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Supplemental information

Microglia/macrophage-derived human CCL18 promotes glioma progression via CCR8-ACP5 axis analyzed in humanized slice model

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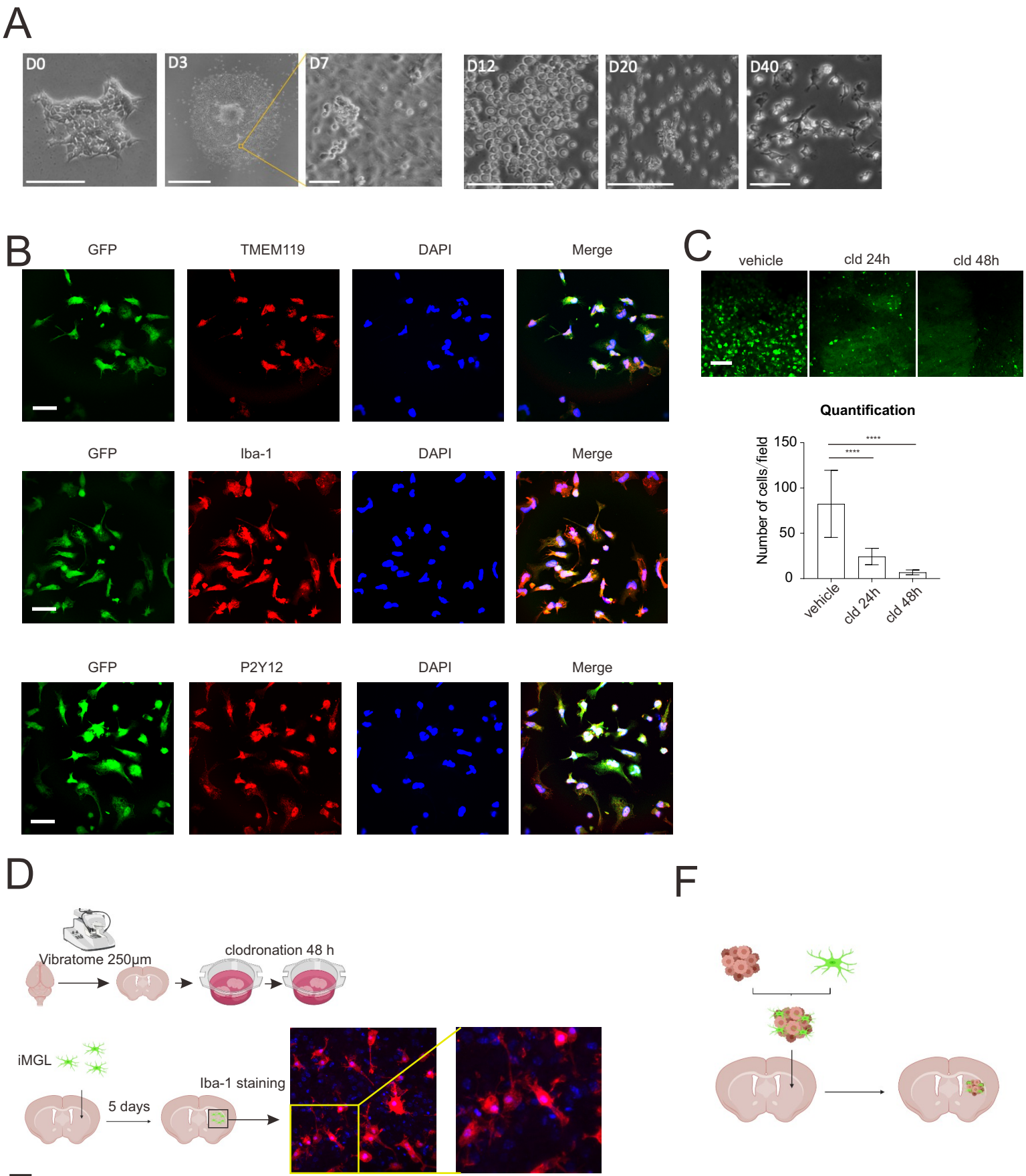

A
D0
D3
D7
D12
D20
D40
B
GFP
TMEM119
DAPI
Merge
GFP
Iba-1
DAPI
Merge
GFP
P2Y12
DAPI
Merge
C
vehicle
cld 24h
cld 48h
Quantification
Number of cells/field
150
100
50
0
****
****
vehicle
cld 24h
cld 48h
D
Vibratome 250µm
clodronation 48 h
iMGL
5 days
Iba-1 staining
F
E
Iba-1
Iba-1+DAPI
TMEM119
TMEM119+DAPI
P2Y12
P2Y12+DAPI

Figure S1. HMGUi001-A -hiPSC derived iMGL differentiation in vitro and establishment of humanized glioma-microglia ex vivo model. Related to Figure 1.

(**A**) Representative images of in vitro microglial differentiation are shown for Xmoo1 hiPSC. HMGUi001-A hiPSC were replated onto a Geltrex-coataed plate at a density of 50 clusters per well and the differentiation was started one day later, changing medium to Medium A of the STEMdiffTM Hematopoietic Kit (Stemcell Technologies). (day 0). (representative cluster shown, bar denotes 200 µm). On Day 3 medium was replaced with Medium B (representative image shown, bar denotes 750 µm). First hematopoetic progenitor cells (HPC) emerged on day 7 (representative image shown, bar denotes 75 µm). Over the next days, HPC increased in number and can now easily be harvested by removing the medium from the well on day 10/12 (representative image of HPC on day 12, bar denotes 150 µm). HPC were replated into serum-free medium that was supplemented with IL-34 (100 ng/ml), TGF-ß1 (50 ng/ml) and M-CSF (25 ng/ml). Cells became increasingly adherent and more ramified over time (representative image at day 20, bar denotes 150 µm). At day 37 and 39, CD200 (100ng/ml) and CX3CL1 (100ng/ml) were added to the medium additionally. iMGL were ready for harvest on day 40 (representative image shown, bar denotes 75 µm).

(**B**) Representative images of of iMGL (GFP labelled) stained with TMEM119 (top), Iba-1 (middle), P2y12 (below) and DAPI. Scale bar = 50 µm.

(**C**) The representative figures on top show the OBS obtained from CSF1R-EGFP (Macgreen) mice in which microglia are GFP labelled. Left is a slice treated with vehicle, middle treated with clodronate liposome for 24 h and right for 48 h. Bar = 100 µm. Quantification of GFP-labelled microglia is presented in the graph below. (n = 6, at least 2 slices per n, **** = $p < 0.0001$).

