Supporting Information

A Microchip Platform for Interrogating Tumor-Macrophage Paracrine Signaling at the Single-Cell Level

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Supporting information includes Supporting Methods, Supporting Figures 1-8, and Supporting Tables 1-2.

Supporting Methods

I. Cell Culture and Preparation

Culture of established cell line. The U-87 MG (HTB-14) human glioma and the U937 human monocytes were purchased from ATCC (American Type Culture Collection). 100 µL of the macrophage immune cell line was stimulated with 1 µL 20 µg/mL phorbol 12-myristate 13-acetate (PMA/Fisher) according to standard protocols for macrophage differentiation. Cells were grown at 37 °C with 5% CO_2. The U-87 cells were detached with trypsin (Gibco, Invitrogen) and maintained in MEM medium (Gibco, Invitrogen), 10% fetal bovine serum (FBS/ATCC). The immune cell lines were cultured in RPMI 1640 medium (Gibco, Invitrogen), 10% FBS (ATCC).

Live cell tracking dye staining. U-87 and U937-differentiated macrophages were grown via standard procedure for 4 days. The cells were detached using trypsin, harvested by centrifugation, and the supernatant was removed. The U-87 cells were resuspended in prewarmed green CellTracker dye solution, while macrophages were resuspeneded in prewarmed red CellTracker dye solution for subsequent identification (CellTracker™, green CMFDA, Red CMTPX, INVITROGEN. CellTracker dye solution consisted of 1 mg of dry dye powder was dissolved in research-grade DMSO (Dimethyl sulfoxide) to a final concentration of 10mM. The final working concentration of cell suspension, including dye, was 25 µM in fresh medium. The cells were incubated for 45 minutes in a standard tissue incubator. Subsequently, the cells were collected by centrifugation. The dye solution was replaced with fresh medium. This washing step was repeated gently three times with fresh medium. Cell viability, proliferation and functionality were not affected by CellTracker dyes compared to non-fluorescence labelled cells (data not shown).

II. Population Analysis Device and Methods

The population secretion assay platform. The simple in-house cell-culturing platform consists of two components- a glass slide and a PDMS slab (A (10): B(1), RTV615, Momentive)- that

were punched to form two single wells and one double-well (D ~ 1cm). The PDMS component was treated with oxygen plasma prior to bonding on a glass slide. The cell-culturing platform allows imaging of the cultures while providing long term culturing conditions for the cells.

Detecting proteins in culture medium. A custom antibody microarray was patterned on a poly-L-Lysine (Sigma) glass slide using a microarraryer (Spotbot3/Arrayit). The printed array consisted of triplicates of 16 antibody spots. The printed antibody glass slide after functionalization was incubated in a wet box overnight for antibody stabilization. To be able to load the collected supernatants from cell suspensions, a PDMS reservoir was bonded to antibody printed glass slide. Upon blocking the assembled microELISA platform with 3% BSA solution for 2 hours, the collected cell supernatants were loaded into different wells and the system incubated for 1 hour. The standard surface-bound immunoassay procedure for protein signal detection was performed and the expression levels of the proteins were analyzed as explained below.

III. Single Cell Level Heterotypic Analysis Microchip and Methods

High density antibody barcode array slide. Antibody patterning of antibody barcode slide (the second component of the single-cell microchip) was achieved via a custom technique of "flow patterning" using a removable microfluidic chip. During flow patterning, antibodies were flown through the channels of the microfluidic chip via inlets in a N2 pressurized setup. The design of the antibody flow pattering microfluidic chip allowed pattering up to 20 different antibodies with 20-µm widths and 40-µm pitches in a custom "spatial encoding" as described in Lu et al. (18), allowing for each microwell to interface with at least 2 full 20-channel barcode sets. The microfluidic chip was fabricated based on soft lithography techniques and the soft polymer PDMS (A (10): B(1), RTV615, Momentive). Fabrication of the antibody barcodes was explained in detail in Lu et al. (23). The PDMS device was sonicated with ethanol 70% and 2-propanol before its thermal bonding (80 °C for 2 hours) with poly-L-lysine (Erie Scientific) glass slide.

2.0 uL of each antibody (**Supplementary Table 1 and 2**) at 0.5 mg/mL was injected into an individual microchannel of the flow patterning device and flown through the microfluidic channels until antibodies were immobilized and functionalized to the poly-L-lysine glass slide via covalent surface chemistry, typically after 6 hours of N2 pressurized flow during the flow patterning technique. The microfluidic flow chip was subsequently removed and discarded. The antibody barcode slide was later blocked as described and used in the single-cell microchip device. Batch uniformity analysis was performed on the manufactured antibody barcode chips as previously described in Lu et al. (18).

Single-cell suspension preparation. Green-stained U-87 and red-stained U937-derived macrophages were mixed 1:1 ratio. The final cell number of the cell suspension were set an optimal concentration range of ~ 10^6 cells/ml. Using a 200 uL pipette, the cells were pipetted on the surface of the single-cell PDMS reservoirs and allowed cells to sediment into individual wells for 5 minutes. By gravity, cells (including single and multicell homotypic and heterotypic combinations following a Possion distribution) randomly fell into microchambers within the 5500 microwell array. For the analysis presented herein, microwells that had 0 cells, single U-87 cells, single macrophages, and heterotypic combinations (mentioned in the main text) were chosen (n= 0-5) for signal extraction after cell counting via the data analysis steps of the assay (see later section).

