kinases, such as RSK family members (1-4). Activated RAS also promotes the activation of class I phosphatidylinositol-3 kinases (PI-3Ks) (5), as do proteins that bind the regulatory subunits (e.g., p85α/β) of class I PI-3Ks, which include some RTKs themselves and scaffolding adaptors, such as IRS (6) and GAB (7-9) family members. The lipid products of PI-3K bind to PH domain-containing proteins and promote activation of the serine-threonine kinase AKT (and other PH-domain containing signaling components) (10). RTKs also can bind to and activate phospholipase C-γ, SRC family kinases (SFKs) and/or STAT family transcription factors (11).

A major question in RTK signal transduction is how activation such a restricted number of downstream pathways can result in such diverse effects. Differential recruitment/activation of signal relay molecules and the cellular context in which RTK activation occurs may explain some of the distinct effects of RTKs. Yet the mere number of RTKs and the paucity of downstream signaling cascades they evoke suggest that other mechanisms contribute to RTK specificity. Several studies show that the kinetics of downstream pathway activation/inactivation are critical determinants of the biological response to RTK activation (12). Consequently, elucidating the mechanisms that control pathway dynamics, including negative feedback pathways, is critical for a detailed understanding of RTK action.
Macrophage-colony stimulating factor (M-CSF) is essential for monocyte/macrophage and osteoclast proliferation, differentiation, viability and motility (13, 14). Accordingly, op/op mice, which lack M-CSF, are deficient in most macrophage populations and develop severe osteopetrosis (15). M-CSF signals via the Class III RTK, the M-CSFR (a.k.a., CSF1R, c-Fms), which, like other RTKs, binds multiple SH2 (and PTB) domain-containing signal relay molecules that activate canonical downstream signaling cascades. M-CSF-evoked RAS/ERK activation reportedly is mediated via binding of SFKs, GRB2, and CBL1 to M-CSFR-Tyr559, -Tyr697/Tyr921 and -Tyr973, respectively (16-19). M-CSF-induced PI3-K/AKT activation is evoked by p85 binding to M-CSFR-Tyr721 (17, 20) and to GAB family adaptor proteins (21, 22). By contrast, M-CSFR-Tyr706 recruits and activates STAT1, whereas c-CBL binding to Tyr973 promotes M-CSFR ubiquitination and subsequent lysosomal trafficking (23) and degradation (24, 25). The prevailing model holds that the RAS/ERK pathway is important for M-CSF-evoked monocytic cell proliferation, whereas the PI3 kinases/AKT pathway promotes cell survival (13) and STAT1 activation is important for macrophage differentiation (26, 27). There is, however, some disagreement over details: for example, some reports implicate ERK activation in differentiation as well as proliferation (28), whereas others implicate PI-3K/AKT activation in proliferation in addition to survival (29).

The SRC homology-2 domain-containing protein tyrosine phosphatase 2 (SHP2), encoded by the PTPN11 gene, is required for optimal RAS/ERK pathway activation in most, if not all, RTK, cytokine and integrin signaling pathways (30, 31). SHP2 has variable effects on the PI-3K/AKT pathway: in at least some cell types, SHP2 negatively regulates EGF-evoked PI-3K/AKT activation by dephosphorylating the PI-3K binding sites on GAB1 (32, 33). However, SHP2 is required for PI-3K activation downstream of other RTKs (34-36). Moreover, SHP2 has cell type-
and receptor-specific effects on STAT, RHO family GTPase, NF-kB and NFAT activation (31, 37, 38). Reflecting its manifold effects on cell signaling, global Ptpn11 deletion in mice causes early embryonic lethality due to trophoblast stem cell death (39), whereas post-natal SHP2 deletion has variably severe effects on the development and function of multiple cell and tissues (30, 31). ES cell differentiation and chimera experiments implicate SHP2 in the earliest stages of embryonic and adult hematopoiesis, yet less is known about its role in specific hematopoietic cells (40, 41). In monocyte/macrophage lineage cells, for example, SHP2 is found in a signaling protein complex evoked by M-CSF and associates with GAB family adaptor proteins upon M-CSF stimulation (21). Yet its precise role in M-CSF signaling, as well as its effects on monocyte/macrophages and osteoclasts in vivo have remained unknown (42).

To address these issues, we generated mice with selective deletion of the Ptpn11 gene in monocyte/macrophages and osteoclasts. As in other RTK signaling systems, we find that SHP2 is required for RAS/ERK pathway activation, although monocyte/macrophage SHP2 deficiency has relatively minor effects, decreasing BMM proliferation and osteoclastogenesis. In the course of these studies, however, we uncovered a novel, ERK-mediated negative feedback pathway that controls phosphorylation of the M-CSFR on specific tyrosyl residues and thereby regulates the dynamics of M-CSF-evoked PI-3K/AKT activation. Loss of this feedback pathway (as a consequence of defective RAS/ERK activation) might mitigate the effect of SHP2 deficiency on monocyte/macrophage lineage cells and could provide potential escape mechanisms for cancer cells treated with SHP2 or MEK inhibitors.
MATERIALS AND METHODS

Reagents M-CSF and GM-CSF were purchased from Peprotech Inc, and erythropoietin (EPO) was purchased from Janssen-Ortho Inc. UO126 and LY294002 were from Calbiochem, and rapamycin was from Sigma. The RSK inhibitor Fmk was synthesized as described previously (43). Rabbit polyclonal antibodies against murine M-CSFR (c-Fms) were a gift from Dr. Sara A. Courtneidge (Sanford-Burnham Institute). Rabbit polyclonal antibodies against the C-terminus of M-CSFR, SHP2 and the p85 subunit of PI3-K were purchased from Santa Cruz Biotechnology, Inc., rabbit polyclonal antibodies against PKA substrates (R/KXXS/T), p-ERK1/2 (Try202Thr208), p-AKT (Ser473), p-p90RSK(Ser380), p-p70S6 kinase, p90RSK2, and p-M-CSFR (Tyr721) were from Cell Signaling Inc., monoclonal anti-phosphotyrosine antibodies (clone 4G10) were from Upstate Biotechnology, Inc., and anti-FLAG-epitope antibodies were purchased from Sigma. HRP-labeled anti-mouse and rabbit secondary antibodies and protein-A and G beads were purchased from Amersham Inc. For LICOR-based western blotting, goat anti-rabbit IR-Dye 800 and 680, goat anti-mouse IR-Dye 800 and 680 were purchased from LICOR. All antibodies were diluted in 1X TBS/5% non-fat milk for immunoblotting experiments. pMX(puro)-based constructs harboring EPO receptor (EPOR)/M-CSFR-WT (FLAG-epitope-tagged) were described previously (18). EPOR/M-CSFR chimeras containing Ser711 to Ala and Ser728 to Ala mutations were constructed by using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene) and the following primers: Forward: 5’-ATATGTGCGCAGGGA<AGCTGGCTTCTCCAGTCAAGGGT and Reverse: 5’-CACCCTGACTGGAGACAGGCCAGCGTCCCTGAGCAC; Forward: 5’-CGTGGAGATGAGCCCTGCGACTTCTTCAAGTGA<CTCC and Reverse 5’-GGAGTCACCTGAAGAAAGT<CGCGACAGGCCTCACACTCCACG. Point mutations were
confirmed by DNA sequencing. Recombinant M-CSFR and RSK2 proteins were purchased from Signal Chem. Inc.

