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Supplemental Information

Pharmacologic or Genetic Targeting of Glutamine Synthetase Skews Macrophages toward an M1-like Phenotype and Inhibits Tumor Metastasis

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SUPPLEMENTAL INFORMATION
Figure S1 related to Fig. 1

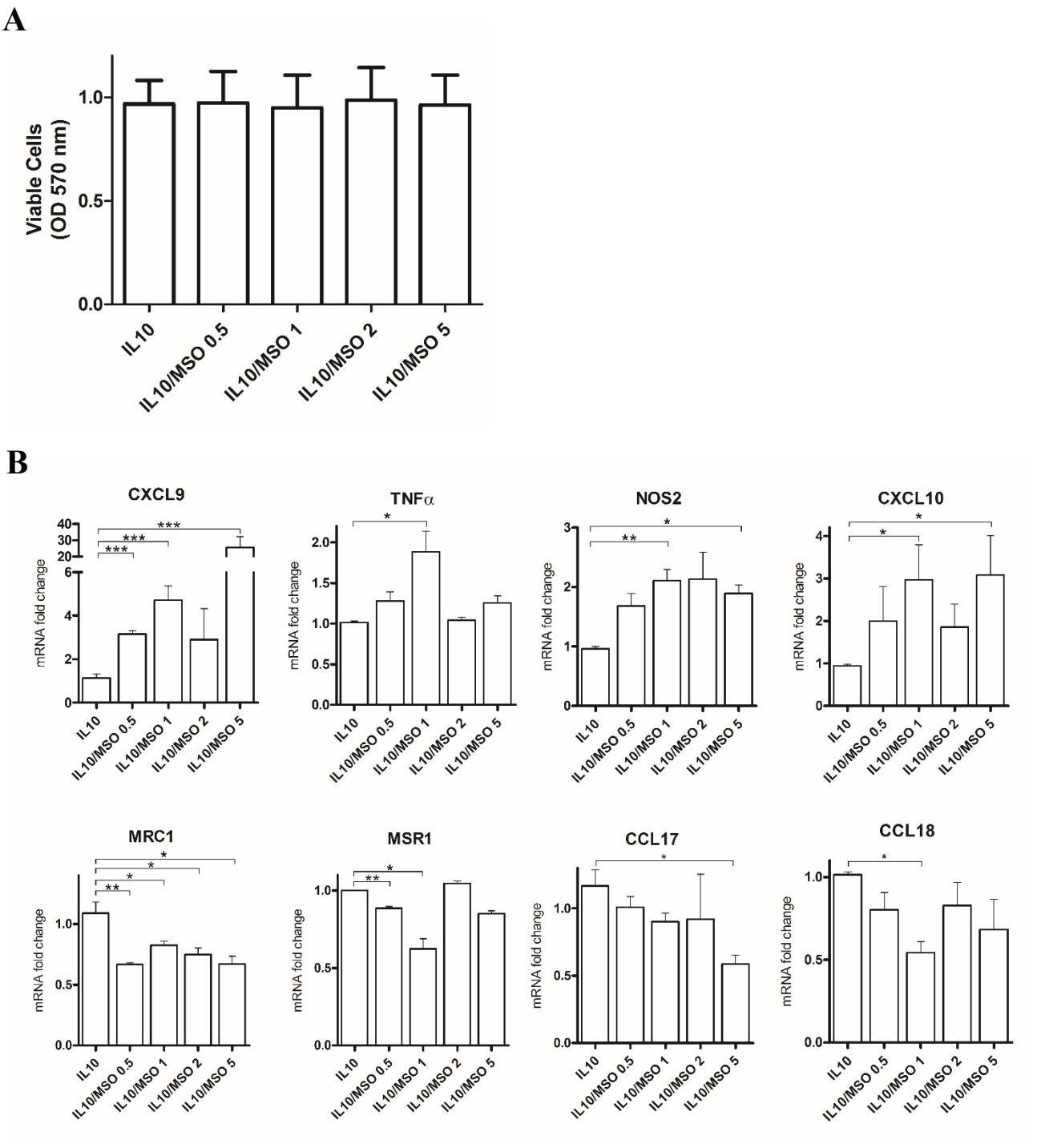

Fig S1 related to Fig. 1
MSO dose response in IL10-treated macrophages
(A) Viability of IL10 macrophages in the presence of MSO at different concentrations.
(B) RT-PCR quantification of M1 or M2 markers in macrophages.
Top, fold increase of M1 markers mRNA in IL10 and MS0-treated IL10 at 4 increasing concentrations of MSO following 24 h of activation (n=3).
Bottom, fold reduction of MSR1, MRC1, CCL17 and CCL18 mRNA in IL10 and MS0-treated IL10 macrophages at 4 increasing concentrations of MSO following 24 h of activation (n=3).

