Colony-stimulating factor 1 receptor blockade inhibits tumor growth by altering the polarization of tumor-associated macrophages in hepatocellular carcinoma

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Abstract

Colony-stimulating factor-1 (CSF-1) and its receptor, CSF-1R, regulate the differentiation and function of macrophages and play an important role in macrophage infiltration in the context of hepatocellular carcinoma (HCC). The therapeutic effects of CSF-1R blockade in HCC remain unclear. In this study, we found that CSF-1R blockade by PLX3397, a competitive inhibitor with high specificity for CSF-1R tyrosine kinase, significantly delayed tumor growth in mouse models. PLX3397 inhibited the proliferation of macrophages in vitro, but intratumoral macrophage infiltration was not decreased by PLX3397 in vivo. Gene expression profiling of tumor-associated macrophages (TAMs) showed that TAMs from the PLX3397-treated tumors were polarized toward an M1-like phenotype compared with those from vehicle-treated tumors. In addition, PLX3397 treatment increased CD8+ T-cell infiltration, whereas CD4+ T-cell infiltration was decreased. Further study revealed that tumor cell–derived CSF-2 protected TAMs from being depleted by PLX3397. In conclusion, CSF-1R blockade delayed tumor growth by shifting the polarization rather than the depletion of TAMs. CSF-1R blockade warrants further investigation in the treatment of HCC.
Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide (1). Many cancers, including HCC, arise from sites of infection and chronic inflammation (2). Inflammation has been recognized as having a role in tumor initiation as well as tumor progression (3,4). A tumor microenvironment largely shaped by inflammatory cells is favorable for tumor growth and progression (5). Tumor-associated macrophages (TAMs) are a major component of inflammatory cellular infiltrates in tumors and play a pivotal role in tumor progression in inflammation-related cancer (6-10). Macrophage activation in response to different agents has long been recognized as M1 and M2 macrophages. Differential cytokine production is a key feature of polarized macrophages(11). The M1 phenotype macrophages typically produce interleukin (IL)-12b and IL-23, whereas M2 macrophages produce IL-10, chemokine (C-C motif) ligand (CCL)17, and CCL22 (6,12). Our previous studies found that colony-stimulating factor 1 (CSF-1) expression and TAM density (CD68 or CSF-1 receptor, CSF-1R) in the adjacent liver tissue are associated with patient survival after resection of HCC (13-15), suggesting that CSF-1/CSF1R may play an important role in tumor progression and macrophage polarization in HCC(16).

CSF-1 is a cytokine that controls the differentiation and function of macrophages via its receptor, CSF-1R. Ligand binding activates the receptor kinase through a process of oligomerization and transphosphorylation. Cellular changes occur in macrophages under different circumstances, which contributes to the heterogeneity and variability of TAMs (17). This plasticity is a hallmark of myeloid cells, particularly those of the monocyte–macrophage lineage (18). Activated forms of
macrophages, known as M1 and M2, are linked to lineage-determining growth factors in T helper 1 (Th1) and T helper 2 (Th2) cells (19). CSF-1 and granulocyte-macrophage colony-stimulating factor (GMCSF, or CSF-2) are often used to obtain mature macrophages, and they are also involved in the polarization of anti-inflammatory/protumorigenic and pro-inflammatory/antitumorigenic macrophages, respectively (11,20,21).

We previously found that depletion of TAMs enhanced the antitumor effect of sorafenib in liver cancer models (22,23), and a clinical trial has been initiated in our hospital to test the safety of a combination treatment using sorafenib with zoledronic acid (ClinicalTrials.gov identifier: NCT01259193). Another approach is the deactivation of TAMs by a CSF-1R inhibitor, which was studied in diffuse-type giant cell tumor (24) and glioblastoma multiforme (25). PLX3397, a competitive inhibitor with high specificity for CSF-1R tyrosine kinase, has been tested in several studies, with promising therapeutic results (26-33).

In the present study, we tested the effect of PLX3397 in HCC mouse models and found that PLX3397 treatment delayed tumor growth. This delay was mediated by altering TAM polarization rather than by causing TAM depletion.

Materials and Methods

Cell lines

Four human HCC cell lines (HepG2, MHCC97-H, MHCC97-L, and HCCLM3), one non-transformed human hepatocyte cell line (L-02), and one mouse HCC cell line (Hepa1-6) were
used in this study. MHCC97-H, MHCC97-L, and HCCLM3 were established at our institute (34,35). HepG2 and Hepa1-6 were obtained from American Type Culture Collection (ATCC, Rockville, MD). Human hepatic stellate cell line LX2 was obtained from Shanghai Advanced Research Institute, Chinese Academy of Sciences. These cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, NY) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL).

