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in Microglia/Brain Macrophages via Toll-like Receptor 4 Signaling**

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Glioma stem cells but not bulk glioma cells upregulate IL-6 secretion in microglia/brain macrophages via Toll-like receptor 4 signaling

Omar Dildar a Dzaye<sup>1\*</sup> MS, Feng Hu<sup>2,1\*</sup> MD/PhD, Katja Derkow<sup>3</sup> PhD, Verena Haage<sup>1</sup> MSc, Philipp Euskirchen<sup>3,4</sup>MD, Christoph Harms<sup>4</sup> MD, Seija Lehnardt<sup>5</sup> MD, Michael Synowitz<sup>6</sup> MD, Susanne A. Wolf<sup>1\*</sup> PhD and Helmut Kettenmann<sup>1\*</sup> PhD

- 1 Cellular Neuroscience, Max Delbrück Centre for Molecular Medicine (MDC), 13125 Berlin, Germany
- 2 Department of Neurosurgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 430030 Wuhan, People's Republic of China
- 3 Department of Neurology, Charité - Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany
- 4 Center for Stroke Research Berlin, Department of Experimental Neurology, Department of Neurology, Charité - Universitätsmedizin, Berlin, Germany
- 5 Department of Neurology and Center for Anatomy, Institute of Cell Biology and Neurobiology, Charité - Universitätsmedizin, 10117 Berlin, Germany
- 6 Department of Neurosurgery, University of Schleswig-Holstein, Campus Kiel, 24105 Kiel, Germany

\*♦ contributed equally

Corresponding authors: H. Kettenmann and S. A. Wolf, Cellular Neurosciences, Max Delbrück Center for Molecular Medicine, Robert Rössle Str. 10, 13125 Berlin, Germany. Phone: +49 30 94063325; Email: kettenmann@mdc-berlin.de; susanne.wolf@mdc-berlin.de

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H.K., S.W. and M.S. conceived the study. O.D.a D. and F.H. performed the majority of the experiments. S.L. and K.D. performed FlowCytomix experiments. V.H. contributed to qPCR experiments. C.H. and P.E. contributed to tumor inoculation. O.D.a D., F.H., S.W. and H.K. wrote the paper.

### **Keywords**

glioma, glioma associated microglia/brain macrophages, glioma stem cells, IL-6, Toll-like receptor 4

### **Abbreviations**

GBM glioblastoma

GAMs glioma associated microglia/brain macrophages

GSCs glioma stem cells

GCM glioma conditioned medium

AC-GL261 adhesive GL261

NS-GL261 neurosphere GL261

TLR Toll-like receptor

## **Abstract**

Peripheral macrophages and resident microglia constitute the dominant glioma-infiltrating cells. The tumor induces an immunosuppressive and tumor supportive phenotype in these glioma associated microglia/brain macrophages (GAMs). A subpopulation of glioma cells has stem cell properties such as self-renewal, multipotency and act as glioma stem cells (GSCs). In the present study we explored the interaction between GSCs and GAMs. Using CD133 as a marker of stemness, we either enriched for or deprived the mouse glioma cell line GL261 of GSCs by FACS. Over the same period of time, 100 CD133<sup>+</sup> GSCs had the capacity to form a tumor of comparable size to the ones formed by 10000 CD133<sup>-</sup> GL261 cells. In IL-6<sup>-/-</sup> mice, only tumors formed by CD133<sup>+</sup> cells were smaller when compared to wild-type. After stimulation of primary cultured microglia with conditioned medium from CD133 enriched GL261 glioma cells, we observed an upregulation in microglial IL-6 secretion while medium from CD133 deprived gliomas did not trigger this release. This upregulation was selective for IL-6 as compared to a battery of other cytokines (e. g. TNF- $\alpha$  or IL-4). This upregulation depended on Toll-like receptor (TLR) 4, a pattern recognition receptor which can trigger pro-inflammatory cytokine release, since the effect was abolished in the TLR4<sup>-/-</sup> mouse, but not in other strains deficient for other TLRs. Our results show that GSCs, but not the bulk glioma cells, initiate microglial IL-6 secretion via TLR4 signaling and that IL-6 regulates glioma growth by supporting GSCs. Using human glioma tissue we could confirm the finding that GAMs are the major source of IL-6 in the tumor context.

## **Introduction**

Glioblastoma (GBM) is the most common and most malignant primary brain tumor in adults with high recurrence rates even after a complete resection. New GBM therapeutic strategies are desperately needed, requiring insights into the biological and molecular mechanisms controlling glioma growth. GBMs are complex tumors that display cellular heterogeneity within the tumor mass. Several studies suggest that GBMs contain a subpopulation with tumorigenic potential and stem cell characteristics(1-5). These glioma stem cells (GSCs) play key roles in the growth, invasion, angiogenesis and immune evasion of glioma(6, 7). They have also been identified as the major cellular entity for conferring chemo- and radioresistance(3, 8, 9), and have thus emerged as a new therapeutic target. GSCs express neural stem cell markers such as nestin, SOX2, and Musashi-1(2). In addition, CD133 is an enrichment marker for GSCs, however several studies have demonstrated its limitations as a specific marker(10, 11). A functional feature of GSCs is their ability to form neurospheres in the culture medium containing B27 supplement and defined growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). To identify GSCs, we tested for their ability to form neurospheres in combination with cell sorting using CD133.

A perivascular niche has been proposed for GSC that determines the characteristics of GSCs and controls the malignant behaviour of tumor cells(12). However, there is only limited knowledge about the composition of the GSC niche. Inflammatory mediators and inflammatory cells are indispensable components of the neoplastic microenvironment(13). Glioma-associated microglia (GAMs), the brain-resident macrophages, together with blood-borne monocytes are the predominant cells, contributing up to 30% of the total tumor mass(14) and their abundance is positively correlated with glioma malignancy(15). Tumor secreted CXCL12 (stromal cell-derived factor-1, SDF-1) is a potent microglia and macrophage recruiting molecule, especially for

attracting GAMs to hypoxic areas (16). Activation of SDF-1 and its receptor CXCR4 have been shown to promote macrophage mobilization and tumor revascularization (17). Despite their cytotoxic and phagocytic potential(18), these GAMs rather support tumor growth. Depletion of microglia reduced glioma invasion in organotypic brain slices(19) and also decreased glioma expansion in vivo(20). Microglia release many factors, including extracellular matrix proteases and cytokines, which directly or indirectly influence tumor migration and proliferation(21). We have previously shown that glioma-triggered microglial MT1-MMP and MMP9 expression via Toll like receptor (TLR)2 is one of the mechanisms for microglia-induced tumor expansion(20, 22, 23). TLRs are the main members of the pattern recognition receptor (PRR) family that are necessary for the induction of an innate immune response to damage-associated molecular patterns (DAMPs) through the activation and maturation of macrophages and dendritic cells(24). Microglia have been reported to be the predominant TLR-expressing cell type in the normal CNS(25) as well as in the glioma tissue(23). Microglia/brain macrophages freshly isolated from human glioma tissue also express substantial levels of TLR2, TLR3 and especially TLR4(26). Interleukin (IL)-6 is a cytokine secreted after TLR activation and its expression has been shown to correlate with glioma invasiveness(27). It also plays a major role in the response to injury or infection and is involved in the immune response, inflammation, and hematopoiesis(28). Glioma cells have been reported to secrete IL-6(29, 30) and express IL-6 receptors(27). Glioma-derived IL-6, working together with other tumor-secreted factors such as TGF- $\beta$  and PGE<sub>2</sub>, polarize glioma-infiltrating microglia toward an anti-inflammatory phenotype(21) and microglia-derived IL-6 has been reported to induce glioma cell migration and invasiveness(30).

In this research we have investigated the link between microglial IL-6 production, TLR expression and the potential of tumor stem cells to form glioma by utilizing two glioma murine models and human samples.

## **Materials and Methods**

### ***Animals***

All experiments were carried out using C57BL/6J WT mice (Charles River Laboratories, Sulzfeld, Germany) or TLRs 2, 4, 7, 9 and MyD88<sup>-/-</sup> on a C57BL/6J background. The TLR knockout mice were generated by Dr. Shizuo Akira and colleagues from the Osaka University, Japan and obtained from Oriental BioServices Inc., Japan(22). The generation of IL-6 knockout mice (IL-6<sup>-/-</sup>) has been described in detail previously(31). Briefly, IL-6 deficient mice had been backcrossed on a C57BL/6J background for >10 generations(32). To broaden the relevance of our findings, we employed another murine model where the tumor is initiated by the overexpression of PDGFb in Nestin-expressing cells in vivo: Ntv-a/Ink4a-Arf<sup>-/-</sup> mice develop high-grade gliomas, which reflect features of the pro-neural subtype in human GBMs(33-35), 6 to 8 weeks following intracranial injection of RCAS-PDGFb-producing DF-1 chicken fibroblast cells at 4.5 to 10 weeks of age(36, 37). The mice were bred and maintained in the animal housing facilities of the Max Delbrück Center for Molecular Medicine and Charité university hospital (Berlin, Germany) as per rules of the local governmental institutions (LaGeSo, G 0268/10, G 0343/10, G 0438/12). The mice were housed with a 12 h/12 h light-dark cycle and received food and water ad libitum.

### ***Human materials***

All human glioma materials in this study were obtained from the Department of Neurosurgery at Charité University Hospital according to the rules of the Ethical Committee (Charité, EA4/098/11).

