Glioblastoma-derived Macrophage Colony Stimulating Factor (MCSF) Induces Microglial Release of Insulin-like Growth Factor-Binding Protein 1 (IGFBP1) to Promote Angiogenesis

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Background: Glioblastoma is highly aggressive and incurable to current treatment modalities. MCSF is regulated by SYK-PI3K-NFkB pathway in glioma and induces secretion of IGFBP1 from microglia to promote angiogenesis. Conclusions: Microglial IGFBP1 is a key mediator of MCSF-induced angiogenesis. Significance: IGFBP1 is a potential target for glioblastoma therapy.

ABSTRACT

Glioblastoma (Grade IV glioma/GBM) is the most common primary adult malignant brain tumor with poor prognosis. To characterize molecular determinants of tumor-stroma interaction in GBM, we profiled 48 serum cytokines and identified Macrophage Colony Stimulating Factor (MCSF) as one of the elevated cytokines in sera from GBM patients. Both MCSF transcript and protein were up-regulated in GBM tissue samples through a Spleen Tyrosine Kinase (SYK)-dependent activation of the PI3 kinase-NFkB pathway. Ectopic overexpression and silencing experiments revealed that glioma-secreted MCSF has no role in autocrine functions and M2 polarization of macrophages. In contrast, silencing expression of MCSF in glioma cells prevented tube formation of HUVEC cells elicited by the supernatant from monocytes/microglial cells treated with conditioned medium from glioma cells. Quantitative proteomics based on Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) showed that glioma-derived MCSF induce changes in microglial secretome and identified Insulin-like Growth Factor-Binding Protein 1 (IGFBP1) as one of the MCSF-regulated protein secreted by microglia. Silencing IGFBP1 expression in microglial cells or its neutralization by an antibody reduced the ability of supernatants derived from microglial cells treated with glioma cell-conditioned medium to induce angiogenesis. In conclusion, this study shows up-regulation of MCSF in GBM via a SYK-PI3K-NFkB-dependent mechanism and identifies IGFBP1 released by microglial cells as a novel mediator of MCSF-induced angiogenesis, of potential interest for developing targeted therapy to prevent GBM progression.

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Microglial IGFBP1 – a novel mediator of MCSF-induced angiogenesis

Glioblastoma (grade IV glioma/GBM) is the most common, malignant adult primary brain tumor with a poor survival (1,2). Despite advances in treatment strategies, the prognosis is only marginally improved, which prompts for further understanding of the disease (3). The tumor is surrounded by microenvironment composed of various stromal elements which include fibroblasts, leukocytes, endothelial cells, pericytes and extra-cellular matrix (4). During cancer progression, the microenvironment also evolves through continuous paracrine communication between tumor and stromal elements, thus suggesting the vital role of tumor-stromal interactions in cancer development (5). In case of glioma, macrophages/microglial cells are present in abundance, accounting for nearly 30% of tumor mass (6). Moreover, macrophages/microglia has been implicated in glioma pathophysiology (7-9). Macrophages/microglia can be either classically activated (M1 phenotype) or alternatively activated (M2 phenotype). M1 phenotype is considered to be anti-tumorigenic while M2 is pro-tumorigenic in nature (10). Tumor-associated macrophages (TAMs) belong to M2 phenotype and promote tumor progression, invasion and angiogenesis (11,12). Cytokines are important mediators of tumor-stroma interactions and are deregulated in numerous cancers (13). Alteration in various cytokines and their receptor expression has been reported in GBM (14).

In the current study, we profiled 48 cytokines in the sera of glioma and normal healthy controls and identified 33 cytokines with differential abundance in GBM sera. Cytokines exhibiting an increased level in GBM serum included Macrophage Colony Stimulating Factor (MCSF), which was also up-regulated in GBM tissue via a mechanism dependent on the SYK-PI3K-NFkB pathway. We also established that MCSF is an independent poor prognostic indicator of GBM. Further, we demonstrate that glioma-secreted MCSF induces angiogenesis in vitro and in vivo via macrophage/microglia-secreted factors. These studies were complemented by quantitative proteomics experiments based on Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC), to identify in microglial secretome molecular substrates of angiogenesis elicited by GBM-derived MCSF.

EXPERIMENTAL PROCEDURES

Cell lines and reagents

Human Glioma cell lines U251, U87, U373, LN299 and A172 were grown in Dulbecco’s Modified Eagle Medium (DMEM). SVG, an immortalized human fetal glial cell line was grown in Minimum Essential Medium (MEM). CHME-3, an immortalized human microglial cell line (15), was a kind gift from Dr. Anirban Basu (NBRC, India) and was cultured in DMEM. All media were supplemented with 10% FBS and antibiotics- penicillin, streptomycin and gentamycin - unless otherwise indicated. Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Life Technologies and cultured under company recommended conditions. For conditioned media (CM) collection, glioma cells were grown in serum containing growth media until they reach 80-90% confluence, later washed thoroughly with 1X PBS and fresh serum-free growth media was added. The CM was collected after 24 hrs of incubation, filtered using 0.2μm membrane filter and stored at -20°C until use. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from buffy coat obtained from normal blood donors at Kidwai Memorial Institute of Oncology, Bangalore, India using ficoll gradient method. Later, monocytes were separated from other cells by plastic adherence method for 2 hrs and cultured in DMEM under different conditions for 7 days as indicated.

The following reagents were used in this study: Recombinant MCSF (Biolegend), MCSF, SYK and IGFBP1 specific siRNA (Dharmacon), MCSFR inhibitor GW2580 (LC laboratories), Bay 11-7082 (Sigma-Aldrich), LY294002, U0126 and Bay 61-3606 (Calbiochem), anti-AKT and anti-pAKT (Cell Signalling, 4691 and 4060 respectively), anti-IGFBP1 (R&D Systems, MAB675), anti-MCSFR (Abcam, ab89907), anti-MCSF (Novus Biologicals, NB110-57176), anti-CD68 (Biogenex, MU416-UC), anti-CD204 (Sigma Aldrich, HPA000272), MCSF and IGFBP1 ELISA kit (R & D systems, DY216 and DY871 respectively) and Luciferase assay reagent (Promega). The human MCSF cDNA construct was a kind gift from Prof. Richard Stanley, Yeshiva University, New York.
The MCSF promoter-dependent luciferase wild type and mutant construct was a kind gift from Prof. Jay Rappaport, Temple University, Philadelphia.

**Tumor samples and Serum collection**

Glioma tumor and blood samples were collected from patients at National Institute of Mental Health and Neurosciences (NIMHANS) and Sri Satya Sai Institute of Higher Medical Sciences (SSSIHMS), Bangalore, India. As control/normal samples, non-tumorous brain tissue obtained from the non-dominant anterior temporal cortex region during surgery for intractable epilepsy was used. Tissue from tumor as well as normal samples was used for both RNA isolation and immunohistochemistry (IHC) studies. A total of 122 glioma tissue samples (10 grade II/Diffuse Astrocytoma/DA, 10 grade III/Anaplastic Astrocytoma/AA and 12 grade IV/Glioblastoma/GBM) and 12 control brain tissues were used in this study. We also used sera samples from 26 normal, 24 DA, 22 AA and 148 GBM patients. All the serum samples were collected prior to surgery. Histological specimens were centrally reviewed and confirmed as different grades of glioma by the neuropathologist as per WHO 2007 classification scheme (16). This study has been approved by the ethics committee of NIMHANS and SSSIHMS. The patient’s written informed consent was obtained before collecting samples. Blood samples were collected from normal healthy individuals at Indian Institute of Science (IISc), Bangalore, India, with prior consent and used as normal controls. The patient and normal blood samples were allowed to clot at 4°C overnight, followed by centrifugation at 4°C for 5 min at 1000 rpm to separate serum (upper phase) from clot. Serum samples were stored at -80°C until use.