**Generation of Ptpn11 conditional deletion allele** The Ptpn11 floxed allele (44), Tg(Rosa26-ZsGreen1)(45) and Tg(LysM-Cre) (46) were described previously. PCR conditions for genotyping are available upon request. All experiments herein used mutant mice and littermate controls on a C57BL/6 X 129/Svj mixed genetic background. To assess the role of SHP2 in myelomonocytic cells including macrophages, mice bearing a Ptpn11 floxed allele were bred with Tg(LysM-Cre) mice to generate Ptpn11\(^{\text{floxed}};\text{Tg}(\text{LysM-Cre})\) (hereafter “SHP2\(_{m\Phi}\) CTR”) and Ptpn11\(^{\text{floxed}};\text{Tg}(\text{LysM-Cre})\) (hereafter, “SHP2\(_{m\Phi}\) KO”) mice, respectively (Fig. 1A). To trace the cell-fate of myelomonocytic cells in the SHP2\(_{m\Phi}\) CTR and SHP2\(_{m\Phi}\) KO mice in vivo and in vitro, Tg(Rosa26-ZsGreen1) reporter was crossed over to SHP2\(_{m\Phi}\) CTR and SHP2\(_{m\Phi}\) KO mice in some studies.

**Cell culture** BMMs from 6-9 week-old wild type (WT), SHP2\(_{m\Phi}\) CTR, and SHP2\(_{m\Phi}\) KO mice were derived and cultured for 7-9 days in DMEM containing 10% fetal bovine serum (FBS) and 1/100 volume of CMG14-12 culture supernatant, as described previously (18). Plat-E packaging cells were cultured in the presence of puromycin and blasticidin, as described (47). RAW 264.7 macrophages were cultured in DMEM supplemented with 10% FBS. All cell cultures contained 1% Penicillin/Streptomycin. To generate BMMs and RAW 264.7 cells expressing EPOR/M-CSFR chimeras, Plat-E cells were transiently transfected with pMX(puro)-based constructs by using Effectene (Qiagen), according to the manufacturer’s instructions. Viruses were collected 48 h post-transfection and used to infect BM cells seeded the previous day in the presence of M-CSF (10
ng/ml) and polybrene (4 µg/ml). Infected cells were subsequently cultured in M-CSF and 2µg/ml puromycin to select and expand transductants (18, 48).

Proliferation, differentiation and cell cycle assays BMMs were washed once in 1X PBS, starved in DMEM with 0.2% FBS for 12 hours, and harvested by treatment with Trypsin (0.25%)-EDTA for 5 min. For growth curves, 2 x 10^5 BMMs were seeded in triplicate in 6 cm dishes, and cell number was counted by using a hemocytometer on 5 successive days. For dose-response curves, cells were seeded in triplicate in flat-bottom 96-well tissue culture plates (2 x 10^4 cells/well) at 37°C. Each well contained 100 µl of DMEM supplemented with 10% FCS and various doses of M-CSF. After 2 days of culture, 10 µl (1/10 vol) of WST-1 reagent (Roche) was added and the absorbance of the formazan product was measured at 440 nm. For CFU-M assays, BM cells were placed in semisolid methycellulose medium containing M-CSF (10 ng/ml), cultures were incubated for 7 days at 37°C and colonies containing ≥50 cells were enumerated using a dissection microscope. After counting, cells were recovered by cytocentrifugation and stained with Wright-Giemsa.

To test the effect of Ptpn11 deletion on differentiation, BMMs were cultured for 5-7 days, harvested with non-enzymatic cell dissociation solution (Sigma) and re-suspended in ice-cold PBS plus 2% FBS. For staining with biotinylated F4/80 and FITC-conjugated CD11b (Mac-1) antibodies, cells were initially incubated in Fcγ-block antibodies (1:100 dilution) on ice for 30 min, followed by incubation with primary antibodies (1:200 dilution) on ice for 30 min. Cells were then washed twice in staining solution and incubated with streptavidin-PE (1:500 dilution) for a further 30 min on ice. For cell cycle assays, BMMs were cultured in DMEM supplemented with 10% FBS and 10 ng/ml M-CSF, and BrdU (10 µM) was added 1 hour before removal of the cells with
non-enzymatic cell dissociation solution. BMMs were then fixed, permeabilized and stained with anti-BrdU antibodies and 7-AAD (1µl/10⁶ cells), and analyzed by using a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

**Biochemical analyses** BMMs were starved for 16h in DMEM supplemented with 0.2% FBS, and then stimulated with the indicated growth factors. After stimulation, cells were lysed in NP-40 buffer (0.5% NP40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.4), supplemented with a protease inhibitor cocktail (1mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, 0.5 µg/ml antipain, and 0.5 µg/ml pepstatin). Immunoprecipitations (IPs) were performed on cleared lysates, as described previously [39]. For immunoblotting, TCLs (30-50 µg) were resolved by SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibodies at 4°C for 2 hours-overnight (according to the manufacturer’s instructions), followed by HRP-conjugated secondary antibodies or goat anti-rabbit/mouse IR-Dye 800 or -600 antibodies for 1 hour. Detection was by enhanced chemiluminescence (Amersham) or LICOR scanning.

