ISCI, Volume *23*

Supplemental Information

A Xenotransplant Model of Human Brain Tumors in Wild-Type Mice

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Supplemental Figures

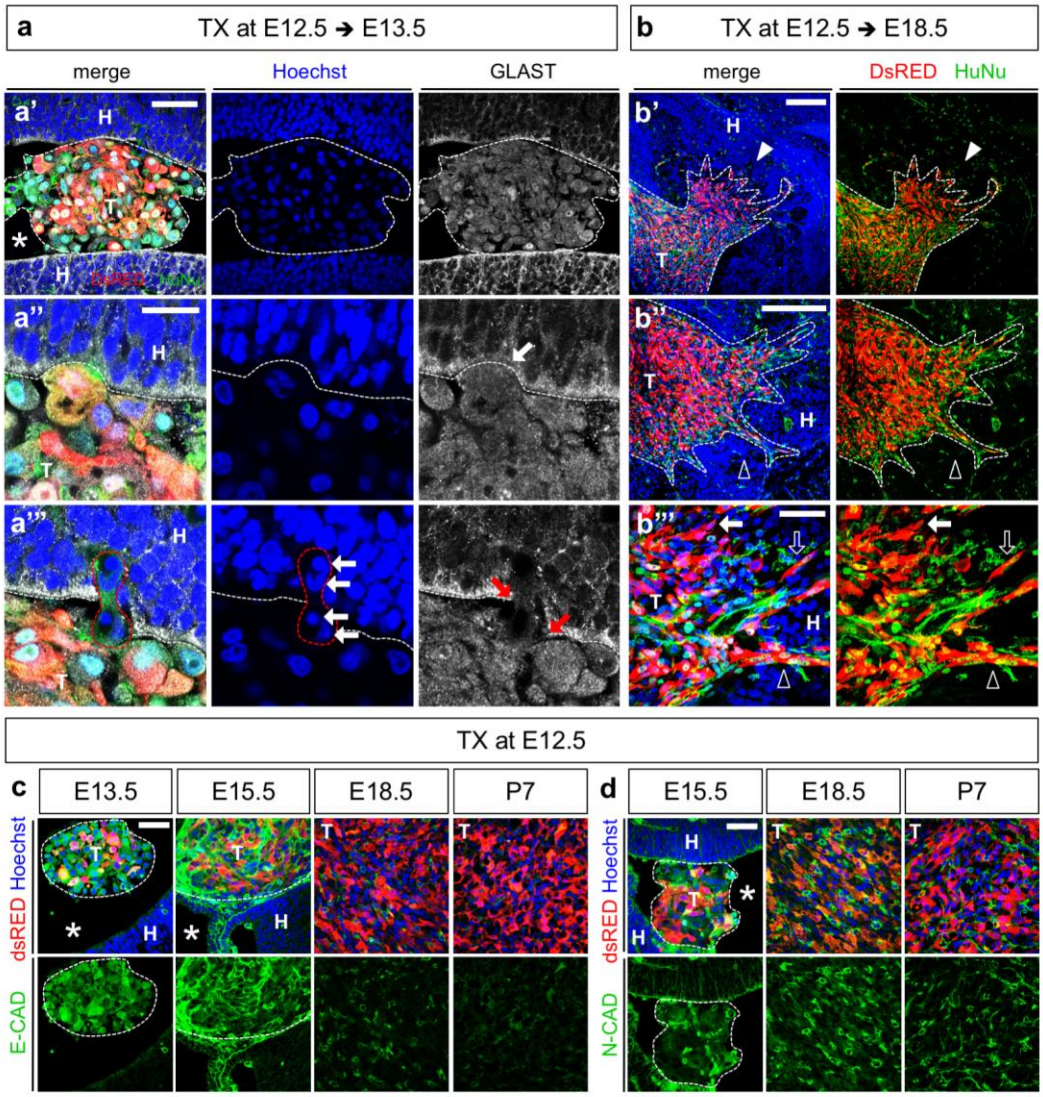

Figure S1. TX show invasive growth pattern and mesenchymal-like features in brains of WT mice, Related to Figure 3.