Single Cell Microchip Operation. The high- density antibody encoded glass slide and casted

PDMS microchamber array were blocked with 3% BSA solution for 2 hours and then rinsed with fresh cell medium prior to loading single cell suspension on the PDMS microchamber array. To isolate cells in the microchambers, 50 – 250 µL cell suspension was pipetted over the microchamber array and spread across the surface to ensure uniform coverage. After cells were settled in the microchambers (5 – 10 minutes), the antibody patterned barcode glass slide was positioned on top of the microwell array with the barcode facing down, allowing each microwell to interface with 2 antibody barcode sets for functional phenotyping by microwell. The assembled device was clamped with 2 custom polycarbonate clamping plates and 6 screws, adjusted to equal clamping force. The device was imaged on the microscope to record the locations of the cells, and then it was transferred to the tissue incubator for 24 hours for protein secretion. Accompanying steps were typical of an ELISA detection sequence after unclamping the sandwich and removing the glass slide.

On-chip immunosandwich detection. A 250 uL mixture of biotinylated detection antibodies (Supplementary Table 1) in 1:200 ratio to 3% BSA were added on the antibody glass slide of the single-cell microchip device and incubated for 45 minutes at RT prior to washing with 3% BSA solution, as typical of standard ELISA procedures. Subsequently, APC dye-labeled streptavidin (eBioscience, 200 µL, 5 µg/mL) was pipetted on the glass slide and allowed 45 minutes of incubation for protein detection, a standard protocol for ELISA antibody sandwich arrays. It should be noted only one fluoroprobe is needed as antibodies are "spatially encoded" on the barcode device by their flow channel number and thus more than one dye or probe is not necessary for multiplexed protein analysis. Afterwards, 3% BSA was used to wash and block the glass slide for 30 min. Following steps included rinsing the glass slide in DPBS (Dulbecco's Phosphate-Buffered Saline) twice, and then rinsing in distilled water twice in 45 mL falcon tubes sequentially. The barcode slide was then gently dried using pressurized air for subsequent imaging and storage. Conditions of this workflow were optimized for high signal- to-noise ratio and signal processing in the accompanying imaging steps.

Optical read-out, data quantification, processing and statistics. Accompanying the assay were two distinct imaging steps for (1) the detection, counting, and spatial localization of cells within the microchambers of the sandwiched single-cell microchip, and (2) the detection of bound proteins after the assay incubation and immunosandwich assay-based detection steps described above. For the cell counting, a brightfield, phase contrast, and fluorescent images were taken following device assembly and before single cell microchip incubation. For the completion of this step, the chip was imaged by a Nikon Eclipse confocal digital microscope with CCD camera array (Nikon) and, due to the large field of view of the chip, a motorized stage (Prior, ProScan III) was used to collect 186 images per microwell array, subsequently stitched together by ELEMENTS software (Nikon). The combined image was used to count the number of cells and their cell line identity as well as determine their spatial location on chip in relation to the high throughput microwell array for subsequent matching to protein detection methods (18). Cell counting was achieved via a custom automated cell counting software (DETECT, IsoPlexis) and, for uniformity, checked via a manual image processing program (ELEMENTS, Nikon).

In the second imaging step, protein signals were detected from the whole-slide antibody barcode array in a microarray scanner (Genepix 4200A, Molecular Devices). FITC (488 – blue) and APC (635 – red) channels of Genepix 4200A scanner (Molecular Devices) were used during scanning acquisition at 500 PMT Gain/50 Laser power. FITC was flown onto the chip during manufacture as a spatial marker for counting antibody channels during the protein signal extraction processing steps. Signal analysis was performed using GenePix Pro software (Molecular Devices) and a

custom automated signal processing software (DETECT, IsoPlexis). Protein signals were extracted by matching a microwell array block features to the flow patterning lines with precise alignment via contrast enhancement such that, for each microwell, features corresponding to each antibody were identified and the mean photon counts per each antibody feature were extracted and associated with the microwell. A resultant data matrix of 16 antibody photon counts by 5000+ microwell blocks was subsequently matched, by spatial location and image overlay, to the microwell cell counts, identities, and cell locations. The matrix was grouped by 0, single macrophage, single glioma, and combination identities for further processing. Zero cell wells were used as on-chip controls for background subtraction per antibody.

Limited noise and background reduction was performed on chip. Local antibody-specific background subtraction in all cell-containing wells using zero cell wells (excluding 5% outliers) as control on chip (Raw photon counts- [zero cell well mean + 2STDV]) was performed for each antibody. The additional 2STDV serves to control spurious background fluctuations and is used as an activity "gate" similar to flow cytometry. Typical background ranges were 200-800 photon counts. Global background subtraction using a non-functionalized region of the chip as a negative control to account for background due to general chip noise (not associated with antibody patterning) was also performed with typical subtractions of 0-70 photon counts. For graphical visualization techniques (i.e. heat maps), data was also normalized by antibody using Z-scores.

