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Clodronate-Liposome Mediated Macrophage Depletion Abrogates Multiple Myeloma Tumor Establishment *In Vivo*

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Abstract

Multiple myeloma is a fatal plasma cell malignancy that is reliant on the bone marrow microenvironment. The bone marrow is comprised of numerous cells of mesenchymal and hemopoietic origin. Of these, macrophages have been implicated to play a role in myeloma disease progression, angiogenesis, and drug resistance; however, the role of macrophages in myeloma disease establishment remains unknown. In this study, the antimyeloma efficacy of clodronate-liposome treatment, which globally and transiently depletes macrophages, was evaluated in the well-established C57BL/KaLwRijHsd murine model of myeloma. Our studies show, for the first time, that clodronate-liposome pretreatment abrogates myeloma tumor development *in vivo*. Clodronate-liposome administration resulted in depletion of $CD169^{+}$ bone marrow–resident macrophages. Flow cytometric analysis revealed that clodronate-liposome pretreatment impaired myeloma plasma cell homing and retention within the bone marrow 24 hours postmyeloma plasma cell inoculation. This was attributed in part to decreased levels of macrophage-derived insulin-like growth factor 1. Moreover, a single dose of clodronate-liposome led to a significant reduction in myeloma tumor burden in KaLwRij mice with established disease. Collectively, these findings support a role for CD169-expressing bone marrow–resident macrophages in myeloma disease establishment and progression and demonstrate the potential of targeting macrophages as a therapy for myeloma patients.

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Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by the clonal proliferation of aberrant plasma cells (PC) within the bone marrow (BM); accumulation of monoclonal immunoglobulin (paraprotein); and end-stage organ damage including osteolytic bone lesions, hypercalcemia, anemia, and renal insufficiency [1]. MM accounts for 1% of all cancers [2], with over 100,000 people diagnosed worldwide each year [3]. In almost all cases, MM is preceded by an indolent, asymptomatic disease known as monoclonal gammopathy of undetermined significance (MGUS), which is characterized by an increase in PC numbers within the BM (<10%) but manifests with few, if any, of the clinical features of symptomatic MM [4]. Despite advances in MM management and therapy, MM remains almost universally fatal.

In response to a chemokine gradient, MM PC home to the BM and colonize discrete endosteal niches within the medullary cavity, adjacent to bone surfaces [5]. Cells within the BM produce factors such as C-X-C motif chemokine ligand 12 (CXCL12; SDF-1) that increase the migration, adhesion, and retention of MM PC [6,7]. Recent data show that while many MM PC home to the BM, very few proliferate and contribute to the tumor burden [8,9]. This phenomenon is, at least in part, determined by the microenvironment in which these cells colonize. However, it is currently unclear what cell types within the BM contribute to the proliferative MM niche and which maintain MM cells in dormancy.[2]

Resident macrophages are heterogeneous immune cells of the mononuclear phagocytic lineage found in most adult tissues [10]. Within the BM, resident macrophages can be partitioned into distinct subpopulations based on their phenotype, anatomical location, and specialized function. Notably, macrophage numbers have previously been shown to increase in the BM of patients with active MM compared with asymptomatic MGUS [11] and have been associated with poor prognosis [12]. In addition, tumor-associated macrophages (TAMs) have been shown to promote angiogenesis, as well as MM PC growth and survival [13]. Interestingly, macrophages have been shown to directly contribute to the myeloma blood vessel network *via* "vasculogenic mimicry" [14] and are well documented to protect MM PC from drug-induced apoptosis [15–17]. Despite these findings, a direct role for BM resident macrophages in the establishment of MM remains unknown.

Interestingly, the depletion of mature macrophages using liposomal clodronate has shown promise as a therapy to inhibit tumor progression in a range of malignancies including lymphoma [18], melanoma [19], lung adenocarcinoma [20], and ovarian cancer [21]. The bisphosphonate clodronate, like other members of its class, is an antiresorptive agent that is rapidly and selectively adsorbed to bone following administration, limiting systemic exposure [22]. Encapsulation of clodronate in lipid vesicles specifically targets clodronate to phagocytic macrophages that engulf and degrade the liposome, leading to clodronate accumulation and subsequent cellular apoptosis [23]. Unlike free clodronate, clodronate-liposomes (clo-lip) globally deplete macrophages and other phagocytic cells [23,24].

In this study, the well-established C57BL/KaLwRijHsd (KaLwRij) murine model of myeloma was utilized in combination with clo-lip–mediated macrophage depletion to assess the role of mature macrophages in the initial establishment of MM PC within the BM. Furthermore, we assessed the efficacy of clo-lip as a potential therapy for MM. We found that clo-lip pretreatment led to a significant reduction in BM MM PC homing and retention, a concomitant increase in the numbers of circulating tumor cells, and decreased tumor burden. In addition, we characterized the effects of clo-lip on the BM microenvironment and investigated BM macrophages *in vitro* in order to elucidate the potential mechanisms by which clo-lip–mediated macrophage depletion impaired MM PC homing.

Methodology

Cell Culture

All cells were cultured under sterile conditions and maintained at 37°C with 5% CO_2. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich, St. Louis, MI. All media were supplemented with additives: 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin. Mouse 5TGM1 MM PC, previously modified to express green fluorescent protein (GFP) and luciferase construct (luc) (5TGM1-eGFP-luc) [25,26], were maintained in complete Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum (FCS) (HyClone, QLD, Australia). A human BM endothelial cell (BMEC) line (TrhBMEC) was maintained in Medium 199 (M199) supplemented with 20% FCS, 0.01% sodium bicarbonate, 1× MEM nonessential amino acids (Life Technologies), 50 μg/ml endothelial cell growth supplement (BD Biosciences, Franklin Lakes, NJ), and 100 U/ml heparin.

Animals

C57BL.KaLwRijHsd (KaLwRij) mice were bred and housed at the South Australian Health and Medical Research Institute (SAHMRI) Bioresources facility. All studies were performed in accordance with SAHMRI Animal Ethics Committee approved procedures. Six- to 8-week-old age- and sex-matched KaLwRij mice were injected i.v. with a single dose of clo-lip or control phosphate-buffered saline (PBS)–liposome (PBS-lip) suspensions (200 μl/20 g mouse) (Liposoma BV, Amsterdam, the Netherlands) or i.p. with 100 μg/kg zoledronate (Novartis Pharma, Basel, Switzerland) and administered i.v. with 5×10^5 or, for the homing assay, 5×10^6 5TGM1-eGFP-Luc cells in 100 μl of sterile PBS. Tumor development was monitored weekly by bioluminescence imaging (BLI) as previously described [27,28]. C57BL/6 mice were injected i.v. with a single dose of clo-lip or control PBS-lip suspensions (200 μl/20 g mouse) 24 hours prior to i.v injection of 1×10^6 Vk*Myc 4929 cells. At experimental endpoints, or weekly for the Vk*Myc model, serum was isolated and serum paraprotein electrophoresis (SPEP) performed on a Sebia Hydragel b1/b2 kit (Sebia, Norcross, GA).

Flow Cytometric Analysis

For BM detection of GFP^+ tumor cells, BM was flushed from the long bones using a 21G needle into PFE (PBS, 2% FCS,2 mM ethylenediamine tetra-acetic acid [EDTA]), cut longitudinally with a scalpel blade, scraped along the inner surface, and finely chopped.