For immune complex PI-3K assays, M-CSFR immunoprecipitates from starved and M-CSF-stimulated BMMs were washed and subjected to lipid kinase assay, as described [49]. Reactions were terminated by adding 200 µl of 1 N hydrochloric acid (HCl), extracted with 200 µl methanol/chloroform (1:1), and 50 µl of the organic phase were resolved by thin layer chromatography (TLC) in methanol/chloroform/H2O/NH4OH (20:23:4:1) on LK6D plates (Whatman). Plates were exposed to X-ray film, and phospholipid species were identified by co-chromatographed standards.
For protein kinase assays, recombinant M-CSFR cytoplasmic domain (500 µg) was incubated with recombinant human RSK2 (0.1 µg) for 30 min in the presence of γ-32P-ATP (3000Ci/mmol) in kinase buffer (25 mM MOPS, pH7.2, 12.5 mM β-glycerophosphate, 25 mM MgCl₂, 5 mM EGTA, 2 mM EDTA and 25 mM DTT). Samples were then boiled, subjected to SDS-PAGE, transferred onto a PVDF membrane and autoradiographed. Bands corresponding to the M-CSFR were excised, hydrolyzed in 5.7N HCl for 1 hr at 110°C, dried in a Speedvac, and resuspended in 5 µl pH 1.9 buffer containing a mixture of non-radioactive phosphoserine, phosphothreonine and phosphotyrosine. Phosphoamino acids were separated by 2-dimensional electrophoresis/chromatography, according to a published protocol (50). For phosphorylation of peptide arrays, 23 peptides spanning all serine residues in the M-CSFR cytoplasmic domain were synthesized (peptide sequences are listed on Fig. 5C) by using standard Fmoc chemistry on a modified cellulose membrane with an Intavis MultiPep SPOT peptide arrayer, as described (51, 52). Peptide-based kinase assays also were performed as described (53).
RESULTS

Generation of mice with conditional *Ptpn11* deletion in myelomonocytic cells Global SHP2 deficiency causes embryonic lethality in mice (39, 54, 55). To assess the role of SHP2 in macrophages, we crossed our *Ptpn11* floxed allele (44) to *Tg(LysM-Cre)* mice (46). As expected, SHP2 levels were reduced by ~50% and >90% in BMMs from SHP2*mf*CTR and SHP2*mf*KO mice, respectively, compared with wild type control levels (Fig. 1A). No truncation variants were detected by immunoblotting with antibodies directed against either the N- or C-terminus of SHP2 (44).

SHP2 is required for myelomonocytic cell proliferation but not for differentiation

SHP2*mf*KO mice were born at the expected Mendelian ratio and appeared healthy. Blood counts and differentials, measured at 6-8 weeks of age, were comparable in SHP2*mf*CTR and SHP2*mf*KO mice. Preliminary experiments showed that there was no difference in phenotype between wild type, SHP2*mf*CTR (hemizygous) and *Ptpn11*<sup>+/−</sup> or *Tg(LysM-Cre)* parental mice; consequently, we used SHP2*mf*CTR and SHP2*mf*KO mice for most experiments. BMMs (Fig. 1B), as well as cells from bone marrow (BM), spleen and peritoneal cavity of SHP2*mf*KO mice (Fig. S1), showed normal expression of myelomonocytic cell surface markers, indicating that macrophage differentiation was unaffected by SHP2 deficiency. Likewise, cells from M-CSF-dependent colonies (CFU-M) showed normal monocytic morphology (Fig. 1C). By contrast, the number and size of CFU-M were reduced in SHP2*mf*KO mice, suggesting that SHP2 is required for optimal M-CSF-driven proliferation (Fig. 1C). Indeed, proliferation in response to a saturating dose of M-CSF was impaired in SHP2*mf*KO BMMs. M-CSF responsiveness also was diminished, as indicated by a rightward shift in dose-response curve (Fig. 1D). Consistent with these data and the
monocyte/macrophage origin of osteoclasts, SHP2mΦKO mice showed defective osteoclastogenesis in response to M-CSF and RANKL in vitro and developed mild osteopetrosis at >7 months of age (Fig. 1E). We conclude that SHP2 is required for optimal proliferation of macrophage progenitors, but is dispensable for their differentiation to mature macrophage populations. SHP2 also contributes to the generation of optimal levels of osteoclasts.

**SHP2 deficiency impairs RAS/ERK activation but enhances the PI3-K/AKT pathway** To explore the molecular basis for defective proliferation of SHP2-deficient BMMs, we assayed known M-CSF-evoked signaling pathways. BMMs from SHP2mΦCTR and SHP2mΦKO mice were starved for 16 hours, and then stimulated with M-CSF (30 ng/ml). As expected, SHP2mΦCTR BMMs showed robust and sustained activation of RAS (assayed by GST-Raf-RBD binding) and ERK (adjudged by immunoblotting with phospho-specific antibodies). By contrast (but similar to the effects of SHP2 deficiency in many other RTK and cytokine signaling pathways; see Introduction), RAS and ERK activation were transient in SHP2mΦKO BMMs (Fig. 2A). In control BMMs, AKT activation (assessed by Ser473 phosphorylation) peaked at 5 min., and then declined to basal levels by 30 min. In SHP2mΦKO BMMs, however, AKT activation was enhanced and sustained. These differences in ERK and AKT activation could not be explained by altered M-CSFR (c-Fms) levels: surface M-CSFR (CD115) expression (Fig. 1B) was unaffected by SHP2 deficiency, and M-CSFR protein, assessed by immunoblotting, declined at a comparable rate following M-CSF stimulation of SHP2mΦCTR and SHP2mΦKO BMMs. SHP2 deficiency also had no effect on M-CSF-evoked STAT3 or STAT1 activation (Fig. S2A).

SHP2 negatively regulates EGF-induced AKT activation by dephosphorylating the p85 binding site on the adaptor protein GAB1 (32, 33, 56, 57). Surprisingly, PI-3K activity associated
with GAB2, the major GAB family member in BMMs, was unaffected in SHP2-deficient BMMs (Fig. 2B). PI-3 kinase also binds directly to the M-CSFR (17, 20), and indeed, M-CSFR-associated PI-3K activity was enhanced and sustained in SHP2mKO BMMs (Fig. 2C). This increase was accompanied by a slight increase in overall tyrosyl phosphorylation of M-CFSR (Fig. 2D, left panel) and a more substantial increase in phosphorylation of the binding site (Tyr721) for p85 on the receptor (17, 20) (Fig. 2E). M-CSFR/p85 association also was enhanced (Fig. 2D, right panel). Taken together, these findings strongly suggest that increased AKT activation in SHP2-deficient BMMs is due to increased M-CSFR phosphorylation on its binding site for p85.