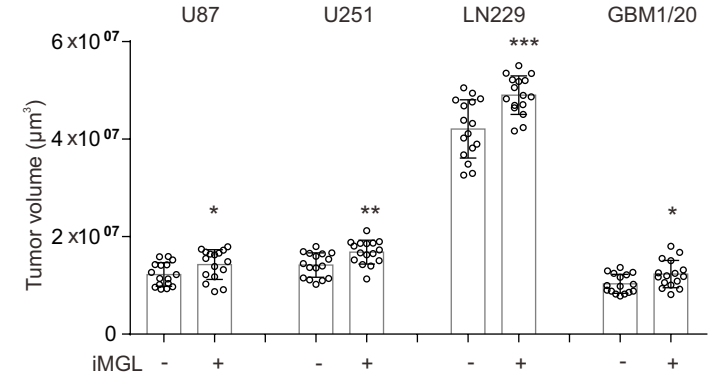
(**D**) Scheme illustrating the procedure to establish the humanized microglia ex vivo model. Briefly,

brain from P14 mice were cut into 250 µm slices using a vibratome and mounted on inserts containing tissue culture medium. Clodronate filles liposomes were added into the medium for 48 h to deplete intrinsic murine microglia. Subsequently liposomes were washed away with normal medium and slices were cultured for 72 h. Then iMGL were injected into the organotypic brain slices for 5 days. Microglia specific markers Iba-1 was used to label iMGL on the organotypic brain slices, and the representative figures are shown on the right (red, Iba-1; blue, DAPI).

(**E**) Representative images of the organotypic brain slices labelled with microglial markers Iba-1 (red, left), P2Y12 (red, middle), TMEM19 (red, right) and corresponding markers merged with DAPI (blue). Scale bar = 50 µm.

(**F**) The scheme illustrates the procedure of generating humanized glioma-microglia ex-vivo model. Organotypic brain slices were prepared as described in C. Fluorescence labelled glioma cells were mixed (1:1) with iMGL and co-inoculated into the organotypic brain slices and cultured for 5 days. Subsequently slices were fixed and the tumor volume was quantified.

A


U87
U251
LN229
GBM1/20
6 x10 07
4 x10 07
2 x10 07
0
Tumor volume (µm3)
*
**
***
*
iMGL - + - + - + - +

B

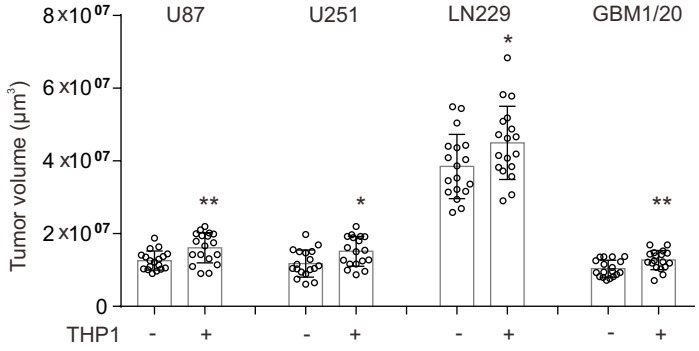
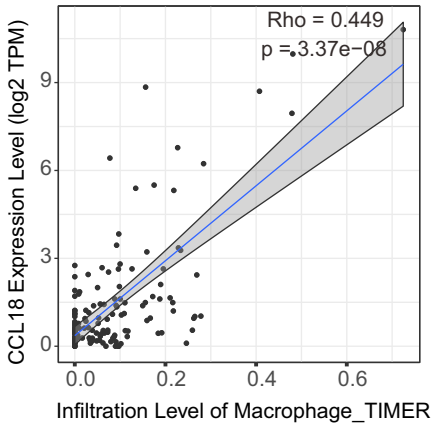

8 x10 07
6 x10 07
4 x10 07
2 x10 07
0
Tumor volume (µm3)
U87
U251
LN229
GBM1/20
**
*
*
**
THP1 - + - + - + - +

Figure S2. The tumor volumes of co-injecting 5000 tumor cells with 2500 iMGL cells on OBS. Related to Figure 1.

Glioma cell lines U87-MGmCherry, U251-MGmCherry and LN229-MGmCherry as well as primary glioma cells GBM1/20 were inoculated alone (-) or in combination with 2500 iMGL (+) shown in (A) or with THP-1 macrophages shown in (B). Tumor volumes were quantified. (n = 7 per group, at least 2 tumors per n, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)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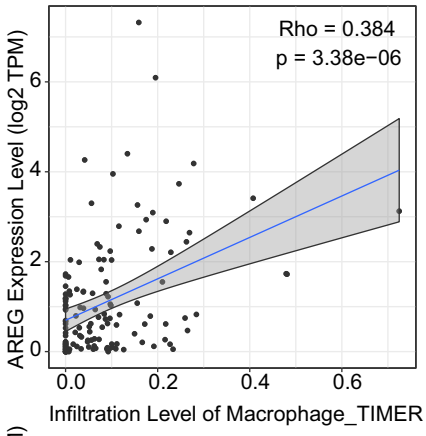
p = 3.37e−08
CCL18 Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER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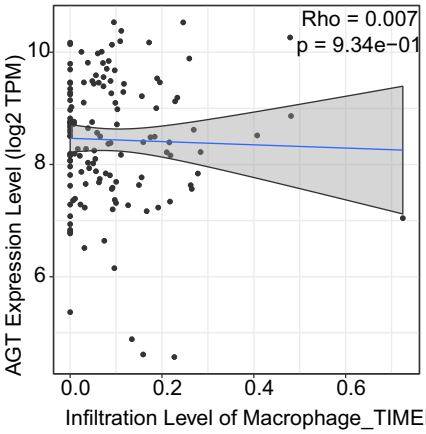
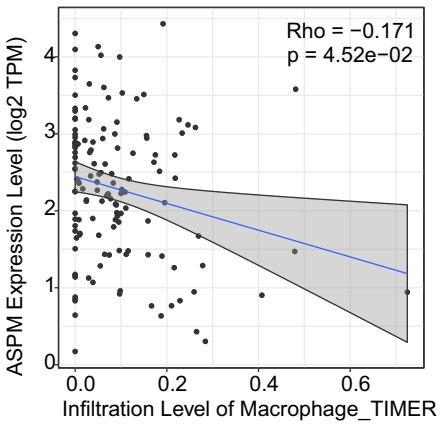
AREG Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER


Rho = 0.007
p = 9.34e−01
AGT Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER


Rho = −0.171
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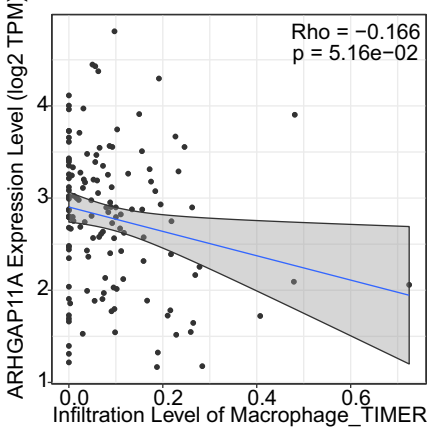
ASPM Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER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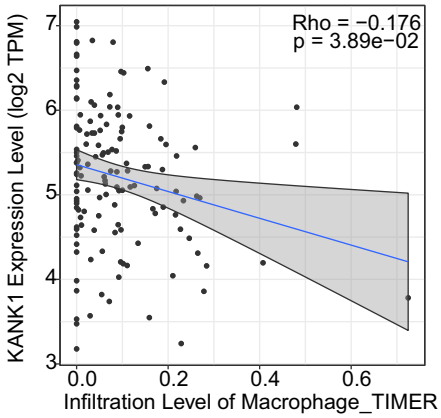
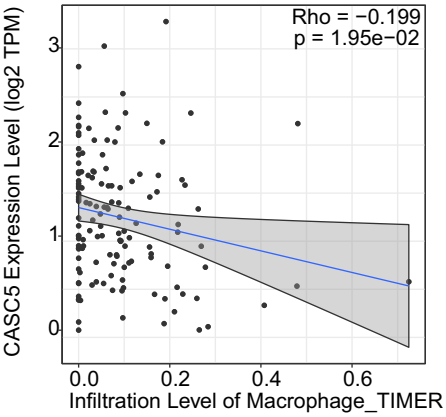
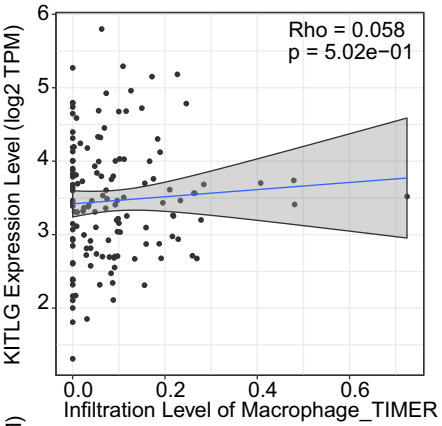
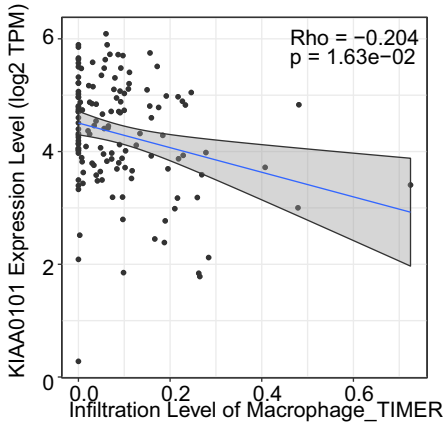
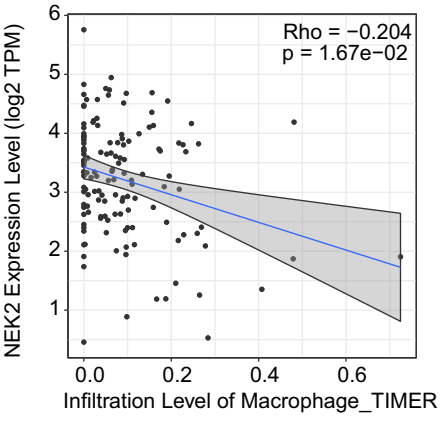
ARHGAP11A Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER


Rho = −0.176
p = 3.89e−02
KANK1 Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER


Rho = −0.199
p = 1.95e−02
CASC5 Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER


Rho = 0.058
p = 5.02e−01
KITLG Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER


Rho = −0.204
p = 1.63e−02
KIAA0101 Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER


Rho = −0.204
p = 1.67e−02
NEK2 Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER

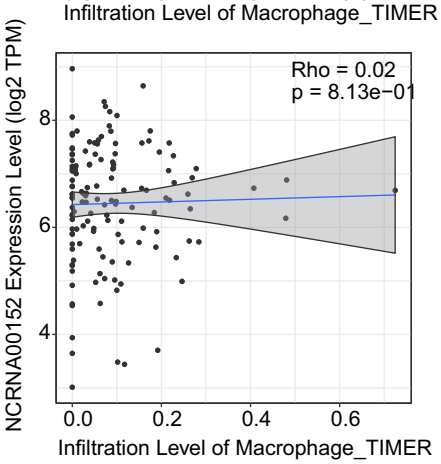

Rho = 0.02
p = 8.13e−01
NCRNA00152 Expression Level (log2 TPM)
Infiltration Level of Macrophage_TIMER

Figure S3. The correlationship between GAMs infiltration level and the expression of a selection of 11 different genes as indicated. Related to Figure 2.

The figures presented the Rho value and p value of the correlation analysis based on Spearman rank correlation test.