**Serum cytokine profiling**

Serum cytokine profiling was done using serum samples from normal (n=26), DA (n=24), AA (n=22), and GBM (n=148) by bead array technology. We used commercially available human cytokine kits: 21-plex and 27-plex (Bio-Rad, MF0-005KM11 and M50-0KCAF0Y respectively) and followed the protocol according to manufacturer’s instructions. The 21-plex included following cytokines: IFNα2, IL1α, IL2ρα, IL3, IL12 (p40), IL16, IL18, CTACK, GROα, HGF, TRAIL, LIF, MCP3, MCSF, MIF, MIG, βNGF, SCF, SCGFβ, SDF1α and TNFβ. The 27-plex included following cytokines: IL1β, IL2, IL1ρα, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12 (p70), IL13, IL15, IL17, Eotaxin, Basic FGF, GCSF, GMCSF, VEGF, IFNg, IP10, MCP1, MIP1α, MIP1β, PDGFB, RANTES and TNFα. The cytokine levels were log2 transformed before using for further analysis.

**RNA isolation and qRT-PCR**

RNA from cells and tissues were isolated using TRI reagent (Sigma) and cDNA was made using the High capacity cDNA reverse transcription kit (Life technologies, USA). Gene specific primers were used to quantify the relative expression by real time PCR. Gene expression study was performed using ABI PRISM 7900 (Applied Biosystems) sequence detection system and Dynamo kit containing SYBR green dye (Finnzyme, Finland). Expression was analyzed using GAPDH, ACTB, RPL35A or AGPAT1 as a reference genes and followed ΔΔCt method (17). Firstly, the average ct value of a gene for a given sample is normalized by subtracting it from average ct value of reference gene, which gives ΔCt. Next, ΔΔCt is calculated by subtracting ΔCt of the test sample with that of control sample for a given gene. Further, ratio of ΔΔCt is calculated and log 2 transformed to obtain log 2 ratio.

**ELISA**

ELISA for MCSF and IGFBP1 were performed according to manufacturer’s protocol. The cell free supernatant (100μl) was used to measure levels of MCSF in all glioma cell lines and expressed as pg/ml. However in experiments where different pathway inhibitor treatment was given followed by MCSF level measurement and in overexpression and silencing condition, the results were expressed as % fold change normalized to respective control samples. Similarly, IGFBP1 levels were expressed as % fold change normalized to respective control samples.
Total protein isolation and Western blotting

Total protein extracts were prepared using RIPA buffer. The extract (100 µg) was resolved on 12% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was blocked with 5% skimmed milk powder in 1X PBST buffer for at least an hr, followed by incubation with primary antibody (1:1000 dilution) at 4°C overnight. The membrane was washed thoroughly and then incubated with HRP-conjugated secondary antibody (1:10,000 dilution, Sigma) for 2 hrs at room temperature. The protein was visualized by chemiluminescence (Pierce).

Immunohistochemistry (IHC)

Paraffin sections (4 µm) from the tumor tissue and control samples were collected on silane-coated slides and IHC was performed on 66 samples that included 5 normal, 10 DA, 10 AA, and 41 GBM tumors. Antigen retrieval was done by heat treatment in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) at three different Watts (W): 850W for 5 mins, 600W for 10 mins and 450W for 5 mins respectively. Slides were cooled to room temperature and rinsed in 1X PBS. After the initial processing steps, sections were incubated overnight with primary antibodies: MCSF (1:100 dilution), CD68 (1:40 dilution), CD86 (1:250 dilution) and CD204 (1:200 dilution). This was followed by incubation with secondary antibody (MACH-1 Universal HRP-Polymer Detection kit). 3,3′-Diaminobenzidine (Sigma-Aldrich) was used as the chromogenic substrate. A visual semiquantitative grading scale was applied to assess the intensity of the immunoreactivity as follows: zero (0) if the staining was absent, 1+ for moderate staining and 2+ if it was strong. Only 2+ staining intensity was considered for analysis in line with our previous studies (18). For each sample >1000 cells were counted and the percentage of cells with 2+ staining was depicted as the labeling index (LI). The staining pattern for MCSF was cytoplasmic in the tumor cells while CD68, CD86 and CD204 showed positive staining in the tumor infiltrating microglial/macrophage cells.

Transfection and Luciferase assay

The LN229 cells were transfected with the construct encoding MCSF or the empty vector using lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions and grown for 24 hrs in selection media containing hygromycin (200 µg/ml). The growth media with drug was replaced every alternate day once until distinct colonies appeared (~3-4 weeks). These resistant cells were pooled and confirmed for MCSF expression by qRT-PCR and ELISA. For silencing experiments, the cells were transfected with 100 nM of either control non-targeting siRNA (siNT) or gene specific siRNA as indicated using Dharmafect I (Dharmacon) according to manufacturer’s instructions. After 72 hrs of transfection, cells were harvested and confirmed for MCSF silencing by qRT-PCR and ELISA. In case of CM collection, 48hr after siRNA transfection, cells were washed and incubated with serum-free growth media for another 24 hrs before CM collection.

For luciferase assay, the cells were transfected with MCSF promoter-dependent luciferase construct (0.5 µg) along with pCMV-beta Gal (0.5 µg). MCSF promoter-dependent luciferase construct contains -1310 to + 48 bp of MCSF promoter region cloned in pGL3-Basic vector (Promega, USA). NFkB mutant has mutation in four NFkB binding sites at -51, -359, -378 and -438 positions (19). After 6hrs of transfection, vehicle control and different pathway inhibitors were added at indicated concentrations followed by 24 hrs of incubation. At the end of incubation period, cells were harvested and extracts made for measuring luciferase activity which was further normalized to beta-galactosidase activity.

HUVEC tube formation assay

In this assay, 96-well plate was pre-coated with matrigel 30µl/well followed by plating HUVEC cells (passage 2-4) at a concentration of 15,000 cells/well under different conditions as indicated. After overnight incubation, tube formation was observed and images were taken from multiple fields of each well using phase contrast microscope. Quantification was
performed by counting the total number of completely enclosed networks in each well.

**Intradermal angiogenesis assay**

In this assay, male nude mice were injected intradermally at ventral skin surface with one million tumor cells in 100μl of 1X PBS containing 2% serum. GW2580 inhibitor or vehicle control treatment was given through oral lavage (160mg/Kg body weight every day) following tumor cell inoculation. Five days after tumor cell inoculation, mice were sacrificed and the tumor containing skin was dissected and imaged using digital camera. The tumor directed capillaries were quantified by counting the number of newly formed blood vessels around tumor-inoculated site.