[2] BLI: bioluminescence imaging; BM: bone marrow; BMEC: BM endothelial cell; clo-lip: clodronate-liposome; HSC: hematopoietic stem cells; M-CSF: macrophage colony-stimulating factor; MGUS: monoclonal gammopathy of undetermined significance; MM: multiple myeloma; MSC: mesenchymal stem cells; PBS-lip: PBS-liposome; PC: plasma cells; SPEP: serum paraprotein electrophoresis; TAM: tumor-associated macrophage

Bone fragments were crushed and syringed several times before straining through a 70-μm cell strainer, and the resulting cell suspension was pooled with the flushed BM. Cells were washed and resuspended in PFE for immediate analysis on an LRSFortessa X20 flow cytometer (BD Biosciences).

For circulating tumor cell analysis, mice were anesthetized by isoflurane inhalation and cardiac blood collected using a 26G needle containing 50 μl 0.5 M EDTA to prevent clotting. Red blood cells were lysed by incubating three times with red blood cell lysis buffer (0.15 mM ammonium chloride, 10 mM potassium bicarbonate, and 1.26 mM disodium EDTA, pH 8.0) for 10 minutes. Cells were washed and resuspended in Hank's buffered saline solution with 5% FCS, filtered, and analyzed immediately on a BD FACS Canto II flow cytometer. In all instances, a BM or blood from a naive (noninjected) mouse was used as a negative control or was spiked with *in vitro*–cultured 5TGM1 cells for a GFP-positive control.

For cell lineage analysis, BM cells were extracted from long bones (femora and tibiae) using a mortar and pestle, stained with Fixable Viability Stain 700 (323 ng/ml; BD Biosciences), and blocked with mouse gamma globulin (117 μg/ml; Abacus ALS, QLD, Australia). For detection of tumor cells in the Vk*Myc model, BM was stained with CD138-PE (BioLegend, San Diego, CA) and B220-PE-Cy7 (eBioscience, San Diego, CA). For detection of BM macrophages, BM was stained with CD11b-APC Cy7, CD169-PE (BioLegend), and F4/80-Pacific Blue (Bio-Rad, Hercules, CA). For hematopoietic cells, mature Lin^+ cells were excluded by incubation with a lineage cocktail of biotin-conjugated antibodies (B220, CD3, CD4, CD5, CD8, Gr1, Ter119 [BioLegend] and Cd11b [eBioscience]) followed by streptavidin-APC (Life Technologies, Carlsbad, CA) secondary. Cells were concurrently stained with Sca-Brilliant Violet-(BV)786, cKit-PE-Cy7, CD135-PE-CF594, and CD34-BV421 (all from BD Biosciences). Mesenchymal cells were quantitated from compact bone as previously described [26]. Cells were either fixed in 1% neutral buffered formalin, 2% glucose, and 0.01% sodium azide in PBS or immediately analyzed on the LRSFortessa X20.

Histological Analysis and TRAP staining

Femora were fixed in 10% paraformaldehyde, processed, and embedded in methacrylate as previously described [29]. Five-micron sections were cut and stained for the presence of tartrate-resistant acid phosphatase 5 (TRAP) to identify osteoclasts, as previously described [29]. Briefly, slides were deplasticized in acetone and incubated in AS-BI phosphate (0.4 mg/ml) in acetate-tartrate buffer (200 mM sodium acetate, 100 mM potassium sodium tartrate, pH 5.2) at 37°C for 30 minutes. The samples were then transferred to hexazotized pararosanoiline solution (1 mg/ml) in prewarmed tartrate-acetate buffer and incubated for 30 minutes at 37°C. Sections were rinsed and counterstained with 0.05% methyl green solution. To enumerate osteoclasts, histomorphometric analysis was performed using the OsteoMeasure7 v4.1.0.2 analysis system (OsteoMetrics, Decatur, GA). Osteoclasts were defined based on TRAP-positive staining and the standard criterion of multinucleated cells (≥3 nuclei) residing along the bone surface.

In Vitro Cell Survival Assay

5TGM1-eGFP-Luc cells (1×10^5) were incubated in IMDM media supplemented with additives and 20% FCS with clo-lip or PBS-lip for 3 days. Cells were stained with Fixable Viability Stain 700 (323 ng/ml) and analyzed on LRSFortessa X20 flow cytometer.

In Vitro Macrophage Maturation

Long bones (tibiae and femora) were excised from 7-week-old KaLwRij mice and BM flushed using a 21G needle. BM cells were seeded into flasks at 2.6×10^5 cells/cm^2 in IMDM media supplemented with additives, 10% FCS, and 25 ng/ml recombinant mouse macrophage colony-stimulating factor (M-CSF; Lonza, Basel, Switzerland), and media were replaced every 2-3 days. Cells were harvested following 6 days of M-CSF treatment using Accutase (Sigma-Aldrich) as per manufacturer's instructions. Macrophages were stained with rat anti-mouse F4/80-FITC (Bio-Rad) and CD169-PE (BioLegend), or FITC rat IgG2b-isotype control and PE rat IgG2a-isotype control (BioLegend), respectively, and analyzed on LRSFortessa X20 flow cytometer (BD Biosciences).

Matured Macrophage Conditioned Media

KaLwRij matured macrophages were seeded at 1×10^5 cells/cm^2 in IMDM media supplemented with additives, 10% FCS, and 12.5 ng/ml M-CSF. After 24 hours, fresh media (without M-CSF) were added. Conditioned media were collected after a further 24 hours of culture, filtered through a 0.22-μm filter, and stored at –80°C until required.

Transendothelial Migration Assay

Migration assays were performed using 8-μm polycarbonate membrane Transwells (Costar, Washington, DC) in a 24-well plate. BMECs were seeded into the upper chamber of Transwells at 1×10^4 cells/well. After 48 hours, media were removed, BMECs were washed with serum free IMDM, 1×10^5 5TGM1-eGFP-luc cells in IMDM media were supplemented with additives, and 10% FCS was added on top of the BMEC monolayer. Macrophage-derived conditioned media diluted to 10% and 50% in IMDM or IMDM medium alone were added to the lower chamber. The number of migrated 5TGM1-eGFP-luc cells was enumerated after 20 hours using an inverted microscope, digital camera (Olympus CKX41), and ImageJ software as described previously [30].

Western Blot

5TGM1-eGFP-luc cells (2.5×10^6) were stimulated for 10 minutes with KaLwRij matured macrophage conditioned media or IGF-1 recombinant protein (ProSpec Bio, East Brunswick, NJ). Cell lysates were prepared, and equivalent amounts of protein (100 μg) were separated on a 10% acrylamide gel and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane and subsequently blocked with 5% w/v skim milk. Immunoblotting was performed with antibodies directed against phospho-IGF-1Rβ (Tyr1135/1136), IGF-1Rβ (Cell signaling technologies, 1:1000), and β-Actin Clone AC-15 (Sigma-Aldrich, 1:2500). Following incubation with the appropriate IgG Dylight conjugated secondary antibodies (Thermofisher, 1: 20,000), proteins were visualized using the Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE, USA).