a-d) Immunofluorescence images of TX of mouse brain cryosections. blue: Nuclear DNA (Hoechst+); green: HuNu, red: DsRED+ U87MG cells. **a'-'''**) images show GLAST staining (white) in TX of mouse brain at E13.5; a'', a''' high magnification. Tumor cells (T); Host tissue (H); dashed line indicates the tumor border; arrows indicate multi-nucleated tumor cell (a'''), tumor cells penetrating apical membrane (a'', arrow); red arrows indicate broken apical membrane after tumor cell invasion; red dashed line indicate invading "leading tumor cell". Scale bar: 100 µm (a'); 10 µm (a''-'''). **b'-''**) images of TX in cross sections through E18.5 mouse brain. b''') high magnification. Scale bars: 200 µm (b', b''); 50 µm (b'''). Arrow, hollow arrow, arrowheads and hollow arrowheads indicate different infiltrative growth pattern of GBMs and routes of invasion. **c-d**) Images show Green: E-CADHERIN (c) or N-CADHERIN (d) of TX in cross sections of mouse brain at indicated times. Note the progressive down-regulation of E-CADHERIN expression. Scale bars: 200 µm.

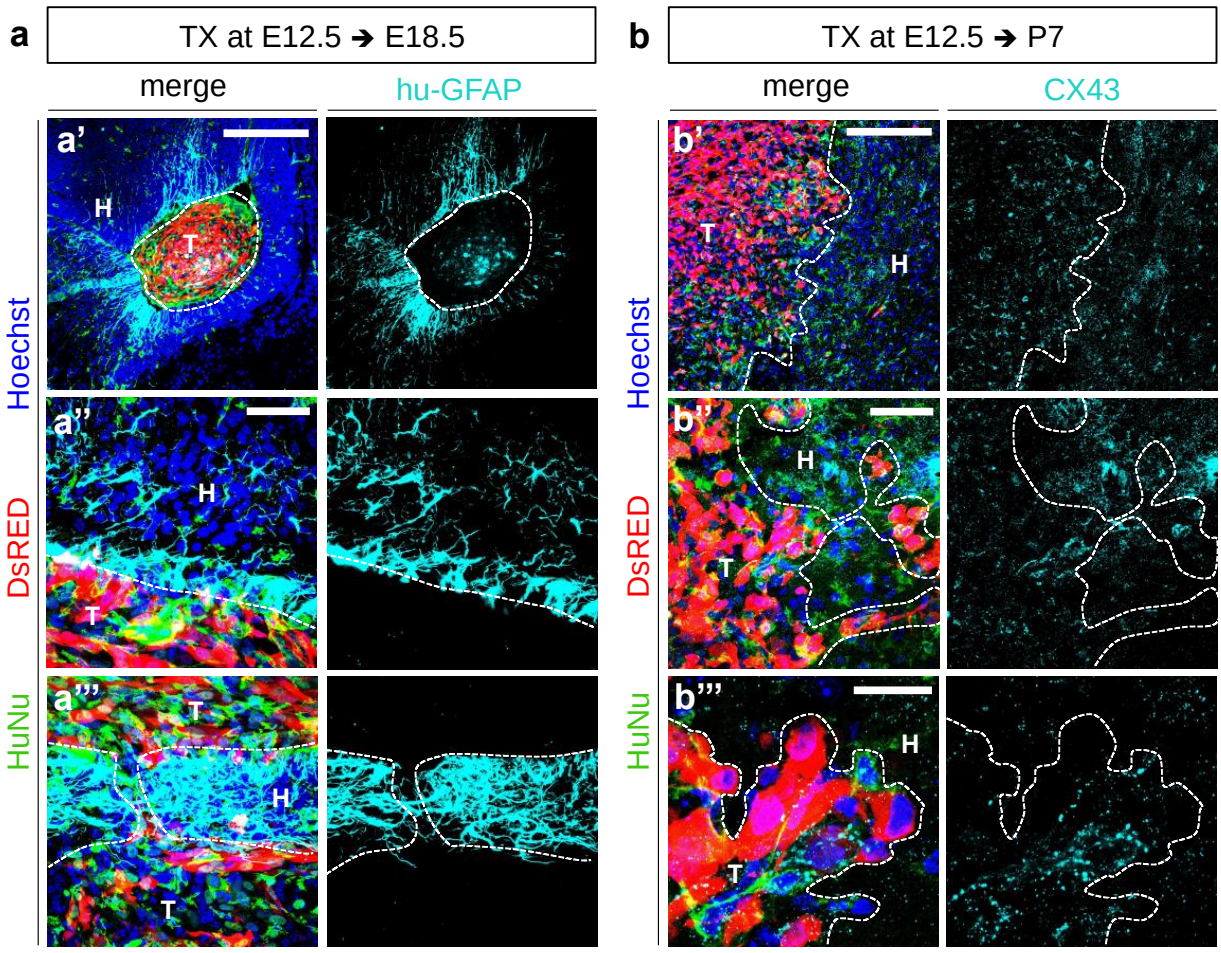

Figure S2. Expression of GFAP in astrocytes and Cx43 in TX, Related to Figure 4.

a) Immunofluorescence images of TX in cryosections of E18.5 embryonic mouse brains; blue: Nuclei (Hoechst+), red: DsRED+ U87MG cells, green: HuNu and cyan: human-specific GFAP. **b)** Immunofluorescence images of TX in cryosections at P7; blue: Nuclei (Hoechst+), red: DsRED+ U87MG cells, green: HuNu and cyan: CONNEXIN-43 (CX43). Tumor (T); Tumor border (dashed lines). Scale bars: 200 µm (a', b'), 50 µm (a'', a''', b'') and 20 µm (b''').