The extracted data was compiled using Excel (Microsoft). Cluster/Treeview (Eisen Laboratory) was used to obtain the heat maps. Prism 5 (GraphPad) and R (R Development Core Team) were used for statistical analysis and other graphical formats.
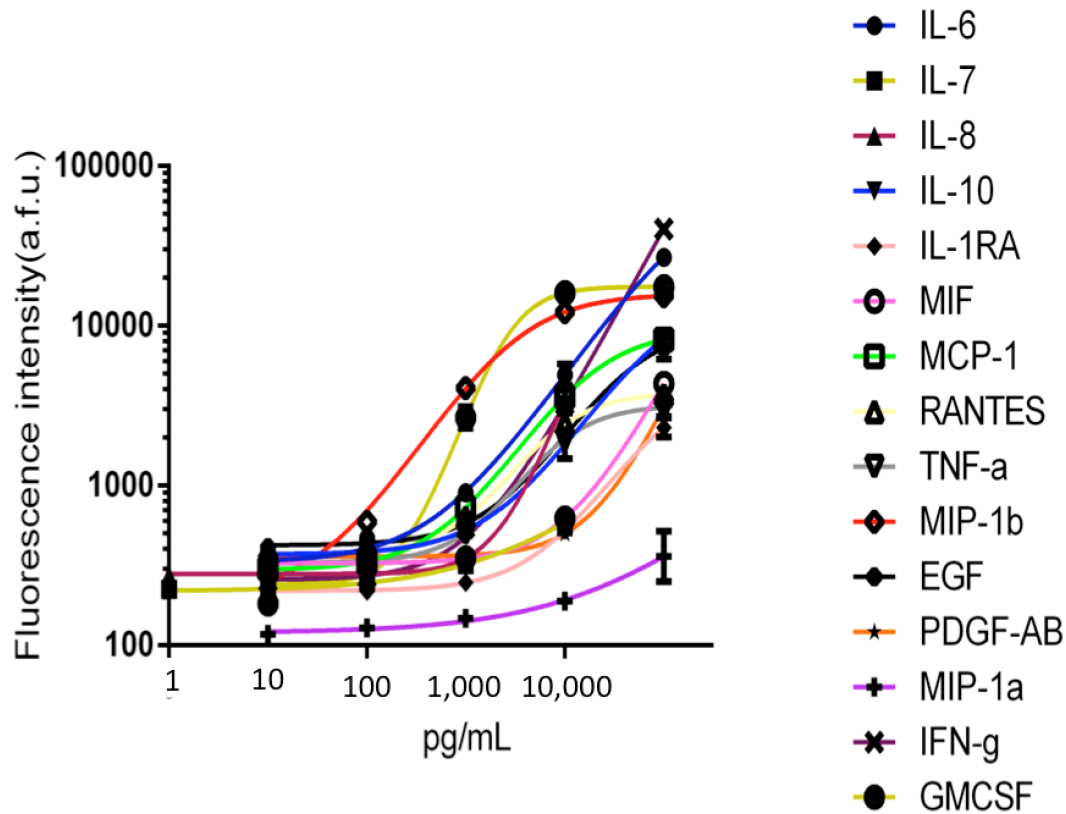
Supporting Figures
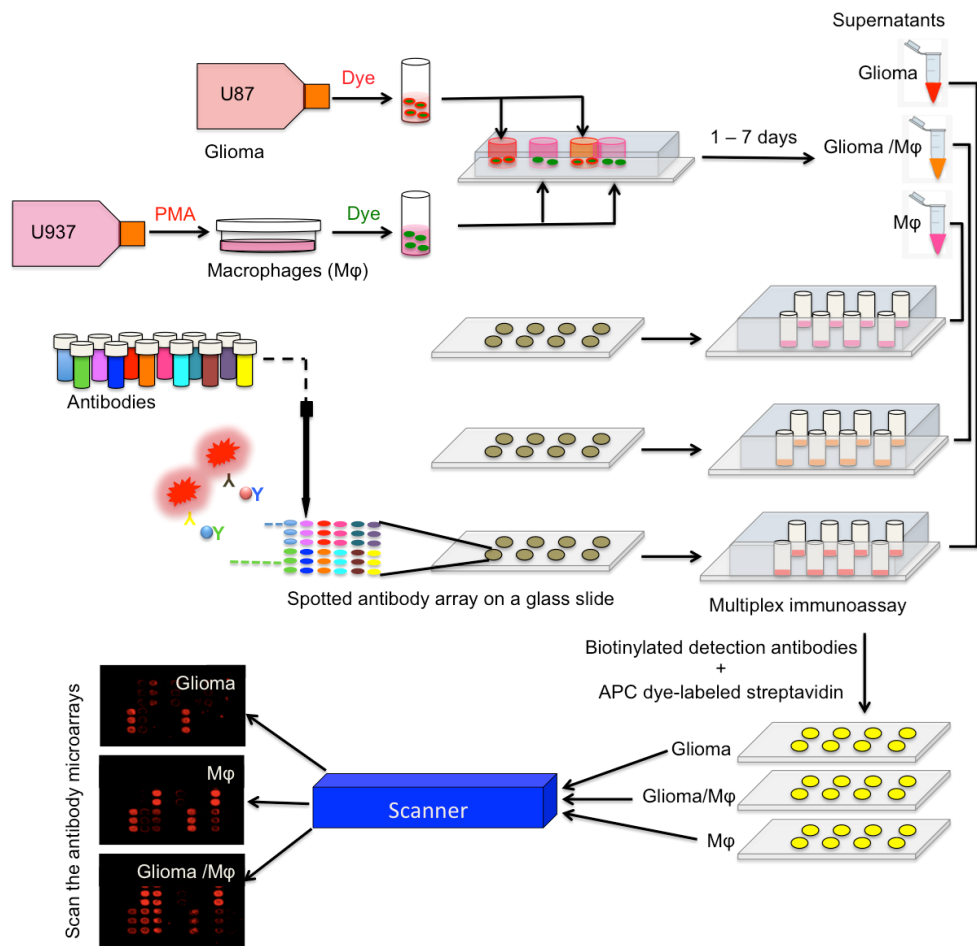
Supporting Figure 1. Work flow for population level secretomic assay. Human U-87 glioma and human U937 monocytes were grown in MEM medium and RPMI-1640 medium, respectively. U937 cell line was differentiated to macrophages (MΦ) using standard differentiation protocol with PMA (phorbol 12-myristate 13-acetate). The glioma cells were stained with green; the MΦ cells were stained with red cell tracker dyes. Stained cells were loaded into the cell-culturing platform that has two isolated wells and a co-culture well for glioma, MΦ, glioma/MΦ communication, respectively. Upon 24 hours of growth, the supernatants were collected from the wells and loaded into a custom microELISA chip with 16 patterned antibodies on a poly-L-Lysine slide and a PDMS slab to load supernatants. Standard ELISA procedures were followed and the slide was then scanned to detect and analyze protein secretions.