Data are means ± SEM. *p<0.05, **p<0.001, ***p < 0.0001 versus WT.

Figure S2 related to Figure 2

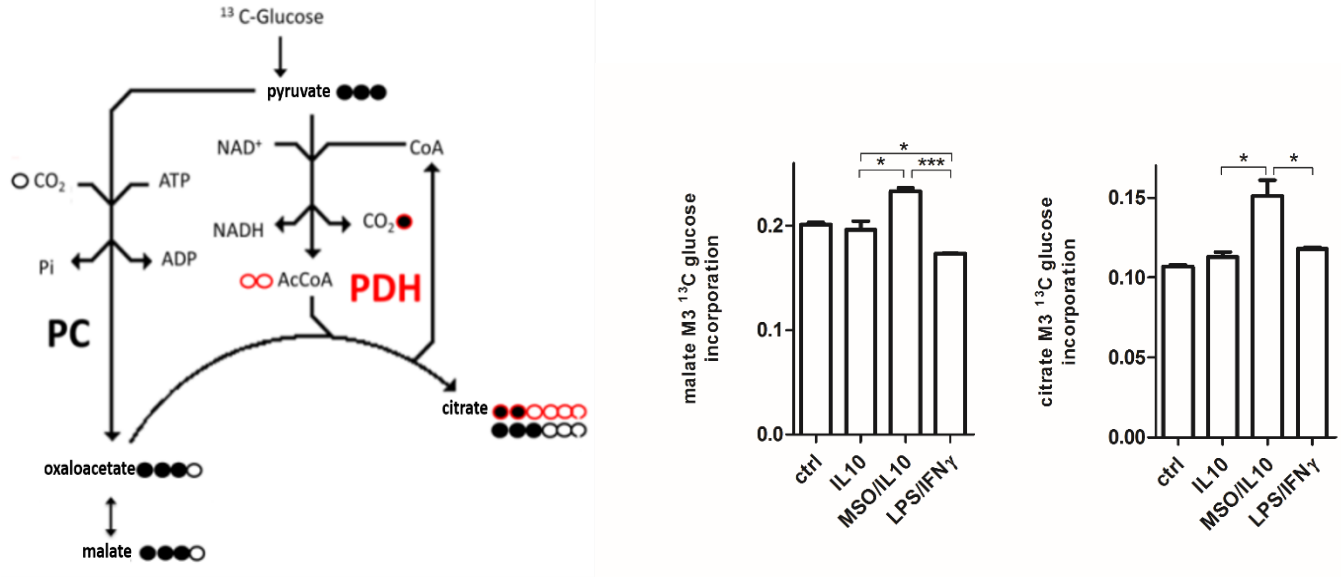

Fig S2 related to Figure 2
GS inhibition reprograms pyruvate metabolism in IL10-treated macrophages

Evaluation of the (M+3) 13C enrichment from [U-13C]-glucose in malate and citrate (n=3).

Data are means ± SEM. *p < 0.05, **p < 0.001, ***p < 0.0001.