RNA Sequencing

Total RNA was extracted from 5TGM1-eGFP-luc cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. 5TGM1-eGFP-luc RNA was confirmed to be of adequate quality (RIN score >8) using a Bioanalyzer 2200 (Agilent), and samples were stored at –80°C. The cDNA libraries were prepared using NEXTflex mRNA-sequencing kit (BIOO Scientific) and

sequenced using a NextSeq500 sequencer (Illumina) at the David Gunn Genomics Facility (SAHMRI, Adelaide). Raw RNA-sequencing data (fastq files) of single-end reads (1 × 75 bp) were analyzed. Briefly, read quality was assessed using FastQC. Overrepresented adapter sequences were trimmed using Trimmomatic version 0.33, and quality assessment was repeated. Filtered reads were mapped to the reference genome hg19 using STAR version 2.5.0b. Aligned output data (BAM files) from individual lanes of the same sample were combined, and the number of mapped reads were counted using featureCounts (part of Rsubread version 1.12.6). Transcripts expressed at levels below five counts per million reads, in at least three libraries, were filtered out from downstream analysis. Relative expression was determined using quasi-likelihood *F* test from edgeR R package to account for variability due to relatively small sample size. Significantly regulated genes were identified using a cutoff of one-fold or greater changes in mean expression and FDR <0.05.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from BM cells and KaLwRij matured macrophages using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Following which, cDNA was synthesized using Superscript IV (Life Technologies) as per manufacturer's protocol. Gene-specific quantitative real-time PCR was conducted on a Bio-Rad CFX 9000 qPCR instrument using RT^2 SYBR Green reagent (QIAGEN, Hilden, Germany) and primer pairs as shown in Supplementary Table I. Resultant gene expression was analyzed using the ΔCt method **($2^{-\Delta Ct}$)** normalized to β-actin.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism v 7.03. Groups were compared using one-way or two-way analysis of variance (ANOVA) with Tukey's or Holm-Sidak's multiple comparisons posttests or unpaired *t* tests as indicated.

Results

Clodronate-Liposome mediated Macrophage depletion

Clo-lip has previously been shown to globally deplete mature, functional macrophages [23,31,32]. Initially, we confirmed the effect of clo-lip on BM macrophages in tumor-naive KaLwRij mice. Mice were treated with a single i.v. injection of clo-lip or a PBS-lip control, and the extent of macrophage depletion was investigated. As clo-lip administration is known to induce apoptosis of all phagocytic cells, including osteoclasts [24], zoledronate was administered to a third group of mice as an osteoclast-depletion control [33]. Flow cytometric analysis revealed a 70% reduction in the total $CD11b^+F4/80^+$ monocyte/macrophage population within the BM of clo-lip–treated animals compared with controls (Figure 1*A*). Notably, this decrease was due solely to the ablation of the $CD11b^+F4/80^+CD169^+$ mature BM macrophage population, which was reduced by 90% in the clo-lip–treated mice compared with PBS-lip and zoledronate controls, while the $CD11b^+F4/80^+CD169^-$ monocyte/macrophage population was unaffected by clo-lip treatment (Figure 1, *A* and *B*). Importantly, the depletion of BM macrophages was maintained for more than 14 days, with the $CD169^+$ macrophage population only returning to 50% of that of control animals 28 days after a single i.v. injection of clo-lip (Figure 1*C*).

Inhibition of MM Tumour Development by Clodronate-Liposome Pretreatment

To investigate the effect of clo-lip–mediated macrophage ablation on MM tumor development, KaLwRij mice were inoculated with 5TGM1 MM PC *via* the tail vein 24 hours after the mice had been treated with clo-lip, PBS-lip, or zoledronate. Zoledronate-treated mice developed tumor at a similar rate to PBS-lip–treated mice, indicating that inhibition of osteoclasts does not affect tumor establishment and growth in this model. In contrast, clo-lip pretreatment resulted in a significantly reduced tumor burden compared with PBS-lip controls (Figure 2). Mice treated with clo-lip displayed >95% lower tumor burden as determined by both BLI (Figure 2, *A* and *B*) and SPEP analysis (Figure 2*C*) after 4 weeks. In addition, flow cytometric analysis at 4 weeks post tumor cell inoculation revealed a significant decrease in the number of GFP^+ tumor cells in the circulation (Figure 2*D*) of clo-lip–treated mice compared with controls. Notably, comparable results were observed in the progressive Vk*Myc MM murine model following upfront clo-lip treatment (Figure S1).

Interestingly, although there was a significant decrease in the total number of GFP^+ tumor cells in the BM of clo-lip–treated KaLwRij mice at day 28, there was no difference in the tumor growth rate between the two treatment groups from day 14 to day 28 [population doublings: PBS-lip, 8.9 ± 0.2; clo-lip, 8.4 ± 0.4 (mean ± SD; P = .24)] (Figure S2). Moreover, 5TGM1 cell viability was not affected by 72 hours of clo-lip treatment *in vitro* (Figure S3). Together, these data suggest that the inhibition of tumor development in clo-lip–treated animals was not due to direct effects on 5TGM1 cell growth or survival.

Effect of Clodronate-Liposomes on MM PC Homing and Retention in the BM

The homing of MM PC to specific niches within the BM that support their colonization and growth is a critical event in the establishment of MM tumors [5,9]. As there was no effect on MM PC proliferation or survival following clo-lip treatment, we next investigated the effect of clo-lip–mediated macrophage depletion on 5TGM1 MM PC homing and retention. 5TGM1 MM PC were injected i.v. into clo-lip– or PBS-lip–treated mice, and the number of GFP^+ tumor cells present within the BM and peripheral blood was assessed after 24 hours by flow cytometry. A 2.7-fold reduction in the total number of tumor cells present within the BM of clo-lip–treated mice was observed (Figure 3*A*), with a concomitant 5.4-fold increase in the number of tumor cells remaining in circulation (Figure 3*B*), compared with PBS-lip–treated controls. Collectively, these data suggest that clo-lip treatment impairs MM development *in vivo,* at least in part, by inhibiting MM PC homing to and/or retention within the BM.

Role of Macrophages in MM PC Migration

As clo-lip treatment inhibited the homing of MM PC to the BM, we next investigated whether macrophages play a specific role in MM PC migration. Macrophages were matured *in vitro* from KaLwRij BM by treatment with macrophage-colony stimulating factor (M-CSF) for 6 days. As confirmed by flow cytometric assessment at day 6, more than 90% of the matured cells were $F4/80^+$, and on average, 42% were $CD169^+$ (Figure S4). Conditioned media from these KaLwRij matured macrophages stimulated 5TGM1 MM PC