| Glioma (U87)	MΦ (U937-derived)	Co-culture	Proteins Detected
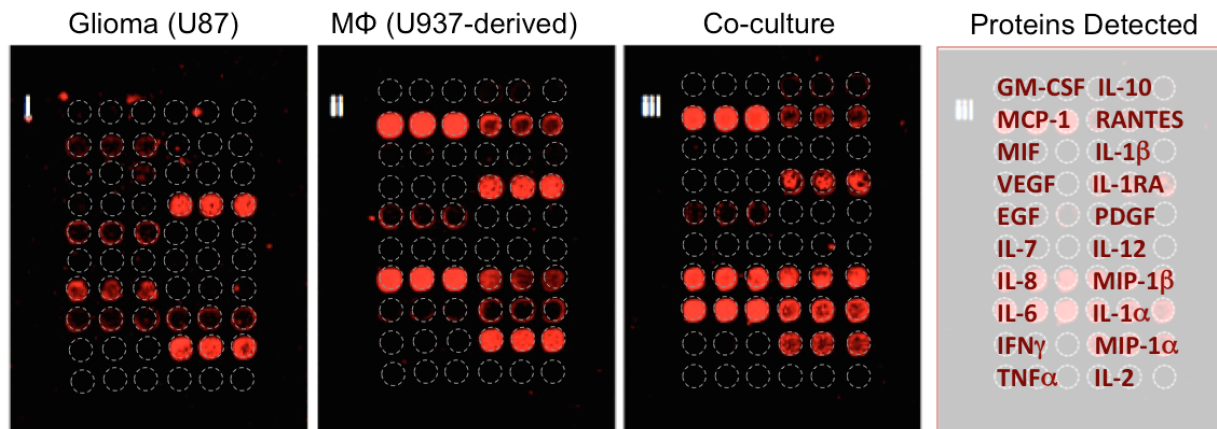
Supporting Figure 2. Raw scanned fluorescence images of the pin-spotted antibody microarrays used to measure population protein secretion. Cell culture media were collected after 24 hours of incubation of (i) glioma, (ii) macrophage (MΦ) and (iii) glioma-macrophage co-culture. Microarrays were read out using APC dye-labeled streptavidin at 635 nm (Red). Spots are 150μm in diameter.

Supporting Figure 3. Cross-reactivity assay. Recombinant proteins used to perform cross-reactivity assay to check whether antibody pairs are of sufficient specificity against each other. The antibodies were flow patterned to poly-L-lysine glass slide as the same way prepared for single-cell secrotemics chip assembly. A PDMS microchannel patterned slab was aligned on top of the flow patterned antibody array and each recombinant antigen at 10 ng/mL was loaded into a separate 100 um microchannel by pressure driven flow, each interfacing with the full antibody barcode. The assembly were kept 4 hours at room temperature and then ELISA based detection performed. Optical readout and data processing was applied similarly to methods for the single cell microchip to determine, across the barcode, levels of protein capture detected per antibody per recombinant protein. The VEGF has slight cross-reactivity compared to other antibodies. Experiments for excluded antibodies did not generate results due to possible low-performance antigens at time of experimentation.