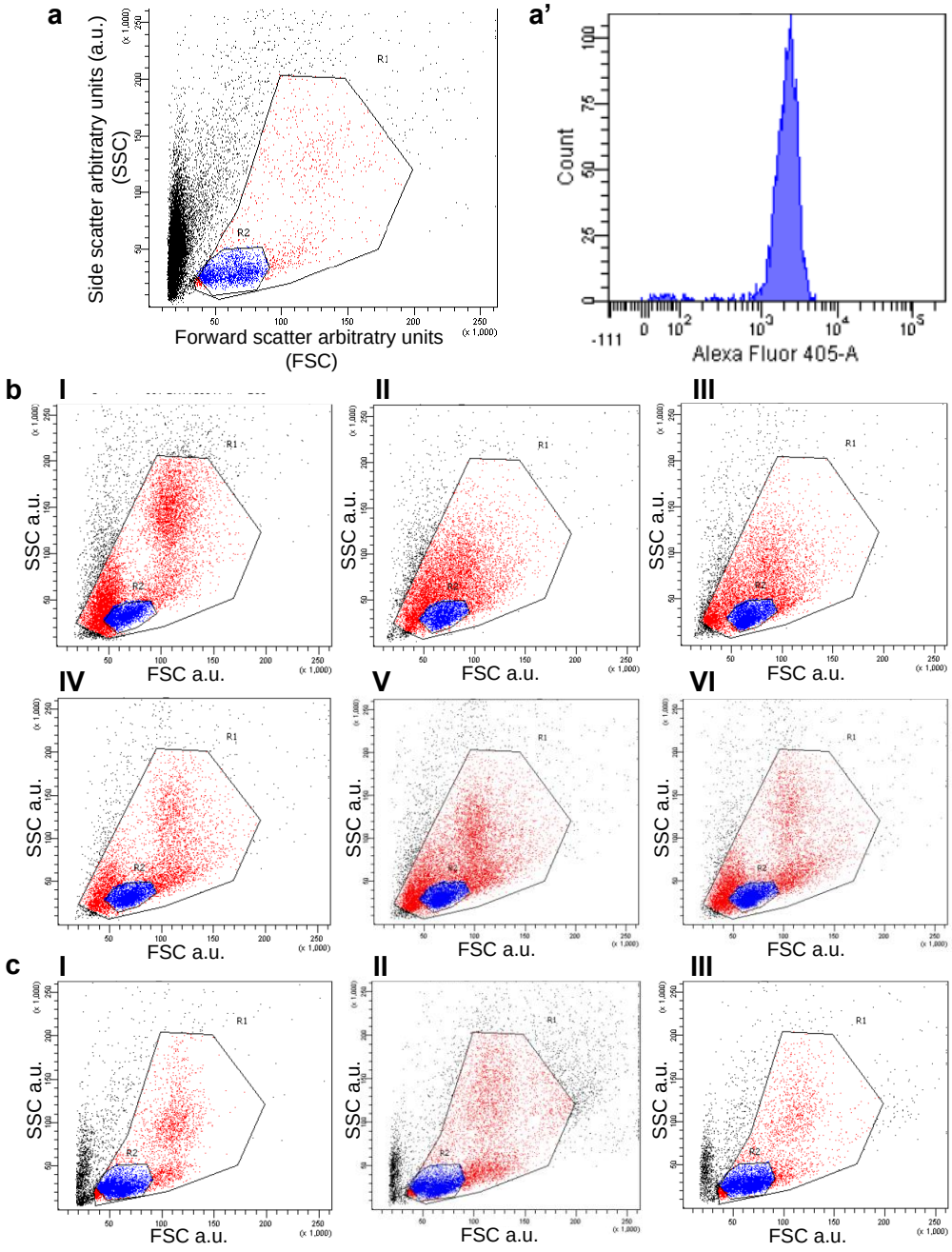

Figure S3. FACS SSC and FCS Scatterplots for BM and Blood Mouse Lymphocytes, Related to Figure 5.

a-c) Fluorescence activated cell sorting (FACS) for bone marrow (BM) and blood lymphocytes. **a)** SSC and FSC scatterplot CD45+ mouse blood sample; R1: population of CD45+ leukocytes (red), R2: population CD45+ lymphocytes (blue); a') Cell count for CD45+ leucocytes gated in R2. **b)** SSC and FSC scatterplots for mouse BM samples at different ages: adult (I), P7 (II), P15 (III), P21 (IV) and of TX (V; P21) and PDX (VI; P21); Representative scatterplots related to quantifications shown in Fig. 5 a, b. **c)** SSC and FSC representative scatterplots for blood samples at P21 of CTL mice (I), TX (II) and PDX (III); Representative scatterplots related to quantifications shown in Fig. 5 c. R1: total cell population gated (red), R2: population of interest - lymphocytes (blue) (for b,c).