### *Cell culture*

The murine GL261 glioma cell line (which is isogenic to C57BL/6J mice; National Cancer Institute, Frederick, MD), rat glioma cell line C6 (American Type Culture Collection, ATCC, Teddington, UK) and human glioma cell line U87 (ATCC) were grown in DMEM with 10% FCS, 200 mM glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin (Invitrogen, Darmstadt, Germany). EGFP GL261 cells were generated as previously described(22). NCH421K is a primary human glioblastoma cell line, which is highly enriched in CD133<sup>+</sup> GSCs(38). NCH421K cells (CLS Cell Lines Service GmbH, Eppelheim, Germany) were grown in stem cell medium consisting of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM / F-12; Invitrogen, Carlsbad, CA) containing supplement (2% B27; Invitrogen), growth factors (20ng/ml EGF and bFGF; from PeproTech, Hamburg, Germany) and additives (100 U/ml penicillin, and 100 mg/ml streptomycin; all from Invitrogen). Neonatal microglial cells were prepared from WT, MyD88<sup>-/-</sup> and TLRs 2, 4, 7 and 9<sup>-/-</sup> mice according to previously established protocols(22). Microglia were also cultured from adult mice (P49–P56) as previously described in detail(39). Briefly, cortical and midbrain tissue was freed of blood vessels and meninges in Hank's balanced salt solution (HBSS), mechanically dissociated into 1-mm<sup>3</sup> pieces and trypsinized in 1% trypsin and 0.05% deoxyribonuclease for 5 min at room temperature, as described for neonatal microglia. Digested tissue was dissociated using a fire-polished pipette and washed twice in HBSS. Cells were then plated on a confluent monolayer of P0 astrocytes in 75-cm<sup>2</sup> flasks. The feeder layer of astrocytes was depleted of neonatal microglial cells using clodronate (200 mg/ml) before the adult microglia were added. The adult mixed glial cultures received fresh complete DMEM medium every other day and were treated with 33% L929-conditioned medium after 7 days or once cells became confluent. Microglia were shaken off 1



week later and were used for experiments within 1 day of plating. For analysis of the inflammatory response in vitro, microglia were plated at  $3 \times 10^4$  cells/96-well. All cells were maintained in a 37°C incubator with a 5% CO<sub>2</sub> humidified atmosphere. The TLR ligands lipopolysaccharide (LPS) and Poly I:C were obtained from Invivogen (San Diego, USA).

### ***Isolation and cell culture of glioma stem cells***

Mouse glioma stem cells were isolated from the GL261 cell line as previously described(40). In summary, bulk cultures of GL261 cells were grown in stem cell medium for at least 4-6 weeks. To broaden the relevance of our findings, we employed the RCAS-PDGFB murine tumor model. RCAS-PDGFB tumors were excised from tumor brains using a scalpel, minced, and incubated with Accutase (eBioscience, San Diego, CA, USA) for 15 minutes at 37°C. Tissue pieces were mechanically dissociated using a 1 ml pipette and washed in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA). Cells were passed through a 70 µm cell strainer and seeded into a T25 cell culture flask. Cells were grown in stem cell medium. Glioma stem cells were isolated by fluorescence-activated cell sorting (FACS) using Phycoerythrin (PE)-conjugated anti-mouse CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) and Allophycocyanin (APC)-conjugated anti-mouse CD133 (eBioscience,) or by magnetic activated cell sorting (MACS) using murine CD133 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). CD133 expression level was analyzed by FACS and the percentage of CD133 cells in the CD133<sup>+</sup> population was more than 90%, while in the CD133<sup>-</sup> population was less than 1% as previously described(40) (Supplemental Digital Content 1 and 2).

### ***Preparation of glioma-conditioned medium***

Neurosphere/adhesive cultured GL261 cells or CD133<sup>+</sup>/CD133<sup>-</sup> FACS sorted GL261 and RCAS cells were seeded at a density of  $0.5 \times 10^6$  cells in Greiner CELLSTAR® cell culture dishes (diam. × H 100 mm × 20 mm; Sigma-Aldrich, Munich, Germany). The stem cell culture medium was left on the cells for 16-18h after seeding before being harvested. The conditioned medium was collected, briefly centrifuged to remove cell debris and filtered using a 0.2 µm filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and used for all further experiments. Protein concentrations were measured in the conditioned media using Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). The protein concentration in the conditioned medium was equal for all conditions (Supplemental Digital Content 3).

### ***Multiple analyte detection and ELISA***

Multiple analyte detection of cytokines and chemokines in supernatants was performed using FlowCytomix (Bender MedSystems, Burlingame, USA). The immunoassay is a bead-based method to detect the concentrations of up to 20 analytes in one sample using a flow cytometer. The mouse/rat basic kit was used in combination with mouse simplex kits. Inflammatory mediators analyzed by the FlowCytomix assay included IL-13, IL-22, IL-2, IL-5, IL-6, IL-1β, IL-23, IFN-γ, TNF-α, GM-CSF, IL-4 and IL-17. Additionally IL-6, TNF-α and IL-1β concentrations in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using the BD OptEIA™ Set Mouse IL-6, TNF-α and IL-1β (BD Biosciences, San Diego, USA) according to the manufacturer's manual.

### ***TLR4 antibody treatment***

For the treatment with a TLR4 antibody, anti-mouse MTS510 or isotype control (both from eBioscience) was applied to the medium after seeding primary cultured neonatal microglia to incubate overnight, and new antibody or isotype was added when the medium was changed to stimulate with GCM.

### ***Magnetic cell separation of human brain tumor tissue***

Glioma associated brain microglia/macrophages were isolated from the human tumor resected tissues. Fresh tissue was dissociated immediately after resection using the neural tissue dissociation kit (MiltenyiBiotec, Bergisch Gladbach, Germany). Erythrocytes were lysed by adding 5 ml ammonium chloride solution for 10 min. Next, cells were resuspended in PBS containing 0.5% bovine serum albumin and 2mM ethylenediaminetetraacetic acid (EDTA). Magnetic sorting for CD11b<sup>+</sup> cells was then performed using a CD11b MicroBead kit (MiltenyiBiotec) following the manufacturer's instruction. Magnetic activated cell sorting (MACS) into CD11b<sup>-</sup> and CD11b<sup>+</sup> enriched cell populations was carried out using several MACS columns in a series. Both CD11b<sup>-</sup> and CD11b<sup>+</sup> fractions were collected. A purity check was performed after MACS separation by flow cytometry analysis of a small fraction of the sorted populations.

### ***Real-time qPCR***

Total RNA was isolated from microglia obtained from WT mice, as well as from CD133<sup>+</sup> and CD133<sup>-</sup> FACS sorted GL261 cells and MACS purified microglia from human GBM tissue using Invitrap Spin Universal RNA mini kit (Invitex GmbH, Berlin, Germany). Quality and yield were determined by NanoDrop ND-1000 (Thermo Scientific, Schwerte, Germany). First strand cDNA

synthesis of RNA was done using the Superscript II (Invitrogen) reverse transcriptase according to the manufacturer's instructions. For mRNA transcription, oligo-dT primers (Invitrogen) were used. Gene amplification was done in duplicates using SYBR Green PCR mix (Applied Biosystems, Foster City, USA) with the following PCR conditions: 95°C for 10 min, 95°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec for 40 cycles using the 7500 Fast Real-Time PCR System (Applied Biosystems). Sequences of primers used were: sense 5' - GCTGGCAGCACCTGAGACC-3' , anti-sense 5' -TCCAAGGAGTGCCCGTGACC-3' (mouse IL-6R); sense 5' - AGAAGGCCAGCAGCATCATT-3' , anti-sense 5' - TGACAGACCCAGAAACGAGC-3' (mouse gp130); sense 5'- CCCTGAAGTACCCCATGAA-3', anti-sense 5'-GTGGACAGTGAGGCCAAGAT'-3' (mouse  $\beta$  actin); sense 5'-GTAGCCGCCCCACACAGA-3', anti-sense 5'-CATGTCTCCTTTCTCAGGGCTG3' (human IL-6) and sense 5'- CACCATTGGCAATGAGCGGTTC-3', anti-sense 5'-AGGTCTTTGCGGATGTCCACGT-3' (human  $\beta$ -actin). Changes in human IL-6, mouse IL-6R and mouse gp130 gene expressions were analyzed by the comparative  $2^{(-\Delta \Delta Ct)}$  method relative to  $\beta$ -actin gene expression levels. For assessing CD133<sup>+</sup>-derived TLR4-ligands, gene amplification was performed in triplicates using SYBR Green PCR mix (Applied Biosystems, Foster City, USA) with the following PCR conditions: 50°C for 2min, 95°C for 10min, 95°C for 15 sec, 60-64°C for 30 sec, 72°C for 1min for 40 cycles using the 7500 Fast Real-Time PCR System (Applied Biosystems). Primer sequences: sense 5'- AGTACGTGGCCCAAGAGTTG-3', anti-sense 5'-AGGGCATTGTGGTTCCAGT-3' (mouse fibrinogen  $\alpha$ -chain); sense 5'-ATGACCATCCACAACGGCAT-3', anti-sense 5'- GATCCGTAGTTACCCAGCCG-3'(mouse fibrinogen  $\beta$  -chain); sense 5'- CAACCCCAAAGCCAGGTAT-3', anti-sense 5'-

GCAGCGCTTCGTATTTTACA-3'(mouse fibrinogen  $\gamma$  -chain); sense 5'-GATGGTGAAGACGACACTGC-3', anti-sense 5'- GAATGGCTGTGGACTGGATT-3'(mouse Fibronectin EDA); sense 5'- GGGAGCCTTGGAAGTGTGT-3', anti-sense 5'-GCTTCTCTTCATGTTTGCCTGA-3'(mouse Hsp22);sense 5'-AAACAAGCATCGGGATTCCAG-3', anti-sense 5'- ACAATGCAGTCTTCCGTGGTG-3'(mouse Lactotransferrin); sense 5'- ATTTCCGGTCAGTGCAGGTAGT-3', anti-sense 5'-GGTCAAAGCCATTCTCGAAGAT-3'(mouse neutrophil elastase); sense 5'-GTAATTGTGTCCACCTTCCAC-3', anti-sense 5'- AGT TGCTCATCACCTTCTGGA-3'(mouse S100A4); sense 5'- CCGTCTTCAAGACATCGTTTGA-3', anti-sense 5'-GTAGAGGGCATGGTGATTTCT-3' (mouse S100A8); sense 5'-CCCTGACACCCTGAGCAAGAAG-3', anti-sense 5'-TTTCCCAGAACAAAGGCCATTGAG-3' (mouse S100A9); sense 5'-GTTTGGAGACCGCAGAGAAGAA-3', anti-sense 5'- TGTCCCCATATCTGCCCATCA-3'(mouse tenascin-C);. Fold changes in CD133<sup>+</sup> gene expression compared to respective CD133<sup>-</sup> samples were analyzed by the comparative  $2^{(-\Delta \Delta Ct)}$  method relative to  $\beta$ -actin gene expression levels.