Fig S3 related to Figure 6

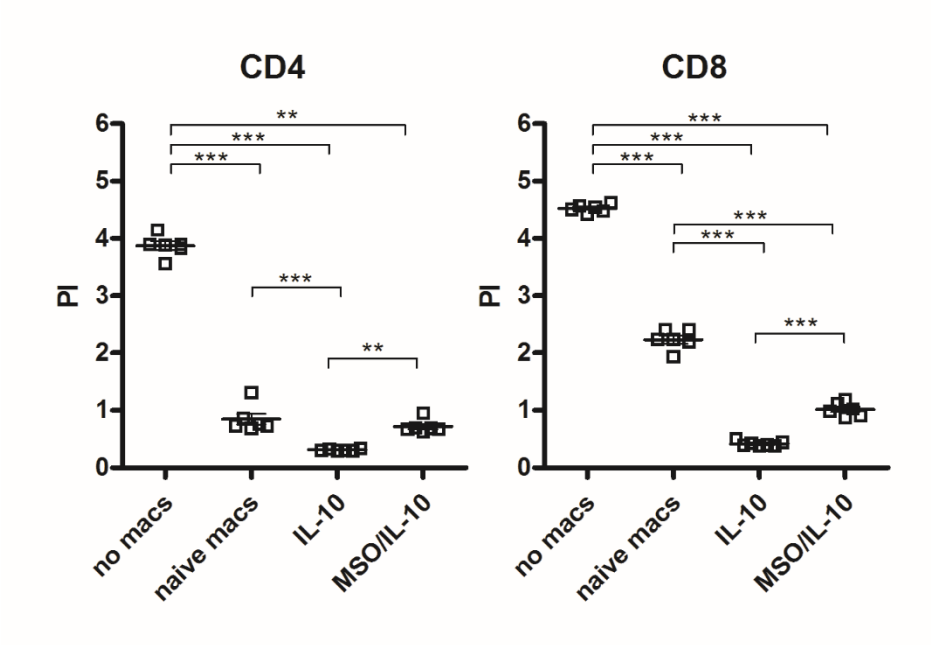

Fig S3 related to Figure 6
Proliferation index of $CD4^+$ and $CD8^+$ cells
Evaluation of the proliferation index (PI) of $CD4^+$ and $CD8^+$ cells co-cultured with IL10 and MSO/IL10 macrophages

Data are means ± SEM. **p < 0.001, ***p < 0.0001 versus no macs

Figure S4 related to Figure 7

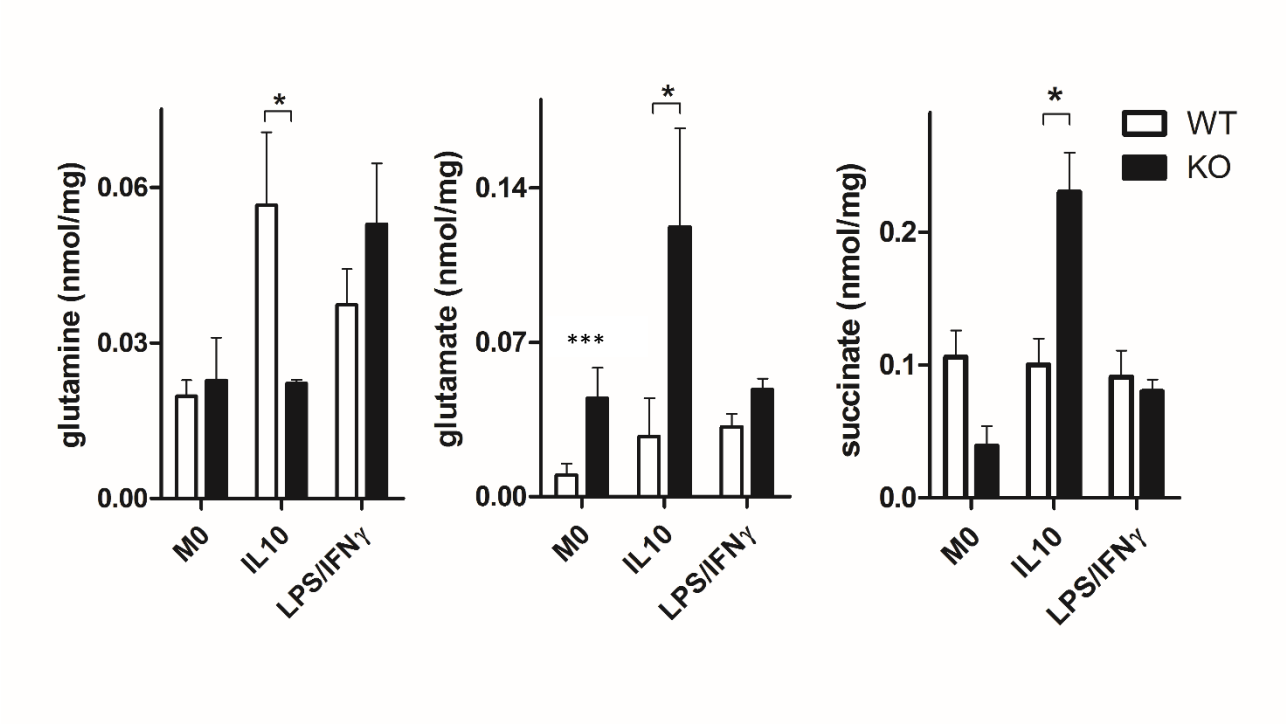

Figure S4, related to Figure 7
Metabolite quantification in WT and cKO BMDMs
Intracellular glutamine, glutamate and succinate levels in BMDMs isolated from WT and cKO mice after 5-day in vivo treatment with tamoxifen (n=3), and treated with IL10 or LPS/IFNγ for 24 h (n=6).

Data are means ± SEM. *p<0.05, ***p < 0.0001 versus WT.