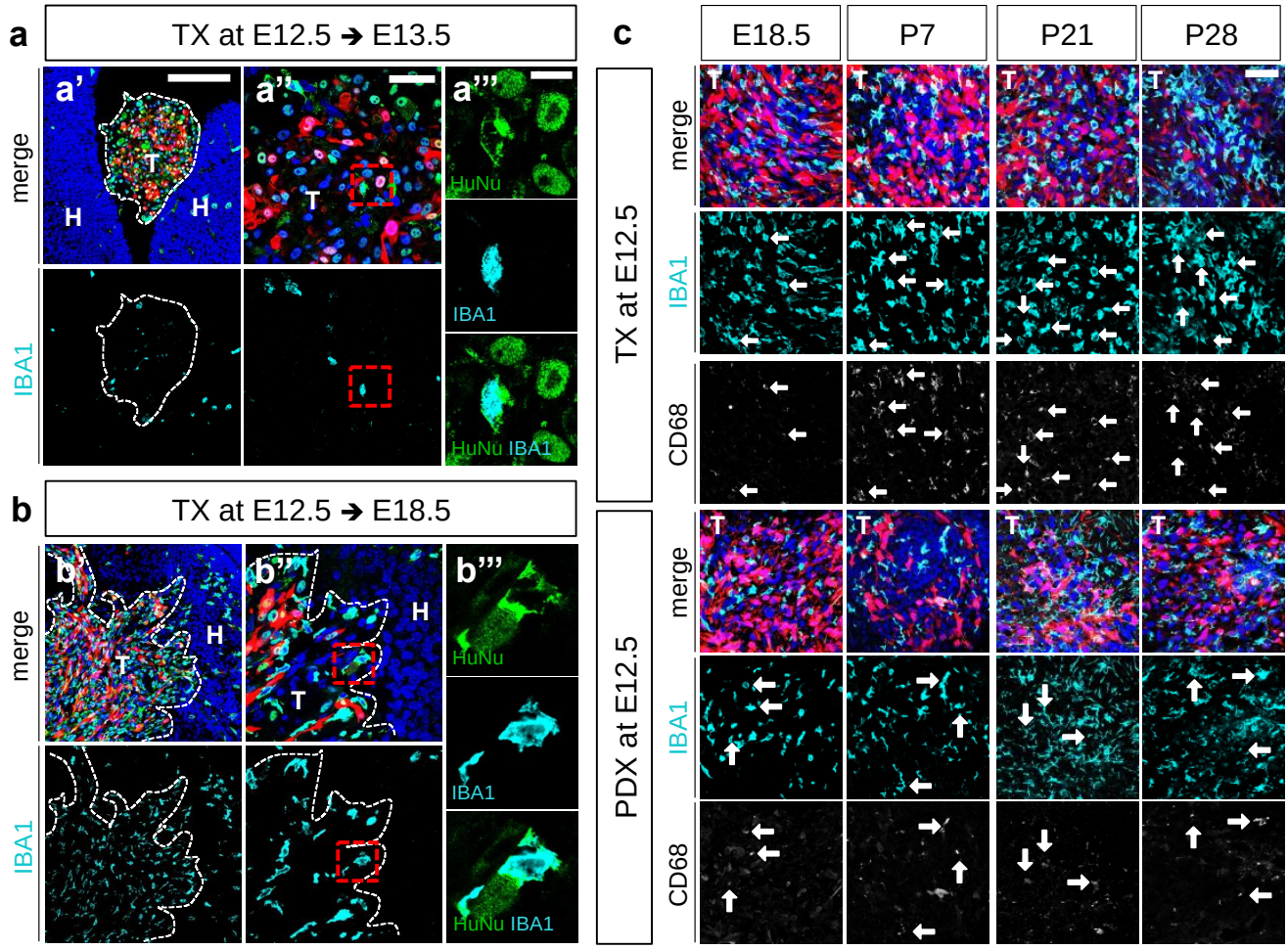

Figure S4. Expression of IBA1 and CD68 in TX and PDX, Related to Figure 5.

a, b) Immunofluorescence images of TX in cryosections through embryonic mouse brains at the indicated times; a''' and b''': high magnification of red dashed ROIs in a'' and b''. **c)** Images related to quantifications shown in Fig. 5 h,i. Scale bars: 200 µm (a', b'), 50 µm (a'', b'', c), 10 µm (a''', b''').

Transparent Methods

Mouse lines

Mice were housed under standard conditions at the animal facility of Istituto Italiano di Tecnologia (IIT), Genoa, Italy. All experiments and procedures were approved by the Italian Ministry of Health (Permits No. 338/2018-PR Ref. # IIT-138 and 176AA.N.U3R Ref. #IIT-129) and IIT Animal Use Committee, in accordance with the Guide for the Care and Use of Laboratory Animals of the European Community Council Directives. WT CD1 females and C57BL6/J males were crossed and mouse embryos used for *in utero* xenotransplantation experiments (TX – U87MG cell transplantation; PDX – patient-derived GBM cell transplantation) at 12.5 days *post coitum* (dpc). For all time-mated animals vaginal plug day was defined as 0.5 (E0.5). None of the animals used in our experiments had been previously used for other procedures. The animals were employed independently of their gender.

Maintenance and transduction of the U87MG cell line

U87MG cells (ATCC, HTB-14TM) were cultured with EMEM (Eagle's Minimum Essential Medium; ATCC®) + 10% FBS (fetal bovine serum, Sigma Aldrich) in T-75 flasks at standard conditions (37°C and 5% CO_2). DsRED+ U87MG cells were obtained with transduction of pLV-CMV-LoxP-DsRed-LoxP-eGFP lentivirus (Plasmid #65726, Addgene) (MOI=5). After viral transduction, positive clones were selected by fluorescence activated cell sorting (FACS) to obtain a pure population. Unlabeled (naïve) and/or DsRED+ cells were used for transplantation experiments.

