

**Vascular targeting of LIGHT normalizes blood vessels in primary brain cancer and induces intratumoral high endothelial venules**

Bo He<sup>1</sup>, Arnaud Jabouille<sup>2</sup>, Veronica Steri<sup>2</sup>, Anna Johansson-Percival<sup>1</sup>, Iacovos P. Michael<sup>3</sup>, Venkata Ramana Kotamraju<sup>4</sup>, Reimar Junckerstorff<sup>5,6</sup>, Anna K. Nowak<sup>7</sup>, Juliana Hamzah<sup>1</sup>, Gabriel Lee<sup>8,9</sup>, Gabriele Bergers<sup>2,10</sup> and Ruth Ganss<sup>1\*</sup>

- <sup>1</sup> The Harry Perkins Institute of Medical Research, Centre for Medical Research, The University of Western Australia, Nedlands, Australia  
<sup>2</sup> Department of Neurological Surgery, Brain Tumour Research Center, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, US  
<sup>3</sup> Swiss Institute for Experimental Cancer Research, School of Life Sciences, Swiss Federal Institute of Technology Lausanne (EPFL), Lausanne, Switzerland  
<sup>4</sup> Cancer Research Center, Sanford Burnham Prebys Medical Research Institute, La Jolla, US  
<sup>5</sup> School of Pathology and Laboratory Medicine, The University of Western Australia, Nedlands, Australia  
<sup>6</sup> PathWest Neuropathology, Royal Perth Hospital, Perth, Australia  
<sup>7</sup> School of Medicine, The University of Western Australia, Nedlands, Australia  
<sup>8</sup> School of Surgery, The University of Western Australia, Nedlands, Australia  
<sup>9</sup> St John of God Subiaco Hospital, Subiaco,  
<sup>10</sup> VIB-Center for Cancer Biology Vesalius and Department of Oncology, KU Leuven, Leuven, Belgium

*\*Correspondence to: Ruth Ganss, The Harry Perkins Institute of Medical Research, 6*

*Verdun Street, Nedlands, West Australia 6009, Australia. Phone: 0061 8 6151 0733. Fax:*

*0061 8 6151 0700. E-mail: ganss@perkins.uwa.edu.au*

**Running title:** Vascular LIGHT targeting in primary mouse and human brain cancers

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**Abstract**

High grade brain cancer such as glioblastoma (GBM) remains an incurable disease. A common feature of GBM is the angiogenic vasculature which can be targeted with selected

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peptides for payload delivery. We assessed the ability of micelle-tagged, vascular homing peptides RGR, CGKRR or NGR to specifically bind to blood vessels in syngeneic orthotopic GBM models. By using the peptide CGKRR to deliver the TNF superfamily member LIGHT (also known as tumor necrosis factor superfamily member 14; TNFSF14) to angiogenic tumour vessels we have generated a reagent which normalizes the brain cancer vasculature by inducing pericyte contractility and re-establishing endothelial barrier integrity. LIGHT-mediated vascular remodelling also activates endothelia and induces intratumoral high endothelial venules (HEVs), specialized blood vessels for lymphocyte infiltration. Combining CGKRR-LIGHT with anti-vascular endothelial growth factor (VEGF) and checkpoint blockade amplified HEV frequency and T cell accumulation in GBM which are often sparsely infiltrated by immune effector cells, and reduces tumour burden. Furthermore, CGKRR and RGR peptides strongly bind to blood vessels in freshly resected human GBM, demonstrating shared peptide binding activities in mouse and human primary brain tumour vessels. Thus, peptide-mediated LIGHT targeting is a highly translatable approach in primary brain cancer to reduce vascular leakiness and enhance immunotherapy.