Figure S5 related to Figure7

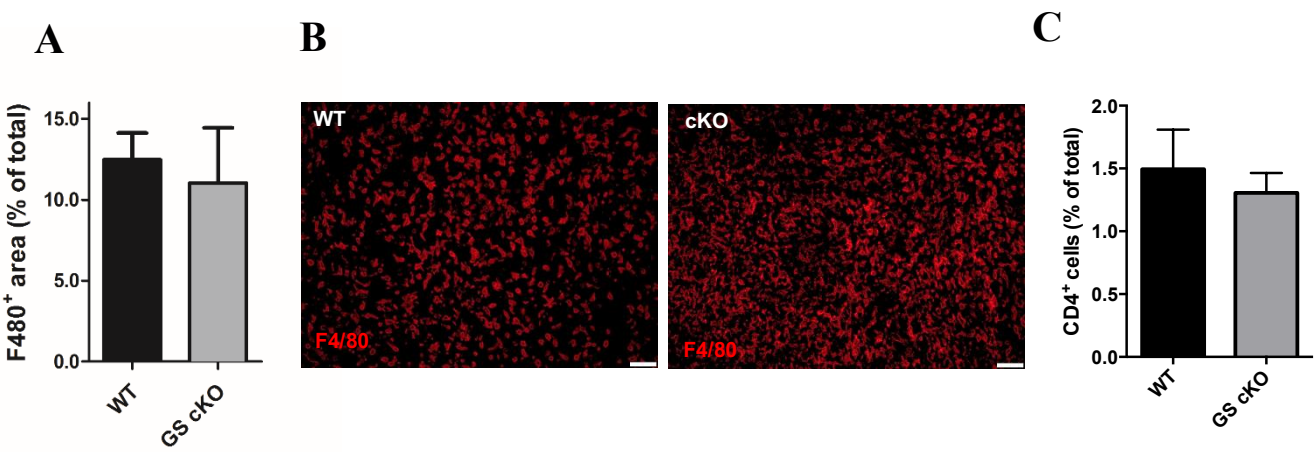

Figure S5, related to Figure 7

F4/80$^+$ tumor areas and CD4+ cells in WT and cKO mice

(A) Quantification and (B) representative images of F4/80$^+$ tumor areas in wild type (HET) and macrophage-specific knock out (cKO) mice (n=8). N represents the number of animals. 6 images per tumor were analyzed. (C) CD4+ cell quantification in wild type (HET) and macrophage-specific knock out (cKO) mice (n=4).

Data are means ± SEM.

Figure S6 related to Fig. 7

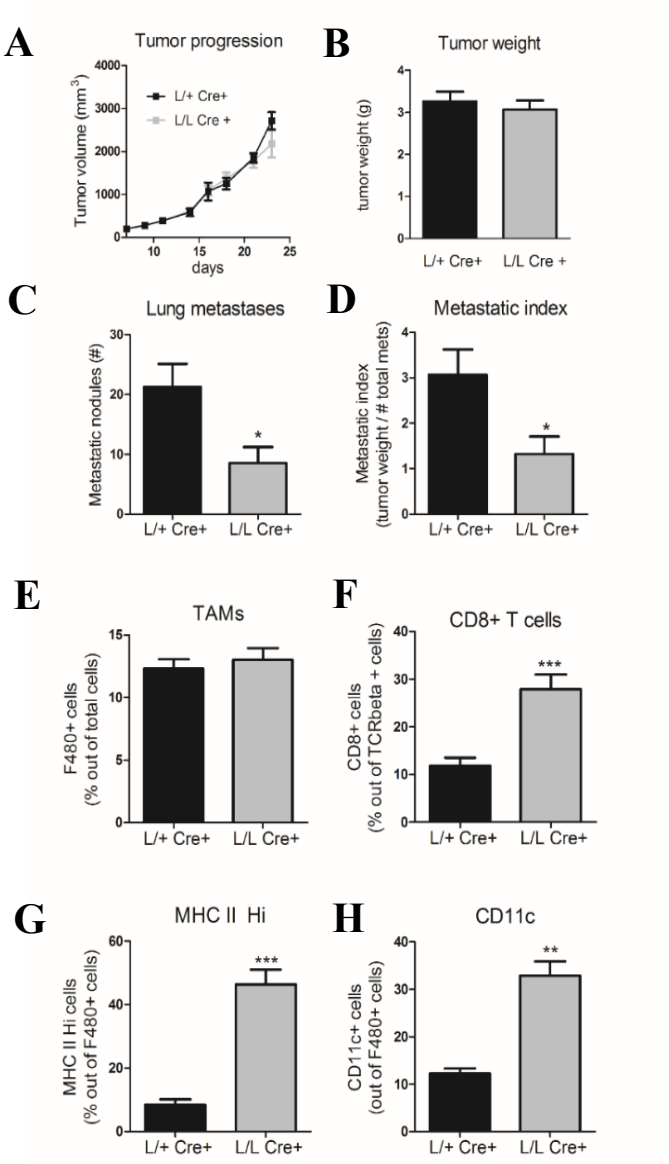

Figure S6. Genetic deletion of GS (L/L Cre+) in TAMs induces the M1-like phenotype, CTL accumulation, and inhibits metastasis.