**Supporting Figure 4. Titration test to validate the antibody pairs for a 16-plex immunoassay.** A set of standard protein samples containing a mixture of all 15 human recombinant proteins (VEGF recombinant protein was not working) were spiked in 1% BSA/PBS and introduced to a microfluidic chip interfaced with an antibody barcode microarray. The microchip has 10 straight microchannels (height ~15um), each of which was used to load a recombinant protein solution at a given concentration. After incubation, the barcode array was developed by introducing fluorophore-conjugated detection antibodies and read out by a laser microarray scanner. Because the fluorophores used in the titration experiment were different from the one used in single-cell experiment (Figs. 2 and 3), this study serves as a validation of antibody pairs for sensitivity and dynamic response, but not for calibration curves (specifically, IL-6,IL-7, IL-8, IL-10, IL-1RA, MIF were detected by Alexa 488; PDGF, EGF, TNF-a, MIP-1b, Rantes, MCP-1 were detected by Alexa 532; IFN-g, MIP-1a, GMCSF were detected by biotinylated antibodies followed by binding to APC-straptavidin)
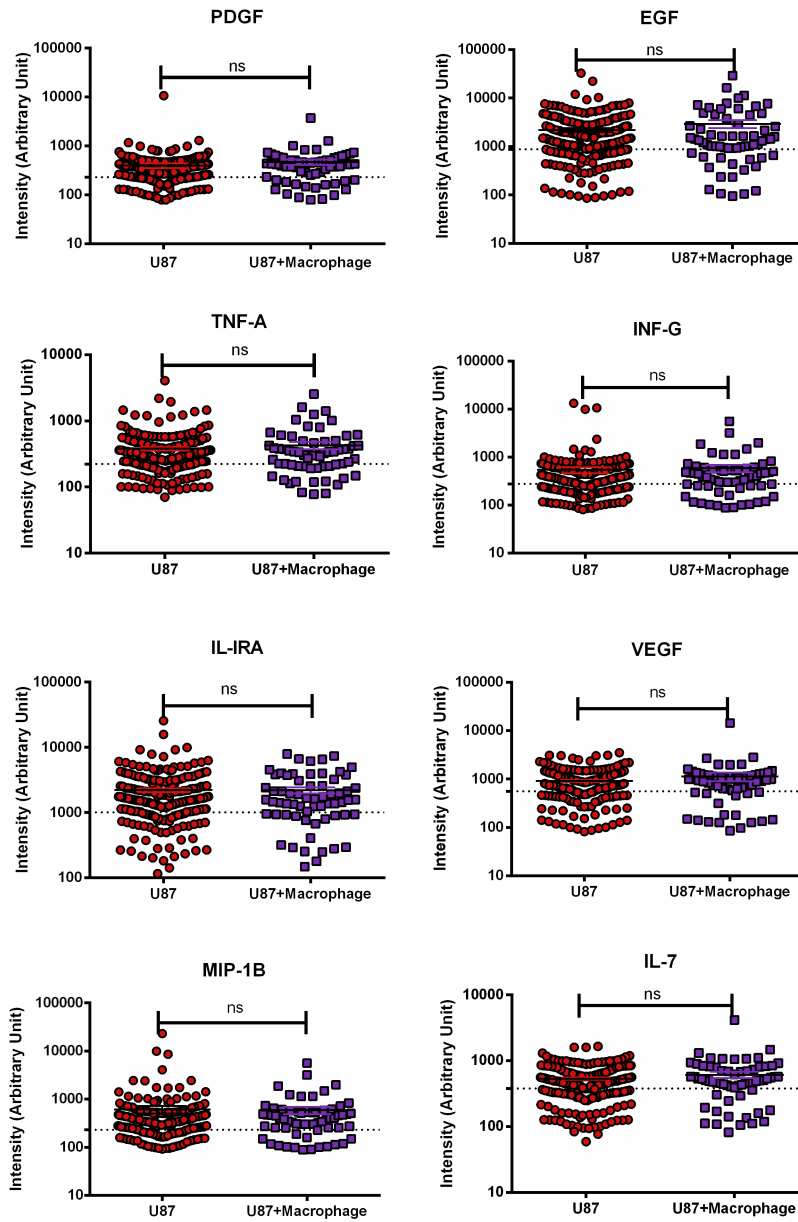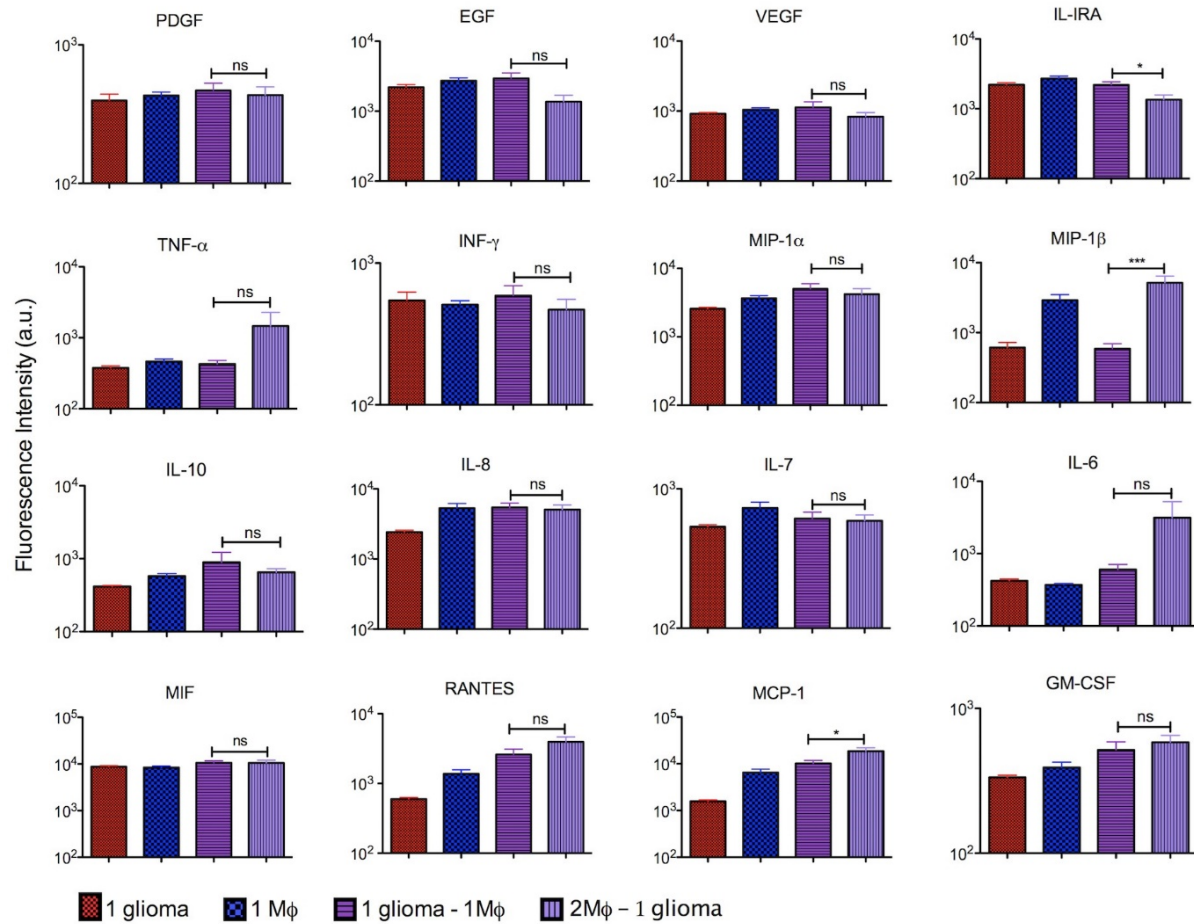
Supporting Figure 5. Scatter plots comparing protein profiles of PDGF, EGF, IL-IRA, VEGF, TNF- α, INF- γ, MIP-1 β and IL-7 from 1 glioma and 1 glioma /1 MΦ microchambers for 20-hour incubation (ns: not significant, n_{glioma} = 245, $n_{1\ glioma/1\ M\Phi}$ = 64). The dashed line shows the threshold gates (mean photon counts measured from empty microchambers as on chip controls + 2STDV) to serve as an active versus non-active secretor indicator similar to flow cytometry gating procedure.
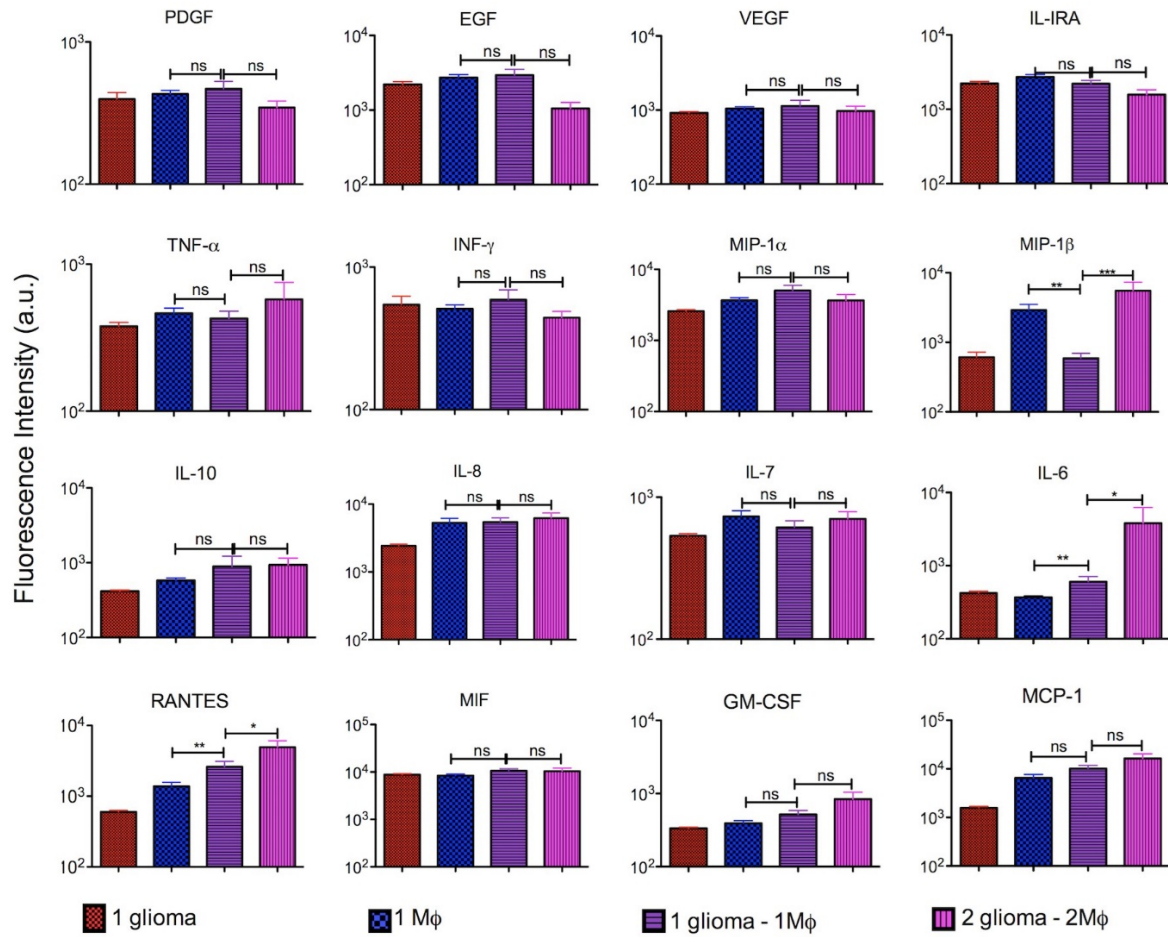
Supporting Figure 6. Effect of glioma-macrophage (MΦ) pair on secreted protein profile of glioma - macrophage communication. Bar plots comparing