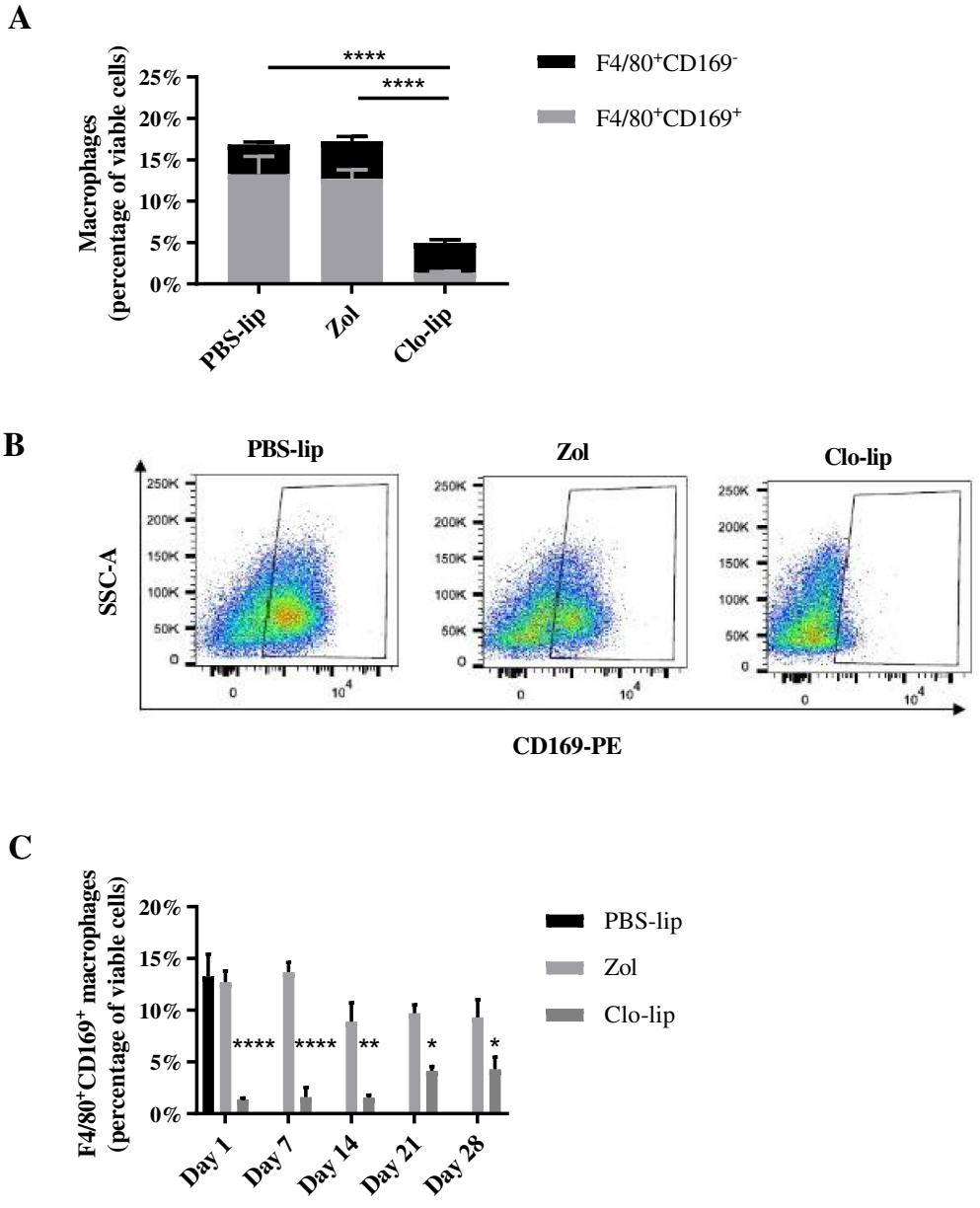

Fig. 1. Clodronate-liposomes deplete CD169-expressing macrophages within the BM.KaLwRij mice were treated once with PBS-lip, zol, or clo-lip. Total BM was isolated from long bones after 24 hours (A, B) or as indicated (C); stained with Cd11b, F4/80, and CD169 fluorescently conjugated antibodies; and analyzed by flow cytometry. (A) $Cd11b^{+}F4/80^{+}CD169^{-}$ monocyte/macrophage population (black; n.s, $P = .694$) and $Cd11b^{+}F4/80^{+}CD169^{+}$ mature macrophages (gray; $^{****}P < .0001$) expressed as a percentage of total viable cells. (B) Representative FACS plots of $Cd11b^{+}F4/80^{+}CD169^{+}$ mature macrophages. (C) Recovery of $Cd11b^{+}F4/80^{+}CD169^{+}$ mature macrophages following a single injection of clo-lip over a 28-day period. $n = 3$/group. Graphs show mean ± SEM, $^{*}P < .05$, $^{**}P < .01$, $^{****}P < .0001$, 2-way ANOVA with Tukey's multiple comparisons test.

migration in a dose-dependent manner (Figure 4*A*), suggesting that macrophages play a direct role in MM PC homing and migration.

To further investigate the potential mechanism by which macrophages increase MM PC migration *in vitro*, the expression of chemokine/cytokine receptors for key secreted factors that play a role in MM pathogenesis was assessed in 5TGM1 cells by RNAseq. Notably, 5TGM1 cells expressed high levels of the insulin-like growth factor 1 (IGF-1) receptor (*Igf1r*), the receptors for BAFF (TNFSF13B) and APRIL (TNFSF13) (*Tnfrsf13b* and *Tnfrsf13c*), the receptor for CXCL12 (*Cxcr4*), the receptor for tumor necrosis factor alpha (TNF-α) (*Tnfrsf1a*), and the genes encoding the interleukin 6 (IL-6) receptor complex (*Il6st* and *Il6ra*) (Figure 4*B* and Supplementary Table II).

Next, the mRNA expression levels of the corresponding ligands were analyzed in KaLwRij matured BM macrophages. Notably, high levels of *Igf1* mRNA (Figure 4*C*), a potent promigratory and proliferative factor for MM PC [34,35], were observed. In contrast, KaLwRij matured BM macrophages expressed low levels of *Tnfa* and *Tnfsf13* and undetectable *Il6, Tnfsf13b*, and *Cxcl12* (Figure 4*C*). Next, we confirmed the expression and activation of IGF-1R in 5TGM1 cells by Western blot. As shown in Figure 4*D*, recombinant IGF-1 protein stimulated a dose-dependent phosphorylation of IGF-