**SILAC sample preparation**

The CHME-3 cells were grown up to five passages in culture medium containing DMEM depleted of L-arginine and L-lysine (SILAC DMEM, Sigma, USA) instead of DMEM and supplemented with isotope-labelled L-arginine and L-lysine: (L-[13C6]arginine (Arg6) and L-[3H4]lysine (Lys4), or L-[13C6-15N4]arginine (Arg10) and L-[13C6-15N2]lysine (Lys8) (All isotopes obtained from Sigma, USA except for Lys 4 from Thermo Scientific). Cells were then treated with either U87 CM derived from non-targeting siRNA transfected cells in presence of Arg 6 and Lys 4 (control condition) or MCSF specific siRNA transfected cells in presence of Arg 10 and Lys 8 (test condition). After 24hrs of incubation with the different CMs (directly added to the culture medium), supernatant from differentially-labeled CHME-3 were collected, mixed and centrifuged at 200 ×g for 5 min and then at 20,000 ×g for 25 min to remove non-adherent cells and cell debris, respectively. Proteins were precipitated using 10% trichloroacetic acid on ice for 30 min. Precipitated proteins were spun down at 10,000 ×g for 20 min and washed three times with diethyl ether to remove any remaining salt from the protein pellets. Precipitated proteins were resuspended in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptopethanol and 0.005% bromophenol blue) for 5 min at 95°C followed by 1hr shaking at room temperature to ensure complete resuspension.

**Protein separation and identification by LC-MS/MS**

Proteins were separated on 12% polyacrylamide gels using the Protean II xi Cell system (Bio-Rad). Gels were stained with Page Blue Protein Staining Solution (Fermentas) and scanned using a computer-assisted densitometer (Epson Perfection V750 PRO). Gel lanes were systematically cut into 16 equal gel pieces and destained with three washes in 50% acetonitrile. After reduction (with 10 mM dithiothreitol at 56°C for 15 min) and alkylation (55 mM iodoacetamide at room temperature for 30 min), proteins were digested in-gel using trypsin (600 ng/band, Gold, Promega), as previously described (20). Digest products were dehydrated in a vacuum centrifuge and reduced to 2 μL. The generated peptides were analyzed online by nano-flow HPLC–nano electrospray ionization using a Q-Exactive mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate Rapid Separation LC (RSLC) system (Dionex, Thermo Fisher Scientific). Desalting and pre-concentration of samples were performed on-line on a Pepmap® pre-column (0.3 mm × 10 mm, Dionex). A gradient consisting of 0–40% B in A for 90 min, followed by 80% B/20% A for 15 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at 300 nL/min was used to elute peptides from the capillary reverse-phase column (0.075 mm × 150 mm, Pepmap®, Dionex). Eluted peptides were electro sprayed online at a voltage of 1.9 kV into the Q-Exactive. A cycle of one full-scan mass spectrum (350-1,500 m/z) at a resolution of 70,000 followed by 10 data-dependent MS/MS spectra was repeated continuously throughout the nano-LC separation. All MS/MS spectra were recorded using normalized collision energy at 17,500 resolution (AGC target 1 x105, 80 ms maximum injection time). Data were acquired using the Xcalibur software (v 2.2). Raw data analysis was performed using the MaxQuant software (v. 1.5.0.0) (21). Retention time-dependent mass recalibration was applied with the aid of a first search implemented in the Andromeda software (22) and peak lists were searched against the UniProt human database.
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(release 2014_06; http://www.uniprot.org, CPS entries), 255 frequently observed contaminants as well as reversed sequences of all entries. The following settings were applied: spectra were searched with a mass tolerance of 7 ppm (MS) and 0.5 Th (MS/MS). Enzyme specificity was set to trypsin. Up to two missed cleavages were allowed and only peptides with at least six amino acids in length were considered. Oxidation on methionine was set as a variable modification. Peptide identifications were accepted based on their false discovery rate (< 1%). Accepted peptide sequences were subsequently assembled by MaxQuant into proteins to achieve a false discovery rate of 1% at the protein level. For protein identification, at least two peptides (amongst which at least one unique peptide) were required. Relative protein quantifications in samples to be compared were performed based on the median SILAC ratios of at least two peptides, using MaxQuant with standard settings.

BiNGO Network analysis

Overrepresentation of GO categories amongst MCSF regulated proteins was analyzed by BiNGO (2.44) (23). Out of the 67 significantly differentially expressed proteins, 11 proteins corresponding to ribosomal sub units were removed and the remaining 56 proteins were used as the input set and the whole annotation set as the reference set. Overrepresentation statistics were calculated by hypergeometric analysis and Benjamini and Hochberg false discovery rate (FDR) correction. The human annotation file was used as custom annotation file. The full ontology was used as custom ontology file with a level of significance of 0.05.

Statistical methods

A nonparametric t-test was performed to find out the significance in difference between two groups. The comparison among multiple groups was performed by one-way ANOVA (nonparametric test) with Tukey test that compares all pairs of columns using Graph Pad Prism 5.01. Supervised hierarchical clustering of significantly differentially expressed cytokines was carried out using the MultiExperiment Viewer (MeV). The volcano graph for 48 cytokines was drawn using R software (version 3.1.0). The log2 fold change expression value of 48 cytokines (x axis) and p value (y axis) were given as input. The prognostic significance was tested by univariate and multivariate Cox proportional hazard analysis using SPSS software (version 19). IHC results were analyzed by using non-parametric test, Kruskal-Wallis one-way ANOVA based on ranks, followed by post hoc test. The results were expressed in the form of mean±SD. A p value of less than 0.05 was considered significant for all analyses.

RESULTS

Cytokine profiling identifies elevated levels of serum MCSF in GBM

We profiled 48 cytokines in the sera obtained from normal healthy individuals (n=26), DA (n=24), AA (n=22) and GBM (n=148) patients using a bead array-based platform. A non-parametric t-test with False Discovery Rate (FDR) correction identified 33 cytokines with different concentrations between GBM and normal sera (Figure 1A; Supplementary table ST1). Further analysis revealed that the transcript levels of 17 cytokines were similarly regulated in GBM tissue compared to normal brain tissue, suggesting that differential expression in GBM is probably the reason for the difference in their concentration in sera (Supplementary table ST1).

Macrophage Colony Stimulating Factor (MCSF), one of the significantly elevated cytokines in GBM sera identified in the present study (Figure 1B), was selected for further investigation given: 1) its well-suggested role in the development and progression of tumors, including glioma (24,25), 2) its influence upon survival, proliferation and differentiation of macrophages/microglial cells which are present in abundance in glioma microenvironment (26,27) and 3) the expression of MCSF receptor (MCSFR) only in microglial cells, but not in glioma cells (28), suggesting that glioma cell-secreted MCSF may function through stromal cells and be one key determinant of glioma-stromal cell interactions underlying glioma progression. MCSF transcript
levels were up-regulated in GBM, compared to normal brain tissue samples, in our cohort and three more cohorts, namely TCGA, REMBRANDT and GSE22866 (Figure 1C). While MCSF transcript levels were significantly higher in all grades of glioma compared to normal brain samples, serum MCSF was only significantly different in GBM when compared to healthy controls (Figure 1B and C Rembrandt dataset). Immunohistochemical (IHC) analysis revealed MCSF positivity in the tumor cells of all grades of glioma with highest expression in GBM (Figure 1D and Table 1). Glioma-derived cell lines showed higher, but varying levels of MCSF transcript and secreted protein levels (except for LN229), compared to SVG, an immortalized astrocytic cell line (Figure 1E and F). These results suggest that MCSF is up-regulated in glioma, particularly in GBM, and that the high levels of MCSF found in GBM serum result probably from its secretion by tumor tissue.