Maintenance and transduction of patient-derived Glioblastoma cells (GBM)

Patient-derived GBM cells (GEFA; GBM2 [37]) obtained from a female patient were grown in media mixture composed of DMEM High Glucose (Dulbecco Modified Eagle Medium; ThermoFisher), DMEM-F12 (Dulbecco Modified Eagle Medium/Nutrient Mixture F12; ThermoFisher) and Neurobasal (ThermoFisher) in 1:1:2 ratio respectively, supplemented with 1% B27 (Life Technologies), 2 mM L-glutamine (Lonza Srl), 15 µg/µl insulin (Sigma), 2 µg/µl heparin (Sigma), 20 ng/ml bFGF (basic Fibroblast Growth Factor) and 20 ng/ml EGF (Epidermal Growth Factor) (PeproTech) (Bajetto, A. *et al.* 2013). Cells were grown on Matrigel (BD Biosciences) as previously reported (Griffero, F. 2009 and Corsaro, A. *et al.* 2016). DsRED+ GBM cells were obtained upon transduction with pLV-CMV-LoxP-DsRed-LoxP-eGFP lentivirus (Plasmid #65726, Addgene) (MOI=5) and positive clones were selected by FACS cells to obtain a pure population. $DsRED^+$ patient derived GBM cells were used for transplantation experiments.

Surgery (TX and PDX)

At the day of injection cultured cells were detached with TryLE Express (ThermoFisher), counted (Neubauer chamber, depth 0.1mm, squares of 0.0025 mm^2 by Marienfeld, Carl Roth) and re-suspended in DPBS (Dulbecco's Phosphate Buffered Saline supplemented with Mg^{2+} and Ca^{2+}, Gibco) at final concentration of 25.000 cells/µl and 10% Fast Green (1% stock solution in H_2O, Fast Green FCF, Sigma Aldrich). Surgery of a pregnant time-mated wildtype dam (12.5 dpc, E12.5) was performed under standard anesthesia conditions (2.5% Isoflurane (Isocare, Animalcare) and 1.5% oxygen). The pregnant dam was placed on a heating plate (37°C) exposing the abdomen. Pre-surgically Voltaren (2.5ml/kg bodyweight, Novartis) was subcutaneously injected (s.c.) at the forelimb. Abdomen was shaved, disinfected with 70% ethanol and wiped with Betadine

(Superfarma). Laparotomy was performed by a 1.5 cm long cutaneous incision and followed by a slightly smaller incision of the abdominal wall next to the *linea alba* to expose the uterus from the abdominal cavity. Single cell suspension was loaded to a pulled-glass capillary (Sutter Instruments, I.D.: 0.69 mm, O.D.: 1.2 mm, Length: 10 cm; BF120-60-10 and Micropipette Puller P-97, Sutter Instruments) and directly connected to the microelectrode/pipette holder (5430-1.0, MPH6S, WPI). Glass capillary was aligned perpendicular to the telencephalon of the mouse embryo *in utero* and injection of the single cell suspension into the lateral ventricle done by 1-2 short manual pulses (vent pressure, ~15 psi (~1.1 bar) (max. 20 psi); Pneumatic PicoPump PV820; WPI) until the ventricle was filled (1 pulse ≙ ~1µl). During surgery the uterus was moistened with filtered PBS (Filter Type 17597, 0.2 µm, Sartorius Stedim Biotech) pre-heated at 37°C. After injections uterine horns were placed back in the peritoneal cavity and the abdominal wall was sutured (VICRYL EP (5/0) V385H; C-3, 13mm, 45cm; Ethicon) and further disinfected with 70% ethanol and Betadine. Pregnant mice were sacrificed by cervical dislocation at the indicated times p.c. and embryos were harvested, dissected and further processed.

Blood and bone marrow (BM) sampling and FACS

Peripheral blood was collected by heart puncture at indicated time points prior to sacrifice (29G needle, U-100, BD Micro-Fine™) and directly transferred into K3 EDTA tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany). Lysis buffer (NH_4Cl 0.15 mM, EDTA 1.26 µM and $NaHCO_3$ 2.8 µM in H_2O at pH 7.4) was used to lyse erythrocytes from whole blood. 50 µl of whole blood was added to 1 mL of the lysis buffer and vortexed. Mix was kept for 5 min at room temperature (RT) and lysis was stopped adding 10ml 1x PBS. Afterwards samples were centrifuged at 1000 rpm for 5 min at RT and after cells were diluted 1:10 in 1x PBS.

