Expanded Online methods

Cell Lines

U87 and U251 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wisent) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Wisent) splitting at a 1:3 ratio with Trypsin 0.25% (Wisent). GSC 8-18 were maintained in GSC specific neural media consisting of DMEM/F12 (Wisent) supplemented with B27 1:50 (Invitrogen), 20 ng/ml EGF (Sigma), and 20 ng/ml bFGF (BD Biosciences). Cells grow as spheres in a suspension culture and are split 1:3 every 5-7days(1).

Cell Transfections

Using a molecular ration of 3:1, 12ul Fugene HD was mixed with 4ug DNA in 500ul Optimem (Gibco), and left to equilibrate for 20min prior to adding to existing fresh media. Alternatively, GSCs, $2x10^6$ cells, were suspended in 90ul of nucleofector and plasmid to final volume of 100ul.. Post transfection media was replaced and supplemented with Puromycin (Wisent) at 1ug/ml. For PBase VEGFTrap system (2, 3). 3 plasmids were required to be transfected at a 1:1:4 ratio. Post transfection, media was replaced and supplemented with neomycin at 1ug/ml. Selected cells were split into 6 well plates and doxycycline was added at 1ug/ml. The dox turns on the VEGFTrap and GFP, allowing GFP+ colonies to be selected. Confirmation of functioning VEGFTrap was carried out with a VEGF ELISA (R&D). VEGFTrap is a fusion protein created from parts of the VEGFR1 and VEGFR2 receptors, it has the ability to sequest both human and mouse free solubilized VEGF (2, 3).

Bone Marrow Reconstitutions

Donor GFP/RFP mice were sacrificed according to institutional guidelines and bone marrow cells were collected from both the tibias and femurs into 1ml of sterile 1X PBS (Wisent) containing

0.1% sterile Bovine Serum Albumin (Calbiochem). 6-8 week old recipient NODSCID mice, (Jackson Labs #001303) were anaesthetized and cranially shielded and placed inside a Gamma Cell 40 irradiator (Nordion International). Mice were exposed to 2.5Gy total body irradiation (TBI) at a rate of 1.02 Gy/min, previously optimized for the strain and size of mouse. Within 24 hours of the TBI, the recipient mice were intravenously injected with $6x10^6$ donor bone marrow previously extracted via intravenous tail vein injection. Recipient mice should be returned to a clean sterile environment and fresh water should be supplemented with 0.3mg/ml Enrofloxacin (Baytril©)(4, 5).

Xengraft Models of GBM

Chimeric BM mice were anaesthetized using 0.5mg/g of intraperitoneal injection of 20mg/ml Avertin (Sigma Aldrich) and 5mg/kg of the pre-surgical analgesic Anafen 1mg/ml (Ketoprofen©) was administered sub-cutaneously. TearGel (Novartis) was applied to the eyes to prevent corneal dehydration and abrasion. Once a toe pinch no longer elicited a response, the scalp was cleaned and hair removed, and a midline incision was made from the ears to the eyes. Underlying periosteium was frozen with 2%Lidocaine (Bimeda MTC) and removed with scissors. A high-speed dental drill with a 2.7mm trephine adaptor (Fine Science Tools) was used to generate a circular bone flap in the right frontal skull which was removed with fine dissection tools (5).

Optionally $4x10^5$ U87:mCherry cells resuspended in 10µl of sterile 1X PBS or $2.5x10^4$ GSC:mCherry cells resuspended in 5ul of sterile 1X PBS were injected 3mm deep into the center of the cranial window using a 10µl 30G Hamilton microsyringe, over the frame of 1minute. Control mice were either a needle sham injection or no injection.

Skull surface was dried and a 3mm glass coverslip (Warner Instruments) was placed over the window, self-curing dental acrylic was used to seal the coverslip in place on the skull surface.

Mice were returned to a fresh sterile heated cage to recover and supplemented with 0.3mg/ml Enrofloxacin in the drinking water (Baytril© Bayer/CDMV, cat. no.102207) (4, 5).