### ***Flow cytometry***

Fluorescence-activated cell sorting (FACS) analysis was carried out on a LSR Fortessa 5Laser (BD Biosciences, Erembodegem, Belgium). Cell sorting was carried out using a FACS-Aria-II (BD Biosciences). Antibodies for fluorescein isothiocyanate (FITC)-conjugated anti-human CD11b, Allophycocyanin (APC)-conjugated anti-mouse CD133, eFluor® 660 conjugated anti-human/mouse Sox2, Alexa Fluor® 488 conjugated anti-mouse Anti-Glial Fibrillary Acidic

Protein (GFAP), Phycoerythrin (PE) conjugated anti-mouse Notch1 and matched isotype controls were all from eBioscience, PE-conjugated anti-mouse CD133 and matched isotype control were from Miltenyi Biotech. Data were analyzed using FlowJo software (Treestar, Ashland, OR, USA).

### ***In vivo glioma implantation***

Wild type and IL-6<sup>-/-</sup> mice were used for the in vivo studies to investigate glioma expansion. To broaden the relevance of our findings, 4.5 to 10-week-old (Ntv-a/Ink4a-Arf<sup>-/-</sup> mice for DF-1 RCAS-PDGFB injection) were used to culture RCAS GSCs. Surgical procedures were performed as described(41, 42). Briefly, mice were anesthetized, immobilized and mounted onto a stereotactic frame (David Kopf Instruments, Tujunga, USA) in the flat-skull position. After skin incision 1 mm anterior and 1.5 mm lateral to the bregma, the skull was carefully drilled with a dental drill or a 20G needle tip. A 1µl syringe with a blunt tip (Mikroliterspritze 7001N, Bonaduz, Switzerland) was inserted to a depth of 4 mm and retracted to a depth of 3 mm from the dural surface into the right caudate putamen. Over 2 minutes, 1 µl glioma cell suspension (2x10<sup>4</sup> cells/µl of EGFP-GL261, 1x10<sup>4</sup> CD133<sup>-</sup> or 100 CD133<sup>+</sup> GL261 cells, 4x10<sup>4</sup> transfected DF-1 cells or 5x10<sup>4</sup> RCAS-PDGFB tumor cells) was slowly injected into the brain. Coordinates for injections of DF-1 cells and RCAS-PDGFB tumor cells into Ntva/Ink4a-Arf<sup>-/-</sup> mice, respectively were bregma 1.5 mm anterior, lateral 0.5 mm (right of midline), and a depth 2.0 mm from the dural surface. The needle was then carefully retracted from the injection canal and the skin was sutured with a surgical sewing cone (Johnson & Johnson International, Langhorne, USA). After surgery, the mice were kept warm until awake. Mice were monitored daily for the first two weeks and twice a day starting from day 15 post-injection for symptoms of tumor development

(lethargy, hydrocephalus, head tilting). The size of resulting tumors ranged from 1.5–2.5 mm (GL261 tumors) and 2.5–3.5 mm (RCAS-PDGFb tumors).

### ***Immunofluorescent staining and image processing***

Free-floating 40  $\mu\text{m}$  thick brain sections from tumor-bearing mice were prepared as previously described(22). Nuclei were visualized using 4,6-diamidino-2-phenylindole (DAPI) from Sigma-Aldrich. The GL261 glioma cells were identified by green fluorescence of the EGFP construct.

### ***Unbiased stereology for tumor volume estimation***

14 days (EGFP-GL261) or 21 days (CD133<sup>-</sup> and CD133<sup>+</sup> GL261 cells) after tumor implantation mice were anesthetized with pentobarbital (Narcofen, Merial, Hallbergmoos, Germany), brains perfused and fixed, and resulting brain slices were subsequently used to analyze glioma expansion in vivo. The tumor volume in brain slices of glioma-bearing mice was quantified according to the Cavalieri principle by determining tumor area in every 12th 40- $\mu\text{m}$ -thick brain slice and then multiplying this area by the factor 12 x 40  $\mu\text{m}$  using the Stereoinvestigator software (MBF Bioscience, Williston, VT). Experimental groups were blinded to the investigator performing the analysis.

### ***Statistical analysis***

All data represent the average of at least 3 independent experiments. Datasets were analyzed statistically with SPSS11.5 software and tested for normality with the Shapiro-Wilks test. The Mann-Whitney U test was used for nonparametric analysis. Parametric testing was done with the Student t test. Comparisons between multiple groups were done using 1-way ANOVA with the

Scheffe' post hoc test. Statistical significance was determined at P values <.05 (\*) and <.01 (\*\*) while "n.s." implied a non significant P value.

## Results

### *IL-6 deficient mice show reduced glioma growth*

To investigate whether ablation of the IL-6 gene locus in the host interfered with tumor expansion in vivo, we implanted EGFP-GL261 cells into WT and IL-6<sup>-/-</sup> mice and measured glioma volume by unbiased stereological estimation (Cavalieri method). After 2 weeks of implantation, the tumor volume in IL-6<sup>-/-</sup> mice was significantly smaller compared to the WT mice (WT 3.84±1.13 mm<sup>3</sup>, IL-6<sup>-/-</sup> 2.14±0.46 mm<sup>3</sup>, P=.0002 Fig. 1A). It has been shown by us and others that 100 CD133<sup>+</sup> glioma cells have a similar tumor forming capacity as 10000 CD133<sup>-</sup> glioma cells(40). To see the impact of host IL-6 on CD133<sup>+</sup> cells versus CD133<sup>-</sup> cells, we injected 100 CD133<sup>+</sup> cells or 10,000 CD133<sup>-</sup> cells into the WT and IL-6<sup>-/-</sup> mice and analyzed tumor growth. After three weeks of tumor growth, we found that in WT mice, 100 CD133<sup>+</sup> cells formed tumors of similar size as compared to the 10,000 CD133<sup>-</sup> cells (WT- CD133<sup>+</sup>: 5.06±0.69 mm<sup>3</sup>, WT- CD133<sup>-</sup>:5.0±0.48 mm<sup>3</sup>, p=0.89, Fig. 1B). However, in IL-6<sup>-/-</sup> animals, inoculation of 100 CD133<sup>+</sup> GL261 cells induced significantly smaller tumors (IL-6<sup>-/-</sup>-CD133<sup>+</sup>:2.65±0.38 mm<sup>3</sup>, p=0,004) as compared to wildtype. Injection of 10,000 CD133<sup>-</sup> cells into IL-6<sup>-/-</sup> mice also resulted in smaller tumors, but this decrease was not significant (IL-6<sup>-/-</sup>-CD133<sup>-</sup>:3.94±0.31 mm<sup>3</sup>, p=0.09). These data indicate that IL-6 from the host cells supports tumor growth by GSCs, but not by bulk glioma cells.



***Microglial IL-6 is upregulated by supernatant from glioma stem cells but not from bulk glioma cells***

To investigate the potential of GSCs versus bulk cells to induce microglial cytokine release, mouse primary neonatal microglia cultures were treated with control medium (stem cell culture medium) or supernatant medium from GL261 cells (GCM) either enriched for CD133 or deprived of CD133. After 24 hours of stimulation, cell supernatant was collected to measure expression levels of 12 cytokines. As shown in Fig. 2, the level of IL-6 in supernatant from microglial cells that were stimulated with CD133<sup>+</sup> conditioned medium was higher than the levels in supernatant from microglial cells that were stimulated with CD133<sup>-</sup> conditioned medium (control: undetectable, CD133<sup>+</sup>: 6.28±1.16ng/ml, CD133<sup>-</sup>: 0.32±0.03ng/ml, p=0.004). However, levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-13, IL-22, IL-2, IL-5, IL-23, IFN- $\gamma$ , GM-CSF, IL-4 and IL-17 in supernatant from microglia did not change between treatment groups.

***Supernatant from glioma stem cells induced IL-6 release in both neonatal and adult microglia***

To further investigate the potential of GSCs versus bulk cells to induce cytokines, mouse primary neonatal and adult microglial cultures were treated with medium only (as controls) or GCM from CD133<sup>+</sup>, CD133<sup>-</sup>, neurosphere or adhesive GL261 cells. The percentage of CD133<sup>+</sup> cells in neurosphere GL261 cells is 30.1% while in adhesive GL261 it is less than 1% as described elsewhere (43) (Supplemental Digital Content 1 and 2). Treatment with LPS served as a positive control. After 24 hours of treatment, cell supernatant was collected to perform ELISA for three pro-inflammatory cytokines: IL-6, TNF- $\alpha$  and IL-1 $\beta$ . As shown in Fig. 3, the level of IL-6 in supernatant from microglial cells that were stimulated with CD133<sup>+</sup> or neurosphere GL261 conditioned medium was higher than the levels in supernatant from microglial cells that were stimulated with CD133<sup>-</sup> or adhesive GL261 conditioned medium (control: 0.67±0.22ng/ml,

CD133<sup>+</sup>: 2.05±0.85ng/ml, CD133<sup>-</sup>: 0.34±0.11ng/ml, neurosphere GL261: 3.47±1.83ng/ml, adhesive GL261: 0.3±0.06ng/ml, p=0.04). The difference between CD133<sup>+</sup> cell populations (enriched from NS-GL-261 cells) and NS-GL-261 cells in Fig. 3A was not significant: neonatal microglia (NS-GL261: 3.47±1.83, CD133<sup>+</sup>: 2.05±0.85, p= 0.27) and adult microglia (NS-GL261: 2.95±1.28, CD133<sup>+</sup>: 1.35±0.29, p= 0.13). However, when we analyzed TNF- $\alpha$  and IL-1 $\beta$  levels in microglia treated in different conditions, there was no significant difference. LPS always triggered an induction of these cytokines. IL-6, IL-1 $\beta$  and TNF- $\alpha$  were not detectable in GCM from all glioma cells.