**SHP2 regulates M-CSFR tyrosyl phosphorylation indirectly, via its effect on ERK activation.** The simplest explanation for the above results would be that SHP2 dephosphorylates Tyr721 (and perhaps other sites) on the M-CSFR. However, SHP2 does not associate with the M-CSFR, nor did we find evidence for SHP2-catalyzed dephosphorylation of the receptor. We wondered whether increased M-CSF-evoked PI-3K/AKT activity in SHP2-deficient BMMs might be the indirect consequence of their defective ERK activation. Indeed, whereas pre-treatment of wild type BMMs with the PI-3K/mTOR inhibitor LY294002 blocked M-CSF-evoked AKT activation, the MEK inhibitor UO126 enhanced AKT activation (Fig. 3A, Fig. S2C). This effect was receptor-specific, as MEK inhibition did not affect AKT activation in response to GM-CSF (Fig. 3B). Similar to the effects of SHP2 deficiency, UO126 pre-treatment resulted in increased M-CSF-evoked PI-3K activation, increased phosphorylation of Tyr721 and increased association of p85 with the M-CSFR (Fig. 3C, D).

Previous studies of other cell types and RTK signaling pathways have shown that ERK or the ERK-activated kinase RSK (1) can phosphorylate and inhibit TSC2, thereby promoting mTOR
activity. mTOR, in turn, activates p70S6 kinase (p70S6K), which can inhibit PI-3K/AKT activation via a well-known negative feedback loop (58, 59). However, treatment of BMMs with rapamycin, which inhibits TORC1 (and p70S6K) activation, did not enhance AKT phosphorylation \((\text{Fig. S2B})\), indicating that abrogation of the p70S6K feedback pathway cannot account for the effects of MEK inhibition on M-CSF-evoked AKT activation in BMMs. Thus, RAS/ERK pathway activation in BMM tempers PI-3K activation via a novel negative feedback pathway that regulates phosphorylation of Tyr721 of M-CSFR.

**RSK2 modulates M-CSFR phosphorylation and AKT activation** We considered the possibility that ERK might phosphorylate M-CSFR and thereby affect Tyr721 phosphorylation and PI-3K association. There are, however, no obvious ERK phosphorylation sites \((Y\text{XXXS/T}P)\) (60-62) in the M-CSFR cytoplasmic domain, nor did we observe interaction between ERK and M-CSFR in co-transfection/co-immunoprecipitation assays. We next asked whether ERK-dependent regulation of M-CSFR signaling might be mediated via an ERK-dependent kinase. Indeed, treatment of wild type BMMs with the RSK-specific inhibitor fluoromethylketone (Fmk) (43) resulted in enhanced M-CSF-evoked AKT activation and M-CSFR Tyr721 phosphorylation to extents similar to that evoked by UO126 treatment \((\text{Fig. 4})\).

Inspection of the M-CSFR cytoplasmic domain revealed two highly conserved serine residues that fall within consensus motifs for RSK phosphorylation; interestingly, these motifs, \(YV\text{RRDS}^{711}\text{G} \) and \(\text{MRPVST}^{728}\text{T}\), also flank Tyr721 \((\text{Fig. 5A})\). Despite substantial effort, we were unable to map RSK-dependent phosphorylation sites on the M-CSFR *in vivo*, most likely because the tryptic peptides containing the putative sites did not fly well in mass spectrometry experiments. However, recombinant human RSK2 phosphorylated the M-CSFR cytoplasmic domain *in vitro*.
Therefore, as an alternative approach to identifying RSK phosphorylation sites, we generated a peptide array spanning the entire M-CSFR cytoplasmic domain, and assessed the ability of recombinant RSK2 to phosphorylate these peptides. Remarkably, only peptides containing Ser711 were phosphorylated by RSK2, whereas Ser711>Ala-substituted peptides were refractory to phosphorylation [Fig. 5C].

**Phosphorylation of Ser711 does not influence the phosphorylation of Tyr721 of M-CSFR and its associated AKT activation** These experiments suggested that RSK2 phosphorylates Ser711 on the M-CSFR. To test this hypothesis and to determine the effect of preventing Ser711 phosphorylation on M-CSF-evoked AKT activation, we used retroviral gene transduction to express chimeric receptors comprising the erythropoietin (EPO) receptor (EPOR) extracellular domain linked to the transmembrane and cytoplasmic domains of WT M-CSFR or M-CSFR carrying a Ser711>Ala mutation [Fig. S3]; both chimeras also contained a FLAG-tag at their amino termini. Use of these chimeras allowed us to probe the effects of M-CSFR mutations in cells expressing endogenous M-CSFR. Initial experiments were carried out with the murine macrophage cell line RAW 264.7. After starvation, transduced cells were stimulated with EPO (to selectively activate the chimeric receptor), and the chimeric receptors were immunoprecipitated using anti-FLAG antibodies and subjected to immunoblotting. Mutation of the Ser711>Ala showed significantly reduced M-CSFR immunoreactivity with phospho-PKA substrate antibodies [Fig. 6A], providing strong evidence that Ser711 is phosphorylated *in vivo*. Notably, Ser711 was phosphorylated even in the absence of EPO stimulation, arguing against MCSF- and RSK-dependent phosphorylation of this site. Nevertheless, M-CSFR Tyr721 phosphorylation and M-CSFR/p85 association were increased/sustained upon M-CSF (EPO) stimulation in cells.
expressing the Ser711A mutant chimeras. Importantly, the WT and S711A chimeras were expressed at comparable levels, as revealed by anti-FLAG immunoblotting, and M-CSF-evoked AKT activation was sustained in cells expressing the S711A mutant, consistent with its increased Tyr721 phosphorylation. These data indicate that S711 phosphorylation constrains Tyr 721 phosphorylation, but phosphorylation of Ser711 does not appear to be M-CSF-/RSK-dependent. Hence, although RSK2 can phosphorylate Ser711 in vitro, and Ser711 phosphorylation constrains the extent and duration of Tyr 721 in cells, RSK does not phosphorylate Ser711 in vivo. How RSK controls Tyr721 phosphorylation remains unclear.