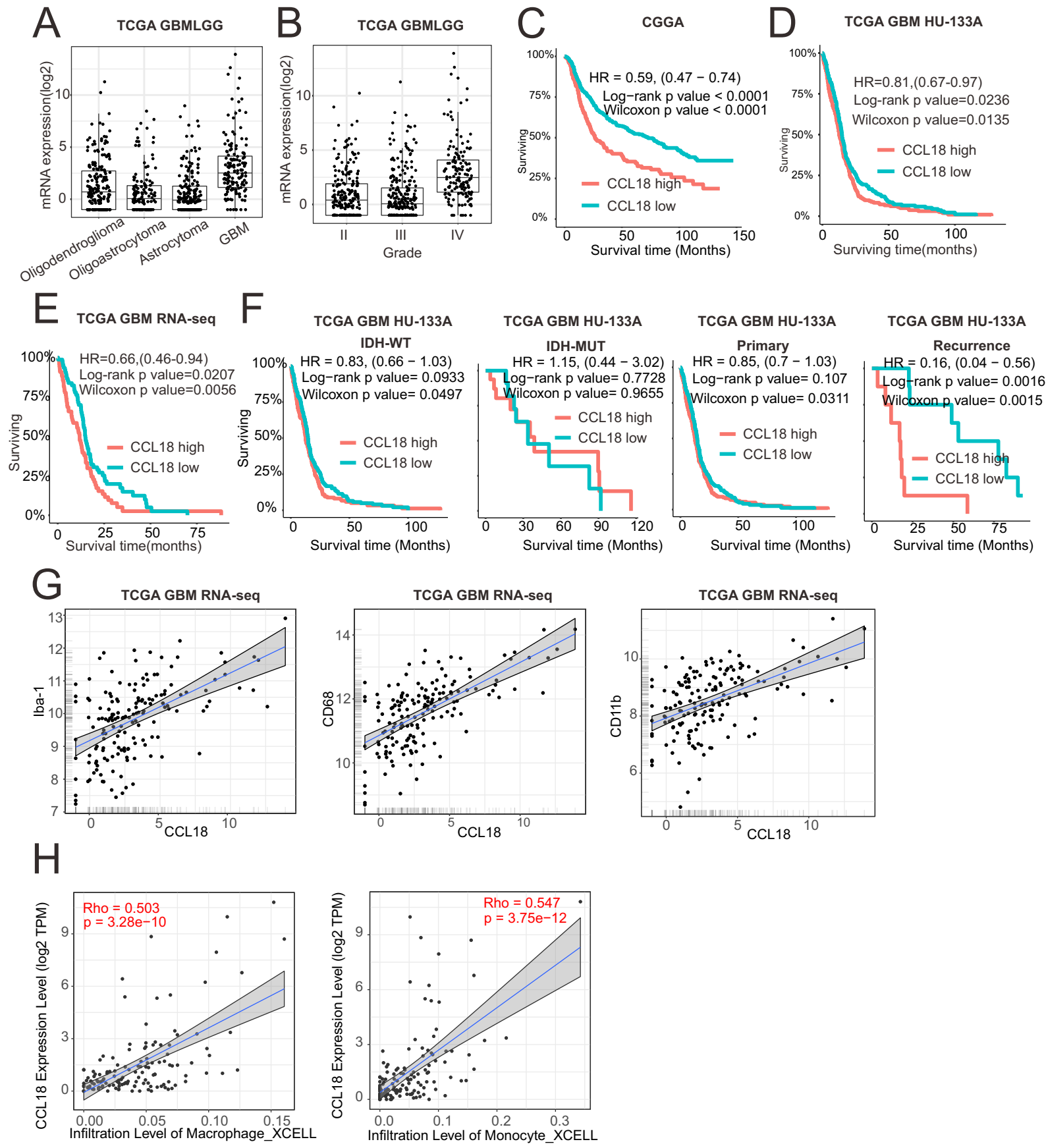

A
TCGA GBMLGG
mRNA expression(log2)
Oligodendroglioma
Oligoastrocytoma
Astrocytoma
GBM
B
TCGA GBMLGG
mRNA expression(log2)
II
III
IV
Grade
C
CGGA
HR = 0.59, (0.47 − 0.74)
Log−rank p value < 0.0001
Wilcoxon p value < 0.0001
Surviving
CCL18 high
CCL18 low
Survival time (Months)
D
TCGA GBM HU-133A
HR=0.81,(0.67-0.97)
Log-rank p value=0.0236
Wilcoxon p value=0.0135
Surviving
CCL18 high
CCL18 low
Surviving time(months)
E
TCGA GBM RNA-seq
HR=0.66,(0.46-0.94)
Log-rank p value=0.0207
Wilcoxon p value=0.0056
Surviving
CCL18 high
CCL18 low
Survival time(months)
F
TCGA GBM HU-133A
IDH-WT
HR = 0.83, (0.66 − 1.03)
Log−rank p value= 0.0933
Wilcoxon p value= 0.0497
TCGA GBM HU-133A
IDH-MUT
HR = 1.15, (0.44 − 3.02)
Log−rank p value= 0.7728
Wilcoxon p value= 0.9655
TCGA GBM HU-133A
Primary
HR = 0.85, (0.7 − 1.03)
Log−rank p value= 0.107
Wilcoxon p value= 0.0311
TCGA GBM HU-133A
Recurrence
HR = 0.16, (0.04 − 0.56)
Log−rank p value= 0.0016
Wilcoxon p value= 0.0015
Surviving
CCL18 high
CCL18 low
Survival time (Months)
G
TCGA GBM RNA-seq
Iba-1
CCL18
TCGA GBM RNA-seq
CD68
CCL18
TCGA GBM RNA-seq
CD11b
CCL18
H
Rho = 0.503
p = 3.28e−10
CCL18 Expression Level (log2 TPM)
Infiltration Level of Macrophage_XCELL
Rho = 0.547
p = 3.75e−12
CCL18 Expression Level (log2 TPM)
Infiltration Level of Monocyte_XCELL

Figure S4

Bioinformatic analysis of CCL18 in TCGA LGG(low grade glioma)/HGG(high grade glioma) data. Related to Figure 2.

(**A**) mRNA expression of CCL18 in different types of glioma using TCGA GBM-LGG datasets.

(**B**) CCL18 mRNA expression level in different WHO grade of glioma using TCGA GBM-LGG datasets.

(**C**, **D** and **E**) Kaplan Meier analysis showing the survival time of glioma patients expressing CCL18 high (red) and low (green) in CGGA GBM (**C**), TCGA GBM HU-133A (**D**) and TCGA GBM RNA-seq datasets (**E**).

(**F**) Kaplan Meier analysis showing the survival time of glioma patients expressing CCL18 high (red) and low (green) (using median cut-off) in IDH-WT, IDH1-MUT, primary and recurrence GBM patients using TCGA GBM HU-133A database.

(**G**) Correlation of microglia/macrophage specific genes (Iba-1, left; CD68, middle; CD11b, right) with CCL18 in TCGA GBM RNA-seq database.

(**H**) The figures presented the Rho value and p value (based on Spearman's test) of macrophage (left) and monocyte (right) infiltration level correlated to CCL18 gene expression based on xCell algorithms using TIMER platform.

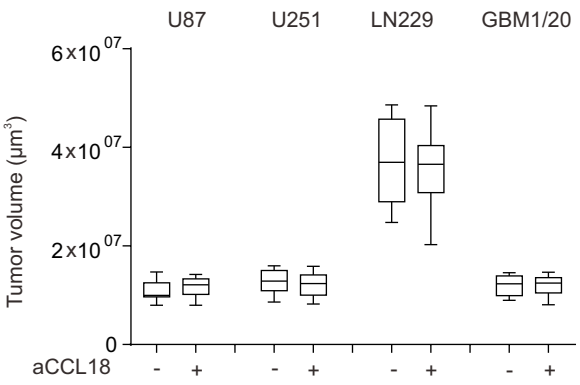

U87
U251
LN229
GBM1/20
6x10 07
4x10 07
2x10 07
0
Tumor volume (µm3)
aCCL18
-
+
-
+
-
+
-
+

Figure S5. The tumor volumes of co-injecting 5000 tumor cells with 2500 iMGL in OBS. Related to Figure 3.

Glioma cell lines U87-MGmCherry, U251-MGmCherry and LN229-MGmCherry as well as primary glioma cells GBM1/20 were inoculated alone (-) or in combination with 2500 iMGL (+) shown in (A) or with THP-1 macrophages shown in (B). Tumor volumes were quantified. (n = 7 per group, at least 2 tumors per n, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