### ***Factors released from glioma stem cells induced microglial IL-6 secretion through MyD88-TLR4 signaling***

We have previously shown that glioma-released versican induced microglial MT1-MMP production through the TLR2 signaling pathway(23). We therefore tested whether IL-6 release is also regulated by TLR signaling. Since MyD88 is the adapter protein for all TLRs except TLR3(44), we stimulated microglia from MyD88<sup>-/-</sup> animals with GSC supernatant. The TLR3 agonist Poly I:C was used as a positive control. As shown in Fig. 4A, in microglial cells deficient for MyD88, IL-6 induction was completely abolished, indicating that GSCs triggered microglial IL-6 induction through TLR signaling. We then screened GSC-triggered microglial IL-6 induction in TLR2, TLR4, TLR7 and TLR9<sup>-/-</sup> animals. Interestingly, only the knockout of TLR4 impaired microglial IL-6 induction (control: 0.2±0.08ng/ml, CD133<sup>+</sup>: 0.24±0.07ng/ml, neurosphere GL261: 0.23±0.04ng/ml, Fig. 4B) while IL-6 upregulation was similar to wild type levels in microglial cells from TLR2-, TLR7- or TLR9-deficient animals (Fig. 4D, 4E and 4F). The TLR4 monoclonal neutralizing antibody MTS510 has previously been shown to functionally block TLR4(45). We first verified that MTS510 blocks microglial TLR4 functionality in vitro.

MTS510 attenuated the TLR4 agonist LPS-induced microglial IL-6 induction (Fig. 4C). To test whether MTS510 impairs microglial IL-6 secretion induced by supernatant from GSCs, microglia were stimulated with either GSC supernatant in combination with MTS510 or GSC supernatant with isotype control antibody for 24 hours. GSC supernatant treatment with isotype control resulted in an increase (control:  $0.64 \pm 0.27$  ng/ml, CD133<sup>+</sup>/Isotype:  $2.78 \pm 0.4$  ng/ml,  $p=0.003$  Fig. 4C) of microglial IL-6 secretion compared to the untreated cells. Treatment with GSC supernatant in combination with MTS510 did not trigger IL-6 secretion (CD133<sup>+</sup>/Anti-TLR4:  $0.62 \pm 0.34$  ng/ml,  $p=0.004$  Fig. 4C), which indicates that MTS510 blocked microglial TLR4 signaling. These data indicate that induction of IL-6 by stimulation with GSCs conditioned-medium was fully dependent on MyD88-TLR4 signaling but not on TLR2, TLR7 and TLR9. In an attempt to narrow down possible TLR4 specific ligands as candidate factors released by the glioma cells we compared the level of eleven TLR4 specific known endogenous ligands in CD133<sup>+</sup> vs. CD133<sup>-</sup> cells (Supplemental Digital Content 5). Fibrinogen alpha, beta and gamma chain, Lactotransferrin, Neutrophil Elastase as well as the S100 proteins S100A8 and S100A9 are not expressed in neither cell population (respective controls with liver and bone marrow revealed primer specificity and validity, data not shown). EDA and HSP22 are expressed by both CD133<sup>+</sup> and CD133<sup>-</sup> at the same level (fold change CD133<sup>+</sup> vs. CD133<sup>-</sup> respectively  $0.76 \pm 0.35$  and  $1.26 \pm 0.66$ ). The specific TLR4 ligand tenascin-C is in average  $3.93 \pm 2.06$  fold significantly higher expressed in CD133<sup>+</sup> compared to CD133<sup>-</sup> cells. Interestingly, we also identified S100A4 as a TLR4 specific ligand that is significantly 64-fold ( $64.16 \pm 20.71$ , data not shown) lower expressed in CD133<sup>+</sup> vs. CD133<sup>-</sup>. However, from this data tenascin-C is the best candidate to be investigated further in order to verify its role in IL-6 upregulation after TLR4 stimulation in microglia cells by soluble factor derived from CD133<sup>+</sup> cells (see scheme in Fig. 6).

### ***GAMs are predominating IL-6 expressing population in gliomas***

To evaluate the potential contribution of IL-6 signaling to the glioma microenvironment, we measured IL-6 release in a series of glioma cell lines as well as mouse microglia and microglia stimulated from conditioned medium of CD133<sup>-</sup> and CD133<sup>+</sup> GL261 cells. While some of the gliomas do secrete IL-6, both CD133<sup>-</sup> and CD133<sup>+</sup> GL261 cells express very low level of IL-6 compared to naïve microglia and microglia primed by CD133<sup>+</sup> GL261 conditioned medium (Fig. 5A). To broaden the relevance of our findings, we employed the RCAS-PDGFb murine tumor model. Both CD133<sup>-</sup> and CD133<sup>+</sup> RCAS cells do secrete low level of IL-6 (CD133<sup>-</sup>: 0.54±0.15ng/ml, CD133<sup>+</sup>: 0.9±0.41ng/ml) compared to microglia primed by CD133<sup>+</sup> RCAS conditioned medium (Fig. 5A). To investigate the potential of GSCs versus bulk cells to induce microglial cytokine release in another murine tumor model, mouse primary neonatal microglia cultures were treated with control medium (stem cell medium) or supernatant medium from RCAS cells (GCM) either enriched for CD133 or deprived of CD133. After 24 hours of stimulation, cell supernatant was collected to measure expression levels of IL-6. As seen in Fig. 5A, the level of IL-6 in supernatant from microglial cells that were stimulated with CD133<sup>+</sup> RCAS conditioned medium was higher than the levels in supernatant from control microglial cells or microglial cells that were stimulated with CD133<sup>-</sup> conditioned medium (control: 0.64±0.27ng/ml, CD133<sup>+</sup>: 3.28±0.5ng/ml, p=0.03). To verify that the IL-6 receptor is expressed on the target cell, we measured IL-6 receptor expression in primary cultured neonatal microglia and freshly FACS isolated CD133<sup>+</sup> and CD133<sup>-</sup> GL261 cells by qRT-PCR (Fig. 5B). CD133<sup>+</sup> cells expressed higher IL-6 receptor and gp130 mRNA levels than CD133<sup>-</sup> cells or microglial cells. These data demonstrate that the expression of IL-6 receptors was elevated on CD133<sup>+</sup> in comparison to CD133<sup>-</sup> cells, and supports the concept of paracrine signaling between GSCs and microglia (Fig. 6). We also purified microglia/brain macrophages from human GBM tissue by

MACS (the purity of CD11b<sup>+</sup> cells was described previously(46)) and tested for the expression of IL-6 by qRT-PCR. In 7 out of 8 samples, the CD11b<sup>+</sup> cells (i.e. GAMs) expressed higher IL-6 than the CD11b<sup>-</sup> cells (mainly tumor cells) (Fig. 5C). These data suggest that GAMs are the main source of IL-6 in gliomas.

## **Discussion**

The interaction between tumor cells and their microenvironment has attracted increasing attention over the last few years. Microglia, as the immune competent cells of the brain, are the key resident cells interacting with glioma. We, along with others, have demonstrated that glioma attract microglia/macrophages and educate them to develop a tumor-supportive phenotype(20, 23, 46-48). Glioma cells in the tumor tissue are a heterogeneous population containing different subgroups. Glioma stem cells (GSCs) are the minority among the glioma cells, but have a strong impact on the disease progression since they are believed to be responsible for glioma relapse and therapy resistance. So far there have only been a few studies addressing the crosstalk between GSCs and microglia/macrophages. GSCs recruit more GAMs than the bulk glioma cells (non GSCs) by releasing higher level of chemoattractants including CCL2 and VEGF-A. In both primary human gliomas and orthotopical transplanted syngeneic glioma, the density of GAMs at the invasive front is increased by the presence of CD133<sup>+</sup> GSCs. The interdependence of these two cell types became evident since GAMs release TGF- $\beta$ 1, which promotes the upregulation of MMP-9 in GSCs and thus tumor invasion(49). In the present study, we found that CD133<sup>+</sup> GSCs trigger IL-6 release from microglia which promotes glioma growth.

Microglia associated with glioma have been assigned the M2 phenotype, since they are tumor-supportive and immune-suppressive(50). However, in a recent microarray study we demonstrated that GAMs share markers of both the M1 and M2 phenotype and thus the genetic profile defines them as a unique phenotype(36). The simple view of separation into the M1 and M2 category has

also recently been challenged for macrophages and a more differentiated scheme has been proposed(51). Indeed, IL-6, as a pro-inflammatory cytokine, is not upregulated in microglia associated with both mouse and human gliomas compared to naive microglia(26, 52). We confirmed this finding by stimulating primary microglia with conditioned medium from GL261 cells, and we also did not detect any induction of pro-inflammatory cytokines. GSCs, however, lead to a selective upregulation of IL-6, but not of a battery of other cytokines including TNF- $\alpha$ . Thus, microglial cells associated with GSCs acquire a phenotype, which is distinct from other glioma-associated microglia. GAMs are thus a heterogeneous population imposed by glioma heterogeneity. Recently it was shown by a single cell sequencing approach, that glioma cells are heterogenic also with respect to the classical diagnostic categories(53).