**Loss of the ERK/RSK-mediated negative feedback loop promotes survival of SHP2-deficient BMM but has minimal effect on cytokine production**

ERK and RSK each phosphorylate several proteins important for cellular proliferation and survival (1, 12, 63) and SHP2-mΦKO BMMs have substantially diminished RAS/ERK/RSK activation. Macrophage progenitors in SHP2-mΦKO mice do show diminished proliferation, yet these defects, and the effects of monocyte/macrophage SHP2 deficiency on the whole organism, are fairly mild. We wondered whether the increase in PI3-K/AKT pathway activation in SHP2_{mΦKO} BMMs (as a consequence of loss of the RSK-mediated negative feedback signaling) might mitigate the effects of SHP2 deficiency on ERK and RSK activation. To test this hypothesis, SHP2_{mΦCTR} and SHP2_{mΦKO} BMMs were treated with the AKT inhibitor A6730 (10 µM) in the presence of murine M-CSF (10 ng/ml) for 48 hours, and cell cycle distribution was assessed. AKT inhibition decreased the percentage of SHP2_{mΦCTR} BMMs in S phase by ~50%, but did not increase the number of sub-G1 (apoptotic) cells. By contrast, under normal growth conditions, SHP2_{mΦKO} BMMs showed a ~2-fold fewer S and G2/M cells, and a slightly increased number of sub-G1 cells, consistent with their decreased
proliferation rate. Addition of the AKT inhibitor (10 µM) caused a further, ~3-fold decrease in S phase cells, and evoked substantial apoptosis, with the sub-G1 population rising to ~19% in SHP2\textsubscript{mΦKO} (compared with ~2% in SHP2\textsubscript{mΦCTR}) BMMs. These data suggest that increased activation of the PI-3K/AKT pathway in SHP2\textsubscript{mΦKO} BMMs (as a consequence of impaired RSK-mediated negative feedback) helps to protect them from the decrease in proliferation and survival that would ordinarily accompany defective RAS/ERK pathway activation. Consistent with this notion, combined inhibition of the RAS/ERK and PI3-K/AKT pathways in WT BMMs (by treatment with UO126 and LY294002) causes a dramatic increase in cell death [Fig. S5], comparable to the effects of AKT inhibition in SHP2-deficient BMM (Fig. 7A).
Discussion

SHP2 is a key mediator of signaling by most, if not all RTKs and cytokine receptors (30, 31), yet its role in specific hematopoietic lineages remains largely unexplored. By studying the effects of selective deletion of a “floxed” Ptpn11 allele in the presence of a LysM-Cre transgene, we found that SHP2, while dispensable for myelomonocytic differentiation, is required for optimal proliferation of monocyte/macrophage progenitors. Because these progenitors also give rise to osteoclasts, SHP2 is required for efficient osteoclast production in vitro, and SHP2mΦKO mice develop mild osteopetrosis as they age. In attempting to elucidate the molecular basis for these mild defects, we identified a novel negative feedback pathway in which the ERK-dependent kinase RSK2 regulates the phosphorylation of the binding site for p85 on the M-CSFR, Tyr721, and possibly other tyrosyl residues (Fig. 6B). This feedback pathway, in turn, controls the extent and duration of M-CSFR-evoked PI-3K and AKT activation, and its absence (and the resultant increase in AKT activation) likely contributes to the relatively mild effects of SHP2 deficiency in the monocyte/macrophage lineage.

SHP2mΦKO mice exhibit significant defects in myelomonocytic cell proliferation in vitro. BM from these mice yields fewer CFU-M than does WT BM, indicating decreased numbers of monocytic progenitors. Moreover, these progenitors have diminished M-CSF responsiveness, as indicated by their rightward-shifted dose-response curve. SHP2mΦKO BMMs show similar proliferative defects, attributable to their diminished M-CSF sensitivity. SHP2-deficient mice also develop mild, age-related osteopetrosis, and show defective osteoclast generation in vitro. Mice lacking the SHP2 binding protein GAB2 also have osteopetrosis, although this phenotype was attributed to defective RANK-L, not M-CSF, signaling (64). Yet overall, despite these in vitro defects, SHP2mΦKO mice are remarkably healthy, with normal peripheral blood counts and no
apparent increased tendency to infection, presumably reflecting the ability of normal homeostatic mechanisms to compensate for the defective M-CSF responsiveness.

As in other RTK signaling pathways and other cell types, SHP2 deficiency in BMMs results in decreased RAS/ERK activation in response to M-CSF stimulation. Previous studies have implicated the RAS/ERK and PI3-K/AKT pathways in M-CSFR-driven proliferation and/or survival \(13\). Given that AKT activation is not diminished in SHP2\(m\phi\)KO BMM (but instead, is enhanced; see below), diminished RAS/ERK activation probably explains, at least in large part, their defective proliferation. Previous work implicated STAT1 and STAT3 in BM cell differentiation towards the myelomonocytic lineage \(65, 66\). SHP2 does not affect M-CSF-evoked STAT1 and STAT3 phosphorylation, and consistent with this finding, macrophage differentiation is apparently unaffected in SHP2\(m\phi\)KO mice.

SHP2-deficient BMMs show increased AKT activity, which, by analogy to studies of other signaling pathways \(32, 33, 57\), we initially expected to be due to increased GAB binding to p85. However, GAB2-associated PI-3K activity (and p85 association) is comparable in SHP2\(m\phi\)CTR and SHP2\(m\phi\)KO BMMs. Instead, we find that M-CSFR-associated PI-3K activation is enhanced in SHP2\(m\phi\)KO BMMs, as a consequence of increased M-CSFR Tyr721 phosphorylation and p85 recruitment.

Several lines of evidence suggest that an indirect, RSK-mediated negative feedback pathway \(\text{Fig. 6B}\) explains the increased Tyr721 phosphorylation and enhanced PI3-K/AKT activation in the absence of SHP2. First, the effects of MEK (U0126) or RSK (Fmk) inhibitors on overall M-CSFR tyrosyl phosphorylation, Tyr721 phosphorylation, p85 association with M-CSFR and AKT activation are comparable to those of SHP2 deficiency. RSK2 deficiency also enhances M-CSF-evoked AKT activation, arguing against off-target effects of the pharmacological
inhibitors and suggesting that of the four known RSK family members (1), RSK2 specifically mediates this negative feedback pathway (Fig. 4B). Second, GM-CSF-evoked AKT activation is unaffected by SHP2 deficiency, which suggests that receptor-proximal events are involved in the negative regulatory mechanism. Although RSK can phosphorylate M-CSFR on Ser711 in vitro, and Ser711 phosphorylation controls the extent and duration of Tyr721 phosphorylation, immunoblotting experiments indicate that Ser711 is constitutively phosphorylated. Furthermore, UO126 treatment does not affect Ser711 phosphorylation (Fig. S4B). Therefore, the RSK-mediated negative feedback pathway must be mediated by another, as yet unidentified protein, and Ser711 must be phosphorylated by another kinase. Protein kinase A is a good candidate for the latter, because BMMs, treated with PKA activator 8-Bromo cAMP, show reduced tyrosyl phosphorylation of M-CSFR (67).