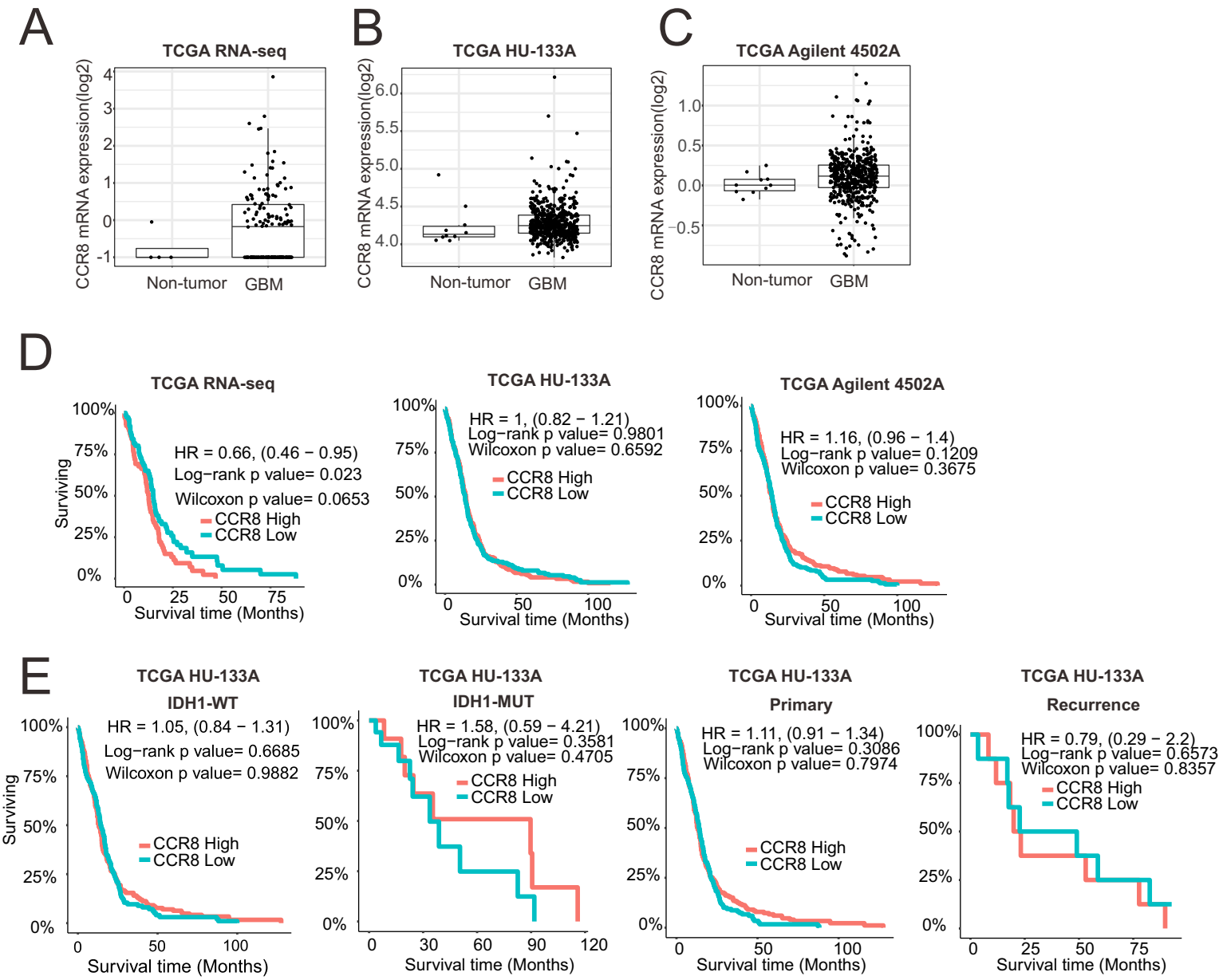

A
TCGA RNA-seq
CCR8 mRNA expression(log2)
Non-tumor
GBM
B
TCGA HU-133A
CCR8 mRNA expression(log2)
Non-tumor
GBM
C
TCGA Agilent 4502A
CCR8 mRNA expression(log2)
Non-tumor
GBM
D
TCGA RNA-seq
HR = 0.66, (0.46 − 0.95)
Log−rank p value= 0.023
Wilcoxon p value= 0.0653
CCR8 High
CCR8 Low
Surviving
Survival time (Months)
TCGA HU-133A
HR = 1, (0.82 − 1.21)
Log−rank p value= 0.9801
Wilcoxon p value= 0.6592
CCR8 High
CCR8 Low
Survival time (Months)
TCGA Agilent 4502A
HR = 1.16, (0.96 − 1.4)
Log−rank p value= 0.1209
Wilcoxon p value= 0.3675
CCR8 High
CCR8 Low
Survival time (Months)
E
TCGA HU-133A
IDH1-WT
HR = 1.05, (0.84 − 1.31)
Log−rank p value= 0.6685
Wilcoxon p value= 0.9882
CCR8 High
CCR8 Low
Surviving
Survival time (Months)
TCGA HU-133A
IDH1-MUT
HR = 1.58, (0.59 − 4.21)
Log−rank p value= 0.3581
Wilcoxon p value= 0.4705
CCR8 High
CCR8 Low
Survival time (Months)
TCGA HU-133A
Primary
HR = 1.11, (0.91 − 1.34)
Log−rank p value= 0.3086
Wilcoxon p value= 0.7974
CCR8 High
CCR8 Low
Survival time (Months)
TCGA HU-133A
Recurrence
HR = 0.79, (0.29 − 2.2)
Log−rank p value= 0.6573
Wilcoxon p value= 0.8357
CCR8 High
CCR8 Low
Survival time (Months)

Figure S6. Bioinformatic analysis of CCR8 in TCGA LGG/HGG data. Related to Figure 4.

(**A**, **B** and **C**) CCR8 expression in TCGA GBM RNA-seq (**A**), TCGA GBM HU-133A (**B**) and TCGA Agilent 4502A (**C**) database.

(**D**) Kaplan Meier analysis showing the survival time of glioma patients expressing CCR8 high (red) and low (green) (using median cut-off) in TCGA GBM RNA-seq (left), TCGA GBM HU-133A (middle) and TCGA Agilent 4502A (right).

(**E**) Kaplan Meier analysis showing the survival time of glioma patients expressing CCR8 high (red) and low (green) (using median cut-off) in IDH-WT, IDH1-MUT, primary and recurrence GBM patients using TCGA GBM HU-133A database.