Mouse femurs were collected at indicated time points and bone marrow cells were flushed from a single femur per animal as previously described (Soleimani, M. & Nadri, S. A., 2009). Briefly, femurs were cleaned from surrounding tissue and intact bones kept in 70% ethanol for 5 mins. Next, femurs were washed with 1x PBS, distal extremities were cut and BM flushed with 1x PBS (27G needle, (0.4mmx30mm), BD PrecisionGlide™). BM was segregated by vigorous pipetting in 1x PBS followed by centrifugation at 1000 rpm for 5 min at RT. BM cells were diluted in 1x PBS to perform cytofluorometric analysis.

Samples were run and analyzed on BD FACS Aria™ IIIu cytofluorometer. 10000 events for each dot plot were analyzed. Physical parameters as forward scatter (FSC) and side scatter (SSC) were shown to indicate the cell populations analyzed.

Immunostaining of blood cells for CD45 was performed according to manufacture's guidelines. Briefly, whole blood was incubated for 30 min at room temperature with CD45 anti-mouse (clone REA737, Myltenyi at 1:50). Cells were washed with 1x PBS and afterwards RBCs were lysed as described previously. Cells were analyzed by flow cytometry.

FITC-Dextran labeling of embryonic Vasculature

4kDA Fluoresceinisothiocyante-Dextran (FITC 3-5 kDA, Sigma-Aldrich, 20mg/ml in PBS at 37°C) was injected intravenously (i.v.) in the tail vein of the pregnant dam whose embryos were subjected to tumor cell injection at E12.5. Analysis was performed 4 h post injection. Brains were post-fixed in 4% PFA (paraformaldehyde; Sigma-Aldrich) and de-hydrated in 30% Sucrose at 4°C. Coronal cryosections (100 µm) were prepared at the indicated age and processed for FITC visualization. Briefly, re-hydrated cryosections were permeabilized with

progressive steps in 0.3% and 0.1% Triton X-100 in 1x PBS (PBST), followed by nuclear counterstain with Hoechst 33258 (IF) (1:300 in 1x PBS from a stock solution of 1 mg/ml in dimethyl sulfoxide, DMSO, Sigma Aldrich) and then extensively washed in 1x PBS and subsequently mounted with ProLong Gold Antifade (Invitrogen), air-dried overnight in the darkness, and sealed with nail polish (Electron Microscopy Sciences). All images were acquired using the Nikon A1. Images were taken with 20x or 60x (oil-immersion) objective.

Immunofluorescence and imaging

Brains were post-fixed in 4% PFA (paraformaldehyde; Sigma-Aldrich) and de-hydrated in 30% Sucrose at 4°C. Coronal cryosections (20 µm (E13.5-P7) and 40 µm (P21 and P28)) were prepared at the indicated ages, and processed for immunofluorescence as previously described (Hoffmann *et al.,* 2018, *see the main text for reference*). Briefly, re-hydrated cryosections, (subjected to antigen retrieval with 10 mM citric acid at pH 6.0 for 10 min at 95°C), were permeabilized with progressive steps in 0.3% and 0.1% Triton X-100 in 1x PBS (PBST). Blocking was performed in 0.1% PBST + 5% normal goat serum for 1 h at room temperature. Sections were afterwards incubated with primary antibodies diluted in blocking solution overnight at 4°C. Afterwards extensively washed in 0.1% PBST and incubated with secondary antibodies diluted in blocking solution for 2 h at RT. Progressive washing steps in 0.1% PBST and then 1x PBS were performed, followed by nuclear counterstain with Hoechst 33258 (1:300 in 1x PBS from a stock solution of 1 mg/ml in dimethyl sulfoxide, DMSO, Sigma Aldrich) for 30 min and immediately mounted with ProLong Gold Antifade (Invitrogen), air-dried overnight in the darkness, and sealed with nail polish (Electron Microscopy Sciences). All images were acquired using the Nikon A1. Images were taken with 20x or 60x (oil-immersion) objective. DsRED is detected at 546 nm and FITC-Dextran at 488 nm.