Our findings have important implications for understanding macrophage and osteoclast regulation by M-CSFR, for RTK signaling in general, and for the potential use of MEK inhibitors for cancer therapeutics. The concomitant loss of the RSK2-mediated feedback pathway probably helps explain the relatively mild effects of impaired RAS/ERK activation in the monocyte/macrophage lineage. Consistent with this notion, SHP2_{mKO} BMMs are strongly sensitized to PI3K or AKT inhibitor treatment (Fig. 7A). It is increasingly clear that the duration and magnitude of downstream pathway activation are key determinants of signal specificity by RTKs and other types of receptors. Feedback pathways probably play an important role in regulating these pathway properties. In this regard, the RSK2-mediated pathway that we have identified is reminiscent of the now well-characterized p70S6K-catalyzed negative feedback regulation of insulin/insulin-like growth factor receptor signaling (4, 58). Finally, the RAS/ERK pathway is up-regulated in many tumors, which has prompted the development of MEK-inhibitors
as potential anti-neoplastic agents (68, 69). If the RSK2-mediated negative feedback pathway (or an analogous MEK-dependent pathway) were operative in cancer cells, they might “escape” from MEK-inhibitor treatment as a consequence of increased AKT activation. A similar escape mechanism via inactivation of the p70S6K-mediated negative feedback pathway has been documented in some cancer cells treated with Tor inhibitors such as rapamycin(70). Alternatively, inactivation of RSK2-mediated negative feedback (by mutations or epigenetic mechanisms) might allow cancer cells to benefit from increased activation of the PI-3K/AKT pathway. Further studies will be required to elucidate the detailed mechanism by which RSK2-mediated negative feedback controls M-CSFR signaling and to assess the generality of its role in RTK regulation and cancer.
Acknowledgements

We thank Dr. Changqi Sun for technical assistance in constructing pMX(puro) expression constructs. This work was supported in part by NIH grants R01CA114945 and R37 CA49152 (B.G.N.), R21AR57156, RO1AR066746 and P20RR025179 (W.Y.). This study was also aided by a grant from Arthritis National Research Foundation (W.Y.). B.G.N. was a Canada Research Chair, Tier 1, and this research was also funded in part by the Ontario Ministry of Health and Long Term Care. The views expressed do not necessarily reflect those of the OMOHLTC.
**Figure Legends:**

**Fig. 1.** SHP2 deficiency compromises myelomonocytic cell proliferation, but is dispensable for differentiation. (A) Diagram showing breeding scheme for generating Ptpn11^{fl/+};Tg(LysM-Cre) (SHP2_{mΦ}CTR) and Ptpn11^{fl/fl};Tg(LysM-Cre) (SHP2_{mΦ}KO) mice. Panels show SHP2 (top) and actin (bottom) immunoblots of BMM lysates (20µg). Actin serves as a loading control. (B) Flow cytometric analysis of cell surface marker expression in SHP2_{mΦ}CTR and SHP2_{mΦ}KO BMMs. (C) Macrophage colony assays (CFU-M) in SHP2_{mΦ}CTR and SHP2_{mΦ}KO mice. Left panels: Phase contrast views of colony morphology and Wright-Giemsa stain of cytospin preparation from colonies. Right panels: M-CSF dose-response curve. Note that SHP2 deficiency results in decreased number and size of CFU-Ms with preserved macrophage differentiation, as well as diminished sensitivity to M-CSF (n=3). *p<0.05 (Student’s t-test). (D) SHP2 deficiency decreases BMM proliferation and M-CSF sensitivity. Shown are proliferation (left panel) and dose-response (WST1 assay, right panel) curves for SHP2_{mΦ}CTR and SHP2_{mΦ}KO BMMs. #p<0.05 (Student’s t test). (E) SHP2 deficiency causes mild osteopetrosis. Representative micro-CT images (top) show increased thickness of cortical bone of eight-month-old SHP2_{mΦ}KO mouse, compared with SHP2_{mΦ}CTR. *In vitro* osteoclastogenesis assay, showing that SHP2_{mΦ}KO BMMs are defective in generating TRAP^{+} osteoclasts in response to M-CSF (10 ng/ml) and RANKL (100 ng/ml). Shown is one of two experiments with similar results; osteoclastogenesis was defective in SHP2_{mΦ}KO mice across a wide range of M-CSF and RANKL doses.

**Fig. 2.** SHP2 promotes M-CSF-evoked RAS/ERK pathway, but negatively regulates M-CSFR-associated PI-3 kinase (PI-3K) and AKT activation. (A) BMMs were starved for 14 hours, then either stimulated with M-CSF (30 ng/ml) for the indicated times or left untreated. TCLs from
SHP2\textsubscript{mΦ}CTR and SHP2\textsubscript{mΦ}KO BMMs were analyzed by GST-RBD assay to assess RAS loading (800 µg) or by immunoblotting (30 µg) with the indicated antibodies. (B and C) GAB2- and M-CSFR-associated PI-3K assays from SHP2\textsubscript{mΦ}CTR and cCKO BMMs. Note that SHP2 deficiency leads to increased M-CSFR-associated (C), but not GAB2-associated (B), PI-3K activity. (D) SHP2 deficiency increases M-CSFR association with PI-3K. Lysates (800 µg) from starved or M-CSF-stimulated BMMs were immunoprecipitated with anti-M-CSFR antibodies, followed by immunoblotting for the p85 subunit of PI-3K (D, left panel), or immunoprecipitated with anti-p85 antibodies, followed by immunoblotting with M-CSFR or anti-pTyr antibodies (D, right panel). Note the increased M-CSFR/PI-3K association and slight increase in M-CSFR tyrosyl phosphorylation in SHP2\textsubscript{mΦ}KO BMMs. (E) Increased phosphorylation of M-CSFR on Tyr721 in SHP2\textsubscript{mΦ}KO BMMs. TCLs (40 µg) from the indicated BMM samples were immunoblotted with p-Tyr721-specific antibodies or M-CSFR antibodies as a control for loading. Data shown are a representative of the three (n=3) independent experiments.