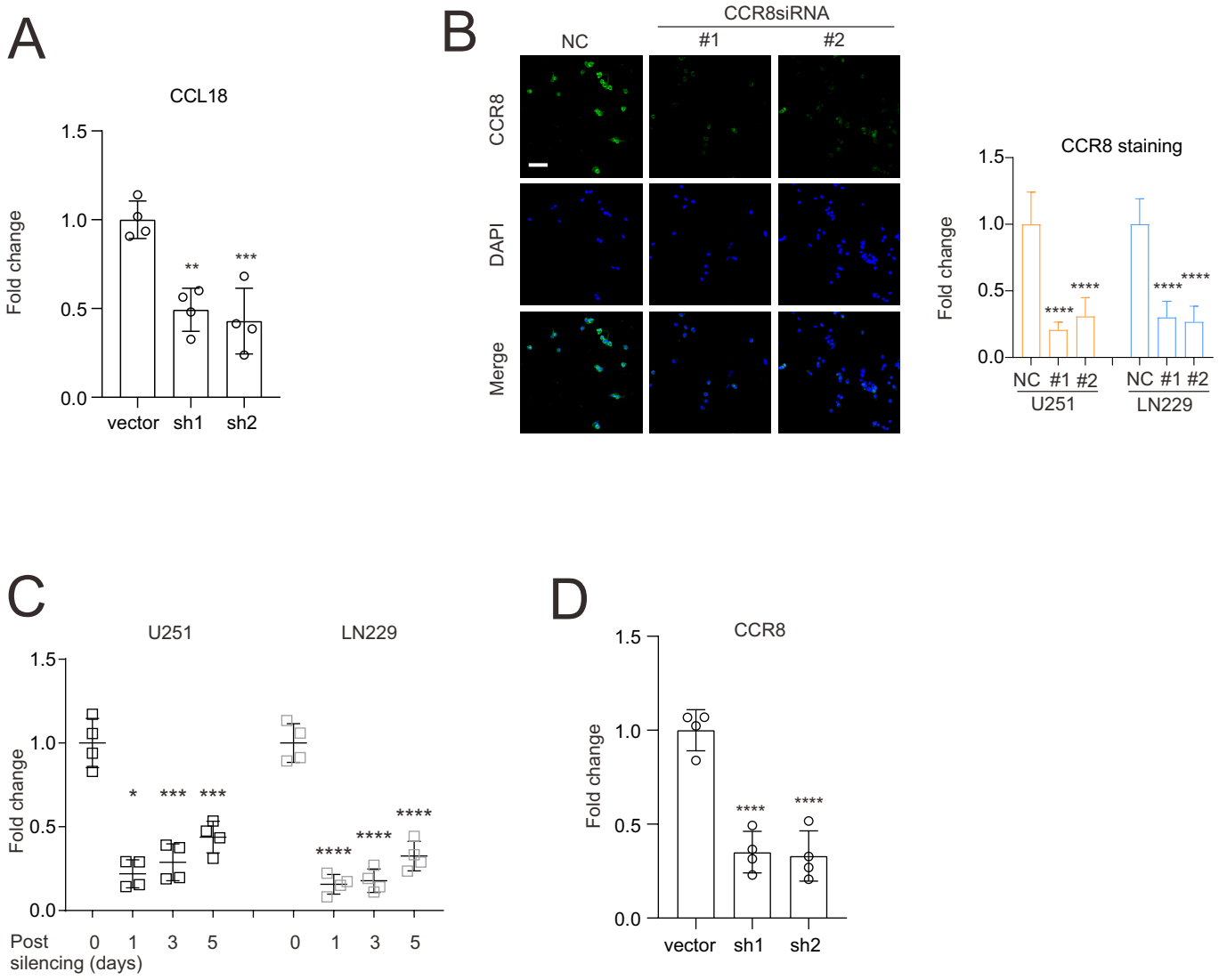

A
CCL18
Fold change
1.5
1.0
0.5
0.0
**
***
vector
sh1
sh2
B
CCR8siRNA
NC
#1
#2
CCR8
DAPI
Merge
CCR8 staining
Fold change
1.5
1.0
0.5
0.0
****
****
****
****
NC #1 #2
U251
NC #1 #2
LN229
C
U251
LN229
Fold change
1.5
1.0
0.5
0.0
*
***
***
****
****
****
Post silencing (days)
0 1 3 5
0 1 3 5
D
CCR8
Fold change
1.5
1.0
0.5
0.0
****
****
vector
sh1
sh2

Figure S7. Knock-down efficiency of CCL18 or CCR8 siRNA/shRNA. Related to Figure 3 and 4.

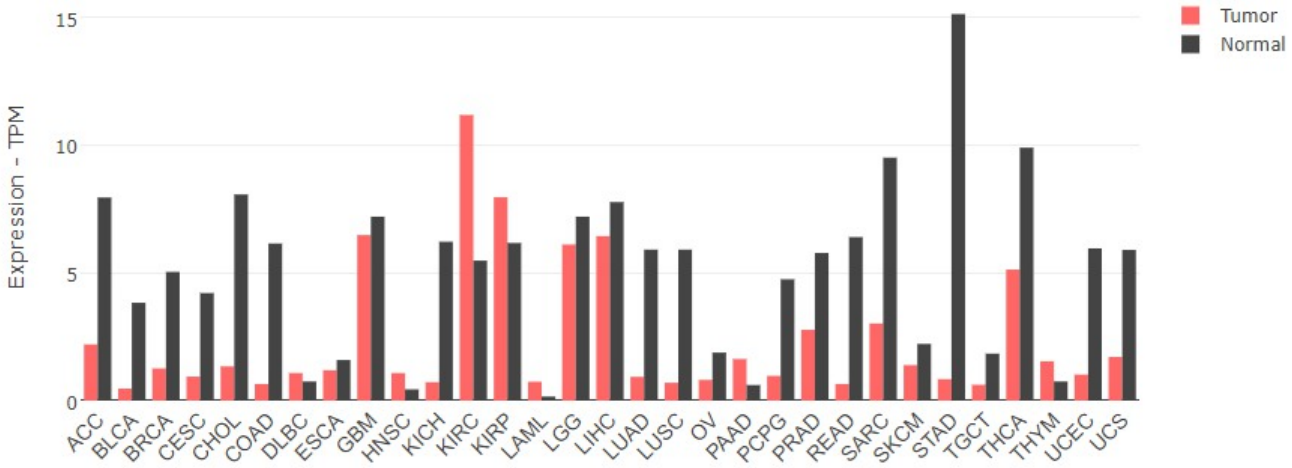
(**A**) CCL18 gene expression of negative control vector (vector) and CCL18 shRNA transfected (sh1 and sh2) THP-1 macrophages. The expression level is relative to the control vector. (n = 4 per group, ** = $p < 0.01$, *** = $p < 0.001$)

(**B**) Representative images show immunofluorescence staining of CCR8 (green) and DAPI (blue) on control (NC) and CCR8 siRNA transfected (#1 and #2) U251-MG glioma cells. Bar = 50 μm. The bar graph below denotes the relative intensity of CCR8 signal to the NC group. (n = 4 per group, **** = $p < 0.0001$)