**MCSF up-regulation depends on SYK-PI3 kinase-NFkB pathway in GBM**

While MCSF expression is known to be regulated by NFkB pathway in myeloid cell lineage (19), its regulation in GBM remains to be elucidated. We found that the wild type MCSF promoter-dependent luciferase activity decreased significantly upon mutation of NFkB-binding sites in U251, U87 and U373 cells (Figure 2A; see experimental procedures for more information). Wild type MCSF promoter-dependent luciferase activity was inhibited by Bay 11-7082, an IKKα inhibitor, in a concentration-dependent manner (Figure 2B) whereas Bay 11-7082 treatment did not alter mutant MCSF promoter-dependent luciferase activity (Figure 2C). Further, treatment with a PI3 kinase inhibitor (LY294002), but not a MAP kinase inhibitor (U0126), inhibited wild type MCSF promoter-dependent luciferase activity and decreased the level of MCSF secreted by U251 and U87 cells, suggesting that MCSF secretion by GBM is regulated by the PI3 kinase-NFkB pathway (Figure 2D and E respectively).

To identify the regulator of MCSF expression upstream to PI3 kinase, we correlated the MCSF transcript levels with the expression level of 171 proteins, measured by Reverse Phase Protein Array (RPPA), in 196 GBMs from the TCGA cohort (29). Among proteins exhibiting significant correlation with MCSF, Spleen Tyrosine Kinase (SYK) had the highest significance and positive correlation (p < 0.0001; r = 0.43) (Figure 2F). Since SYK is shown to activate PI3 kinase pathway (30-34), we investigated the possibility that SYK could be the activator of PI3 kinase contributing to MCSF up-regulation in glioma. SYK transcript levels were found to be up-regulated in GBM when compared to normal brain samples in TCGA, REMBRANT and GSE22866 cohorts (Figure 2G). Since RPPA was not done in normal brain samples, SYK protein up-regulation in GBM samples could not be ascertained. However, we found a significant positive correlation between SYK transcript and protein levels (Figure 2F), suggesting that SYK protein level may also be up-regulated in GBM. We also found that SYK and MCSF transcript levels had a significant positive correlation (Figure 2F). Notably, SYK transcript levels were up-regulated in DA and AA (Figure 2G, Rembrandt dataset), consistent with the up-regulation of MCSF transcript levels in lower grades of glioma (Figure 1C, Rembrandt dataset). Collectively, up-regulation of SYK in glioma, in particular GBMs, and the positive correlation between SYK and MCSF, suggests that SYK may be the upstream regulator of MCSF expression. Further supporting the role of SYK in MCSF up-regulation, treatment of cells with Bay 61-3606, a pharmacological inhibitor of SYK, inhibited MCSF promoter-dependent luciferase activity and decreased secreted MCSF levels as well as AKT phosphorylation (Figure 3A, B and C respectively). In addition, silencing SYK expression by siRNA also reduced the levels of secreted MCSF (Figure 3D and E).

Survival correlation using univariate Cox proportional hazard regression analysis revealed that both SYK and MCSF are poor prognostic indicators in GBM (Figure 3F). However, multivariate survival analysis along with age found that MCSF but not SYK is an independent predictor of survival (Figure 3F). MCSF transcript, SYK transcript and SYK protein levels were found significantly higher in mesenchymal subtype which is known to be associated with poor survival compared to other subtypes (Figure 3H, I and J respectively). Corroborating these findings,
A subgroup of GBM with high MCSF mRNA was significantly enriched in mesenchymal subtype (Figure 3G). Collectively, these results confirm that MCSF is an independent poor prognostic indicator in GBM and MCSF up-regulation in GBM is dependent on the SYK-Pi3k-NFkB pathway.

MCSF secreted by glioma cells induce angiogenesis through a macrophage/microglial cell-dependent mechanism

MCSF induces tumor cell proliferation in renal cell carcinoma in an autocrine way and might contribute to M2 polarization of macrophages in glioma (35,36). To explore the autocrine functions of MCSF in glioma, various properties like proliferation, chemosensitivity, anchorage-independent growth, migration and invasion were monitored after addition of recombinant MCSF (rMCSF), ectopic over expression (Figure 4A and B) or silencing MCSF by siRNA (Figure 4C and D). These experiments showed no changes in any of these properties suggesting the absence of an autocrine function for MCSF in glioma (data not shown). The absence of autocrine function for MCSF in glioma is supported by the unchanged levels of MCSFR transcript in GBM tumor tissue compared to normal brain tissue and also undetectable levels of MCSFR in glioma cell lines compared to the microglial cell line CHME-3 (Figure 4E and 4F respectively). In line with the possible role of MCSF in M2 polarization of macrophages in glioma (35), we next investigated by IHC the presence of M1 and M2 polarized macrophages/microglial cells in all grades of glioma with highest expression in GBM when compared to normal brain samples (Figure 4G and Table 1). However, IHC staining using M1 macrophage specific (CD86) and M2 macrophage specific (CD204) markers showed that the majority of the infiltrated macrophages are of M2 type in GBM (Figure 4G and Table 1). To further investigate the role of tumor-secreted MCSF in M2 polarization of macrophages, we monitored the ability of conditioned medium (CM) derived from glioma cell lines to polarize undifferentiated monocytes derived from human PBMCs and microglial cell line CHME-3. Glioma CM (from U251 and U87 cell lines) treatment of monocytes resulted in the down-regulation of M1 specific markers (TNFα and CXCL10) and up-regulation of M2 specific markers (IL10 and CD204) (Figure 4H). Similarly, treatment of the microglial cell line CHME-3, with glioma CM (from U251, U87, LN229, U373 and A172 cell lines) resulted in the down-regulation of M1 specific markers (TNFα and CXCL10) and up-regulation of M2 specific markers (IL1Rα and CD204) (Figure 4I). However, when microglial cells were treated with CM derived from glioma cells silenced for MCSF or in the presence of MCSFR inhibitor (GW2580), M2 polarization was not significantly affected (Figure 4J and K respectively). Collectively, these experiments suggest that while glioma cell-secreted factors polarize macrophages/microglial cells to M2 type, tumor secreted MCSF is not required for M2 polarization. We conclude from these experiments that glioma-secreted MCSF is not required for autocrine functions and M2 polarization of macrophages/microglial cells.

Since rMCSF has been shown to induce VEGFA expression in monocytes, which in turn promotes angiogenesis (37), we next investigated the role of glioma-secreted MCSF in tumor angiogenesis. To address this question, we performed tube formation assay (an in vitro angiogenesis assay) using Human Umbilical Vein Endothelial Cells (HUVEC) (Figure 5A control experiment). The supernatant from rMCSF-treated monocytes was more efficient than the supernatant from untreated monocytes to promote the tube formation in HUVEC (Figure 5B). The addition of rMCSF or BSA directly to HUVEC failed to induce tube formation (Figure 5B). Further, the supernatants from monocytes treated with CM derived from either U251 or siNT-transfected U251 cells, but not the supernatant from monocytes treated with CM from siMCSF-transfected U251 cells induced HUVEC tube formation (Figure 5C). Similarly, the supernatant from CHME-3 cells treated with U87 CM induced HUVEC tube formation more efficiently, compared to the supernatant from untreated CHME-3 cells (Figure 5D).

To demonstrate the role of MCSF in inducing angiogenesis in vivo, an intra-dermal
angiogenesis assay was performed. LN229/MCSF cells, stably overexpressing MCSF attracted significantly more blood vessels compared to LN229/Vector stable cells (Figure 5E). Similarly, siMCSF-transfected U251 cells showed a decreased number of tumor-directed capillaries compared to siNT-transfected U251 cells (Figure 5E). In addition, mice treated with the MCSFR inhibitor GW2580 showed significantly reduced tumor-directed capillaries compared to mice treated with vehicle (Figure 5E). Collectively, these results suggest that MCSF secreted by glioma cells induces the release by monocytes/microglial cells of extracellular factors, which in turn induce angiogenesis.