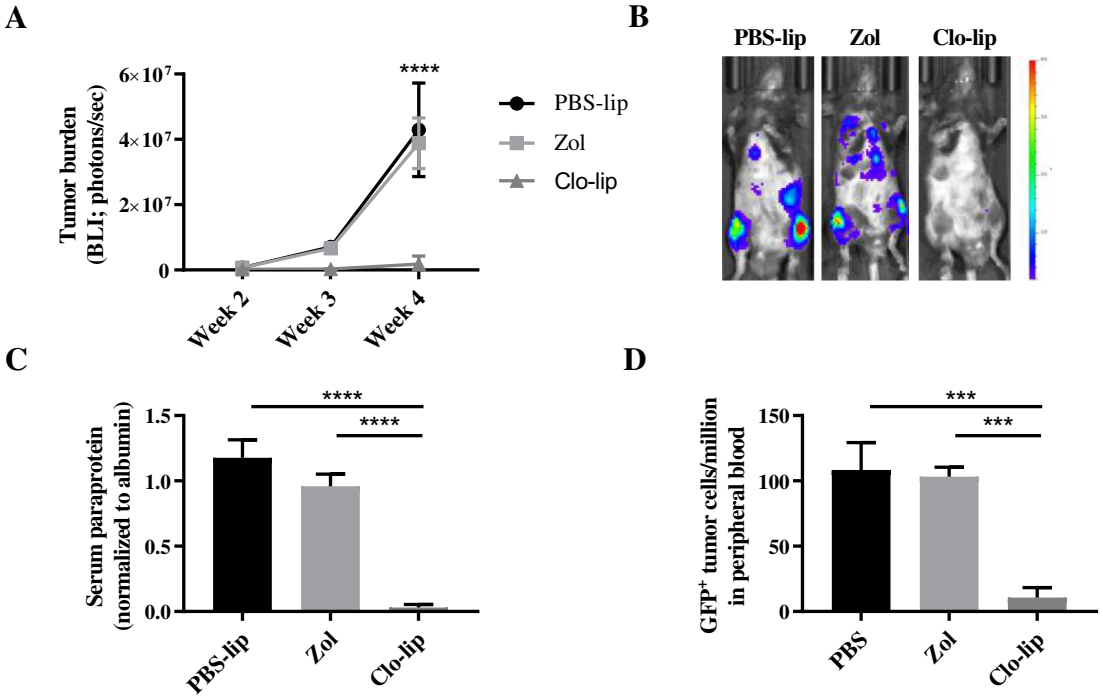

Fig. 2. Clodronate-liposome pretreatment inhibits MM tumor development *in vivo*.KaLwRij mice were treated once with PBS-lip, zol, or clo-lip 24 hours prior to i.v. injection of 5TGM1 MM PC. (A) Tumor burden was measured by BLI at 2, 3, and 4 weeks post tumor cell inoculation. Graph shows mean ± SEM, ****$P < .0001$, two-way ANOVA with Tukey's multiple comparisons test. (B) Representative BLI images for each treatment group at 4 weeks. (C) Serum paraprotein quantitation at 4 weeks. (D) Flow cytometric analysis of GFP+ 5TGM1 within the peripheral circulation at 4 weeks. n = 3–8/group. Graphs show mean ± SEM, ***$P < .001$, ****$P < .0001$, 1-way ANOVA with Tukey's multiple comparisons test.

1R in 5TGM1 cells. Moreover, stimulation with matured macrophage conditioned medium also resulted in IGF-1R phosphorylation (Figure 4*E*), confirming that macrophage conditioned medium contains IGF-1. Taken together, these data suggest that macrophage-derived IGF-1 may play an important role in 5TGM1 MM PC migration.

Clodronate-Liposome Treatment Decreases BM-Expressed Igf1 and Cxcl12 In Vivo

In order to investigate whether clo-lip treatment alters the mRNA expression profile within the BM, the expression of MM-associated chemokines and cytokines, identified above, was assessed by qPCR on total BM 24 hours after clo-lip or PBS-lip treatment. Interestingly, there was a significant decrease in *Igf1* mRNA levels in clo-lip–treated animals compared with PBS-lip controls (Figure 5*A*). In addition, BM expression of *Cxcl12*, which plays an important role in MM PC homing, retention, and growth [36], was significantly decreased following clo-lip treatment (Figure 5*B*). In contrast, no change in the mRNA levels of *Tnfsf13b*, *Tnfsf13*, *Tnfa*, or *Il6* was observed (Figure 5, *C-F*). These findings suggest that MM PC migration may be impaired, at least in part, *via* clo-lip–mediated reduction in BM levels of IGF-1 and CXCL12.

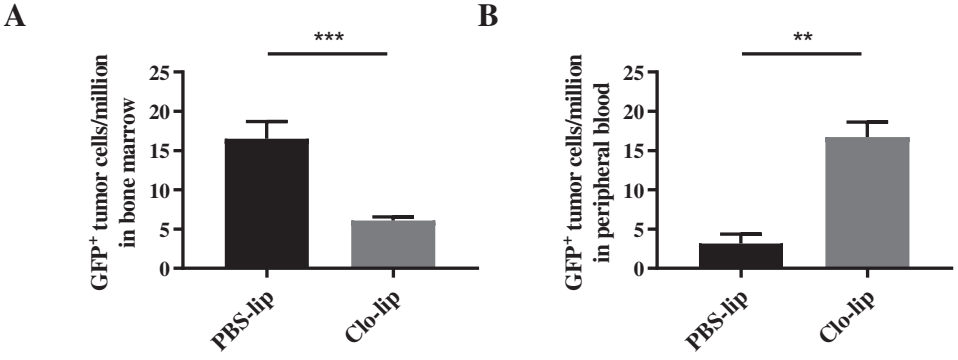

Fig. 3. Clodronate-liposome pretreatment impairs MM PC homing/retention *in vivo*. GFP^+ 5TGM1 MM PC within the (A) BM or (B) in the circulation 24 hours after tumor cell inoculation were analyzed by flow cytometry in PBS-lip– or clo-lip–treated mice. n = 3-5/group. Graph shows mean ± SEM, **$P < .01$, ***$P < .001$, unpaired t test.