contribution of "1 glioma -1 MΦ" and "2 glioma -2 MΦ" pairs on PDGF, EGF, VEGF, IL-IRA, TNF-α, INF-γ, MIP-1α, MIP-1β, IL-10, IL-8, IL-7, IL-6, Rantes, MIF, GM-CSF, and MCP-1 protein profiles (ns: not significant, * p < 0.05, ** p< 0.001, ***p < 0.0001, n_{glioma} = 245, $n_{1\,MΦ}$ = 106, $n_{1\,glioma-1\,MΦ}$ = 64, $n_{2\,glioma-2MΦ}$ = 19).
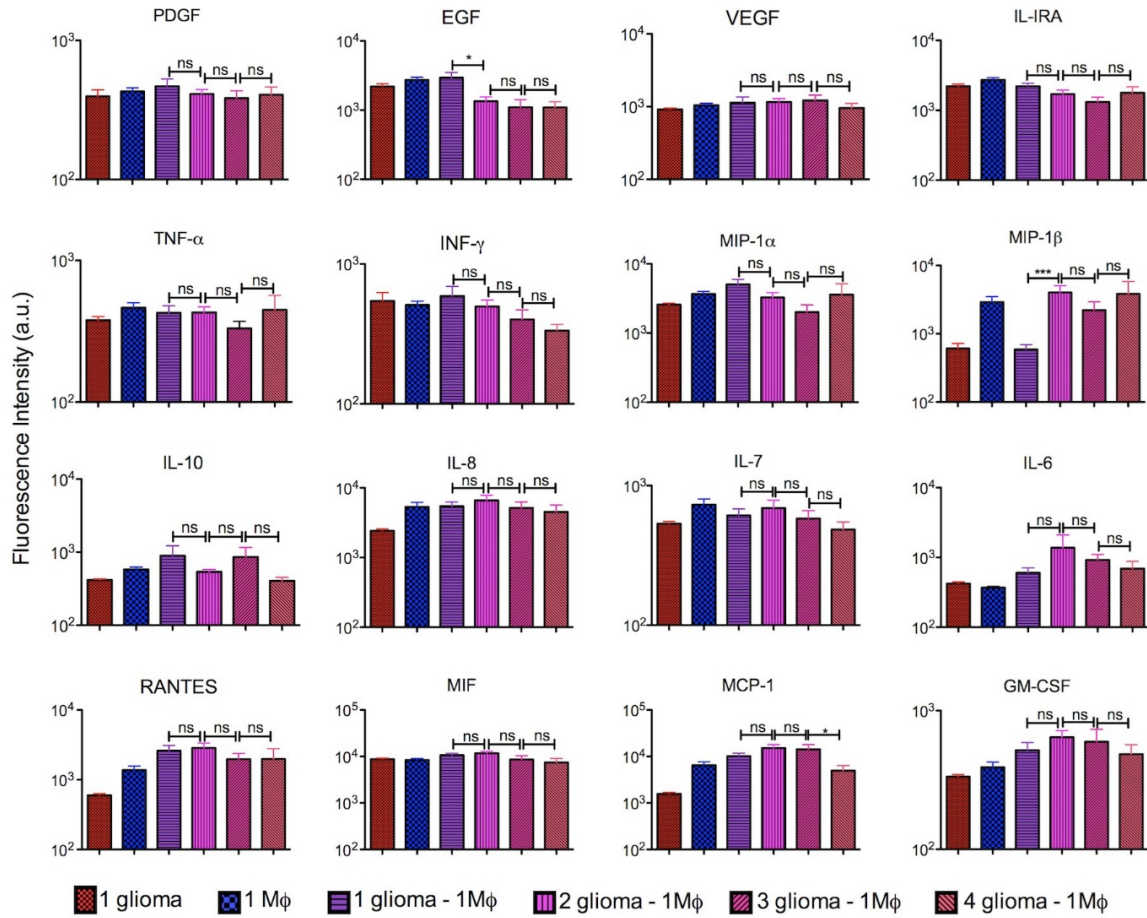
Supporting Figure 7. Effect of glioma number on secreted protein profile of glioma – macrophage communication. Bar plots comparing contribution of 1 glioma -1 MΦ, 2 glioma -1 MΦ, 3 glioma -1 MΦ, 4 glioma -1 MΦ pairs on PDGF, EGF, VEGF, IL-IRA, TNF-α, INF-γ, MIP-1α, MIP-1β, IL-10, IL-8, IL-7, IL-6, Rantes, MIF, GM-CSF, and MCP-1 protein profiles (ns: not significant, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, $n_{1\ glioma} = 245$, $n_{1\ MΦ} = 106$, $n_{1\ glioma-1\ MΦ} = 64$, $n_{2\ glioma-1MΦ} = 36$, $n_{3\ glioma-1MΦ} = 20$, $n_{4\ glioma-1MΦ} = 21$).