Transgenic Mouse Models

RASB8 mice were generated through embryonic stem cell complementation methodology to integrate a V12 RASB8 mutation (IRES LACz) under the control of GFAP-promoter into an ICR background strain mouse. The positive RASB8 male mice are bred heterozygously to ICR females, as the homozygous crosses are lethal before P14 (6). Genotyping is carried out for both the RASB8 mutation and the LACz reporter construct, in addition LACz IHC is carried out to ensure full protein transcription. The nature of this model does not allow for direct ICW and 2PLM imaging and so was restricted to histological analysis. Chimeric RasB8 mice, with GFP^+ or RFP^+ BM, were created as detailed above to study BM in this model.

Targeted Radiation Therapy

Xrad225 small animal irradiator was used to administer image guided RTx to specifically target the ICW. Mice were anaesthetized using isofluorane and placed on a custom made frame designed to immobilize the head, the frame was then placed inside a microirradiator (Precision Xrad 225Cx). A 360o CT scan was taken of the mouse, which after the insertion of a 8mm*11mm collimator was used to adjust the platform to ensure the iso-center of the RTx beam was targeted to the window center. RTx was administered with the Xray tube running at 225kVp and 13mA through a 0.93mm Copper screen from both the AP direction and the PA direction. Treatment regimens ranged from 3*2Gy – 3*5Gy fractionated on consecutive days, and from 2Gy to 15Gy single doses. (5, 7)

Drug Therapy

TMZ (Sigma) was prepared in DMSO 20% final volume and administered at 60mg/kg daily for 5 days as an oral gavage, one hour prior to the RTx therapy at all doses. VEGFTRap was activated through the supplementation of the food with Doxycycline mouse chow, 3mg/ml pellets (Bioserve) activation requires a minimum of 3 days continual Doxycycline chow. AMG386 (Amgen), angiopoietin inhibitor was administered intravenously twice a week at a dose of 10mg/kg diluted in PBS at the beginning of either RTx or VEGFtrap therapy.

Real-time in-vivo Imaging

Mice were anaesthetized using 0.5mg/g of intraperitoneal injection of 20mg/ml Avertin (Sigma Aldrich) and TearGel (Novartis) was applied to the eyes. 15 minutes prior to imaging, either Alexa647-Dextran 10Kda (Invitrogen) used at 0.6µg/g, or APC-CD31 (BD Pharmingen) used at 0.24µg/g were injected intravenously via tail veins. Mouse was inverted onto the specialized restrainer ensuring head was stable and perpendicular to the laser, then mounted onto the 2PLM automated stage. Positioning was carried out using the APC and a tiled reconstruction of the entire window was taken at 5X magnification with all channels in use to allow alignment of future higher resolution images (5)

MRI

A 7-T Bruker model BioSpec 70/30 MR system with B-GA12 gradient coil, 7.2-cm-diameter linear radiofrequency (RF) trans- mitter coil, murine head RF receiver coil, and murine slider bed was used for serial imaging. Mice under isoflurane anesthesia were positioned on the MR bed with a bite block and water warming system to maintain body temperature during imaging. serial multiparametric MRI protocol was carried out as previously described (7).

PFA Perfusion Sacrifice

Mice were anaesthetized with 0.5mg/g of intraperitoneal injection of 20mg/ml Avertin (Sigma Aldrich). After opening the mouse's chest the vena cava was snipped and a butterfly needle injected into the left ventricle ensuring a single puncture wound. 40ml ice cold 0.9% PBS was pushed through the heart with a strong steady pressure to flush out blood. Next 40ml ice cold fresh 4% PFA in 0.2M sodium Phosphate was pushed through, movement of the cadaver is indicative of successful perfusion. After 24 hours in PFA brains were put in 30% sucrose solution in 0.1M sodium phospate pH 7.3 until dehydrated. OCT blocks of the tissue were made and immediately placed into -80 freezer.