**Fig. 3.** Blocking ERK activation increases M-CSF-evoked M-CSFR-Tyr721 phosphorylation and PI-3K activation. (A and B) WT BMMs were starved, pre-treated with the MEK inhibitor U0126 (10µM), the PI-3K/mTOR inhibitor LY294002 (10 µM), or vehicle (DMSO) for 1 hour, and then were either left unstimulated (-) or stimulated (+) for 10 min with 30 ng/ml M-CSF (A) or 50 ng/ml GM-CSF (B). AKT and ERK activation were assessed in TCLs (30 µg) by immunoblotting with the indicated p-specific antibodies. Blocking ERK activation by MEK inhibitor treatment increases M-CSF-, but not GM-CSF-evoked AKT activation, whereas inhibiting M-CSF or GM-CSF-evoked PI-3K activation does not affect ERK activation. (C and D) MEK inhibitor treatment increases M-CSFR-associated PI-3K activity and M-CSFR Tyr721 phosphorylation and
association with the p85 subunit of PI-3K. WT BMMs were starved, pre-treated for 1 hr with vehicle (DMSO), UO126 (10µM) or LY294002 (10µM), and then stimulated with M-CSF (30ng/ml) for the indicated times. M-CSFR immunoprecipitates (from 1 mg TCL) were subjected to PI-3K assays (C). M-CSFR and p85 immunoprecipitates also were immunoblotted with the indicated antibodies (D). Data shown are a representative of the three (n=3) independent experiments.

**Fig. 4** RSK2 regulates M-CSFR Tyr721 phosphorylation and AKT activation. (A) WT BMMs were starved, pre-treated with UO126 (10 µM), the RSK inhibitor Fmk (fluoromethylketone) (6µM), or vehicle (DMSO) for 1hr, and then either stimulated with M-CSF (30 ng/ml) or left unstimulated. TCLs (30 µg) were immunoblotted with the indicated antibodies. (B) BMMs from Rsk2-/- mice or littermate controls (WT) were starved, and then stimulated with M-CSF (30 ng/ml) for the indicated times (+) or left unstimulated (-). TCL (30 µg) were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies. Data shown are a representative of the three (n=3) independent experiments.

**Fig. 5.** RSK2 phosphorylates M-CSFR on Ser711 *in vitro*. (A) M-CSFR is inducibly phosphorylated on site(s) that conform(s) to RSK consensus motif. Lysates (1 mg) from SHP2<sub>wt</sub>CTR or SHP2<sub>wt</sub>KO BMMs that had been starved and stimulated with M-CSF (30 ng/ml) for the indicated times (+) or left unstimulated (-) were immunoprecipitated with anti-M-CSFR antibodies and then immunoblotted with anti-p-PKA substrate antibodies (which recognize the sequence R/KXXpS/pT). (B) Sequence of section of M-CSFR cytoplasmic domain, showing position of two potential RSK sites (consensus motif R/KXXS/T) flanking the p85 binding site,
Tyr721. Note that these sites are conserved in mouse (m), human (h), monkey (rh), rat (rt), guinea pig (gp) and dog (dg). (C) M-CSFR phosphorylates RSK2 in vitro. Recombinant M-CSFR cytoplasmic domain was incubated with recombinant RSK2 in the presence of γ³²P-ATP, as described in Materials and Methods. The reaction products were resolved by SDS-PAGE, and the phosphorylated M-CSFR band was subjected to phosphoamino acid analysis. (D) Peptide library-based kinase assays. Peptides corresponding to the 23 serine residues of M-CSFR cytoplasmic domain were subjected to spot blot kinase assays with recombinant RSK2. Phosphorylated peptides were visualized and quantified by using a Phosphorimager. Note that only peptides containing Ser711 were phosphorylated to any significant extent.

**Fig. 6** Loss of RSK2-mediated feedback pathway helps promote proliferation and survival of SHP2-deficient BMMs. (A) Mutation of Ser711 on M-CSFR leads to increased phosphorylation on M-CSFR Tyr721 and enhanced M-CSF-evoked AKT activation. RAW264.7 cells expressing the indicated EPOR/M-CSFR chimera were starved (-), and then stimulated with 5U/ml erythropoietin (EPO) for the indicated times (+). Cell lysates were immunoprecipitated with anti-FLAG antibody, and then immunoblotted with the indicated antibodies. Note that the Ser711Ala mutant shows decreased reactivity with anti-pPKA antibodies and increased reactivity with anti-pTyr and -pTyr721 antibodies. Data shown are a representative of the three (n=3) independent experiments. (B) Model for RSK-mediated negative feedback pathway in comparison with p70S6 kinase-mediated feedback signaling loop. See text for details.

**Fig. 7** SHP2 deficiency sensitizes BMM to apoptosis evoked by AKT inhibitor A6730. Randomly growing BMMs from SHP2<sup>wt</sup>CTR and SHP2<sup>wt</sup>KO mice were treated with DMSO or the AKT
inhibitor A6730 (10 µM). BrdU (10 µM) was added to the culture medium for 1 hour, and cells were stained with anti-BrdU antibodies and 7-AAD and subjected to flow cytometric cell cycle analysis. Regions 1, 2, 3 and 4 (R1, 2 3, and 4) represent sub-G1, G0/G1, S phase and G2/M cell populations, respectively. Note that, compared with SHP2 <sup>mΦ</sup> CTRs, SHP2 <sup>mΦ</sup> KO BMMs are hypersensitive to AKT inhibition. Data shown are a representative of the three (n=3) independent experiments.
Supplementary information

Figure S1. (A) Flow cytometric analysis demonstrating the comparable level of macrophage markers (Mac1 and F4/80) in the indicated cell populations from SHP2<sub>mΦ</sub>CTR and SHP2<sub>mΦ</sub>KO mice. Spl, Spleen; BM, bone marrow. (B) Fluorescent Images showing the similar morphology and number of BMMs derived from SHP2<sub>mΦ</sub>CTR;Rosa26<sup>ZSG</sup> and SHP2<sub>mΦ</sub>KO;Rosa26<sup>ZSG</sup> mice. Data shown (A, B) are a representative of the three (n=3) independent experiments.