**Insulin-like growth factor-binding protein 1 (IGFBP1) present in microglial cell secretome is a novel mediator of MCSF-induced angiogenesis**

To identify novel factors present in the microglial secretome responsible for MCSF-induced angiogenesis, we used SILAC based on two different sets of heavy amino acids: Arg6 and Lys4 (medium label) for CHME-3 cells treated with U87 siNT CM and Arg10 and Lys8 (heavy label) for CHME-3 cells treated with U87 siMCSF CM (Figure 6A). The supernatants from differentially labelled CHME-3 cells were mixed in equal proportion and subjected to mass spectrometry. A total of 1,196 proteins were identified (Supplementary table ST2). Of these, 580 proteins (48.5% of the identified proteins) displayed a SecretomeP score >0.5 and/or were predicted to have a signal peptide by the Signal P (V.4.1) algorithm (Supplementary table ST2). While most proteins quantified in CHME-3 supernatants did not show significant change between the two experimental conditions, a total of 67 proteins showed significantly different SILAC ratios based on significance B with a p value of 0.01 (Figure 6B; Supplementary table ST3) (21). Thirty-nine of them were down-regulated (negative H/M ratio), while twenty-eight proteins were up-regulated (positive H/M ratio) in supernatants derived from CHME-3 cells treated with U87 siMCSF CM compared to supernatants derived from CHME-3 cells treated with U87 siNT CM (Supplementary table ST3).

Gene ontology analysis of MCSF-regulated proteins by BINGO showed enrichment in several processes which emphasize their important role in regulating angiogenesis (Supplementary tables ST4). Proteins exhibiting difference in abundance in the supernatant of CHME-3 treated with U87 siMCSF CM and U87 siNT CM included VEGFA, a known angiogenesis inducer (log 2 H/M ratio = -1.998) (Figures 7A). The reduced VEGFA levels in CHME-3 cells treated with U87 siMCSF CM, compared to CHME-3 cells treated with U87 siNT CM, was further confirmed at the transcript levels (Figure 7C).

In order to identify novel mediators of MCSF-induced angiogenesis in CHME-3 supernatant, we chose IGFBP1 (identified with five peptides by MS/MS analysis, Figure 6C) for further investigation for the following reasons: 1) it was not differentially expressed between GBM and control brain tissues, 2) its level was reduced in microglial supernatant treated with U87 CM derived from MCSF silenced condition (log 2 H/M ratio = -1.266) and 3) it is a known to be a secreted protein (Figures 7B, 7K and Supplementary table ST3). The reduced IGFBP1 expression in CHME-3 cells treated with U87 siMCSF CM was first verified at the level of transcripts (Figure 7D) and then confirmed by ELISA in an independent set of experiments (Figure 7E and F). Next, we carried out experiments to explore the role of CHME-3-secreted IGFBP1 in angiogenesis. Adding an IGFBP1 antibody substantially inhibited the ability of the supernatant from CHME-3 cells treated with U251 siNT CM to induce tube formation compared to addition of IgG control antibody (Figure 7I compare third bar with second bar). As expected, the supernatant derived from CHME-3 cells treated with U251 siMCSF CM failed to show any increase in tube formation (Figure 7I compare fourth bar with second bar). To further confirm the role of IGFBP1 in angiogenesis, we carried out experiments using supernatant from IGFBP1-silenced CHME-3 cells. The supernatant derived from CHME-3 cells transfected with siNT and subsequently treated with U251 CM was more efficient to promote tube formation, compared to the supernatant from CHME-3 cells transfected with siIGFBP1 and subsequently treated with
U251 CM (Figure 7G, H and J). Collectively, these results identify IGFBP1 secreted by microglial cells in response to MCSF as an important mediator of angiogenesis.

DISCUSSION

The study of the stromal components and the host immune response in addition to tumor cells led to the better understanding of cancer development (38). Cytokines play an important role in tumor-stroma interaction, thus facilitating tumor progression and aggressiveness (39). In the present study, we identified 33 cytokines exhibiting difference in abundance in GBM sera with 17 of which likely to be secreted by tumor tissue. The high proportion of profiled cytokines exhibiting differences in abundance in sera from patients with GBM found in the present study is consistent with previous investigations aimed at establishing distinctive cytokine signatures of various pathological situations, including breast cancer, which showed a deregulation of more than half of the cytokines tested (40-42).

MCSF, one of the cytokines present at high levels in glioma sera, in particular GBM, was further studied in detail in line with its important role in survival, proliferation and differentiation of macrophages/microglial cells which are present in abundance in glioma microenvironment (26,27). Although several reports showed elevated levels of MCSF in various cancers including glioma, the regulation and the function of MCSF in GBM remains poorly characterized (25,43,44). In this study, we confirm that MCSF is up-regulated in glioma, particularly in GBM, both tumor tissue and serum. It is interesting to note that while an elevated levels of MCSF transcript and protein are seen in all grades of glioma (DA, AA and GBM), serum MCSF was found elevated only in GBM. This might be due to the fact that there is a selective blood-brain barrier disruption reported in GBM compared to low-grade glioma (45).

In an effort to identify the mechanism underlying MCSF up-regulation in tumor cells, we found that it is critically dependent on the SYK-PI3 kinase-NFkB cascade. SYK plays a major role in survival signaling through multiple classes of immune recognition receptors in hematopoietic cells (46). However, SYK has been shown to act as both tumor promoter and tumor suppressor in different cancers (47). While SYK activation generally involves recruitment through its SH2 domains to a phosphorylated immune receptor tyrosine-based activation motif (ITAM), it can also be activated by other mechanisms such as elevated levels and its association with integrins (47). However, the impact of SYK in glioma has not been reported so far. Here, we demonstrate that both SYK transcript and protein levels are upregulated in glioma. To identify the possible upstream activators of SYK, analysis of expression of various integrins, which are known to be activated in GBM (48), from TCGA data revealed eleven integrin α, seven integrin β and three integrin interacting proteins including integrin-linked kinase to be up-regulated in GBM compared to normal brain samples (Supplementary table ST5). The PI3 kinase-AKT pathway plays a major role downstream of activated SYK (47). Further, several reports suggest that PI3K and NFkB pathways are activated by SYK (30-34). Our study demonstrates that SYK activates the PI3 kinase-AKT pathway which further leads to NFkB-dependent up-regulation of MCSF in GBM. Collectively, we conclude that SYK-PI3K-NFkB pathway, likely activated through integrins, is essential for MCSF up-regulation and may play a tumor promoter role in GBM.

We also found that both SYK and MCSF levels are higher in mesenchymal subtype of GBM compared to other subtypes. A very high level of MCSF seen in mesenchymal subtype is particularly interesting because mesenchymal GBMs are significantly enriched in high-risk GBMs with poor survival (49). Correspondingly, we also found that MCSF mRNA level was a poor prognostic indicator in GBM and that a subgroup with high MCSF mRNA is enriched with mesenchymal GBM patients. The high levels of MCSF seen in mesenchymal subtype, which result from SYK up-regulation, may favor angiogenesis (see below) and explain the poor prognosis of mesenchymal GBMs.