Histology

OCT sections were air-dried and endogenous peroxidase and biotin was inhibited. 5% serum derived from the secondary antibody source was used to block. Sections were incubated for one hour at room temperature with the primary antibodies. Biotinylated secondary (Vector labs) or fluorescent secondary (Invitrogen) to the primary antibodies were next applied for one hour. IHC stains underwent a 30min horseradish peroxidase-conjugated ultrastreptavidin labeling (ID labs) treatment and color was developed using freshly prepared DAB solution (Vector labs), slides were lightly counterstained with hematoxylin, dehydrated in alcohols, cleared in xylene and mounted in Permount (Fisher). IF stained slides were mounted using a DAPI aqueous mount (Vector Labs) and were stored below 0^{o}C.

Immunostaining Antibody concentration

ANG1 (Santa Cruz 1:500), ANG2 (Santa Cruz 1:500), B220 (Ebiosciences 1:100), BrDU (Chemicon, 1:200), CD11b (BD Pharmingen, 1:50), CD11c (BD Pharmingen, 1:50), CD3 (Abcam 1:100), CD31 (BD Pharmingen, 1:500), GFAP (Dako, 1:4000), IBA1 (Wako, 1:200), Ki67 (Dako 1:50), MAC3 (BD Pharmingen, 1:100), SMA (Abcam, 1:1000), TIE2 (Santa Cruz 1:500)

Image analysis

For 2PLM data quantification 10 high-powered fields from minimum 5 samples were blindly analyzed using Image J software. Briefly, RGB images were split and converted to 8bit gray scale images. Threshold levels were adjusted (45pi<x<225pi) to mask the 'BMDCs', resulting images were converted to binary for analysis. Particle analysis was completed based upon size restrictions of $8pi^2$–infinity leaving morphology unspecified. IHC and IF image analysis was carried out manually on 10 high-powered fields from minimum 3 samples in kati-kat cell counting software by 3 blinded counters.

Laser Capture Microscopy (LCM)

Frozen 5µm sections were loaded onto the flat surface of the LCM. The slides were dehydrated with 5min in each alcohol 70%, 95%, 100%, 100% followed by a 15 min treatment in Xylene then left to air dry for 15min. The LCM slide was loaded into the microscope (MMI Cellcut system mounted on a Zeiss Axiovert 200M microscope). Vessels were isolated based on their GFP BM contribution and position to tumor. Laser was set to 100% power to cut twice. Additional cuts were needed especially in the case of folded or marred tissue. A minimum of 15 vessels were collected.

RNA was extracted from LCM samples as per the Picopure RNA extraction kit (Applied Biosciences), and cDNA synthesized using superscript VILO kit (invitrogen). cDNA was diluted 1:3 prior to use in 50ul SYBR green qPCR reactions (Invitrogen). ROX internal control was added to all samples.

QPCR primers were taken from previously published sources or produced using QuantPrime QPCR primer design web-tool. All primers were validated by checking their linear standard curves and amplification efficiency following gradient PCR, on both mouse and human control

samples, to check cross reactivity. MBactin (F-GATGCAGAAGGAGATCACTGC R-GTACTTGCGCTCAGGAGGAG), HBactin (F-CAATGTGGCCGAGGACTTTG R-CATTCTCCTTAGAGAGAAGTGG), MAng1 (F-GTCGGAGATGGCCCAG R-CTGTGAGCTTTCTGGTC), HAng1 (F-CTGCAGAGAGATGCTCCACA R-GCCATCTCCGACTTCATGTT), MAng2 (F-CGACTACGACGACTCAGTGC R-TTGGATCTCCACCATCTCCT), HAng2 (F-CCTACGTGTCCAATGCTGTG R-GCCACTGAGTGTTGTTTTCC), MTie2 (F-CCGGCTTAGTTCTCTGTGGA R-ATGCAGGTGAGGGATGTTTC), HTie2 (F-GCATGGACTCTTTAGCCAGC R-AGAGGCAATGCAGGTGAGAG).

Statistical Analysis

All experiments were performed in triplicate with mean and standard error of the mean reported where appropriate. Analysis of variance (ANOVA) was conducted for multi group comparisons followed by a post-Dunnetts test (groups compared to one control group) or post-Tukey (to identify differences among sub-groups). Where appropriate, direct comparisons were conducted using an unpaired two-tailed Student's t-test. *Significance was defined as $p<0.05$. This applies to all image analysis and QPCR data.