Human monocyte THP-1 cells obtained from ATCC were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% FBS and supplemented with 10 mM Hepes (Gibco BRL). THP-1 was differentiated into macrophages by 24-h incubation with 160 nM phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) followed by 24-h incubation in RPMI medium. Macrophages were further polarized to M1 macrophages by incubation with 10 pg/ml of lipopolysaccharide (LPS; Sigma) and 20 ng/ml of interferon (IFN)-γ (R&D Systems, MN) and are referred to as M(LPS+IFN-γ) cells. M2 macrophages were obtained by incubation with 20 ng/ml of interleukin (IL)-4 (R&D Systems) and are referred to as M(IL4) cells (36).

A human umbilical vein endothelial cell (HUVEC) line was purchased from Allcells (Shanghai, China) and was grown in Allcells completed medium supplemented with 10% FBS. All cell lines obtained from the cell bank were tested for authentication using short tandem repeat fingerprinting and were passaged for fewer than 6 months.

**Isolation of TAMs and T cells from tumor tissues**

Isolated TAMs were obtained from orthotopically implanted hep1-6 tumors in C57BL/6 mice. Specimens were minced with scissors and digested by incubation for 1 h at 37°C in high-glucose media.
DMEM (Life Technologies, CA) containing 0.1% collagenase IV (Sigma). After being washed in medium plus 10% FBS, the cell suspension was forced through a graded series of meshes to obtain single-cell suspensions and centrifuged at 400 g for 30–40 minutes at 18–20°C on layers of Lympholyte-M (Cedarlane Labs, Burlington, Ontario, Canada), after which the upper layer was removed, thus leaving the lymphocyte layer undisturbed at the interface. TAMs were sorted using Fluorescence-activated cell sorting (FACS, Becton Dickinson, Franklin Lakes, NJ) with CD45+, CD11b+, and F4/80+ (37) (Becton Dickinson) according to the protocol listed at http://www.immgen.org/Protocols/ImmGen%20Cell%20prep%20and%20sorting%20SOP.pdf. TAMs growing in DMEM supplemented with 10% FBS were tested to determine their biological characteristics.

CD8+ T and CD4+ T cells were purified using the CD8+ or CD4+ T Cell Isolation Kit from Miltenyi Biotec, respectively. The purified T cell suspension was labeled with CFSE (Invitrogen). 1×10^5 CFSE labeled CD8+ or CD4+ T cells were added to CD3/CD28 coated plates (1.0 μg/ml each, eBioscience) to induce T cell proliferation. Analysis of cells was performed on a BD LSRFortessa cell analyzer after labeling with fluorescent antibodies and 7AAD (BD Bioscience) to exclude dead cells.

**ELISA**

IFN-γ concentrations in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer’s instruction. We collected the total cell protein to assess the different cell numbers of the different groups. An equal volume of lysis buffer was added before the total cellular protein extracted, and then bicinchoninic acid (BCA)
assay was used to measure the protein concentration. Thereafter, the IFN-γ concentration was normalized to the total cellular protein.

**Flow cytometry analysis**

Lymphocytes isolated from tumors were washed in PBS containing 0.5% (w/v) bovine serum albumin (BSA). After cells were counted, single-cell suspensions in FACS buffer (1% IgG free BSA in PBS; Jackson Immunoresearch, West Grove, PA) were incubated with 1 µl of Fc Block (Becton Dickinson) for every 1 million cells for at least 15 min at 4°C. Surface staining was performed in the dark for 30 min at 4°C in staining buffer. Cells were then incubated for 30 min at 4°C with the appropriate antibody or with a control in PBS containing 0.5% (w/v) BSA. Cells were analyzed on a fluorescence activated cell sorter (BD LSRFortessa; Becton Dickinson). Surface markers used for these experiments included F4/80 clone REA126 FITC (1:100, Miltenyi Biotec, Germany), CD11b clone M1/70 PE-Cy7 (1:125, BD Pharmingen, NJ), Ly6G clone 1A8 AlexaFluor700 (1:125, BD Pharmingen), and MHC Class II clone M5/114.15.2 APC (1:100, Miltenyi Biotec). Directly conjugated mouse immunoglobulin G1k was used for isotype controls. Cells were then washed twice with staining buffer followed by fixation in 1% paraformaldehyde (VWR, West Chester, PA). Cells were analyzed on a BD LSRFortessa cell analyzer and gating was performed with FlowJo analysis software (TreeStar, Ashland, OR). Biexponential transformation was adjusted manually when necessary.