We found that TLR4 signaling in the microglia/macrophages is essential for IL-6 secretion since release was abolished in TLR4-deficient mice and in a mouse line deficient for the TLR-adaptor protein MyD88, which is an essential component for TLR signaling. Moreover, the anti-TLR4 monoclonal antibody MTS510 inhibited GSC conditioned medium-driven microglial IL-6 secretion. We thus propose that glioma cells release ligands that activate TLR4 signaling. We measured the levels of eleven TLR4 specific ligands in CD133<sup>+</sup> and CD133<sup>-</sup> cells and identified tenascin-C (TNC) as a possible candidate mediating the IL-6 release in microglia via TLR4 signaling. This is in line with recent findings where TNC was reported as a stem cell marker in human glioma samples(54). Whereas TNC is produced by stromal fibroblasts in the majority of solid tumors, brain tumor cells themselves are the main source of extracellular matrix TNC in glial malignancies(55, 56). The intensity of TNC expression is shown to correlate with glioma grade and patient prognosis(55, 57). Due to its expression in solid tumors, TNC has been used as a tumor-associated antigen to deliver antibody-conjugated radiotherapeutic agents to GBM(58). While the classic ligand for TLR4, LPS, triggers the release of several pro-inflammatory

cytokines including IL-6 and TNF- $\alpha$ , we thus assume that the ligand released from glioma CD133<sup>+</sup> cells triggers a selective release of IL-6. It is possible that other factors such as epigenetic regulations like miRNA deregulation, histone modification or DNA methylation specifically suppress the transcription of other pro-inflammatory cytokines(59). Recently, it was shown that a miR-142-3p-driven autocrine and paracrine positive loop epigenetically regulates the progression and cancer stem-like property of glioblastoma by targeting the secretion of the pro-inflammatory cytokine IL-6(60). The exact mechanism of how tumor cells educate bulk cells in order to serve in favor of tumor growth and invasiveness remains to be elucidated. At this point we like to stress, that due to the global deletion of IL-6 in our mouse model we cannot exclude primary or secondary effects of IL-6 on tumor growth apart from the microglia/macrophage population. However, our in vitro data strongly support the importance of the CD133-TLR4 -IL6 pathway.

Zhang et al. describe another mode of interaction between glioma and microglia mediated by IL-6 independent of TLR4. They used human glioma lines and non-transformed human microglia isolated from surgically resected epileptic brain tissue and described that gliomas release the chemokine CCL2. Overexpression of CCL2 in the U87 glioma line stimulated microglia to release IL-6(30).

Our results are in line with a study where it was demonstrated that interfering with IL-6 signaling in GSCs led to reduced growth and neurosphere formation capacity, and to an increase in the apoptosis rate(27). From a clinical perspective, the expression of IL-6 and its receptors in the human glioma tissues is inversely correlated to patient survival. The median survival times were 16 months in patients with negative IL-6 expression and 7 months in those with positive IL-6 expression(61). An IL-6 neutralizing antibody attenuated microglia-stimulated glioma

invasiveness and reduced glioma growth in vivo(27, 30). Our data further corroborates the important role of microglial IL-6 in glioma growth. Specifically, we propose that TLR4 signaling is an important component of GSC-microglia crosstalk and propose tenascin-C released by glioma CD133<sup>+</sup> cells as a regulator of this pathway (Fig. 6).

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## Figure legends

**Fig. 1 Host IL-6 interferes with glioma expansion by influencing GSCs.** (A) EGFP-GL261 cells were intra-cerebrally implanted into WT and IL-6<sup>-/-</sup> mice, tumor volume in WT versus IL-6<sup>-/-</sup> animals was evaluated based on unbiased stereology. (B) WT and IL-6<sup>-/-</sup> mice were intra-cerebrally implanted with 100 CD133<sup>+</sup> or 10000 CD133<sup>-</sup> GL261 cells and after 3 weeks tumor volume was evaluated based on unbiased stereology.

**Fig. 2 Cytokine release by multiple analyte detection in microglia stimulated with conditioned medium from GSCs and non-GSCs.** Neonatal primary cultured microglia were stimulated with conditioned medium from CD133<sup>-</sup> and CD133<sup>+</sup> GL261 cells for 24 hours and the release of the cytokines IL-13, IL-22, IL-2, IL-5, IL-6, IL-1 $\beta$ , IL-23, IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF, IL-4 and IL-17 were analyzed by FlowCytomix. We also measured the cytokine levels of the conditioned medium from CD133<sup>-</sup> and CD133<sup>+</sup> GL261 before application to microglia.

**Fig. 3 Factors released from glioma stem cells induced IL-6 but not TNF- $\alpha$  and IL-1 $\beta$  secretion from both neonatal and adult primary microglia.** (A) Neonatal and adult primary cultured microglia were stimulated with conditioned medium from adhesive (AC-), neurosphere (NS-), CD133<sup>-</sup> and CD133<sup>+</sup> GL261 cells for 24 hours and IL-6 release was analyzed by ELISA. TNF- $\alpha$  (B) and IL-1 $\beta$  (C) were also analyzed in a similar way. LPS was used as a positive control. We also determined the cytokine levels of the conditioned media from different cells before application to microglia.

**Fig. 4 IL-6 release induced by GCM in microglia from MyD88- and TLR-deficient mice. (A)**

Neonatal microglia from MyD88<sup>-/-</sup> mice were stimulated with conditioned medium from adhesive (AC-), neurosphere (NS-), CD133<sup>-</sup> and CD133<sup>+</sup> GL261 cells for 24 hours and IL-6 release was analyzed by ELISA and compared to an un-stimulated control. Similarly, IL-6 release was analyzed in MyD88- (A), TLR4- (B), TLR2- (D), TLR7- (E) and TLR9<sup>-/-</sup> mice (F). For MyD88<sup>-/-</sup> and TLR4<sup>-/-</sup>, Poly I:C was used as a positive control while LPS was used as a positive control in other groups. (C) Monoclonal antibody MTS510 inhibited GSCs conditioned medium-driven microglial IL-6 secretion. Primary microglial cells were stimulated with CD133<sup>-</sup> and CD133<sup>+</sup> conditioned medium together with 10 µg/mL isotype, and 10 µg/mL MTS510 for 24 hours. We also determined the cytokine levels of the conditioned media from different cells before application to microglia.

**Fig. 5 IL-6 expression in GAMs and gliomas. (A)**

IL-6 release was analyzed in conditioned medium from U87, C6, NCH421K, AC-, NS-, CD133<sup>-</sup> and CD133<sup>+</sup> from GL261 and RCAS cells, primary cultured microglia and microglia treated with GSCs conditioned medium were used as controls. (B) IL-6 receptor and gp130 expression were analyzed in microglia, FACS sorted CD133<sup>-</sup> and CD133<sup>+</sup> cells from GL261 by qRT-PCR. (C) MACS freshly isolated CD11b<sup>+</sup> cells and CD11b<sup>-</sup> (i.e. mainly glioma cells) from 8 human GBM (Supplemental Digital Content 4) samples were analyzed for IL-6 by qRT-PCR. Dashed line represents IL-6 expression in CD11b<sup>+</sup> cells in each sample, solid bars represent folder changes of IL-6 expression in flow through cells compared to CD11b<sup>+</sup> cells.

**Fig. 6 Overview of the paracrine relationship between glioma stem cells and microglial cells**

GSCs, but not the bulk glioma cells, initiate microglial IL-6 secretion via TLR4 signaling and IL-6 regulates glioma growth by supporting GSCs.