References

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Supplemental Figure 1 Proliferative index of tumors

IHC Ki67 staining, as demonstrated by thumbnails demonstrates the number of actively proliferating nuclei in the field. Once quantified as a percentage of total nuclei it is visible that there is no significant difference between early and late tumor indices. Additionally there is no significant change upon the administration of RTx at a clinically relevant dose. There is a significant reduction in proliferation at late stages of tumor growth when higher doses of radiation are applied (3*5Gy) P***= 0.0001. Data represented as mean ± SEM, n=10.

Scalebars in microns.

Supplemental Figure 2 Role of BMDCs as Scavengers

IF confocal image analysis demonstrates BMDC engulfing $mCherry^+$ U87 GBM cells, suggesting a scavenger role. All principal cell type that BMDC differentiate into **(A)** $IBA1^+$ **(B)** $MAC3^+$ and **(C)** CD11b participate in scavenging of GBM tumor cells (arrowheads); GFP^+BMDC Green, $mCherry^+$ GBM Tumor Cells Red, IF Stain Blue). High magnification Z-stack projections confirm the relationship of GFP+ BMDCs and Cherry+ tumor cells in the tumor environment.

The scavenging population of **(D)** IBA1$^+$ **(E)** MAC3$^+$ and **(F)** CD11b$^+$ BMDCs can be seen to be engulfing mCherry$^+$ tumor cells only in the tumor stroma and not in the perivascular niche. (Xenograft: GFP$^+$ BMDC Green, mCherry$^+$U87 GBM Tumor Cells Red, IF Stain Blue). **(G)** Quantification of IF images shows that 10% of stained BM-derived microglia and inflammatory cells act as scavengers. Following RTx treatment (3*2Gy) the percentage of scavenging BMDC increases with statistical significance in the MAC3$^+$ (*p=0.0263) and CD11b$^+$ (***p=0.0008) subpopulations. Data represented as mean ± SEM, n=10.

Scalebars in microns.

Supplemental Figure 3 AMG386 ANG2 inhibition

IF images of ex vivo tissue demonstrates a regain of BMDCs in RTx treated tissue (**Ai**; GFP+ BMDC Green, CD31+ Red). The addition of AMG386 to inhibit ANg2 in un-irradiated tissue results in a slight loss in recruited BMDC to central vessels **(Aii)** whilst its addition following RTx significantly reduces BMDC recruitment (**Aiii**; GFP$^+$ BMDC Green, Tumor Red, CD31$^+$ Blue), similarly this pattern was seen following AMG386 administration following VEGFtrap (**Aiv**; Nuclei Cyan, Tumor Green, RFP$^+$BMDC Red, CD31$^+$ Blue). Quantification of the images demonstrates the significant increase in BMDC recruitment seen in central vessels following RTx (**p=0.001) or VEGFTrap (**p=0.0043) is significantly reduced when AMG386 was administered immediately after RTx (**p=0.0012) and VEGFTrap (**p=0.0019) **(B).** Data represented as mean ± SEM, n=10.

Scalebars in microns.

Supplemental Figure 4 Schematic of hypothesis

At early stages of tumor growth BMDCs exist in a differentiated state through the central and peripheral regions. At later stages the differentiated BMDCs are lost in the vasculature in the central regions but retained in the periphery (A). Following inhibition of VEGF through direct

drug therapy, VEGFtrap (C), or indirectly, RTx (B), there is a compensatory up-regulation of ANG2, which in turn initiates the recruitment of differentiated BMDCs back to central vessels**.**

Legend as attached.

Supplementary Video 1 Time Lapse Imaging of BMDCs

Time Lapse images capturing a 150msec time window demonstrates the ability of the imaging to track dynamically the movement of BMDCs and reconstituted blood through the vessels, (Green: BMDCs)

10X magnification.

Supplementary Video 2 Z-stack imaging of BMDCs

Z-Stack images capturing a 150μm depth section of the window every 10μm demonstrates the ability of the imaging to enhance the three dimensional morphology of BMDCs and their relationship to the vessels, (Green: BMDCs, Blue: CD31)

10X magnification.