Supporting Figure 8. Effect of macrophage number on secreted protein profile of glioma – macrophage communication. Bar plots comparing contribution of 1 glioma -1 MΦ, 2 glioma -1 MΦ, 3 glioma -1 MΦ, 4 glioma -1 MΦ pairs on PDGF, EGF, VEGF, IL-IRA, TNF-α, INF-γ, MIP-1α, MIP-1β, IL-10, IL-8, IL-7, IL-6, Rantes, MIF, GM-CSF, and MCP-1 protein profiles (ns: not significant, * $p < 0.05$, ** $p < 0.001$, ***$p < 0.0001$, $n_{1\ glioma} = 245$, $n_{1\ MΦ} = 106$, $n_{1\ glioma-1\ MΦ} = 64$, $n_{2\ MΦ-1\ glioma} = 31$).

Supporting Tables

Supporting Table 1: List of all 16 proteins assayed in single cell and population microchips and their functions in human physiology.

Protein	Function	Protein	Function
PDGF *(Platelet-derived growth factor)*	Proliferation, angiogenesis, migration.	**IL-10** *(Interleukin-10)*	Anti-inflammatory cytokine.
EGF *(Epidermal growth factor)*	Cellular proliferation, differentiation, and survival.	**IL-8** *(Interleukin-8)*	Chemotaxis and angiogenesis.
IL-IRA *(Interleukin-1 receptor antagonist)*	Inhibits and modulates a variety of interleukin 1 related immune and inflammatory responses.	**IL-7** *(Interleukin-7)*	Proliferation, differentiation.
VEGF *(Vascular endothelial growth factor)*	Angiogenesis, vasculogenesis.	**IL-6** *(Interleukin-6)*	Acute phase response.
TNF-α *(Tumor necrosis factor alpha)*	Acute phase reaction.	**MIF** *(Macrophage migration inhibitory factor)*	Regulation of macrophage function.
INF-γ *(Interferon gamma)*	Macrophage activation.	**RANTES** *(Regulated on activation, normal T cell expressed and secreted)*	Chemotaxis.
MIP-1β *(Macrophage inflammatory protein-1beta)*	Chemoattractant.	**MCP-1** *(Monocyte chemotactic protein 1)*	Recruits immune cells.
MIP-1α *(Macrophage inflammatory protein-1alpha)*	Acute inflammatory response.	**GM-CSF** *(Granulocyte-macrophage colony-stimulating factor)*	Granulocyte and monocyte production.

Supporting Table2. List of primary and secondary antibodies used in this study

Primary antibody (vendor: clone)(catalog No.)	Detection antibody (vendor: clone)(catalog No.)
Mouse Anti-Human IFN gamma (ebio: NIB42)(14-7318)	Anti-Human IFN gamma biotin(ebio: 4S.B3)(13-7319)
Anti-Human TNF alpha Purified(ebio: MAb1)(14-7348)	Anti-Human TNF alpha biotin(ebio: MAb11) (13-7349)
Mouse anti-human RANTES(R&D)(DY278)	Goat anti-human RANTES(R&D) (DY278)
Rat Anti-Human IL-6(ebio: MQ2-13A5)(14- 7068)	Rat Anti-Human IL-6 biotin(ebio: MQ2-39C3)(13-7068)
Rat Anti-Human IL-10(ebio: JES3-9D7)(14- 7108)	Rat Anti-Human IL-10 biotin(ebio: JES3-12G8)(13-7109)
Anti-Human GMCSF(BD)(555126)	Anti-Human GMCSF biotin(BD)(555126)
Mouse Anti-CCL2 (MCP-1)(ebio: 5D3-F7)(14- 7099)	Armenian Hamster Anti-CCL2 (MCP-1) biotin(ebio: 2H5)(13-7096)
Mouse Anti-Human EGF(R&D)(DY236)	Goat Anti-Human EGF Biotin (R&D) (DY236)
Mouse Anti-Human PDGF-AB (R&D)(DY222)	Goat Anti-Human PDGF-AB Biotin (R&D) (DY222)
Mouse Anti-Human CXCL8/IL-8 (R&D)(DY208)	Goat Anti-Human CXCL8/IL-8 (R&D) (DY208)
Mouse Anti-Human VEGF (R&D)(DY293B)	Goat Anti-Human VEGF Biotin (R&D) (DY293B)
Mouse Anti-Human MIF (R&D)(DY289)	Goat Anti-Human MIF Biotin (R&D) (DY289)
Mouse Anti-Human IL-7 (R&D)(DY209)	Goat Anti-Human IL-7 (R&D) (DY209)
Goat Anti-Human CCL3/MIP-1 alpha (R&D)(DY270)	Goat Anti-Human CCL3/MIP-1 alpha (R&D)(DY270)
Mouse Anti-Human CCL4/MIP-1 beta (R&D)(DY271)	Goat Anti-Human CCL4/MIP-1 beta (R&D)(DY271)