**Obtaining CSF1- and CSF2-stimulated macrophages from bone marrow–derived**
macrophages (BMDMs)

Femur and tibia bones were harvested from 8-week-old wild-type (WT) C57BL/6 mice. Bone marrow was flushed out into cold PBS (Life Technologies) plus 2% heat-inactivated FBS, passed through a needle five times to dissociate the cells, and then passed through a 70-μm cell strainer (Becton Dickinson) to remove cell clumps, bone, hair, and other cells/tissues. After addition of three volumes of NH₄Cl solution (0.8% NH₄Cl solution; Beyotime Institute of Biotechnology, Jiangsu, China), the mixture was incubated on ice for 10 min to remove red blood cells; the cells were then spun down and resuspended in cold PBS with 2% FBS (38). The harvested cells were cultured in DMEM containing 10% FBS and supplemented with 10 ng/ml recombinant mouse CSF1 (R&D Systems) or 10 ng/ml recombinant mouse CSF2 (R&D Systems) for 7 days to obtain CSF1- or CSF2-induced BMDMs: M(CSF1) or M(CSF2) cells, respectively.

Immunohistochemical assay

Frozen sections (5 μm) of tumor samples were used to determine the cells with dual expression of CD68 (1:250, AbD Serotec, CA; 1:200, Abcam, Cambridge, UK) and Fizz1 (1:100; Abcam), cells with dual expression of CD68 and MHC-II (1:100; Abcam), or cells with dual expression of CD68 and CSF-1R (1:100; Genetex, TX) by immunofluorescent staining and were then imaged by confocal laser scanning microscopy.

Macrophages growing on glass coverslips were fixed in 4% paraformaldehyde for 15 min, rinsed three times with PBS for 5 min each time, and incubated in a protein-blocking solution for 30 min at room temperature. After incubation with the primary antibody against CD68 and F4/80 (1:100;
Abcam), CD68 and CSF-1R (1:100; Novus Biologicals, CO, USA), CD68 and CSF2Ra (1:100; Abcam), or CD68 and CSF2Rβ (1:100; Abcam) overnight at 4°C followed by incubation with the secondary antibody (Alexa Fluor 488 donkey anti-rat, Alexa Fluor 546 donkey anti-rabbit, 1:300; Jackson Immunoresearch) at 37°C for 2 h, the cells were counterstained with DAPI (Beyotime). Cells on slides not incubated with primary antibodies served as negative controls.

Immunohistochemical assay was performed on 6-μm sections of paraffin-embedded Hepa1-6 tumor tissues using antibodies against CD8 (1:100; Abcam) and CD4 (1:100; Abcam). For observing the staining for each antibody, a uniform setting was applied for all slides. CD68- or CSF-1R-positive areas in the photographs were measured by the Leica Qwin Plus, and the macrophage density in each photograph was calculated as CD68-positive area/total area. The positive staining area values were determined as described previously (13).

Quantitative real-time polymerase chain reaction analysis

Total RNA from L-02, HCC cell lines (MHCC97-H, MHCC97-L, HCCLM3, and HepG2) and Hepa1-6, mouse M(CSF1), M(CSF2), M(LPS+IFN-γ), and M(IL4) cells was extracted and reverse-transcribed onto single-stranded cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). For quantitative real-time polymerase chain reaction (qPCR), primers were designed by Sangon Biotech (Shanghai, China), and their efficiency was tested on a genomic DNA dilution series. qPCR was performed with the Applied Biosystems 8100 HT Sequence Detection System (Applied Biosystems, Carlsbad, CA). Expression of the glyceraldehyde-3 phosphate dehydrogenase gene was used to normalize the expression of each
gene. The primer sequences used to determine the expression of the target genes are listed in Supplementary Tables S1 and S2.

**Western blot analysis**

Cells were lysed with RIPA Lysis Buffer (Santa Cruz Biotechnology, Dallas, TX) containing protease inhibitors (Beyotime Institute of Biotechnology). The protein concentration was determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology) and equalized before loading. Aliquots of 25–50 μg of protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked and blotted with the relevant antibodies. Horseradish peroxidase–conjugated secondary antibodies were detected with an enhanced chemiluminescence reagent (Beyotime). Antibodies against phosphorylated CSF-1R (Cell Signaling Technology, CST, Danvers, MA), CSF-1R (CST), and CSF2rβ (Abcam) were used to determine their expression. GAPDH was used as a loading control. All antibody dilutions were 1:1000, except for the GAPDH antibody, which was used at a dilution of 1:5000.

**Cell proliferation assay**

Aliquots of the cell suspension were inoculated into a 96-well plate (3 × 10³ to 5 × 10³ cells in 100 μl/well) and then incubated in a humidified incubator (37°C, 5% CO₂). 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and the plate was the incubated for 2–4 h (37°C, 5% CO₂). Absorbance at 450 nm was measured by a microplate reader.
Animal studies

Male C57BL/6 mice (6 weeks old) and male BALB/c mice (6 weeks old) were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science, and housed under specific pathogen-free conditions. The experimental protocol was approved by the Shanghai Medical Experimental Animal Care Commission.