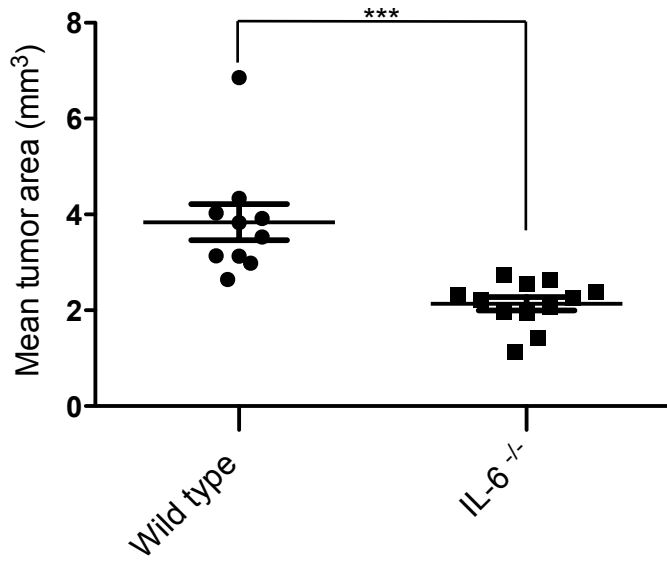
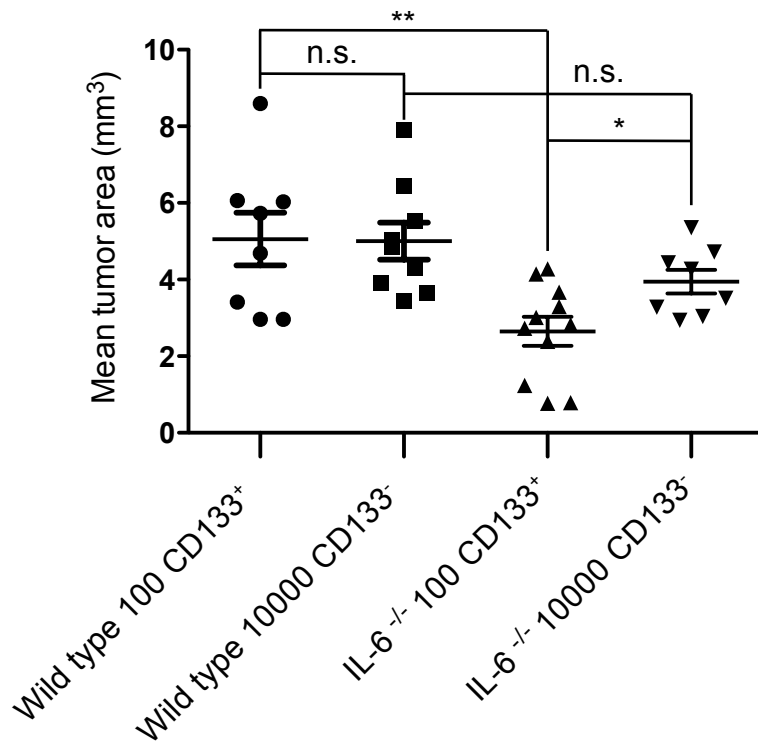
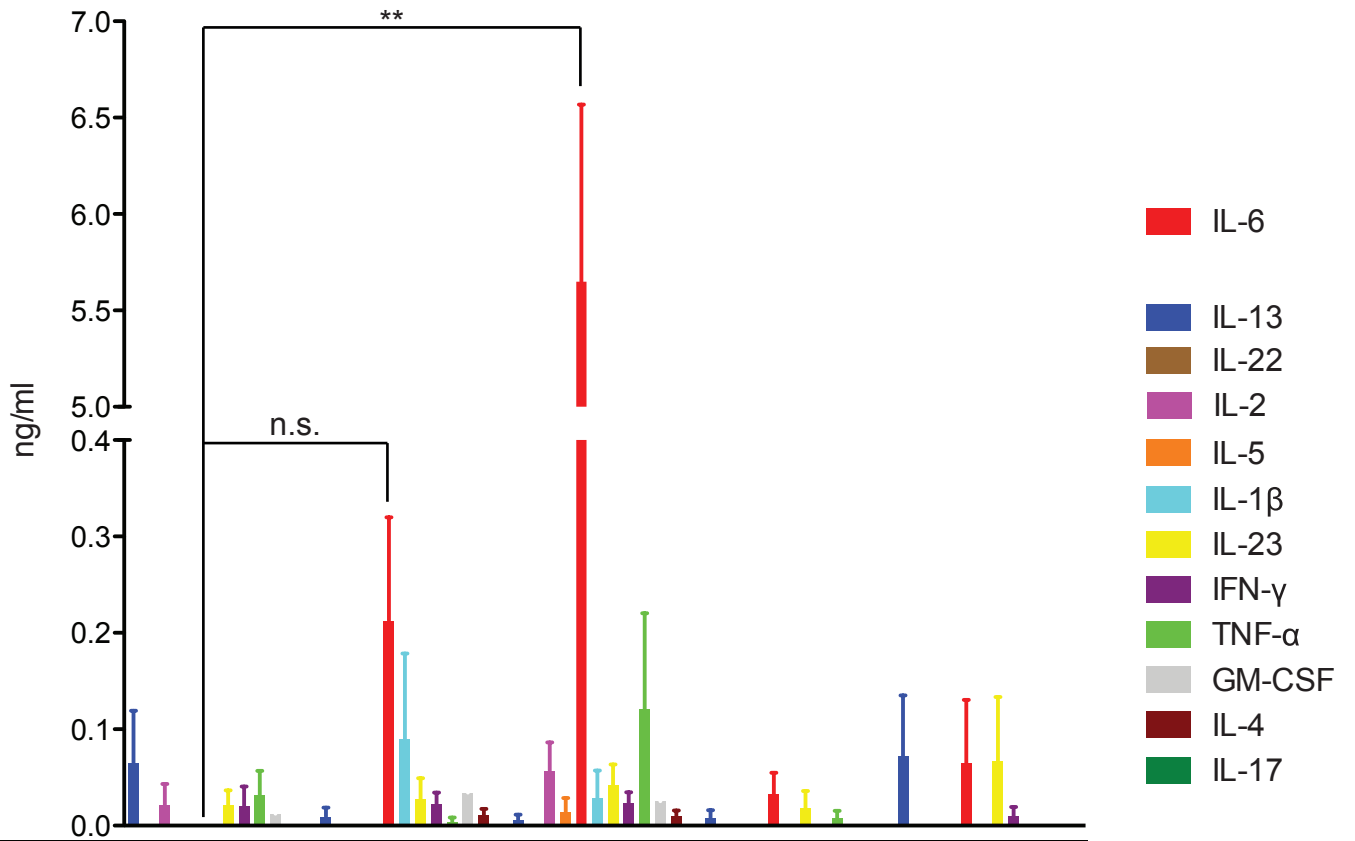
**A****B**

Figure 1



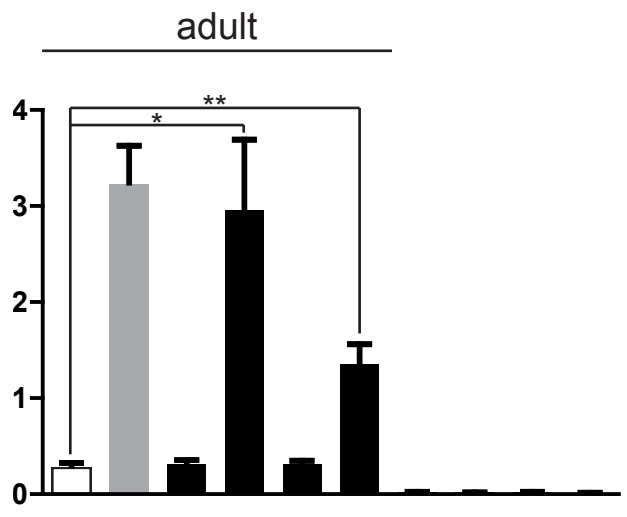
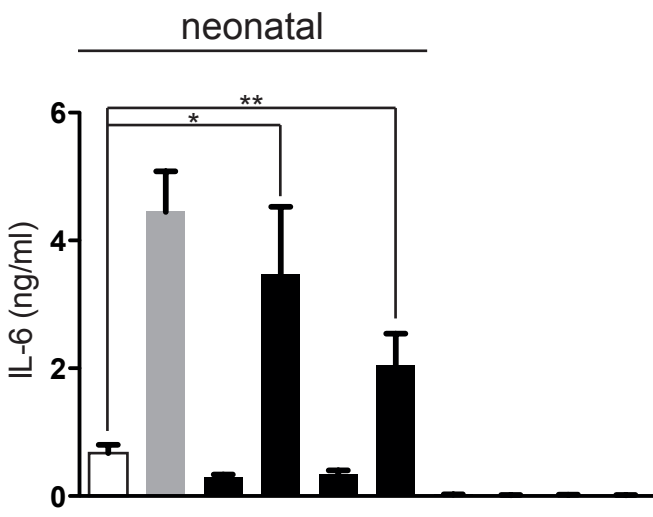
neonatal



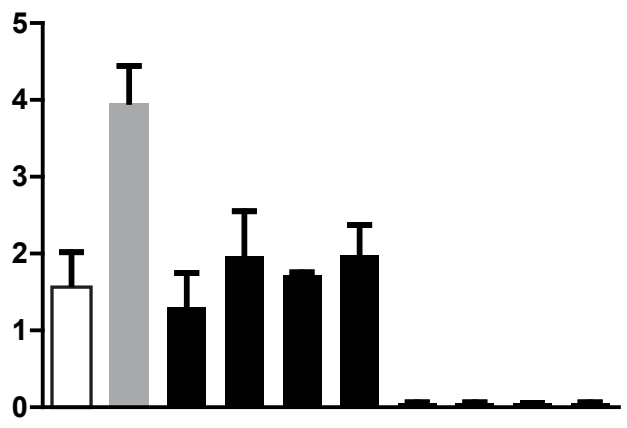
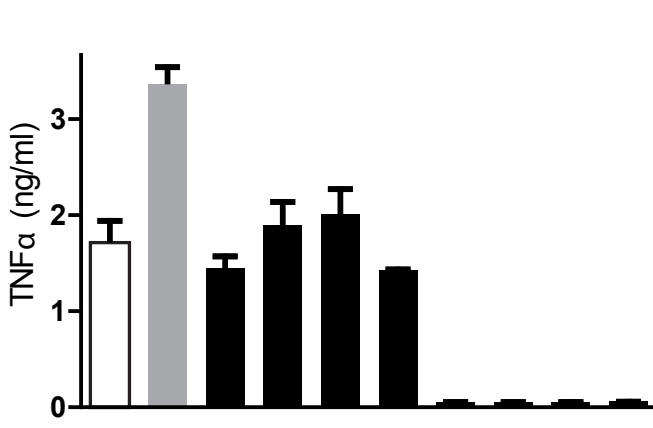
microglia	+	+	+	-	-
CD133 <sup>-</sup>	-	+	-	+	-
CD133 <sup>+</sup>	-	-	+	-	+