In breast cancer and renal cell carcinoma, MCSF and its receptor MCSFR are co-expressed in tumor cells, allowing an autocrine action and thereby increasing malignancy and proliferation.
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(36,50). Likewise, data indicate expression of both MCSF and its receptor MCSFR in glioma, suggesting autocrine and paracrine effects (43). However, we found that glioma-secreted MCSF has no function in promoting tumor cell properties directly in an autocrine fashion. Consistently, we found that the MCSFR is not expressed in glioma tumor and cell lines. However, the CHME-3 microglial cell line expresses high levels of MCSFR, suggesting that MCSF may function by activating certain pathways in microglial cells. A role for MCSF-MCSFR signaling in M2 polarization of macrophages has also been proposed, based on a report indicating that MCSFR inhibition resulted in reduced tumor growth with decreased M2 markers in macrophages (28). While this report proves the importance of MCSFR in M2 polarization of macrophages, a direct role for MCSF in M2 polarization remained to be established. This is particularly important because MCSFR is also activated by IL34 (51). Our results demonstrate that while glioma cell CM contained factors that could induce M2 polarization of monocytes/microglial cells, MCSF present in the glioma CM is not required for M2 polarization.

Recombinant human MCSF induces angiogenesis through macrophages by promoting VEGFA expression (37). Our results demonstrate that MCSF present in the glioma cell CM acts on monocytes or microglial cells and promotes secretion of factors, which induce angiogenesis in vitro. We also provide evidence that angiogenesis elicited by monocyte/microglia exposed to glioma cell CM is dependent on activation of MCSF-MCSFR signaling in macrophages. Further, a search of novel mediators of angiogenesis in the microglial secretome using an unbiased proteomic strategy identified 67 proteins significantly regulated by MCSF.

Gene ontology analysis of the MCSF-regulated proteins in the microglial secretome underscored their important role in angiogenesis. While cellular components category revealed the importance of extracellular matrix, extracellular region, extracellular space and proteins therein, biological process ontology recognized the proliferation of endothelial cells, angiogenesis and wound healing in addition to the process of coagulation. Temporal and spatial regulation of extracellular matrix remodeling events plays a key role in angiogenesis (52). Tissue factor-initiated coagulation through thrombin activated PAR family of G-protein-coupled receptor signaling has been found to promote tumor angiogenesis (53,54). The molecular function gene ontology identified the importance of cell surface receptors, calcium ion and phospholipid binding. Intracellular calcium and downstream signal transduction has been shown to regulate angiogenesis (55,56). Phospholipids have been shown to regulate tumor angiogenesis (57).

Consistent with previous findings (37), we demonstrate that VEGFA level is increased in the microglial cell secretome through a MCSF-dependent mechanism. Likewise, glioma-derived MCSF increased IGFBP1 level in the secretome of microglial cells. Furthermore, neutralizing IGFBP1 by a specific antibody in the microglial secretome after treatment with glioma cell CM, strongly reduced tube formation in HUVEC, indicating that MCSF-induced increase in IGFBP1 secretion by microglial cells was essential for angiogenesis. Similarly, silencing IGFBP1 in microglial cells before treatment with glioma cell CM significantly reduced the ability of microglial cell secretome to induce tube formation in HUVEC. Notably, there was no significant difference in IGFBP1 transcript levels between GBM and normal brain (Figure 7K), suggesting that IGFBP1 secreted by microglial cells in response to glioma-secreted MCSF is probably the major source for tumor angiogenesis.

There are several reports in the literature connecting IGFBP1 to angiogenesis. IGF1, which is regulated by its binding to IGFBP1, regulates migration and angiogenesis of human endothelial cells (58). IGFBP1, which can also function in an IGF-independent manner, induces angiogenesis and binds to the pro-survival factor BAK, thereby inhibiting growth inhibitory functions of p53 (59). IGFBP1 also induces endothelial nitric oxide synthase (eNOS) activity through PI3 kinase signaling, resulting in increased nitric oxide (NO) production (60-63). IGFBP1 secreted from human chondrocytes upon activation by lysophosphatidic acid (LPA) through LPA receptor has been found to induce angiogenesis (57).
Collectively, these findings and our data suggest that IGFBP1 released by microglial cells is an important effector contributing to angiogenesis elicited by glioma-derived MCSF. Therefore, they point to the possible role of factors other than VEGF that may promote angiogenesis and cancer stem cell-derived endothelial cells through transdifferentiation (64). This is of potential clinical interest as bevacizumab, a VEGF targeting antibody approved by FDA, failed to show any significant improvement in overall and progression free survival in the recently conducted trails (65,66). Thus, IGFBP1 could be a potential alternate candidate for developing a targeted therapy for GBM.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author’s contribution: ASH, BAC, AA, VS and KS conceived and coordinated the study. MBN, PM and KS wrote the paper. MBN, SU and PM designed, performed and analyzed the experiments shown in Figure 6. MBN and VP designed, performed and analyzed the experiments shown in Figure 1, 2 and 3. SDS and KS designed, performed and analyzed the experiments shown in Figure 1D and 4G. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES
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et de la Recherche Médicale (INSERM), la Fondation pour la Recherche Médicale (Equipe FRM 2009) and la Région Languedoc-Roussillon.

The abbreviations used are: GBM—Glioblastoma; MCSF—Macrophage Colony Stimulating Factor; SYK—Spleen Tyrosine Kinase; SILAC—Stable Isotope Labeling by Amino Acids in Cell Culture; VEGFA—Vascular endothelial growth factor A; IGFBP1—Insulin-like growth factor-binding protein 1.

FIGURE LEGENDS

FIGURE 1: MCSF levels in sera, GBM tumor tissue and glioma cell lines.

A. Heat map of the 33 differentially abundant cytokines in Normal and GBM sera. A dual-color code was used, with yellow and blue indicating high and low abundance, respectively. A total of 24 cytokines were present in higher abundance and 9 at lower abundance in GBM sera compared to normal sera. The red line separates Normal from GBM samples.

B. Scatter plot representation of MCSF levels in Normal (n=26), DA (n=24), AA (n=22) and GBM (n=148) sera estimated by the bead array method. Statistical analysis (one-way ANOVA) was performed and the p values are indicated. The horizontal line represents mean value, ns= not significant.

C. MCSF transcript levels in normal brain tissue (n=10), and GBM tumor tissue (n=61) estimated by qRT-PCR. Microarray data for MCSF gene expression from TCGA consisting of normal (n=10) and GBM (n=572) samples, Rembrandt dataset consisting of normal (n=28), DA (n=65), AA (n=58) and GBM (n=227) samples and GSE22866 dataset consisting of normal (n=6) and GBM (n=40) samples are shown as scatter plot. Non-parametric t-test was performed and the p values are indicated. The horizontal line in each plot represents mean value.

D. Representative images (Original Magnification x160) of MCSF immunostaining in normal brain tissue (n=5), DA (n=10), AA (n=10) and GBM tumor tissue (n=41). Normal brain is negatively stained whilst DA, AA and GBM show variable staining, with maximum staining in GBM.

E. MCSF transcript levels in various glioma cell lines estimated by qRT-PCR method and normalized to SVG (an immortalized glioma cell line).

F. MCSF secreted protein levels in the culture supernatant of various glioma cell lines and SVG measured by ELISA method.

FIGURE 2: Regulation of MCSF expression in glioma.

A. Glioma cells were transfected with either wild type MCSF promoter-dependent luciferase construct or with mutant construct wherein all four NFkB binding sites are mutated. MCSF promoter activity was measured by luciferase assay and normalized to β-Galactosidase activity.