Hepa1-6 cells (6 × 10^6 cells) in 200 μl of normal saline were implanted by subcutaneous injection to obtain subcutaneous tumors. Twenty C57BL/6 mice were treated by orthotopic implantation of 1mm³ tumor into the liver from a subcutaneously growing one. Three days after implantation, the mice were randomized into two PLX3397-treated groups (50 mg/kg/day, oral administration) or two vehicle-treated groups. Each group included five mice. The mouse body weights were measured every week. One PLX3397-treated group and one vehicle-treated group were continuously observed for survival analysis. In the other two groups, tumors were resected after 5 weeks to obtain TAMs; the remaining tumor tissues were stored in 4% paraformaldehyde solution for further study. Tumor volume was calculated according to the following formula: tumor volume = (largest diameter × perpendicular height^2)/2.

HepG2 cells (5 × 10^5 cells) or HCCLM3 (5 × 10^5 cells) in 200 μl of normal saline were implanted by subcutaneous injection to obtain subcutaneous tumors. Nude mice were treated by orthotopic implantation of 1mm³ tumor into the liver from a subcutaneously growing one. Twelve Balb/c nude mice with orthotopic HepG2 or 10 Balb/c nude mice with orthotopic HCCLM3 tumors were randomized into PLX3397-treated and vehicle-treated groups. Three days after implantation,
PLX3397 50 mg/kg/day or vehicle solution was orally administered by gavage. Body weights were measured every week. Tumors were removed after 5 weeks and stored in a 4% paraformaldehyde solution.

**Molecular characteristics of macrophages**

THP-1 derived macrophages, including M(LPS+INFγ) and M(IL4) cells, were processed in Trizol and then analyzed using the Affymetrix U133 Array platform. M(CSF1) cells, M(CSF2) cells, and TAMs from mouse tumors were processed in Trizol within 4 h after sorting and then analyzed using whole-mouse genome Affymetrix Mouse Gene 1.0 ST Arrays. Data files are available at the GEO database [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE95407](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE95407). The M1 macrophage-related genes for MHC II (HLA-DRA), TNF, IL1B, IL12B, IL23A, CD80, CD86, IL6, and CXCL10 and the M2 macrophage-related genes for CCL17, MRC1, IL10, and IL4R were used to characterize macrophage polarization (19). Hierarchical clustering analysis using the aforementioned panel of genes was performed using MeV v4.6.0 software ([http://www.tm4.org/](http://www.tm4.org/); TM4, Boston, MA). The matrix was presented graphically by coloring each gene expression on the basis of a measured color range: lower limit “−6” was blue, upper limit “6” was red, and midpoint value “0” was white. A hierarchical clustering algorithm usually required two main steps, which were repeated to find the strains that were the most similar: the M1/M2-associated gene result values from the same strain were assigned to its own cluster, and the two clusters that were closest to each other were merged until only one large cluster resulted.

**Reagents and antibodies**
Other reagents and antibodies used in this study are shown in Supplementary Table S3.

**Statistical analysis**

Statistical analysis was performed with SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL). Quantitative variables were analyzed by the independent samples $t$-test. Overall survival and time to recurrence were assessed using the Kaplan–Meier method and compared with the log-rank test. Statistical significance was defined by $P<0.05$. Western blots were analyzed with ImageJ (National Institutes of Health, Bethesda, MD). Cell migration was analyzed by Image-Pro Plus as described previously (39). Pearson correlation was used as a distance metric and the complete linkage method was used in hierarchical clustering.

**Results**

**Bone marrow–derived monocytes were polarized toward the M2-like or M1-like phenotype by CSF1 or CSF2 stimulation**

M(CSF1) cells (CSF1-induced BMDMs) had a spindle appearance with a colony growth pattern, whereas M(CSF2) cells (CSF2-induced BMDMs) had a more rounded appearance with a pattern of individual growth (Supplementary Fig. S1A, B). FACS assay showed that more than 95% of M(CSF1) and M(CSF2) cells were positive for F4/80 (Supplementary Fig. S1). Immunocytofluorescence assay further confirmed that M(CSF1) and M(CSF2) cells were positive for the markers specific to macrophages, including CD68, F4/80, and CSF-1R (Fig. 1A, B). CSF2Rα and CSF-2Rβ expression was found on M(CSF1) and M(CSF2) cells by immunocytochemistry assay (Fig. 1C, D). However, BMDMs without CSF-1 stimulation were not
positive for CD68, F4/80, and CSF-1R and were suspended in the culture medium.