Figure S2. (A) M-CSF-evoked STAT1 and STAT3 activation are unaffected by SHP2 deficiency. SHP2<sub>mΦ</sub>CTR and SHP2<sub>mΦ</sub>KO BMMs were starved, and then either left unstimulated (-) or stimulated with M-CSF (+) for the indicated times. Lysates were resolved by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. (B) Blocking M-CSF-evoked mTOR activation by Rapamycin treatment has no apparent effect on AKT activation. Wild type BMMs were starved, then treated with DMSO, UO126 (10µM) or Rapamycin (10 nM) for 1 hour, and then either stimulated with M-CSF (30 ng/ml) for the indicated times (+) or left unstimulated (-). Cell lysates were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. ERK2 (on a duplicate blot) served as an internal loading control. (C) Blockade of ERK activation by UO126 enhanced M-CSF-evoked AKT activation in wild type (WT) BMMs. BMMs were starved, pretreated with UO126 (10µM) and stimulated with M-CSF (30ng/ml) for the indicated times (+) or left unstimulated (-). Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Data shown (A-C) are a representative of the three (n=3) independent experiments.
**Figure S3.** Sequence data demonstrating the conversion of Ser711 to Ala in the EPOR/M-CSFR chimeric receptor. The pMX vector was used as the backbone vector.

**Figure S4.** (A) M-CSF-evoked ERK and AKT activation are comparable in RAW264.7 cells expressing EPOR/M-CSFR chimeras. The cell clones in Fig. 6B were stimulated with M-CSF (30ng/ml) for the indicated times (+) or left unstimulated (-). Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Note that, by contrast to EPO-stimulated cells, M-CSF stimulation results in a comparable magnitude and duration of AKT activation. (B) RAW264.7 cells expressing wild type EPOR/M-CSFR chimera were starved (-), and then treated DMSO or UO126 for 2 hours and stimulated with 5U/ml erythropoietin (EPO) for the indicated times (+). Cell lysates were immunoprecipitated with anti-FLAG antibody, and then immunoblotted with anti-pPKA substrate antibodies. Note that the blockade of ERK/RSK activation by UO126 had no effect on EPOR/M-CSFR phosphorylation. Data shown are a representative of the three (n=3) independent experiments.

**Figure S5.** M-CSF-evoked ERK and PI-3 kinase activation are required for BMM survival. Randomly growing WT BMMs were treated with DMSO, UO126 (10µM) and LY294002 (10 µM) for 14 hours. Cells were then harvested, stained with propidium iodide and analyzed by flow cytometry. Note the relative insensitivity of BMM to U0126 or Ly294002 treatment, and the marked increase in apoptotic (sub-G1) cells in the presence of both inhibitors.
References:

17. van der Geer, P., and Hunter, T. (1993) Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. Embo J 12, 5161-5172


Figure 1

A) 

- **Ptpn11^fl/+; Tg(LysM-Cre)**  
- **Ptpn11^fl/fl**  

- **Ptpn11^fl/+; Tg(LysM-Cre)** (SHP2^mΦCTR or mΦCTR)  
- **Ptpn11^fl/fl** (SHP2^mΦKO or mΦKO)

B) 

- **F4/80**  
- **Mac1**  
- **CD115**

Events

C) 

- **Phase contrast**  
- **Wright-Giemsa**

C) 

- **SHP2^mΦCTR**  
- **SHP2^mΦKO**

D) 

- **Cell No.**  
- 10 8 6 4 2 0  
- **OD (450NM)**  
- 1 0.8 0.6 0.4 0.2 0.1 0  
- **M-CSF (10 ng/ML)**

- **mΦCTR**  
- **mΦKO**

E) 

- **μCT**  
- **TRAP**

- **SHP2^mΦCTR**  
- **SHP2^mΦKO**
Figure 2

A  |  SHP2<sub>mΦ</sub>CTR  |  SHP2<sub>mΦ</sub>KO
---|------------------------|------------------------
-  |  + + + +               |  - + + +                
0  |  |                        |                        
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15 |  |                        |                        
30 |  |                        |                        

M-CSF Min
- RasGTP
- Pan-RAS
- pERK/2
- ERK1/2
- pAKT Ser473
- AKT
- SHP2
- M-CSFR

B  |  GAB2 IP
---|------------------------
SHP2<sub>mΦ</sub>CTR  |  SHP2<sub>mΦ</sub>KO
-  |  +                     |  - +                    
0  |  |                        |                        
15 |  |                        |                        

- PIP3

C  |  M-CSFR IP
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SHP2<sub>mΦ</sub>CTR  |  SHP2<sub>mΦ</sub>KO
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M-CSF Min
- PIP3

D  |  M-CSFR IP  |  P85 IP
---|-------------|---------
SHP2<sub>mΦ</sub>CTR  |  mΦCTR  |  mΦKO  
-  |  +         | - +     
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15 |  |          |          

M-CSF Min
- pTyr
- p85

E  |  SHP2<sub>mΦ</sub>CTR  |  SHP2<sub>mΦ</sub>KO
---|------------------------|------------------------
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M-CSF Min
- pM-CSFR Tyr721
- M-CSF1R
- SHP2
Figure 3

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Legend:

- DMSO
- UO126
- Fmk
**Figure 5**

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C. 

- Free phosphate
- pSer
- pThr
- pTyre

D. 

Load

**Substrate Sequence**

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Figure 6
Figure 7

SHP2<sub>mφ</sub> CTR

SHP2<sub>mφ</sub> KO

BrdU

DMSO

AKT inhibitor A6730

7-AAD
Figure S1

A

Fluorescent Images of BMMs

B

Fluorescent Images of BMMs
Figure S2

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Figure S3

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GCT
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TGTCGACACC
TAC
GTGGAGATGAGGCCTGTC
TCG
ACTTCTT

S711A
Y721

S728
Figure S4

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- EpoR/M-CSFR
- (Ser711Ala)
- pERK1/2
- pAKT Ser473
- ERK1/2
- AKT
- FLAG
- IP: anti-FLAG

B

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- pPKA substrate
- EpoR/M-CSFR (WT-FLAG)
Figure S5

- DMSO
- Kinase inhibitor
- LY294002
- UO126
- DNA (PI)

Events