B. Glioma cells were transfected with wild type MCSF promoter-dependent luciferase construct. MCSF promoter activity was measured by luciferase assay and normalized to β-Galactosidase activity in presence of vehicle control and two different concentrations of Bay 11-7082.

C. Glioma cells were transfected with mutant MCSF promoter-dependent luciferase construct. MCSF promoter activity was measured by luciferase assay and normalized to β-Galactosidase activity in presence of vehicle control and 20uM Bay 11-7082.
D. Glioma cells were transfected with wild type MCSF promoter-dependent luciferase construct. MCSF promoter activity was measured by luciferase assay and normalized to β-Galactosidase activity in presence of different pathway inhibitors as indicated.

E. MCSF secreted protein levels measured by ELISA method in presence of different pathway inhibitors as indicated in U251 and U87 cell lines.

F. Spearman correlation analysis between MCSF transcript and SYK protein levels; SYK transcript and SYK protein levels; MCSF and SYK transcript levels were analyzed using the dataset derived from TCGA, r and p values are indicated in the graph. The straight line inside the graph indicates the mean.

G. Scatter plot representation of SYK transcript levels using- TCGA dataset consisting of normal (n=10) and GBM (n=572) samples; Rembrandt dataset consisting of normal (n=28), DA (n=65), AA (n=58) and GBM (n=227) samples; GSE22866 dataset consisting of normal (n=6) and GBM (n=40) samples. Non-parametric t-test was performed and the p values are indicated. The horizontal line in each plot represents mean value.

FIGURE 3: MCSF is regulated by SYK-PI3K-NFkB pathway and acts as poor prognostic indicator in glioma.

A. U87 glioma cell line was transfected with wild type MCSF promoter-dependent luciferase construct. MCSF promoter activity was measured by luciferase assay and normalized to β-Galactosidase activity in presence of the SYK inhibitor Bay 61-3606. The luciferase activity was expressed as % of the activity present in untreated control cells.

B. MCSF secreted protein levels measured by ELISA in presence of SYK inhibitor Bay 61-3606. MCSF levels were expressed as % of the levels present in untreated control cells.

C. Western blot representation of AKT and phospho-AKT (pAKT) proteins in U87 cell line treated with and without SYK inhibitor Bay 61-3606. β-actin was used as loading control.

D. SYK transcript levels estimated by qRT-PCR method in U87 glioma cell line transfected with either siNT or siSYK, (48 hr). The expression values were normalized to siNT sample.

E. MCSF secreted protein levels measured by ELISA in SYK silenced condition. MCSF levels were expressed as % of the levels present in cells transfected with siNT.

F. Cox proportional hazard regression analysis using MCSF and SYK transcript levels and survival data from TCGA dataset in a cohort of 245 GBM patients. The patient inclusion criteria were, at least 30 days of survival from the time of surgery, KPS ≥ 70 and any kind of chemotherapy received.

G. TCGA patients (n= 245) were divided into gene expression subtypes- classical, mesenchymal, neural and proneural. Patients of each subtype were further sub-divided based on MCSF transcript levels (low MCSF vs. high MCSF). The significance of distribution was analyzed by 2-sample z-test using R software (version 3.1.0). The patient inclusion criteria were, at least 30 days of survival from the time of surgery, KPS ≥ 70 and any kind of chemotherapy received.

H, I and J. Scatter plot representation of MCSF transcript, SYK transcript and SYK protein levels in different subtypes of GBM using TCGA dataset, respectively. Statistical analysis (one-way ANOVA) was performed and the p values are indicated. The horizontal line in each plot represents mean value.

FIGURE 4: MCSF levels modulation in glioma cell lines and role of MCSF in macrophage polarization.
Microglial IGFBP1 – a novel mediator of MCSF-induced angiogenesis

A and B. MCSF transcript and protein levels in LN229 cell line stably expressing vector control or MCSF cDNA estimated by qRT-PCR and ELISA methods, respectively. The expression levels in LN299/MCSF were normalized to respective LN229/Vector control samples.

C and D. MCSF transcript and protein levels in glioma cell lines transfected with either siNT or siMCSF estimated by qRT-PCR (48 hr) and ELISA (72 hr) methods, respectively. The expression levels were normalized to respective siNT samples.

E. MCSFR transcript levels in normal brain tissue (n=8), and GBM tumor tissue (n=71) estimated by qRT-PCR. Non-parametric t-test was performed to find the significance between two groups. The horizontal line represents mean value, ns= non-significant.

F. Western blot representation of MCSFR protein levels in various glioma cell lines and microglial cell line CHME-3. β-actin was used as a loading control.

G. Representative images (Original Magnification x160) of normal brain and GBM tissue sections stained for macrophage marker (CD68), M1 marker (CD86) and M2 marker (CD204).

H. Transcript levels of M1 (TNFα and CXCL10) and M2 (IL10 and CD204) phenotype genes measured by qRT-PCR in blood derived monocytes which were treated with U251 and U87 CM. for 7 days. The expression levels were normalized to untreated cells.

I. Transcript levels of M1 (TNFα and CXCL10) and M2 (IL1Rα and CD204) phenotype genes measured by qRT-PCR in microglial cell line CHME-3 which was treated with various glioma cell line CM for 24 hrs. The expression levels were normalized to untreated cells.

J. Transcript levels of M1 (TNFα and CXCL10) and M2 (IL1Rα) phenotype genes measured by qRT-PCR in microglial cell line CHME-3 which was treated with U87 CM derived from either control or MCSF silenced condition for 24 hrs. The expression levels were normalized to untreated cells.

K. Transcript levels of M1 (TNFα and CXCL10) and M2 (IL1Rα) phenotype genes measured by qRT-PCR in microglial cell line CHME-3 which was pre-treated with vehicle or GW2580 for 1 hr and later treated with U87 CM for 24 hrs. The expression levels were normalized to control cells.

FIGURE 5: In vitro and in vivo angiogenesis assay.

A, B, C and D. Representative images (Original magnification x 100) of the tube formation assay performed in HUVEC under different conditions as indicated:

A. Negative control- HUVEC growth media alone; Positive control- HUVEC growth media with positive angiogenic inducers.

B. Monocyte sup.- supernatant from monocytes; rMCSF/Monocyte sup.- supernatant from monocytes treated with recombinant MCSF; rMCSF-Recombinant MCSF; BSA-purified bovine serum albumin.

C. U251 CM/Monocyte sup.- supernatant from monocytes treated with U251 CM; U251 siNT CM/Monocyte sup.- supernatant from monocytes treated with U251 CM derived from non-targeting siRNA transfected cells; U251 siMCSF CM/Monocyte sup- supernatant from monocytes treated with U251 CM derived from MCSF siRNA transfected cells.

D. CHME-3 sup.-supernatant from microglia; U87CM/CHME-3 sup.-supernatant from microglia treated with U87 CM.
Microglial IGFBP1 – a novel mediator of MCSF-induced angiogenesis

The right side of the panel shows the quantification of the corresponding images. T-test was performed and the p values are indicated.

E. Representative images of dissected skin of the mice near intra-dermal glioma tumor showing tumor-directed capillaries and the quantification graphs in different conditions as indicated: LN229/Vector- cells with vector backbone; LN229/MCSF-cells overexpressing MCSF; U251/siNT - cells transfected with non-targeting siRNA; U251/siMCSF - cells transfected with MCSF siRNA; U251/Vehicle- animals treated with vehicle control; U251/GW2580- animals treated with MCSFR inhibitor (160mg/Kg body wt/day). T-test was performed and the p values are indicated. The dashed-circle within the images shows the tumor location.