(A,B) Subcutaneous LLC tumor growth over the time (A) and end-stage tumor weight (B) in L/+ Cre+ (HET) and macrophage-specific knock out (L/L Cre+) mice (n=12). (C,D) Number of lung metastases (C) and lung metastatic index (which is the number of lung metastatic nodules divided for the corresponding tumor weight) (D) in L/+ Cre+ (HET) and macrophage-specific knock out (L/L Cre+) mice (n=12). (E-H) FACS quantification of total F4/80^{+} TAMs (E), CD8^{+} cytotoxic T cells (F), M1-like MHC-II^{high} TAMs (G) and CD11c^{+} TAMs (H) in L/+ Cre+ (HET) and macrophage-specific knock out (L/LCre+) mice (n=6).

Figure S7 related to Fig. 7

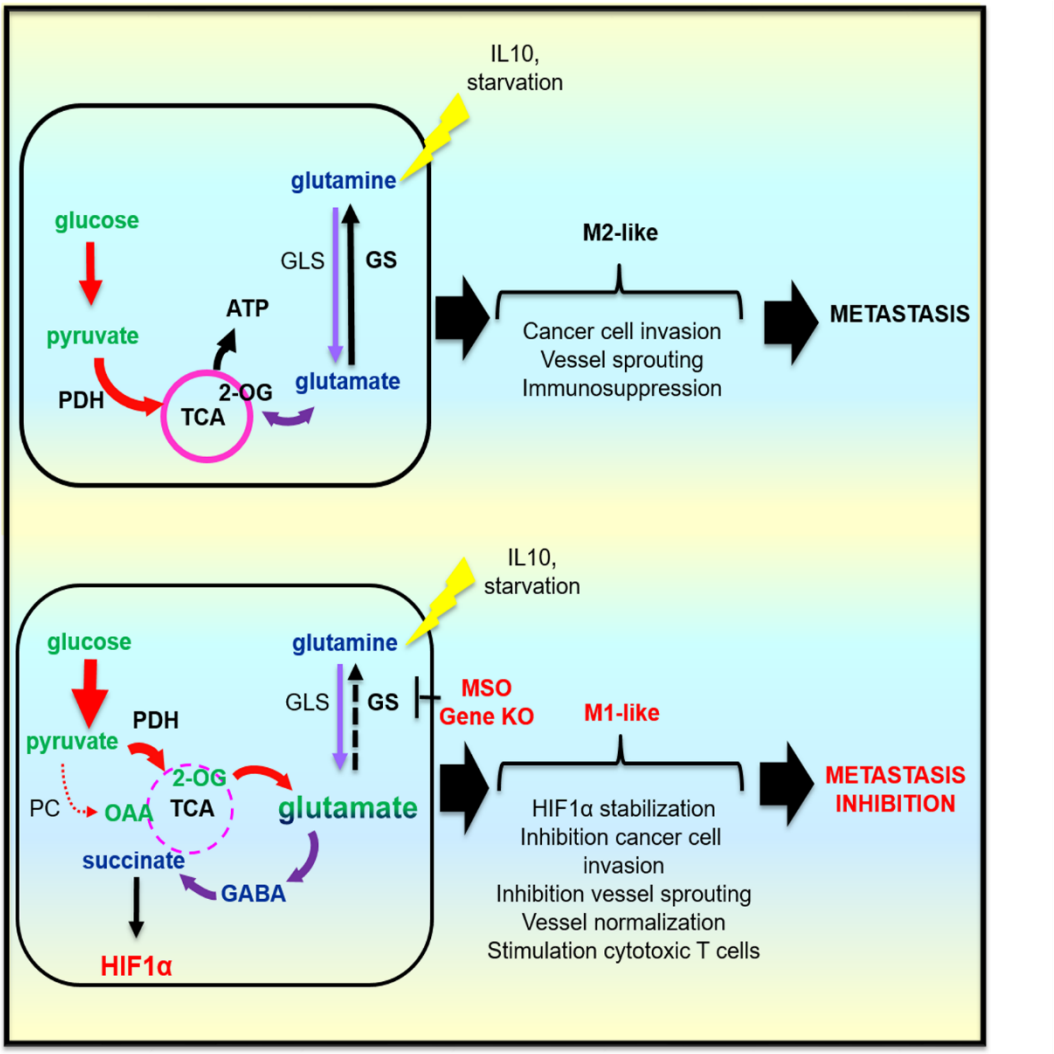

Figure S7. Functional role of metabolic reprogramming following GS inhibition in IL10 macrophages.