Figure 2

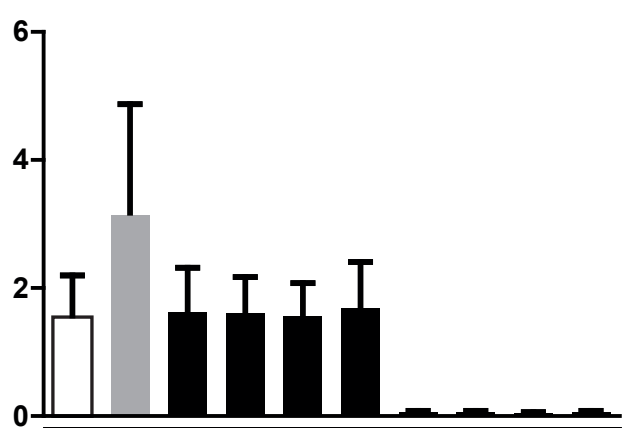
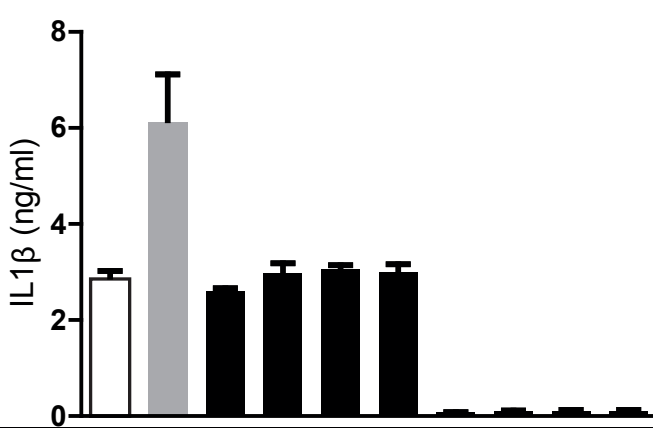
**A**



**B**



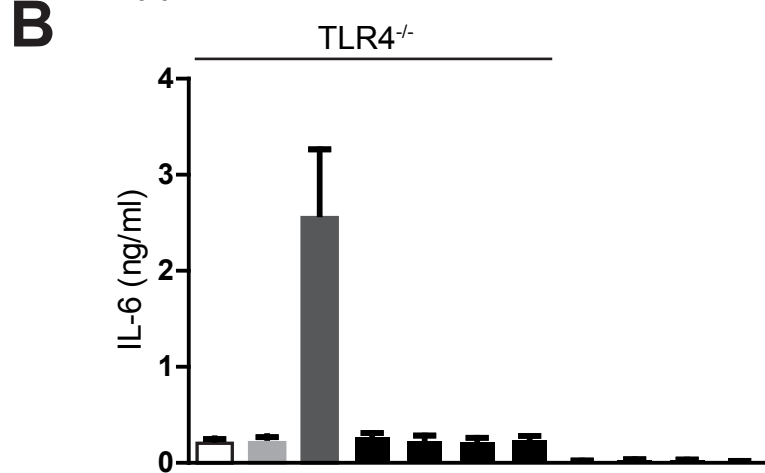
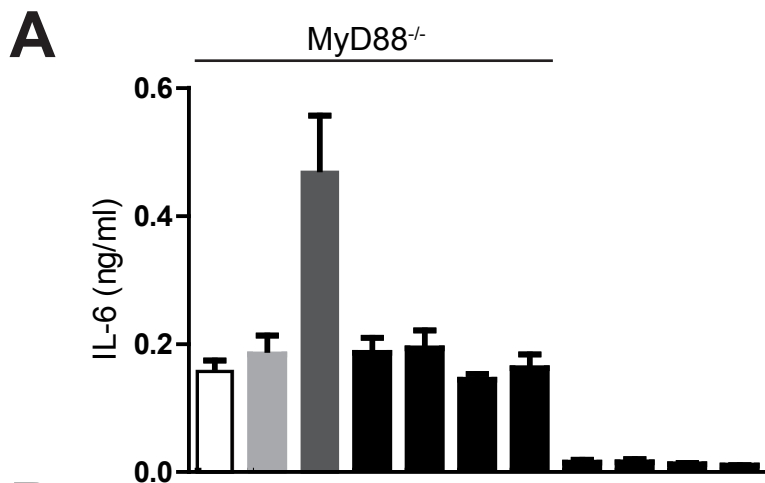
**C**



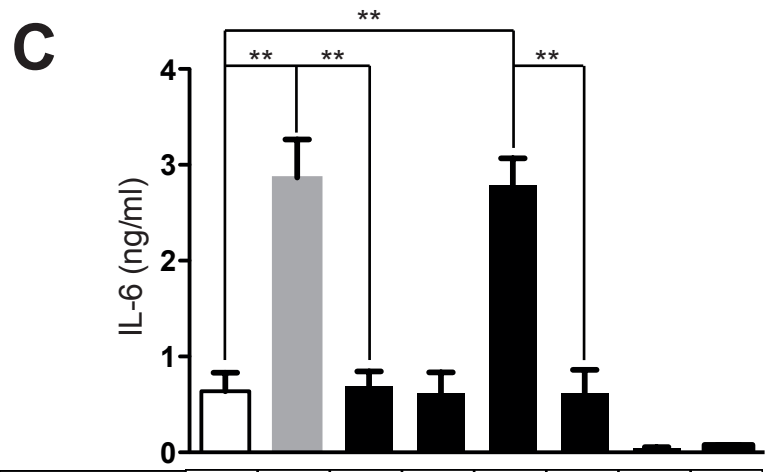
microglia	+	+	+	+	+	+	-	-	-	-
LPS	-	+	-	-	-	-	-	-	-	-
AC-GL261	-	-	+	-	-	-	+	-	-	-
NS-GL261	-	-	-	+	-	-	-	+	-	-
CD133 <sup>-</sup>	-	-	-	-	+	-	-	-	+	-
CD133 <sup>+</sup>	-	-	-	-	-	+	-	-	-	+

+	+	+	+	+	+	-	-	-	-
-	+	-	-	-	-	-	-	-	-
-	-	+	-	-	-	+	-	-	-
-	-	-	+	-	-	-	+	-	-
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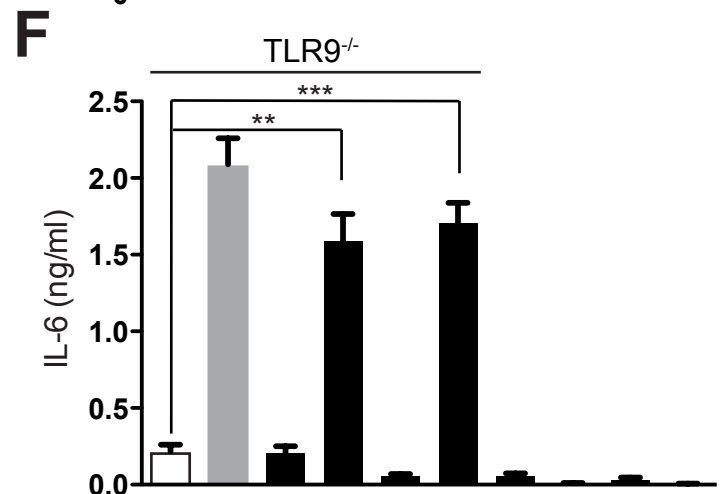
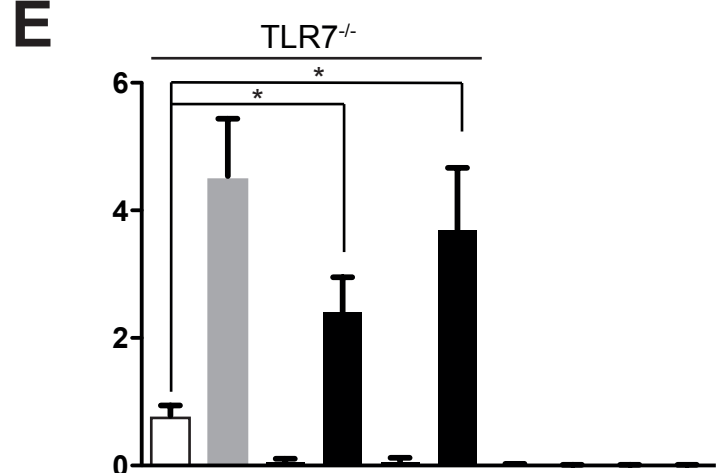
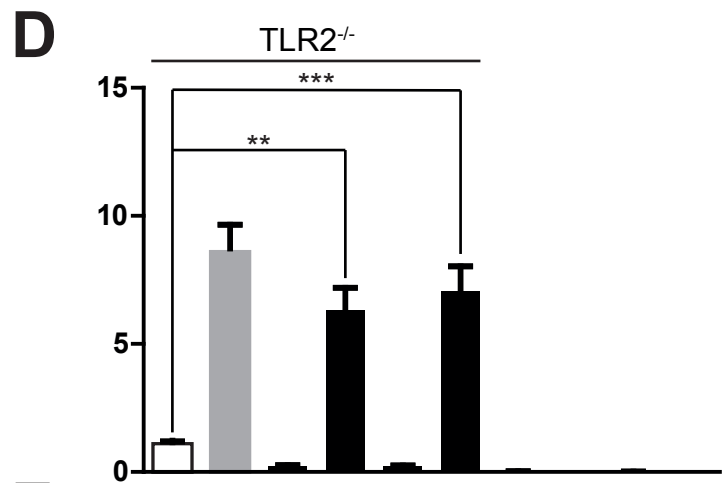
Figure 3



microglia	+	+	+	+	+	+	+	-	-	-	-
LPS	-	+	-	-	-	-	-	-	-	-	-
Poly I:C	-	-	+	-	-	-	-	-	-	-	-
AC-GL261	-	-	-	+	-	-	-	+	-	-	-
NS-GL261	-	-	-	-	+	-	-	-	+	-	-
CD133 <sup>-</sup>	-	-	-	-	-	+	-	-	-	+	-
CD133 <sup>+</sup>	-	-	-	-	-	-	+	-	-	-	+



microglia	+	+	+	+	+	+	-	-
LPS	-	+	+	-	-	-	-	-
Isotype	-	10µg/ml	-	-	10µg/ml	-	-	-
Anti-TLR4	-	-	10µg/ml	-	-	10µg/ml	-	-
CD133 <sup>-</sup>	-	-	-	+	-	-	+	-
CD133 <sup>+</sup>	-	-	-	-	+	+	-	+



microglia	+	+	+	+	+	+	-	-	-	-
LPS	-	+	-	-	-	-	-	-	-	-
AC-GL261	-	-	+	-	-	-	+	-	-	-
NS-GL261	-	-	-	+	-	-	-	+	-	-
CD133 <sup>-</sup>	-	-	-	-	+	-	-	-	+	-
CD133 <sup>+</sup>	-	-	-	-	-	+	-	-	-	+