To establish molecular signatures shared by human and mouse macrophages, we conducted a whole-genome expression profiling assay (40,41), which identified differentially expressed genes in M1-polarized macrophages (THP-1–derived macrophages stimulated by LPS+IFNγ) and M2-polarized macrophages (stimulated by IL4). Of these, expression of M1-associated genes such as *HLA-DRA*, *TNF*, *IL1B*, *IL12B*, *IL23A*, *CD80*, *CD86*, *IL6*, and *CXCL10* was higher, while expression of M2-associated genes such as *CCL17*, *MRC1*, *IL10*, and *IL4RA* was lower in comparison with M(LPS+IFNγ) and M(IL4) cells, which is consistent with previous reports (11,19,42).

Whole mouse genome expression profiling identified 1631 genes that were up-regulated and 2539 down-regulated genes in M(CSF2) cells compared with M(CSF1) cells. Of these, expression of M1-polarized macrophage-related genes such as *MHC-II*, *Tnf*, *Il1b*, *Il12b*, *Il23a*, *Cd80*, *Cd86*, *Il6*, and *Cxcl10* was increased and expression of M2-polarized macrophage-related genes such as *Ccl17*, *Mrc1*, *Il10*, and *Il4ra* was decreased in the M(CSF2) cells, compared with their counterparts, suggesting that M(CSF2) cells were more likely to be M1-polarized macrophages, whereas M(CSF1) cells were more likely to be M2-polarized macrophages. The expression of the typical markers for M1- or M2-polarized macrophages was validated by qPCR to confirm the gene expression levels (Supplementary Fig. S2A, B).

**PLX3397 suppressed tumor growth without depletion of TAM infiltration *in vivo***
In vitro, a population doubling assay showed that the IC_{50} of PLX3397 (33) (Fig. 2A), a highly selective CSF-1R inhibitor, for M(CSF1) cells was 22 nM (Fig. 2B), which is consistent with a previous report (43). A similar inhibitory effect of exogenous CSF-1R antibody on M(CSF1) cells was observed (Fig. 2C). Marked suppression of CSF-1R phosphorylation (p-CSF-1R) in M(CSF1) cells could be achieved with PLX3397 (Fig. 2D). In contrast, the same doses of PLX3397 showed no antiproliferation effect on Hepa1-6, MHCC97-H, HCCLM3, HepG2, HUVEC, T cells, fibroblasts cells, and M(CSF2) cells (Supplementary Fig. S3A–G). In accordance with these results, very low CSF-1R expression was detected in hepatocyte (L-02), hepatoma (MHCC97-H, MHCC97-L, HCCLM3, Hepa1-6, and HepG2), or endothelial cell lines (HUVEC) as compared with M(CSF1) and M(CSF2) cells (Supplementary Fig. S3H).

In an orthotopic C57BL/6 model with Hepa1-6 tumor cells, PLX3397 treatment suppressed tumor growth by 70% without affecting mouse body weight compared to the vehicle-treated mice (Fig. 3A–C). PLX3397 treatment also prolonged survival of the tumor-bearing mice (median survival time, 8.0 weeks versus 11.3 weeks; P = 0.026; Fig. 3D).

We next examined the key mechanism of the antitumor effects of PLX3397. The number of CD68-positive or CSF-1R-positive macrophages in the tumors from the PLX3397-treated and vehicle-treated mice were not statistically different (Fig. 4A, B). The number of cells with CD31 (P = 0.589) or α-SMA (P = 0.913) expression did not differ between the two groups (Supplementary Fig. S4A–F), suggesting that PLX3397 may have no effect on the infiltration of macrophages or tumor angiogenesis in vivo.
Tumor-derived CSF-2 promoted TAM polarization toward an M1-like phenotype under treatment with PLX3397

As PLX3397 treatment did NOT deplete TAM infiltration in vivo, whether the phenotype of TAMs changed or cytokines protect TAM from depletion in the microenvironment was further investigated. To study whether the phenotype of TAMs was affected by PLX3397, we conducted gene expression profiling on the isolated TAMs. We found 4342 up-regulated genes and 4570 down-regulated genes in the PLX3397-treated TAMs compared with the vehicle-treated TAM. The expression profile in TAMs isolated from the PLX3397-treated tumors was similar to that of M1-polarized macrophages, whereas the gene expression profile of TAMs from the vehicle-treated tumors was similar to that of M2-polarized macrophages (Supplementary Fig. S5A, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE95407). The expression of the typical markers for M1- or M2-polarized macrophages were validated by qPCR (Supplementary Fig. S5B).