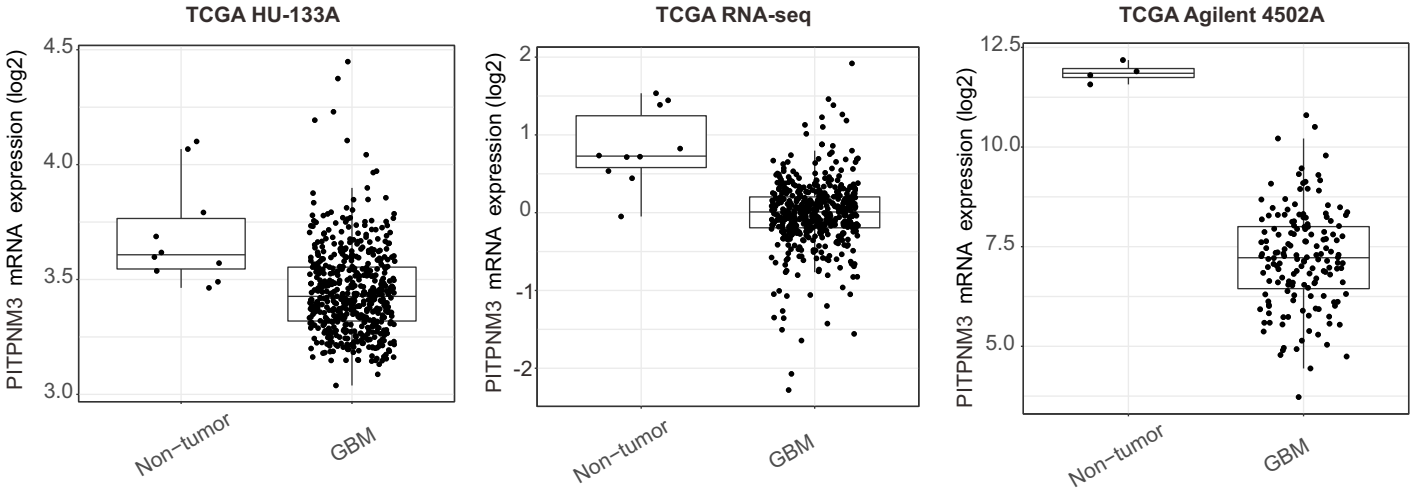
(**C**) CCR8 siRNA (#1) was used to silence CCR8 in U251 and LN229 glioma cells. CCR8 mRNA levels were determined 1, 3 and 5 days after treatment by qPCR and compared to levels prior to treatment. (n = 4 per group, * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$)

(**D**) Relative CCR8 gene expression of negative control vector (vector) and CCR8 shRNA transfected (sh1 and sh2) U251-MG glioma cells. (n = 4 per group, **** = $p < 0.0001$)

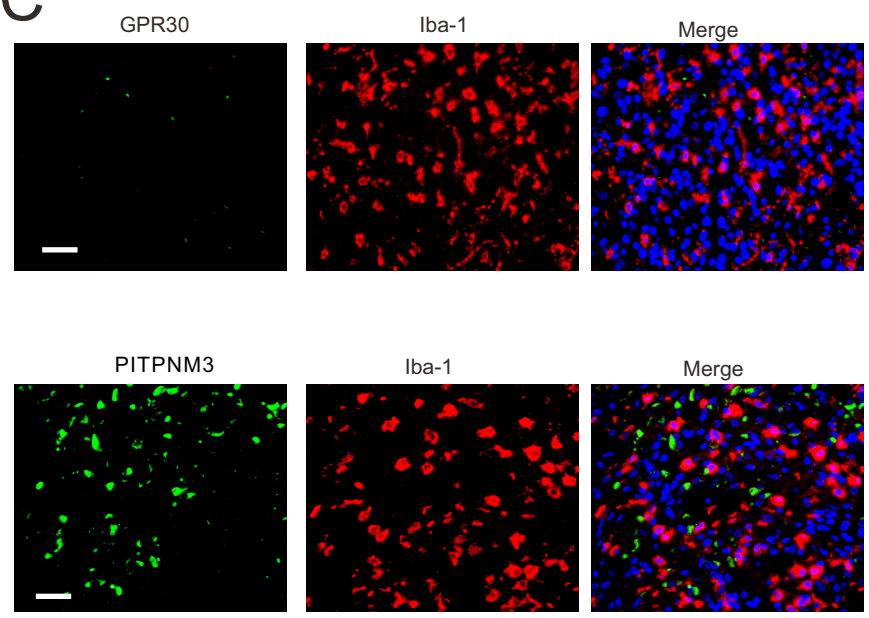
A


Tumor
Normal
Expression - TPM
15
10
5
0
ACC
BLCA
BRCA
CESC
CHOL
COAD
DLBC
ESCA
GBM
HNSC
KICH
KIRC
KIRP
LAML
LGG
LIHC
LUAD
LUSC
OV
PAAD
PCPG
PRAD
READ
SARC
SKCM
STAD
TGCT
THCA
THYM
UCEC
UCS

B


TCGA HU-133A
TCGA RNA-seq
TCGA Agilent 4502A
PITPNM3 mRNA expression (log2)
Non−tumor
GBM

C


GPR30
Iba-1
Merge
PITPNM3
Iba-1
Merge

D

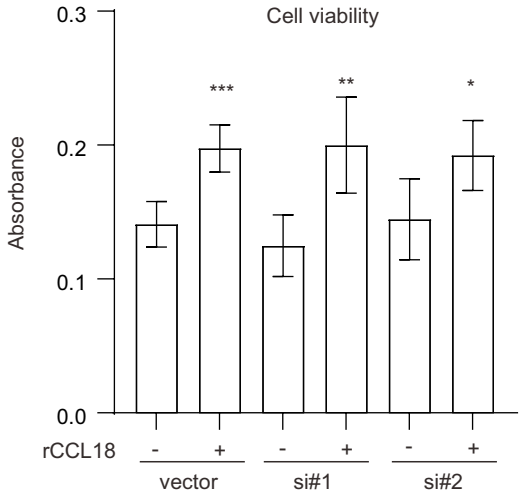

Cell viability
Absorbance
0.3
0.2
0.1
0.0
***
**
*
rCCL18
-
+
vector
si#1
si#2

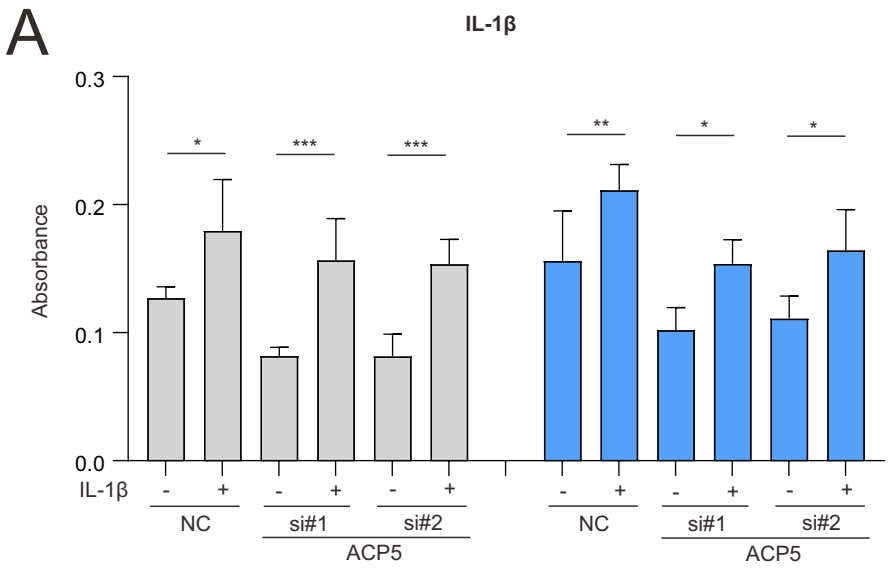
Figure S8. Bioinformatic analysis of GPR30 and PITPNM3 in TCGA HGG data. Related to Figure 4.