(Upper panel) IL10 treatment increases GS expression and activity. Channeling of nutrients toward the TCA cycle for ATP production is favored. Since GS is sensitive to nutrient starvation, decreased nutrient availability by cancer cell might enhance GS expression.

(Lower panel) In vitro GS inhibition lowers intracellular glutamine while increasing glutamate levels. Reduction of glutamine levels increases glucose flux toward glutamate, which strongly accumulates in the cell. Glutamine uptake is not decreased but channeled toward succinate synthesis through GABA. Functionally, GS inhibition or gene deletion reduces M2 and increases M1 markers through stabilization of HIF1α, leading to lower T cell suppression, reduction of angiogenesis with features of tumor vessel normalization, and inhibition of cancer cell invasiveness. Altogether, this translates in a strong inhibition of metastasis formation in mice.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell preparation

Human blood samples were obtained from healthy individuals under an Institutional Review Board approved protocol and the informed consent was obtained from all subjects involved.
Human monocytes were obtained from healthy blood donor buffy coats by two-step gradient centrifugation using ficoll followed by an additional step using the magnetically isolation with CD14 MicroBeads (Miltenyi Biotec Inc.) as previously described (Palmieri et al., 2015). Finally, monocytes (98% CD14+, 13% CD16+) were seeded in RPMI 10% FBS, 2 mM L-Glutamine with Pen/Strep at a concentration of 4×105 cells/ml for 6 days in the presence of either 1000 U/ml rHuGM-CSF (M1) or 100 ng/ml rHuM-CSF (M2). Then 50% by volume of fresh M1 or M2 medium was added. Macrophage polarization was obtained by culturing cells for an additional 24 h in RPMI 1640 stimulated with 100 ng/mL LPS plus 20 ng/mL IFNγ (for M1 polarization) or either 20 ng/mL IL-4, 20 ng/mL IL-13, 10 ng/mL IL-10 or a combination of both IL-4 and IL-10 (for M2 polarization).
Changes in cell morphology were assessed by phase contrast microscopy (Axiovert 135, Zeiss). Phenotypical and functional characterization of MDM was performed after either 6h, 24h, 48h after activation with cytokines.
For MSO treatment, a stock 200mM MSO solution was prepared in ultrapure water and stored in aliquots at -80°C. ACF was dissolved in water to a stock concentration of 10 mM and stored at -20°C. Dimethylglutamate (DMG) was dissolved in water to a stock concentration of 200 mM and stored at -20°C. Where indicated the cells were stimulated with IL10 with and/or without previous 2h incubation with 5mM DMG.
Cell viability was determined after 72 h of incubation, using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega) as described in (Menga et al., 2017; Palmieri et al., 2017).

Murine bone marrow-derived macrophages (BMDMs) were derived from bone marrow precursors as described before (Meerpohl et al., 1976). Briefly, bone marrow cells (1.6 x106 cells/ml) were cultured in DMEM supplemented with 20% FBS and 30% L929 conditioned medium as a source of M-CSF. After 3 days of culture, an additional 3 ml of differentiation medium was added. At day 7, macrophages were treated with 10 ng/mL IL10 or with 100 ng/mL LPS plus 20 ng/mL IFNγ for 24 h and finally harvested with ice cold Ca^{2+} and Mg^{2+}-free PBS.
Spleens were flushed with 200 U ml−1 collagenase III (Worthington) and left for 30 min at 37 °C. Afterwards, spleens were filtered and red blood cells were removed using erythrocyte lysis buffer.

Flow cytometry

Expression of CD14, CD163, CD80, CD3, CD4, CD8, CD25 on either macrophages or T cell surfaces was studied by flow cytometry. Purified cells following specific treatments were washed with FACS buffer and 3×10^5 cells labeled with fluorescence-labeled anti-human antibody (8 µl) for 30 min at 4°C in dark as per manufacturer's recommendation. After labeling, cells were washed with PBS, resuspended in 300 µL of the same saline solution until acquisition which was performed using a 8 colors flow cytometer (Navios, Beckman Coulter). Data analysis was performed with Kaluza software (Beckman Coulter). Suitable negative iso-type controls were used to rule out the background fluorescence for the used fluorochromes (FITC, PE, PE-Cy5, PE-Cy7 and APC). To ensure that analyses were made only of viable cells and not debris, all events labelled with 7AAD were excluded. Percentage of each positive population and mean fluorescence intensity (MFI) were determined using quadrant statistics.