Figure 4

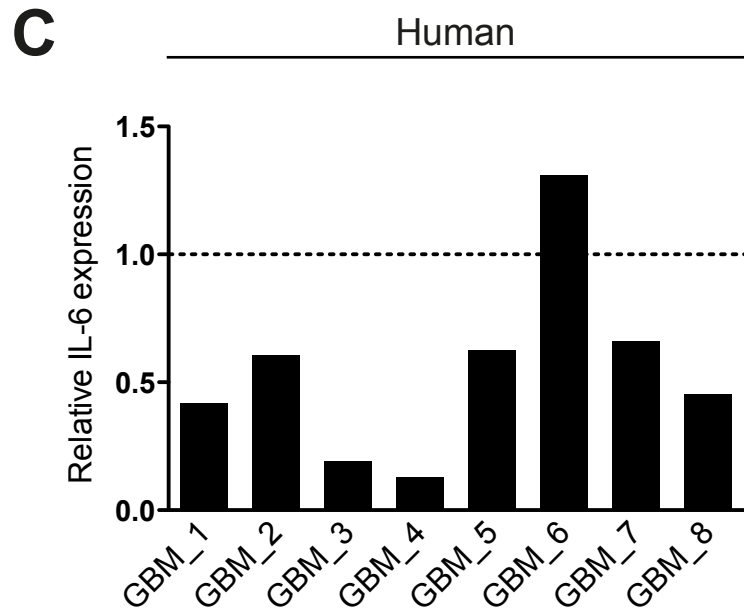
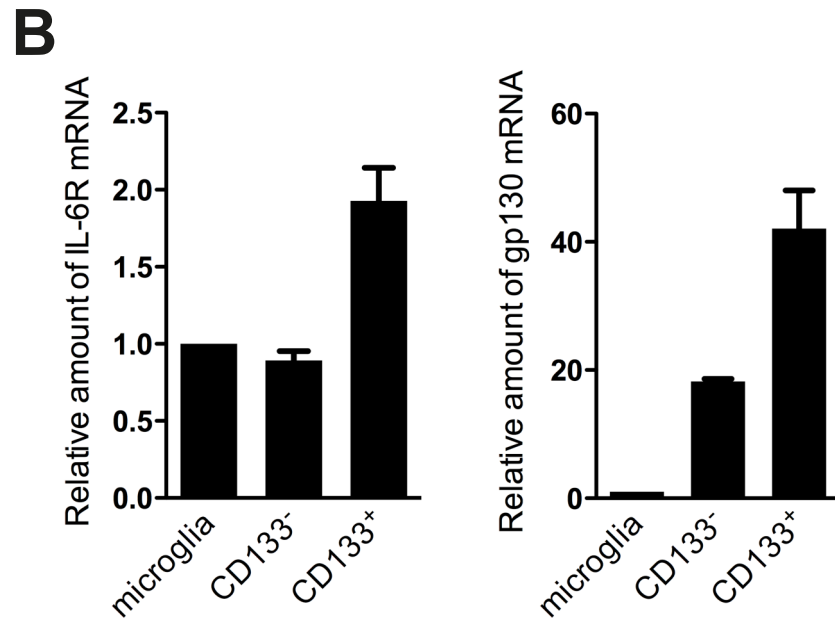
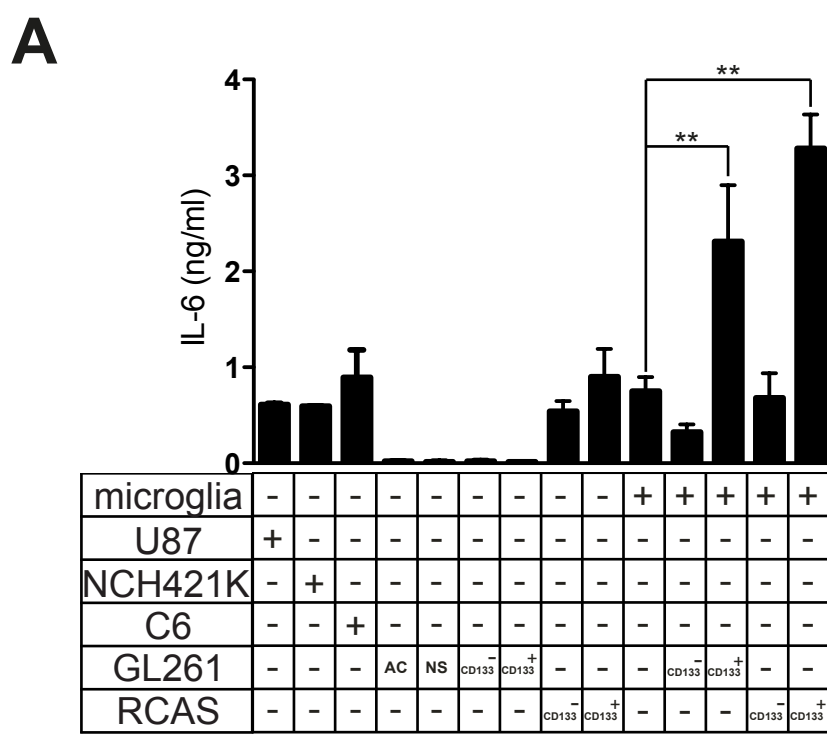


Figure 5

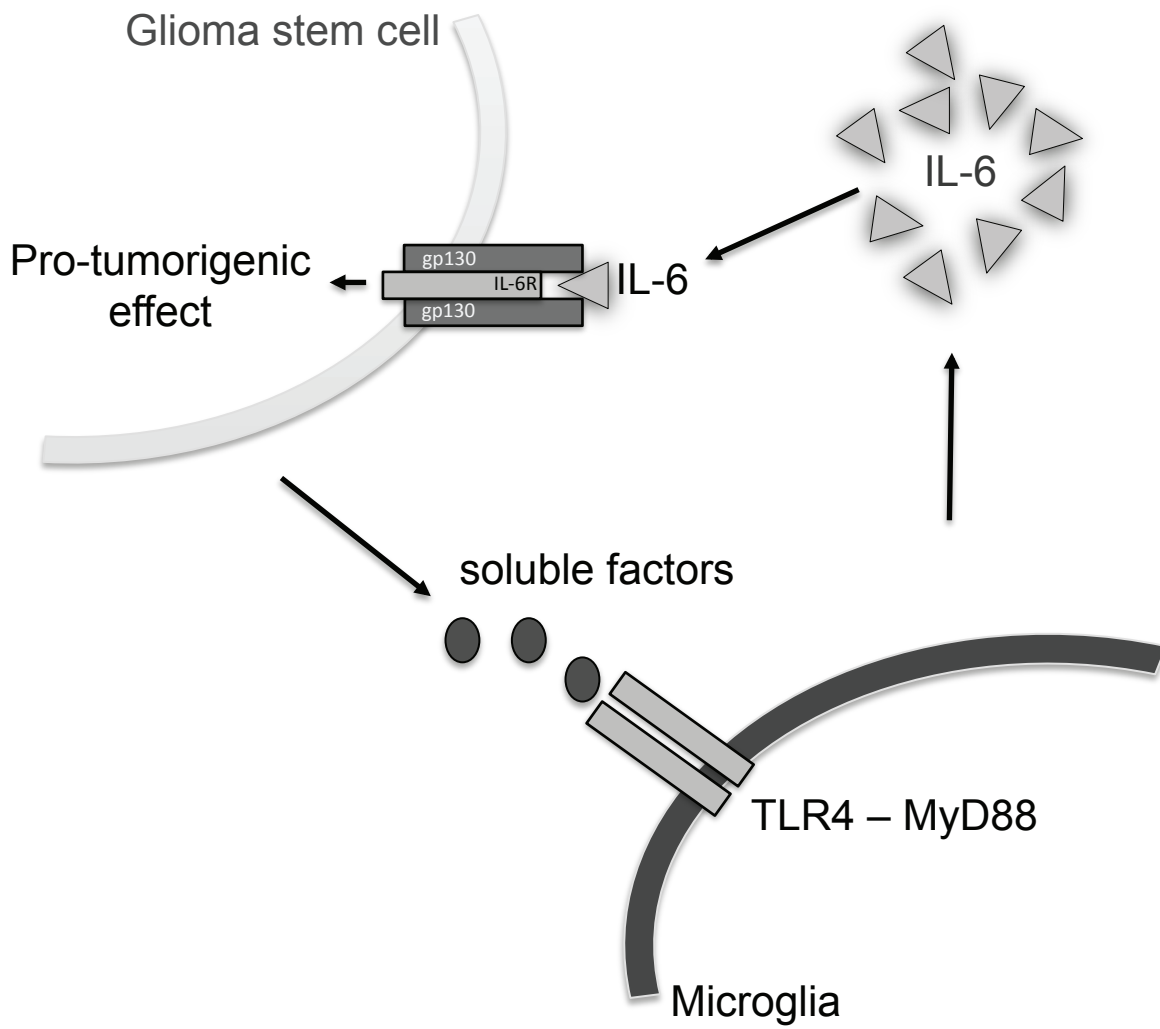


Figure 6