(**A**) Gene expression of GPR30 in TCGA GBM RNA-seq database using GEPIA platform

(**B**) Gene expression of PITPNM3 in TCGA GBM HU-133A (left), TCGA GBM RNA-seq (middle) and TCGA Agilent 4502A (right) database.

(**C**) The upper images show the labelling with GPR30 (left), Iba-1 (middle) and combined with DAPI, the lower images are corresponding labelling with PITPNM3 in GBM specimens. Bar = 100 μm.

(**D**) Negative control (NC) and PITPNM3 silenced glioma U251-MG cells (si#1 and si#2) were treated with recombinant CCL18 (100 ng/ml) for 48 h and the cell viability was determined by CCK-8 kit. (n = 5 per group, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)


A
IL-1β
Absorbance
0.3
0.2
0.1
0.0
*
***
***
**
*
*
IL-1β
- + - + - + - + - + - +
NC
si#1
si#2
ACP5
NC
si#1
si#2
ACP5

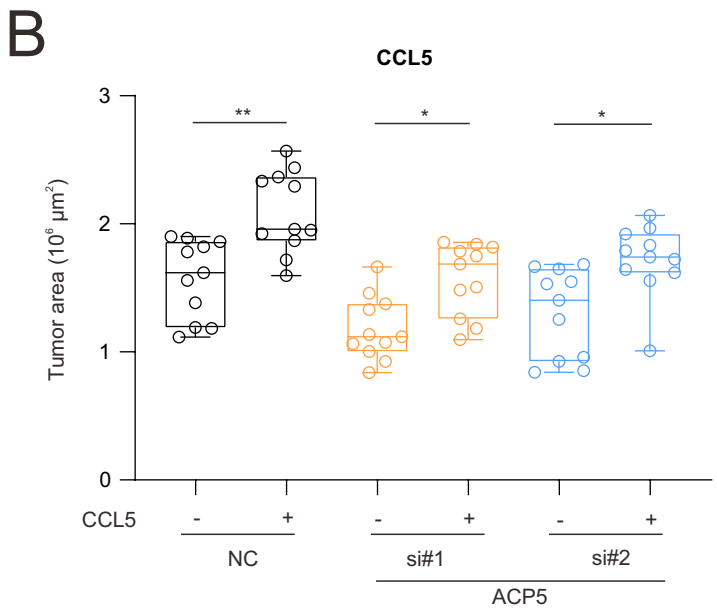

B
CCL5
Tumor area (10⁶ μm²)
3
2
1
0
**
*
*
CCL5
- + - + - +
NC
si#1
si#2
ACP5

Figure S9. ACP5 knock-down on glioma cells had no impact on IL-1beta and CCL5 induced tumor promoting effect. Related to Figure 5.

(A) Negative control (NC) and ACP5 silenced glioma cells U251-MG (si#1 and si#2) were treated with recombinant IL-1β (100 ng/ml) for 48 h and the cell viability (absorbance) was determined by CCK-8 kit. (n = 5 per group, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)

(B) Negative control (NC) and ACP5 silenced glioma cells U251-MG (si#1 and si#2) were inoculated into OBS and followed by recombinant CCL5 treatment (200 ng/ml) for 5 days. Tumor volume were quantified. (n = 5 per group, at least 2 tumors per group, * = $p < 0.05$, ** = $p < 0.01$)

Table S1. Oligonucleotide information. Related to STAR Methods.

Oligonucleotides		
Primer- CCL5: sense 5′- CCAGCAGTCGTCTTTGTCAC-3′, antisense 5′- CTCTGGGTTGGCACACACTT -3′	Biotex	N/A
Primer- OPN: sense 5′- GAAGTTTCGCAGACCTGACAT -3′, antisense 5′- GTATGCACCATTCAACTCCTCG-3′	Biotex	N/A
Primer- CCL18: sense 5′- AGCTCTGCTGCCTCGTCTAT-3′, antisense 5′- GGCCTCTCTTGGTTAGGAGG -3′	Biotex	N/A
Primer- MMP14: sense 5′- GGCTACAGCAATATGGCTACC-3′, antisense 5′- GATGGCCGCTGAGAGTGAC-3	Biotex	N/A
Primer- VEGF: sense 5′- AGGGCAGAATCATCACGAAGT -3′, antisense 5′- AGGGTCTCGATTGGATGGCA-3′	Biotex	N/A
Primer- TBP: sense 5′- AGCGCAAGGGTTTCTGGTTT-3′, antisense 5′- CTGAATAGGCTGTGGGGTCA -3′	Biotex	N/A
Primer- ARG1: sense 5′- TGGACAGACTAGGAATTGGCA-3′, antisense 5′- CCAGTCCGTCAACATCAAAACT-3′	Biotex	N/A
Primer- IL10: sense 5′- GACTTTAAGGGTTACCTGGGTTG-3′, antisense 5′- TCACATGCGCCTTGATGTCTG-3'	Biotex	N/A
Primer- TGFbeta: sense 5′- CAATTCCTGGCGATACCTCAG-3′, antisense 5′- GCACAACTCCGGTGACATCAA-3′	Biotex	N/A
Primer- ACP5, sense 5- GACTGTGCAGATCCTGGGTG-3′, antisense 5′- GGTCAGAGAATACGTCCTCAAAG-3′	Biotex	N/A
Primer- CCR8: sense 5′- CTCACTGCTGTGTGAACCCT-3′, antisense 5′- CACAGCTCTCCCTAGGCATT -3′	Biotex	N/A
siRNA targeting CCR8: si#1, sense 5‘- AUUUGUCUGAAUAAGUUCCGC -3‘, antisense 5‘- GGAACUUAUUCAGACAAAUGG -3‘; si#2, sense 5‘- AUGUUCAUUUUGAAGUUGGUG -3‘; antisense 5‘- CCAACUUCAAAAUGAACAUUU -3‘	AuGCT	N/A
siRNA targeting ACP5: si#1, sense 5‘- AUCAGUAACAGAAAGAUGCUU-3‘, antisense 5‘- GCAUCUUUCUGUUACUGAUGU-3‘; si#2, sense 5‘- AGAAAGAUGCUUGAUUUAGGA-3‘, antisense 5‘- CUAAAUCAAGCAUCUUUCUGU-3‘	AuGCT	N/A
siRNA targeting PITPNM3: si#1, sense 5‘- AAUACUGAAAGUUACACACUU-3‘, antisense 5‘- GUGUGUAACUUUCAGUAUUAU-3‘si#2, sense 5‘- AUGUCAAUGUUGCUUUUGCUG-3‘, antisense 5‘- GCAAAAGCAACAUUGACAUCU-3‘	AuGCT	N/A

Negative control siRNA	Thermofisher scientific	Cat# 4404021