For surface marker characterization of effector cell subsets, CD4+ and CD8+ T cells were activated with anti-CD3/CD28 Dynabeads (Life Technologies) for 5 days in the presence or absence of macrophages differentiated with different stimuli and stained with the following antibodies: anti-CD25 BV650 (Biolegend), anti-CD69 APC, anti-CD4 PE, and CD8 PE (Becton Dickinson). Cells were analyzed on an LSRII or a Fortessa. Live cells were analyzed by gating on Sytox green negative cells (Life Technologies) and single cells were determined using forward scatter height by area gating strategy. To compensate for possible differences in cell sizes, geometric mean or median fluorescence intensities (MFI) were normalized to forward scatters.

^{13}C tracing experiments

For ^{13}C-carbon incorporation from glucose and glutamine in metabolites, cells were incubated for 48 hours (day 6 of differentiation) with labeled substrates (confirmation of steady state). The exposure to cytokines for macrophage activation was performed in the last 24 hours. For mass spectrometry analysis cells were scraped in 80% methanol and phase separation was achieved by centrifugation at 4°C and the methanol-water phase containing polar metabolites was separated and dried using a vacuum concentrator. For media extraction, 50µL of medium was extracted with methanol

and treated as above. The dried metabolite samples were stored at −80°C. Isotopomer distributions and metabolite levels were measured with a 7890A GC system (Agilent Technologies) combined with a 5975C Inert MS system (Agilent Technologies, Inc.) (Schoors et al., 2015) or with a UPLC system (Acquity, Waters) interfaced with a Quattro Premier mass spectrometer (Waters)
(Palmieri et al., 2015; Palmieri et al., 2014).

T cell purification and expansion

CD4+ naïve autologous T cells were isolated by negative isolation from the PBMC fraction using the CD4+ T Cell Isolation Kit II, human (Miltenyi) according to the manufacturer's instructions. This kit removes cells expressing CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a (Glycophorin A). Autologous CD8+ were isolated by negative selection in a similar manner using CD8+ T Cell Isolation Kit, human (Miltenyi). Fresh cells after isolation were then washed with PBS and labeled with 10 µM Cell Trace (CTV: CellTrace™ Violet Cell Proliferation Kit - Life Technologies) as previously described (Quah and Parish, 2012) and resuspended in RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES, 2 mM glutamine and non-essential amino acids. Labeled cells were used for setup of suppression assays. To address the immunopromoting function of macrophages, cultured primary macrophages either naïve/resting or polarized into M2(IL-10) and M2(IL-10) plus MSO seeded in 96- well plates ($2x10^5$ /ml) were cocultured either with CD4+ or CD8+ T cells in stimulator– responder ratios of 1:2. Macrophage stimulants were washed away extensively (two washings each with addition of 200µl of macrophage media) and complete removal of the medium from the cells was performed before adding T cell suspensions in a final volume of 200µl. Cells were cocultured for 5 days in the presence of anti-CD3/CD28 Dynabeads (Life Technologies)(Volpe et al., 2008) and rhIL-2 25U/ml for activation. As controls, $1x10^5$ $CD4^+$ or $CD8^+$ T cells/well were also plated without macrophages and left alone throughout the entire procedure. T cells were collected after 5 d in culture, washed in MACS buffer, labeled for flow cytometry, and suppression was analyzed for CVT-based proliferation and activation was assessed by staining for CD69. Proliferation index was calculated according to (Burt et al., 2010)

T cell migration assay

Migration of $CD8^+$ cells was assessed by using Transwell Permeable supports with 5-µm Polycarbonate Membrane (Costar). To determine cell migration in response to soluble factors secreted by macrophages, the last were precultured in the lower chamber for 7 days in RPMI 10% FBS and then activated for 8h with LPS/IFNγ or IL10 with or without MSO. Then the medium has been changed and after 18 hours autologous $CD8^+$ cells at 48h hours culture after isolation in the presence of anti CD3 (1µg/mL) and anti CD28 (1µg/mL) were placed in the upper chamber ($2x10^5$ cells in 70 µl of medium with 10% FBS). Macrophages and $CD8^+$ cells were incubated for 3h at 37°C and migrated cells were collected and counted under the microscope (Finisguerra et al., 2015).