Antibodies for Immunofluorescence

Primary antibodies: rat monoclonal anti-CD31 (PECAM-1) (BD Pharmingen™, 550274, 1:50), rat monoclonal anti-mouse CD68 (Bio-Techne, MCA1957GA, 1:100), rabbit polyclonal anti-Connexin 43 (GJA1) (Abcam, ab11370, 1:250), mouse monoclonal anti-E-CADHERIN (Abcam, [M168], ab76055, 1:250), rabbit monoclonal anti-human E-CADHERIN (Abcam, [EP700Y], ab40772, 1:500), rabbit polyclonal anti-GFAP (Dako, Z0334, 1:500), rabbit monoclonal anti-human GFAP (Abcam, ab33922, 1:100), rabbit polyclonal anti-GLAST (EAAT1) (Abcam, ab416 , 1:200), mouse monoclonal anti-HuNu (Abcam, ab191181, 1:250), rabbit polyclonal anti-IBA1 (Wako, 019-19741, 1:500), rabbit polyclonal anti-KI67 (Abcam, ab15580, 1:250), mouse monoclonal anti-N-CADHERIN (Abcam, [5D5], ab98952, 1:200), mouse anti-S100β (Abcam, ab66028, 1:500), rabbit monoclonal anti-VEGFA (Abcam, ab52917, 1:400), rabbit polyclonal anti-Snail+Slug (Abcam, ab85936, 1:200), and rat monoclonal anti-CD44 (Abcam, ab11934, 1:100).

Secondary antibodies: ThermoFisher: goat polyclonal anti-mouse Alexa Fluor®488 (A32723, 1:1000), goat polyclonal anti-rat Alexa Fluor®488 (A-11006, 1:1000) and goat polyclonal anti-rabbit Alexa Fluor®647 (A32733, 1:1000)

Image Analysis and Measurements

All images were analyzed with Nikon software version 4.11.0 (NIS Elements Viewer) and processed with ImageJ version 1.48v (Wayne Rasband, National Institutes of Health, USA). Number of tumors was assessed by counting fluorescent tumor masses, formed after DsRED labeled U87MG cells or GBM cells injected in the

lateral ventricles of mouse embryos at E12.5, and observed under stereo microscope (Olympus SZX16 with Olympus U-RFL-T) upon dissection.

Tumor volume: was measured in coronal sections of embryonic brains by the diameter (d) of each tumor in its greatest dimension and volume (V) was estimated with the following sphere formula: $V=⅙*\pi*d^3$. DsRED+ GBM and HuNu+ cells defined tumor masses.

Cell number measurements: all Hoechst+ nuclei were counted within tumor mass (defined by DsRED and HuNu) in all available sections of all tumors in different brains (*n*= number of tumors) and total cell number was finally estimated using the Abercrombie formula (Abercrombie, M. & Johnson, M. L. 1946). Proportions of DsRED+ or HuNu+ or IBA1+ cells: were calculated as DsRED+ or HuNu+ or IBA1+ of total Hoechst+ nuclei/field (*n*= number of brains). Proportions of KI67+ cells: was calculated as KI67+DsRED+ of total DsRED+ cells/field (*n*= number of brains). Proportions of CD68+IBA1+ cells (activated Microglia): was calculated as CD68+IBA1+ of total IBA1+ cells/field (*n*= number of brains).

Tumor composition: was calculated as Hoechst+DsRED+ (red; tumor cells), Hoechst+IBA1+ (cyan; Microglia/TAMs) and Hoechst+DsRED-IBA1- (blue; tumor infiltrating cells) of total Hoechst+ nuclei/field. A field was considered as 60x acquisition with Nikon A1 confocal microscope and several fields depending on tumor size were counted per each *tumor (i.e. between 3 and 19)*.

Three-dimensional reconstruction of PDX tumors: Coronal sections were scanned, and images imported into Neurolucida software (MBF bioscience); contour of whole brain and tumors was performed on every 7 section (intersectional distance 140 µm) and partial three-dimensional images of GBM tumors within the mouse brain were obtained.

Statistical analysis

Data are expressed as standard error mean (s.e.m.) for all quantifications and assays. Number of brains or tumors analyzed in each experiment are indicated in figure legends. Differences between groups were tested for statistical significance, using unpaired Student's *t*-test, one-way-ANOVA followed by Tukey's multiple comparisons test. Significance was expressed as follows in all figures: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001 n.s.: not significant.

Supplemental references

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