An immunocytofluorescence assay was conducted to examine the expression of Mrc1, MHC-II, and CD68 in the PLX3397- or vehicle-treated tumors. The results showed increased expression of MHC-II-positive macrophages and decreased expression of Mrc1-positive macrophages (Supplementary Fig. S5C-D). Taken together, these results indicated that PLX3397 promoted a shift in polarization of macrophages from M2 to M1.

Because TAMs from the PLX3397-treated tumors showed an M1-like phenotype, we hypothesized that this transition was promoted by the cytokines secreted in the tumor microenvironment. The
isolated macrophages from the PLX3397- and vehicle-treated tumors were not different in proliferation (Fig. 5A). The remaining non-macrophage cells from the tumor tissue were cultured for 48 h, and the conditioned medium (CM) was collected. The CM of non-macrophage cells from PLX3397-treated mice, but not from the vehicle-treated mice, protected the M(CSF1) cells from the antiproliferative effects of PLX3397 in vitro (Fig. 5B). These results indicated that some cytokines from the intratumoral microenvironment protected TAMs from the antiproliferative effects of PLX3397.

An antibody array (AAM-ANG-1; Raybiotech, Norcross, GA) was conducted to identify the differentially expressed cytokines in the conditioned medium (CM) of non-macrophage cells from PLX3397-treated and vehicle-treated tumors. The results showed that the expression of CSF2, granulocyte colony-stimulating factor 3 (CSF3), IL3 (multi-CSF), and IFN-γ were higher in the CM of non-macrophages from the PLX3397-treated mice than from the vehicle-treated mice (Fig. 5C,D). Furthermore, a population doubling assay showed that CSF2, IL3, and IFN-γ protected M(CSF1) cells from the inhibitory effects of PLX3397. Among these cytokines, CSF2 was the most potent (Fig. 5E–H). These results suggested that CSF2 signals could be an important survival factor for macrophages under a CSF-1R blockade. In accordance with these results, PLX3397 did not inhibit M(CSF2) cells in a population doubling assay at concentrations of 22 nM and 50,000 nM (Supplementary Fig. S6A); however, IC_{50} of PLX3397 for M(CSF1) cells in medium containing 10 ng/ml CSF1 was 22 nM, whereas IC_{50} of PLX3397 for M(CSF1) cells in medium containing 10 ng/ml CSF2 was 19,495 nM (Supplementary Fig. S6B, C), suggesting that CSF2 protected macrophages from PLX3397 treatment.
PLX3397 treatment changed the intratumoral microenvironment

We also studied the effects of PLX3397 on immune cells in the mouse model by immunostaining the antigen presenting macrophages (F4/80⁻MHC II⁺), myeloid-derived suppressor cells (MDSCs, CD11b⁻Gr1⁻) cells as well as the CD8⁺ and CD4⁺ T cells. We found that the proportion of antigen presenting macrophages was elevated (P = 0.041), while myeloid-derived suppressor cells were decreased (P = 0.029) in the tumor tissues (Supplementary Fig. S7A,B). Furthermore, PLX3397 treatment increased the number of CD8⁺ cell (P = 0.023) and decreased the number of CD4⁺ cells in tumors compared to the vehicle treatment (P = 0.002) (Supplementary Fig. S7C,D). Furthermore, as mentioned above, we found that PLX3397 did not affect the proliferation of fibroblasts and T cells directly in vitro (Supplementary Fig. S3 H, J-M).

PLX3397 treatment inhibited tumor growth in two xenograft models of HCC

In xenograft models derived from two human hepatoma cell lines, HepG2 and HCCLM3, PLX3397 treatment suppressed tumor growth by 33% and 84%, respectively (P = 0.026 and P = 0.047, respectively; Supplementary Fig. S8A–D). In addition, there was no difference in CD68⁻ pan-macrophages, but there was an increased expression of M1-associated marker MHC-II and a decreased expression of M2-associated marker MRC1 in the PLX3397-treated tumors compared to the vehicle-treated tumors (Supplementary Fig. S9).

Discussion

The present study showed that PLX3397 treatment induced the transition of M2-polarized macrophages to M1-polarized macrophages in tumors, which was mediated by inhibition of CSF-1R on tumor-associated macrophages, delayed tumor growth, and prolonged survival of
tumor-bearing mice.

Our findings support the idea that M(CSF2) cells are similar to M1-macrophages, whereas M(CSF1) cells are similar to M2-macrophages, which is consistent with a previous report (25). Other authors have indicated that significant differences in transcriptome level remained in M(CSF1) cells and M2 macrophages (19,21,44); however, the transcriptome data from the present study showed that the gene expression signature determined by a panel of genes in M(CSF1) cells was similar to that of M2 macrophages, and that M(CSF2) cells was similar to M1 macrophages. Using the same signature, we showed that TAMs from the PLX3397-treated tumors were similar to the M(CSF2) cells and TAMs from the untreated tumor were similar to the M(CSF1) cells. We also showed that the M2-polarized macrophages were transformed to M1-polarized macrophages. These results are in agreement with previous data (40,41).