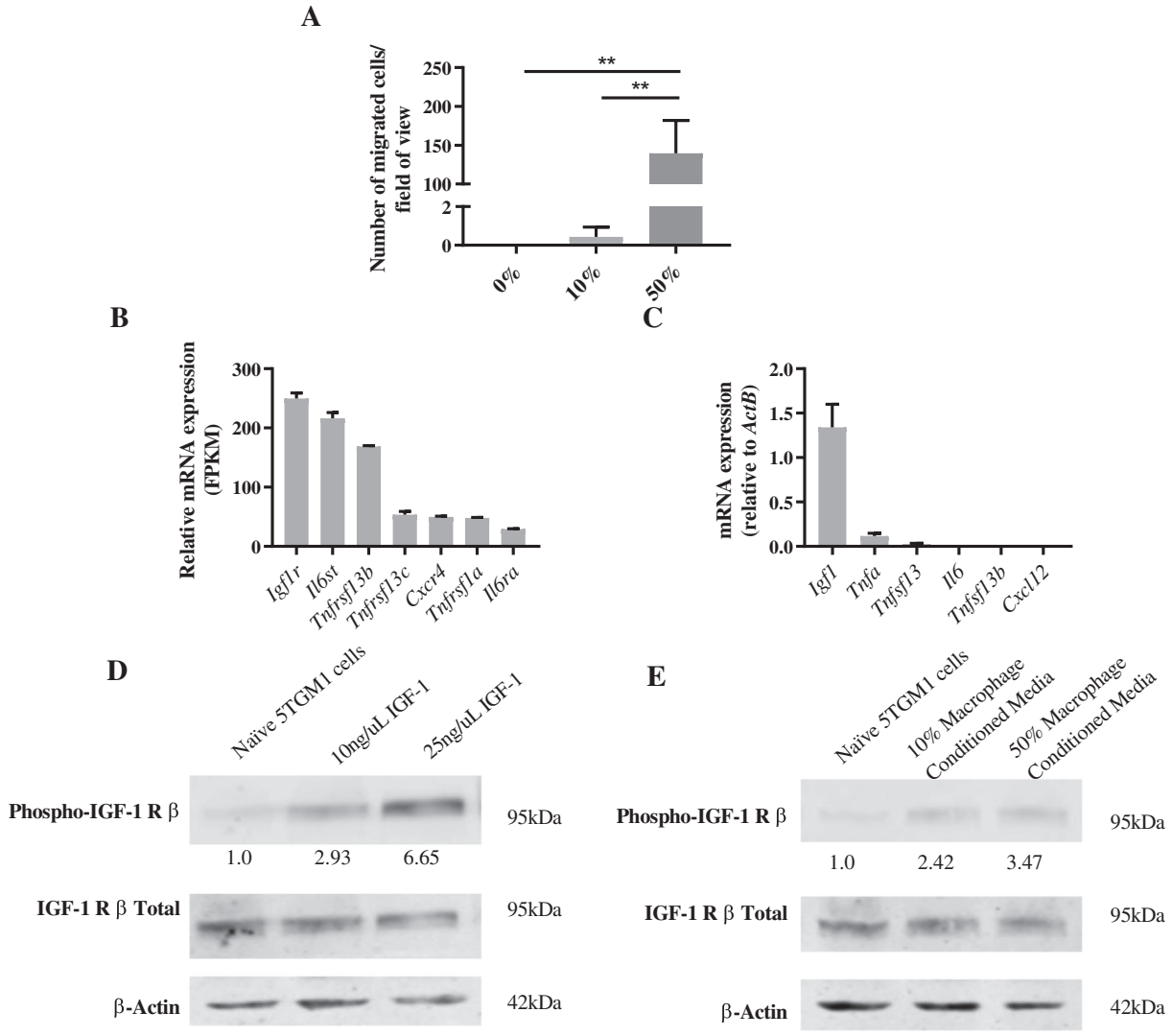

Fig. 4. IGF-1-expressing KaLwRij matured macrophages enhance MM PC migration *in vitro*.(A) Transendothelial migration of 5TGM1 MM PC toward increasing concentrations of KaLwRij matured BM macrophage conditioned media. Graph shows mean ± SD, $n = 3$; $^{**}P < .01$, 1-way ANOVA with Tukey's multiple comparisons test. (B) Relative mRNA gene expression of cell surface receptors on 5TGM1 MM PCs by RNAseq analysis. (C) Relative mRNA gene expression of KaLwRij matured BM macrophages analyzed by qPCR. Graphs show mean ± SD, $n = 3$. (D) Western blot analysis of 5TGM1 MM PC stimulated for 10 minutes with increasing concentrations of IGF-1 recombinant protein or (E) KaLwRij matured BM macrophage conditioned media. Numbers indicate fold change in phospho-IGF-1R relative to total IGF-1R, normalized to naive. Images are representative of three independent experiments.

Clodronate-Liposomes Decrease Osteoblast Numbers In Vivo

As both macrophages and osteoclasts are an abundant source of IGF-1 [37,38], we assessed osteoclast numbers 24 hours post clo-lip and PBS-lip administration. There was no significant difference in the number of osteoclasts (No.Oc/B.Pm) in clo-lip–treated mice compared to PBS-lip controls ($P = .71$, t test, Figure S5*A*). Notably, CXCL12 is not expressed by matured BM macrophages (Figure 4*C*) but is produced in the BM by cells of the mesenchymal lineage, including MSCs and osteoblasts [36]. Changes in MSC, osteoprogenitor, and osteoblast numbers within the compact bone following 24 hours of clo-lip exposure were investigated by flow cytometry. Following exclusion of hematopoietic (CD45/Lin) and endothelial (CD31) cells, mesenchymal cell populations were resolved based on their expression of CD51 and Sca1 [26,39]. While clo-lip treatment had no effect on MSC [$CD45^-Lin^-CD31^-CD51^-Sca\text{-}1^+$] numbers, a 6.5-fold decrease in osteoprogenitors [$CD45^-Lin^-CD31^-CD51^+Sca\text{-}1^-$] and 7.5-fold decrease in osteoblasts [$CD45^-Lin^-CD31^-CD51^+Sca\text{-}1^+$] were observed in clo-lip–treated mice compared with PBS-lip–treated controls (Figure S5*B*). These data suggest that decreased osteoblast numbers may account for the reduction in *Cxcl12* mRNA observed in the BM of clo-lip–treated mice.

Hematopoietic Lineage Cells Increase In Vivo Following Clodronate-Liposome Treatment

As shown above, clo-lip administration dramatically decreased osteoblast and osteoprogenitor cell numbers, which in addition to playing a role in myeloma pathogenesis are also a key component of the hematopoietic stem cell (HSC) niche [40]. Therefore, we also investigated clo-lip–mediated changes to hematopoietic progenitor cell numbers within the BM. Hematopoietic cell populations were resolved based on their expression of CD135 and CD34. Clo-lip treatment resulted in a significant 5-fold increase in hematopoietic