Cancer cell invasion assay

The upper side of a Transwell chamber with a 8.0 µm-porous polycarbonate membrane filter (Costar®) was coated with 5 µg Matrigel (Matrix Growth Factor Reduced from Corning®). Different polarization states and MSO pretreated macrophages were detached in cold PBS and then added ($5 X 10^4$/well) together with calcein-labeled A549 cells ($1 x 10^5$/well) to the upper chamber in RPMI medium supplemented with 2% FBS. RPMI medium supplemented with 10% FBS was added to the lower well to create a gradient of serum as chemoattractant stimulus. The migration of A549 cells was assayed after 24 h of incubation. At this time point, the membranes were removed and stained with DAPI, and the number of cells that had invaded the lower chamber was counted in three randomly selected fields under a fluorescence microscopy.

Tumor model

$1x10^6$ Lewis lung carcinoma (LLC) adherent growing murine cells were injected subcutaneously at the right side of the mouse in a volume of 200 µl of PBS. Tumor volumes were measured three times a week with a caliper and calculated using the formula: $V=\pi x(d2 x D)/ 6$, where d is the minor tumor axis and D is the major tumor axis. At the end stage, tumor weight was registered and samples were collected for histological examination. Lung metastasis nodules were contrasted after intratracheal injection of 15% India ink solution. Superficial metastatic nodules were assessed under a stereomicroscope.

FACS analysis and flow sorting of tissue- and tumor-associated macrophages

LLC tumor-bearing mice were sacrificed by cervical dislocation and tumors were harvested. Tumors or other organs were minced in RPMI medium containing 0.1% collagenase type I and 0,2% dispase type I and incubated in the same solution for 30 minutes at 37°C. Samples from spleen were mechanically dissociated. The digested or dissociated tissue was filtered using a 70 µm pore sized mesh and cells were centrifuged 5 min at 1000 rpm. Red blood cell lysis was performed by using Hybri-Max^{TM} (Sigma-Aldrich). For flow sorting, the myeloid cell population in the tumor single cell suspension, and when appropriate in flushed splenocytes, was enriched by coating with CD11b-conjugated magnetic beads (MACS, Miltenyi Biotec) and separation through magnetic columns (MACS, Miltenyi Biotec). Cells were resuspended in FACS buffer (PBS containing 2% FBS and 2 mM EDTA) and incubated for 15 minutes with Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb (BD-pharmingen) and stained with the following antibodies for 20 minutes at 4 °C: anti-F4/80, anti-CD3, anti-CD4, anti-CD8, anti-MHCII, anti-CD206, anti-CD11c (all eBioscience), and anti-CD31 (BD-pharmingen). Cells were subsequently washed and resuspended in cold FACS buffer before FACS analysis or flow sorting by a FACS Verse or FACS Aria (BD Biosciences), respectively.

Histology and immunostainings

For serial sections cut at 7 µm thickness, tissue samples were fixed in 2% PFA overnight at 4°C, dehydrated and embedded in paraffin. Paraffin slides were first rehydrated to further proceed with antigen retrieval in citrate solution (DAKO). The sections were blocked with the appropriate serum (DAKO) and incubated overnight with the following antibodies: rat anti-CD31 (BD Pharmingen) 1:200, rabbit anti-FITC (Serotec) 1:200, rat anti-F4/80 (Serotec) 1:100, rabbit anti-hypoxyprobe (HPI) 1:100, goat anti-CD105 (R&D Systems) and rat anti-TER119 (R&D Systems). Appropriate secondary antibodies were used: Alexa 488, 647 or 568 conjugated secondary antibodies (Molecular Probes) 1:200, biotin-labeled antibodies (Jackson Immunoresearch) 1:300 and, when necessary, TCA fluoricine or TSA Plus Cyanine 3 System amplification (Perkin Elmer, Life Sciences) were performed according to the manufacturer's instructions. Whenever sections were stained in fluorescence, ProLong Gold mounting medium with or without DAPI (Invitrogen) was used. Microscopic analysis was done with an Olympus BX41 microscope and CellSense imaging software.

Hypoxia assessment

Tumor hypoxia was detected 1h after i.p. injection of 60 mg/kg pimonidazole hydrochloride into tumor-bearing mice. Mice were sacrificed and tumors harvested. To detect the formation of pimonidazole adducts, tumor paraffin sections were immunostained with Hypoxyprobe-1-Mab1 (Hypoxyprobe kit, Chemicon) following the manufacturer's instructions.

Additional References

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