Several studies have suggested that CSF1 is the major chemoattractant in cancers, attracting TAMs to the neoplastic microenvironment and differentiating them to protumorigenic types (13,45,46). In the present study, we demonstrated that blockade of CSF-1R signaling in TAMs affects tumor progression in multiple hepatoma models by polarization of TAMs toward the M1 phenotype in animal hepatoma models. TAMs were not depleted, and they survived CSF-1R inhibitor treatment, which was consistent with findings showing that M(CSF1) cells could survive in CSF2-containing medium with CSF-1R inhibition. The cell shape and sensitivity to the CSF-1R inhibitor also changed in line with adding CSF2 to the medium. One interesting finding was that expression of CSF2, IL3, and IFN-γ in the tumor microenvironment was increased when PLX3397 treatment
was applied to tumors. In the present study, we did not attempt to identify the source of CSF2 in the tumor microenvironment. Other authors have reported similar findings (25,47) supporting other survival factors that were increased when the tumor was treated with CSF-1R inhibitors. The authors suggested that tumor cells might be a source of CSF2 that was affected by CSF-1R inhibitor treatment. Notably, Swierczak et al. have reported that inhibition of CSF-1R/CSF-1 signaling by AFS-98 (a CSF-1R antibody) increased the granulocyte CSF (G-CSF; CSF3) level in the serum of mice with breast tumors (47) and was associated with increased metastasis of mammary tumors in the animal model. It was also reported that increased CSF2 promoted tumor growth through paracrine action on stromal cells in skin carcinoma (48) and promoted epithelial–mesenchymal transition and metastasis in breast cancer (49). Furthermore, Ries et al. reported RG7155 (a CSF-1R antibody) depletes macrophages in vitro and in vivo (50). These discrepancies may indicate that the CSF1/CSF-1R system may play different roles in different types of cancers.

A recent study demonstrated a collaborative interaction between macrophage and stromal cells (fibroblasts) in forming a favorable environment for neuroblastoma development(51). Although we have showed that fibroblasts were not directly affected by PLX3397 in the present study, it would be interesting to explore if the effect of PLX3397 on tumor growth is partially mediated by fibroblasts or other mesenchymal stromal cells(52), which may be affected by polarized macrophages.

PLX3397 treatment results in more antigen presenting macrophages and CD8+ cells and fewer MDSCs and CD4+ cells in tumors. These findings suggested antitumor immunity in the microenvironment has been ameliorated by CSF-1R inhibition, which is consistent with depletion
of TAMs come out with more activated CTLs (53).

In summary, we found that treatment with CSF-1R inhibitor PLX3397 delayed tumor growth in both xenograft and allograft models, and this delay was probably mediated by the transition from M2 macrophages to M1 macrophages in a TAM population induced by blockade of the CSF1/CSF-1R signal pathway (Fig. 6). It would be interesting to investigate the effects of the CSF-1R inhibitor on HCC patients because systemic treatment for HCC is not yet sufficient.
Acknowledgments

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References


Figure Legends

Figure 1. Expression of a series of macrophage markers were found in CSF1- and CSF2-induced BMDMs. Confocal laser scanning microscopy images of CSF1-induced BMDMs (M(CSF1) cells) and CSF2-induced BMDMs (M(CSF2) cells). F4/80 (green), CSF-1R (green), and CD68 (red) were co-expressed in M(CSF1) cells (A) and M(CSF2) cells (B). CSF2Rα (green), CSF2Rβ (green), and CD68 (red) were also co-expressed in M(CSF1) cells (C) and M(CSF2) cells (D). Scale bar = 100 μm.

Figure 2. CSF-1R blockade led to CSF1-induced BMDM (M(CSF1) cells) death by phosphorylated CSF-1R inhibition.

(A) Chemical structure of PLX3397. PLX3397 is an oral, potent receptor tyrosine kinase inhibitor of CSF-1R. Molecular Weight: 417.81. Chemical Formula: C20H15ClF3N5. (B) A population doubling assay showed that IC50 concentration of PLX3397 in M(CSF1) cells was 22 nM. (C) Culture medium with different conditions were performed to determine M(CSF1) survival. A neutralizing antibody against CSF-1R (10 μg/ml, GeneTex, AFS98) led to M(CSF1) cell death in DMEM containing 10% FBS and supplemented with 10 ng/ml recombinant mouse CSF1 in a similar manner with 22 nM, 220 nM PLX3397 and the culture medium without CSF1, compare with M(CSF1) culture in DMEM containing 10% FBS, as determined by CCK8 assays (n = 3 replicates, *, P<0.01). (D) Western blot analysis of M(CSF1) cells, which were cultured in medium without CSF-1 for 12 h before stimulation then followed CSF-1 addition for the time points indicated...
(1.5, 3, and 5 min). A progressive increase of phosphorylated CSF-1R expression resulted, which was effectively inhibited by 22 nM PLX3397; CSF-1R expression was not changed. In lane 1, marked by #, M(CSF1) cells were continuously cultured with CSF-1. GAPDH was used as a loading control.