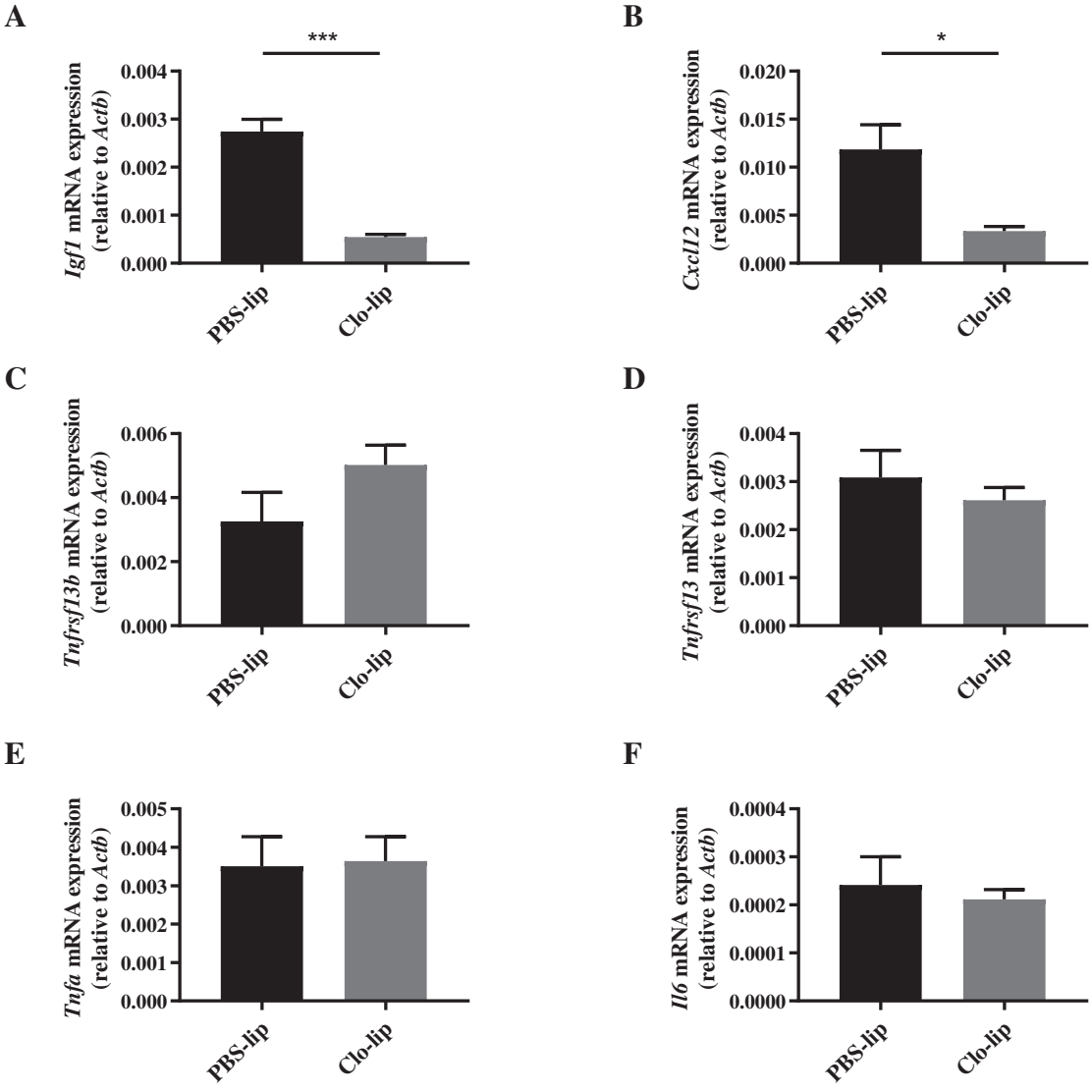

Fig. 5. Clodronate-liposome pretreatment affects the mRNA expression profile of the BM.KaLwRij mice were injected i.v. with PBS-lip or clo-lip, and 24 hours later, BM was harvested from tibiae and femora for assessment of (A) *Igf1*, (B) *Cxcl12*, (C) *Tnfsf13b*, (D) *Tnfsf13*, (E) *Tnfa*, and (F) *Il6* gene expression by qPCR. Graphs show mean ± SEM, n = 3-4/group; *P < .05, ***P < .001, unpaired t test.

stem/progenitor cells (HSPC) [$Lin^-Sca\text{-}1^+cKit^+$], a 2-fold increase in long-term HSC (LT-HSC) [$Lin^-Sca\text{-}1^+cKit^+CD135^-CD34^-$], and a 12-fold increase in short-term HSC (ST-HSC) [$Lin^-Sca\text{-}1^+cKit^+CD135^-CD34^+$] numbers when compared with PBS-lip–treated controls (Figure S5*C*). These data suggest that, in addition to MM tumor inhibition, clo-lip–mediated macrophage ablation may have downstream effects on the cellular composition of the BM microenvironment.

Reduction of MM Tumor Development Following Clodronate-Liposome Treatment In Vivo

To assess whether clo-lip–mediated ablation of macrophages may display therapeutic efficacy in the established disease setting, KaLwRij mice were inoculated with 5TGM1 MM PC and, 2 weeks later, injected with clo-lip or PBS-lip. Notably, clo-lip–treated mice demonstrated a 2.7-fold reduction in tumor burden at 4 weeks, compared with PBS-lip controls, as assessed by BLI (Figure 6, *A-B*) and SPEP (Figure 6*C*). Additionally, flow cytometric analysis demonstrated a corresponding 4.5-fold decrease in the number of GFP^+ 5TGM1 tumor cells within the peripheral circulation at 4 weeks post tumor cell inoculation in clo-lip–treated mice compared with controls (Figure 6*D*). Interestingly, even in the presence of tumor, macrophage ablation was maintained, with the $CD11b^+F480^+CD169^+$ BM-resident macrophage population depleted by 90% 2 weeks post clo-lip treatment (Figure S6).

Discussion

The migration of MM PC to the BM and their subsequent proliferation and survival are well established to be dependent on supportive elements within the BM microenvironment. MM PC leave the peripheral lymphoid organ, enter the circulation, transendothelially migrate, and home to the BM in response to a chemokine gradient wherein they colonize discrete endosteal niches. Here, the MM PC interact with cells of the BM microenvironment, which secrete pro-proliferative and antiapoptotic cytokines that favor MM PC growth and survival within the BM [5,36]. While macrophages

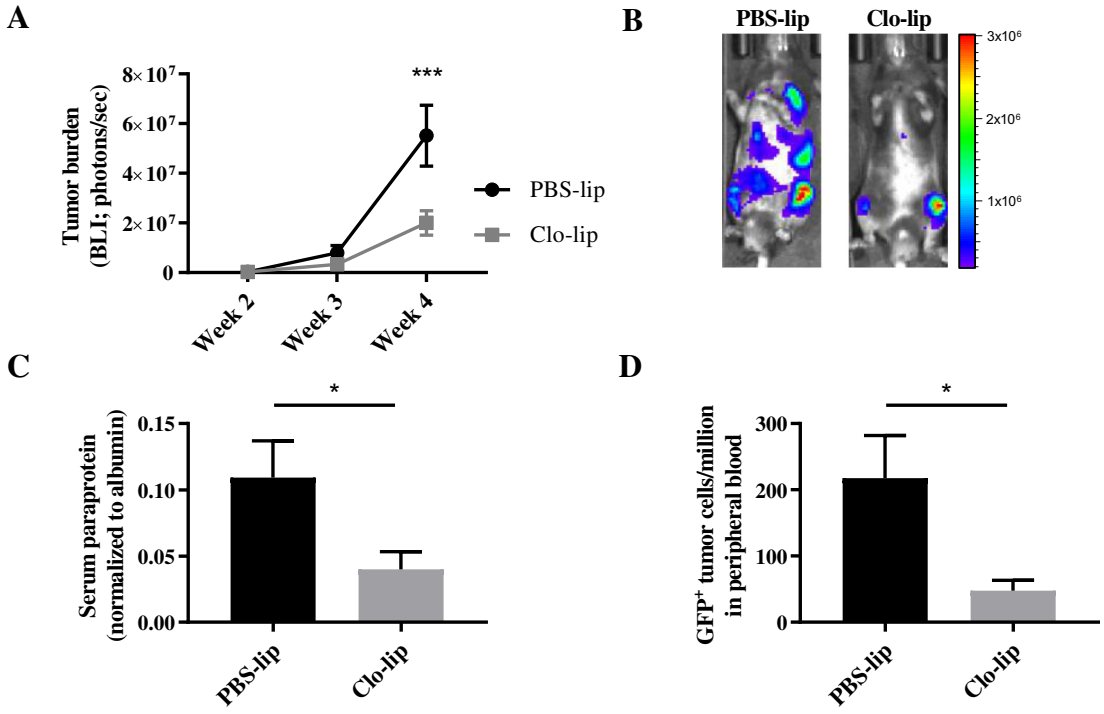

Fig. 6. MM tumor development is significantly reduced *in vivo* in clodronate-liposome–treated mice.KaLwRij mice were inoculated i.v. with 5TGM1 MM tumor cells, and tumor was allowed to establish for 2 weeks, followed by a single treatment with PBS-lip or clo-lip (n = 7/group). (A) Tumor burden was measured by BLI at 2, 3, and 4 weeks post tumor cell inoculation. Graph shows mean ± SEM, ***P < .001, two-way ANOVA with Sidak's multiple comparisons test. (B) Representative BLI images for each treatment group at week 4. (C) Serum paraprotein quantitation 4 weeks post tumor cell inoculation. (D) Flow cytometric analysis of GFP^+ 5TGM1 within the peripheral circulation at 4 weeks post tumor cell inoculation. Graphs show mean ± SEM; *P < .05, unpaired t test.

have been suggested to play a role in this process, infiltrating the established MM tumor [13], the role of BM-resident macrophages in MM PC colonization and disease establishment has not been fully elucidated. Liposome-encapsulated clodronate, which depletes macrophages and other phagocytic cells, has previously been shown to inhibit tumor progression in a range of cancers [18–21]. In this study, we demonstrate, for the first time, that clo-lip–mediated ablation of the mature $CD11b^+F4/80^+CD169^+$ BM macrophage population in the 5TGM1/KaLwRij murine model of MM significantly impairs MM tumor establishment, suggesting that $CD169^+$ BM-resident macrophages may play a pivotal role in MM pathogenesis.