FIGURE 6: Microglial secretome analysis by SILAC.

A. Schematic representation of the workflow used to study the variations in the CHME-3 secretome induced by CM of U87 silenced or not for MCSF using SILAC.

B. Volcano plot representation of SILAC results. The X-axis shows log 2 H/M ratio derived by taking ratio of mean intensity of heavy label to mean intensity of medium label for each protein. The Y-axis shows p value expressed in minus log 10 scale. A total of 67 proteins were significantly differentially regulated, of which 28 were up-regulated and 39 were down-regulated. Each dot represents one protein in the plot. Red and green color dots indicate up- and down-regulation, respectively. The location of VEGFA and IGFBP1 is indicated.

C. Annotated MS/MS spectra of five different IGFBP1 peptides

FIGURE 7: Microglial IGFBP1 is a mediator of MCSF-induced angiogenesis.

A and C. VEGFA protein and transcript levels in CHME-3 measured by SILAC and qRT-PCR methods, respectively. The expression levels of VEGFA in CHME-3 upon treatment with CM from MCSF-silenced U87 was normalized to levels present in CHME-3 which was treated with CM from siNT- transfected (control) U87.

B and D. IGFBP1 protein and transcript levels in CHME-3 measured by SILAC and qRT-PCR methods, respectively. The expression levels of IGFBP1 in CHME-3 upon treatment with CM from MCSF-silenced U87 was normalized to levels present in CHME-3 which was treated with CM from siNT- transfected (control) U87.

E and F. IGFBP1 levels, measured by ELISA, in CHME-3 cell supernatant with different CM treatment as indicated. The IGFBP1 levels were expressed as % of the levels measured in the supernatant of the CHME-3 cells which was treated with CM from corresponding siNT transfected glioma cells. T-test was performed and the p values are indicated.

G and H. IGFBP1 transcript and protein levels in the CHME-3 upon transfection with either siNT or siIGFBP1, as measured by qRT-PCR (48 hr) and ELISA (72 hr) methods, respectively. The expression was normalized to siNT samples.

I and J. Tube formation assay in various conditions as indicated below:

I. CHME-3 sup./IgG Ab- supernatant from CHME-3 cells in presence of IgG antibody; U251 siNT CM/CHME-3 sup./IgG Ab-supernatant from CHME-3 cells treated with U251 CM derived from non-targeting siRNA transfected cells in presence of IgG antibody; U251 siNT CM/CHME-3 sup./IGFBP1 Ab-supernatant from CHME-3 cells treated with U251 CM derived from non-targeting siRNA transfected
cells in presence of IGFBP1 antibody; U251 siMCSF CM/CHME-3 sup./IgG Ab- supernatant from CHME-3 cells treated with U251 CM derived from MCSF silenced cells in presence of IgG antibody;

J. U251 CM/CHME-3 siNT sup.-supernatant from CHME-3 cells transfected with siNT and subsequently treated with U251 CM; U251 CM/CHME-3 siIGFBP1 sup.-supernatant from CHME-3 cells transfected with siIGFBP1 and subsequently treated with U251 CM.

T-test was performed and the p values are indicated.

K. Scatter plot representation of IGFBP1 transcript levels using TCGA dataset which consisted of normal (n=10) and GBM (n=572) samples. Non-parametric t-test was performed and the p value is indicated. The horizontal line represents mean value, ns= non-significant.
Table 1: Expression of different variables in all grades of glioma by IHC.

| Variables | Mean±SD | Kruskal-Wallis p value | Posthoc p value |
|-----------|---------|-----------------------|-----------------|
|           | Normal (n=5) | DA (n=10) | AA (n=10) | GBM (n=41) |                   |                   |
| MCSF      | 0.00±0.00 | 12.00±14.76 | 13.50±12.70 | 18.41±13.94 | 0.025 | 0.068; 0.02; 0.005; 0.638; 0.222; 0.295 |
| CD68      | 15.00±5.0 | 16.50±6.26 | 21.00±4.59 | 28.54±12.76 | 0.002 | 0.651; 0.036; 0.016; 0.102; 0.003; 0.04 |
| CD204     | 0.00±0.00 | 3.50±5.79 | 4.50±5.98 | 25.85±13.96 | <0.001 | 0.188; 0.114; 0.001; 0.687; <0.001; <0.001 |
| CD86      | 10.00±0.00 | 2.50±5.4 | 3.5±5.79 | 11.46±10.2 | 0.01 | 0.016; 0.04; 0.586; 0.01; 0.021 |

a Normal vs. DA, b Normal vs. AA, c Normal vs. GBM, d DA vs. AA, e DA vs. GBM, f AA vs. GBM
Classical Mesenchymal Neural Proneural

SYK Protein expression level

| Variables | p value | Regression coefficient (B) | Hazard ratio |
|-----------|---------|-----------------------------|--------------|
| Univariate analysis |
| MCSF      | 0.02    | 0.42                        | 1.53         |
| SYK       | 0.03    | 0.20                        | 1.22         |
| Age       | <0.0001 | 0.03                        | 1.02         |
| Multivariate analysis |
| MCSF      | 0.02    | 0.44                        | 1.55         |
| Age       | <0.0001 | 0.03                        | 1.03         |
| SYK       | 0.15    | 0.14                        | 1.14         |

Cox proportional hazard regression analysis

Low MCSF (n= 61)  High MCSF (n= 184)

p<0.0001  p=0.0013

ns

Univariate analysis

MCSF 0.02 0.42 1.53
SYK 0.03 0.20 1.22
Age <0.0001 0.03 1.02

Multivariate analysis

MCSF 0.02 0.44 1.55
Age <0.0001 0.03 1.03
SYK 0.15 0.14 1.14

Mamatha et al., 2015; Figure 3
Mamatha et al., 2015; Figure 4

A) MC5 transcript (Log 2 ratio) vs. LN229/Vector and LN229/MCSF

B) Secreted MCSF (% of vector) vs. LN229/Vector and LN229/MCSF

C) MCSF Tractad (Log 2 ratio) vs. siNT and siMCSF

D) Secreted MCSF (% of siNT) vs. siNT and siMCSF

E) MC5R transcript (Log 2 ratio) vs. Normal and GBM

F) U251, U87, LN229, A172, T98, SV, CHME-3, U373, A172, CM

G) Normal GBM

H) U251 CM, U87 CM, TNFα, CXCL10

I) U251 CM, U87 CM, LN229 CM, U373 CM, A172 CM, CD204

J) U87 siNT CM, U87 siMCSF CM, TNFα, IL1Rα

K) Control U87 CM, TNFα, IL1Rα
A

B

C

D

E

Mamatha et al., 2015; Figure 5
Control

Medium label (Arg 6 and Lys 4)

Heavy label (Arg 10 and Lys 8)

Labeling (5 passages)

Add U87 siNT CM (Arg 6 and Lys 4)

Add U87 siMCSF CM (Arg 10 and Lys 8)

24 hrs

24 hrs

Supernatant mix 1:1

SDS-PAGE, in gel trypsin digestion nano-LC-FT-MS/MS

MaxQuant analysis

CHME-3 siMCSF/CHME-3 siNT SILAC ratio

Mamatha et al., 2015; Figure 6
Glioblastoma-derived Macrophage Colony Stimulating Factor (MCSF) Induces Microglial Release of Insulin-like Growth Factor-Binding Protein 1 (IGFBP1) to Promote Angiogenesis

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