**Figure 3. CSF-1R inhibition restricted tumor growth and prolonged mouse survival in an allograft C57BL/6 mouse model with orthotopic implanted hepa1-6 cells.** (A) After 5-week treatment with PLX3397 or vehicle, the mice were killed and the liver tissue was obtained. (B) The mean tumor volume in the PLX3397-treated group was significantly lower than in the vehicle-treated group ($P = 0.045$). (C) Mice treated with PLX3397 had a similar body weight compared with those in the control group at 5 weeks ($P = 0.712$). (D) The survival times were compared between the mice continuously treated with PLX3397 and those that received the vehicle. The cumulative survival plots showed that PLX3397 significantly prolonged the survival time of the tumor-bearing mice ($P = 0.026$). The mice of treated PLX3397 and vehicle group were die through cancer cachexia and no lung, peritoneal cavity metastasis was found.

**Figure 4. PLX3397 suppressed tumor growth without depletion of TAM infiltration but promoted TAM polarization toward an M1-like phenotype.** In the allograft mouse models, the densities of CD68- and CSF-1R-positive cells in the tumors treated with PLX3397 or vehicle were not significantly different ($P = 0.915$ and $P = 0.842$, respectively.) (A and B).
Figure 5. Tumor-derived CSF-2 promoted TAM polarization toward an M1-like phenotype under the treatment of PLX3397. (A) A population doubling assay found that TAMs from PLX3397 and vehicle treatments had no difference in proliferation. (B) The remaining non-macrophage cells from the tumor tissue were cultured for 48 h, and the conditioned medium (CM) was collected. A population doubling assay found that PLX3397-treated non-TAM CM could support M(CSF1) cell survival with 22 nM PLX3397, while vehicle non-TAM CM could not support M(CSF1) cell survival with 22 nM PLX3397. (C, D) Antibody array identified that CSF2, CSF3, IL3, and IFN-γ were higher in PLX3397-treated non-TAM CM than the vehicle group. (E–H). A population doubling assay found that 10 ng/ml CSF2, 10 ng/ml IFN-γ, and 10 ng/ml IL-3 could support M(CSF1) cell survival with 22 nM PLX3397. In these protective factors for M(CSF1) cells treated with CSF-1R inhibitor, CSF2 was the most potent among them.

Figure 6. A diagrammatic illustration summarized the findings of this study. Both CSF1 and CSF2 can support the cell differentiation of BMDMs. However, BMDMs stimulated by the CSF1/CSF-1R pathway (M(CSF1) cells) showed an M2-like phenotype (alternative activation), whereas BMDMs stimulated by CSF2/CSF2Rα and CSF2Rβ pathway (M(CSF2) cells) showed an M1-like phenotype (classical activation). When the CSF1/CSF-1R signaling pathway was blocked, the CSF2/CSF-2R signaling pathway alternatively dominated the differentiation of M(CSF1) cells, and these cells showed an M1-like phenotype.
Figure 1

(A) M(CSF1) Cells

(B) M(CSF2) Cells

(C) M(CSF1) Cells

(D) M(CSF2) Cells
Figure 3

A. Hepa1-6 orthotopic allografts

Vehicle n=5

PLX3397 n=5

B. Tumor volume (mm²)

Vehicle

PLX3397

P=0.045

C. Body weight (g)

Vehicle

PLX3397

P=0.712

D. Percentage of overall survival

PLX3397 Treatment start

Vehicle treatment

PLX3397 treatment

P=0.026

Primary Endpoint

Time after orthotopic tumor planted (weeks)

Time after implantation (days)
Figure 4

**A** 
Vehicle  

**B** 
PLX3397

**Figure 4**

**A** 
Vehicle  

**B** 
PLX3397

**Legend**

- **DAPI**
- **CSF1R**
- **CD68**

**Graphs**

**Cd68**

- **Vehicle**
- **PLX3397**

**P** = 0.915

**CSF-1R**

- **Vehicle**
- **PLX3397**

**P** = 0.842
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Jian-Yang Ao, Xiao-Dong Zhu, Zong-Tao Chai, et al.

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