In support of these findings, a recent study has shown that depletion of monocyte/macrophage lineage cells in MM tumor-bearing mice, by targeting CSF1R genetically or with a function-blocking anti-CSF1R antibody, significantly decreased MM tumor burden [16]. While our study confirmed that macrophages play a role in disease progression, we have also demonstrated a novel role for mature macrophages resident within the BM microenvironment in the initial stages of MM establishment. Moreover, we demonstrated that these mature BM-resident macrophages specifically express CD169 and play a critical role in MM PC homing and colonization within the BM.

In addition to a drastic reduction in tumor burden following upfront treatment with clo-lip, we also observed a significant decrease in tumor burden following the administration of clo-lip in the established disease setting. The dissemination of MM PC to sites throughout the BM is an essential process in MM disease progression and relapse, and parallels various features of solid tumor metastasis to bone [41]. We propose that the decreased tumor burden in the established setting may be attributed to a reduction in the dissemination of MM PC to secondary sites. Several studies have shown that liposomal clodronate results in decreased metastasis of solid tumors [42,43]. Moreover, clo-lip treatment in an intratibial model of prostate cancer perturbed tumor growth within the bone [44]. Together, these studies suggest that macrophages resident within the BM may play an important role in the development of solid tumor metastases within the bone.

To our knowledge, we present for the first time that BM macrophage conditioned medium was a potent inducer of 5TGM1 MM PC migration *in vitro*, suggesting that macrophage secreted factors may play a direct role in increasing the homing of 5TGM1 MM PC to the BM *in vivo*. While 5TGM1 MM PC express a number of receptors for cytokines and growth factors that may increase migration and proliferation, the predominant factor expressed at the mRNA level in KaLwRij BM-derived macrophages was *Igf1*, which is consistent with previous studies [38]. Notably, *Igf1* mRNA levels were also significantly reduced in the total BM of clo-lip–treated mice compared with PBS-lip controls, suggesting that clo-lip negatively affects MM PC homing and establishment, in part, by downregulation of BM IGF-1 levels, likely through direct depletion of IGF-1–expressing macrophages. IGF-1 is an important mitogenic and survival factor for MM PC [34] and has been suggested to be an important promigratory and adhesion factor in MM [35,45].

Consistent with previous studies, we have shown that global phagocytic cell depletion has secondary effects on other BM cells, inducing a rapid decrease in osteoblast and osteoprogenitor numbers

and a dramatic increase in HSCs [31,32,46,47]. IGF-1 has previously been shown to induce osteoblast differentiation [48], and as such, a decrease in *Igf1* may also account for the reduction in osteoblast and osteoprogenitor numbers observed in this study. Although interesting, this decrease in osteoblast number following clo-lip treatment is unlikely to account for the reduction in tumor burden, as studies show that osteoblasts may inhibit MM cell proliferation and survival [49]. Moreover, dormant MM PC reside near osteoblasts *in vivo*, suggesting that osteoblasts play a role in inducing MM PC quiescence rather than proliferation [9].

However, the decrease in osteoblasts observed here may result in changes in BM cytokine levels that decrease MM PC homing *in vivo*. Consistent with previous studies [31], clo-lip treatment was associated with a decrease in BM expression of *Cxcl12* mRNA. Although not expressed by macrophages, CXCL12 is expressed by MSCs and osteoblasts [36], suggesting that the decrease in BM CXCL12 levels may be secondary to the decrease in osteoblast numbers observed in this study. Additionally, BM macrophages have been shown to produce secreted factors that increase CXCL12 production by BM stromal cells *in vitro* [31], suggesting that ablation of BM macrophages may also decrease BM stromal cell CXCL12 production. CXCL12 secretion from stromal cells is known to play a key role in the BM recruitment and retention of MM PC from the peripheral blood [6,7] and CXCL12 induces 5TGM1 cell migration *in vitro* [27]. Interestingly, IGF-1 and CXCL12 have striking synergistic effects on the migration of human MM cell lines *in vitro* [50], suggesting that the decrease in the BM levels of both IGF-1 and CXCL12 may account for the dramatic effect on the BM homing of MM PC seen here in clo-lip–treated mice.

In addition to affecting the production of macrophage- and mesenchymal cell–derived chemokines, macrophage depletion may affect MM PC establishment in the BM by increasing the abundance of other cells in the BM that may crowd out MM PC. Flow cytometric analysis revealed a dramatic increase in the number of long term, short term, and progenitor HSCs within the BM of clo-lip–treated mice compared with PBS-lip controls. This finding supports previous studies that demonstrated a role for BM macrophages in maintaining the HSC niche [31,32,46,47]. Moreover, CXCL12 has been shown to be important in maintaining the quiescent HSC pool [51], and therefore, decreased *Cxcl12* levels within the BM following clo-lip treatment may result in HSC proliferation. While the MM PC proliferative niche is incompletely characterized, it is thought that there are overlaps between the niches occupied by HSCs and proliferating MM PC in the BM [36,52]. We speculate that the dramatic increase in the number of hematopoietic lineage cells within the BM following clo-lip treatment may disrupt the ability of MM PC to colonize supportive niches in the BM; however, further studies are required to confirm this.

This is the first study to demonstrate that CD169-expressing BM-resident macrophages play an instrumental role in the initial homing and establishment of MM disease. Moreover, we confirmed a requirement for macrophages in MM disease progression. Our findings also demonstrate that macrophages produce factors, including IGF-1, that increase the migratory capacity of MM PC. Further, we show that clo-lip–mediated macrophage depletion leads to demonstrable changes to the mesenchyme, leading to a reduction in *Cxcl12* expression, a factor important in MM PC homing and retention. Taken together, these studies highlight the potential of targeting BM-resident macrophages as a novel therapy for MM in the established and relapsed disease setting.

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Contributions

K. S. O. and J. E. N. performed majority of the research. K. C. C., E. A. C., D. R. H., and K. M. M. assisted with data generation. K. S. O., K. V., and J. E. N. performed data analysis. A. C. W. Z., J. E. N., K. V., and K. S. O. designed experiments and contributed substantially to the research project. A. E., N. S., and P. I. C. assisted with experimental design. K. S. O. wrote the manuscript. K. V., D. R. H., K. M. M., P. J. P., J. E. N. and A. C. W. Z. reviewed the manuscript, and J. E. N., P. J. P., and A. C. W. Z. supervised the study.

Declarations

The authors declare that they have no competing interests.

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