**Key words:** angiogenesis, vascular targeting, LIGHT, TNFSF14, glioblastoma, immunotherapy

## Introduction

WHO grade IV glioma (glioblastoma or GBM) is a highly aggressive malignancy and the most common form of primary human brain cancer. Standard treatment for GBM includes surgical resection followed by radiation and has remained unchanged for decades; the recent addition of concurrent and adjuvant chemotherapy with temozolomide only marginally improved the poor survival outcomes. GBM is a highly angiogenic tumour in part due to elevated levels of vascular endothelial growth factor (VEGF). Features of abnormal angiogenesis, leaky blood vessels and irregular blood flow create a hypoxic tumour environment, high interstitial fluid pressure and brain oedema [1]. Cancer-associated oedema and use of corticosteroids for treatment contributes to overall patient morbidity and constitute a serious clinical problem [2]. Pharmacological inhibition of VEGF by bevacizumab has been FDA approved since 2009, but thus far has not clearly delivered survival benefits [3-4]. More recently, it has been demonstrated that therapeutic resistance following prolonged VEGF blockade and vessel destruction is associated with increased tumour hypoxia, invasive cancer growth and mobilization of innate immune cells into the tumour environment [5-7]. Nevertheless, anti-angiogenic therapy can induce a transient state of vessel “normalization” which improves tumour perfusion, alleviates brain oedema and thus provides a temporary opportunity for drug delivery [8]. In normalized tumour blood vessels endothelial cells and surrounding support cells, so called pericytes, are better aligned leading to a tighter, less leaky and more functional vasculature [9-10]. Whilst there is currently no single reagent which can induce long-lasting vessel normalization, clinical data from anti-VEGF treatment, in particular in GBM, support the notion that vessel normalization might be therapeutically beneficial [8,11].

To specifically deliver payloads to the angiogenic vasculature, a variety of peptides have been identified which bind to abnormal tumour blood vessels [12-14]. Among these vascular

targeting peptides (VTPs) is the so called RGR peptide (CRGRRST), which has been identified in a preclinical model of pancreatic neuroendocrine tumours and also binds blood vessels in breast cancer [10,15]. Other peptides such as CGKRK and NGR (CNGRCG) bind to tumour blood vessels in murine breast, melanoma, squamous cell carcinoma and brain cancer models [16-19]. Thus far, NGR is the only VTP which has advanced into clinical trials to deliver the anti-angiogenic agent TNF $\alpha$  into end stage malignancies [20-21].

We have recently developed a compound which combines a VTP with the TNF superfamily cytokine LIGHT (also known as TNFSF14), called LIGHT-VTP [10]. LIGHT is a ligand for the lymphotoxin  $\beta$  receptor (LT $\beta$ R) and herpes virus entry mediator (HVEM) and which stimulates T cells, promotes vascular inflammation and is involved in lymph node neogenesis [22-24]. When targeted to angiogenic blood vessels in preclinical models of neuroendocrine pancreatic or breast cancers LIGHT normalizes the tumour vasculature and also induces specialized endothelial cells, so called high endothelial venules (HEVs) [10,25]. HEVs express peripheral node addressin (PNAd) and facilitate lymphocyte trafficking into secondary lymphoid organs [26]. In cancer, HEVs can arise spontaneously and are often associated with better clinical outcome [27-28]. Importantly, therapeutic induction of HEVs in solid cancers increases spontaneous anti-tumour immunity and enhances active immunotherapy [25,29].

We hypothesized that LIGHT-VTP treatment may induce angiogenic vessel remodelling in GBM with potential future applications for oedema alleviation and combinatorial immunotherapies [30]. Here, we evaluated vascular peptide binding in two distinct orthotopic murine brain tumour models and LIGHT-VTP-induced stromal remodelling *in vivo*. Furthermore, we assessed vascular peptide binding capacities in different types of human brain malignancies in order to inform potential clinical translation of the LIGHT-VTP technology.

## Materials and Methods

### *Cell lines, mouse models and human specimens*

NSCG cells (Ink4/Arf-deficient neural stem cells expressing the EGFRvIII mutant of human EGFR, kindly provided by J. Phillips, University of California, San Francisco, CA, USA) [31] were cultured in Minimum Essential Medium Alpha (MEM-A) supplemented with 20% FCS, 100 units/ml penicillin/100 µg/ml streptomycin, 10 mM HEPES and 0.5% glucose. NFpp10 cells (generated from embryonic C57BL/6 neural stem cells transfected with shP53-shNF1 and shPTEN lentiviral constructs and EGFP [32]) were grown in Ham's F12 Medium supplemented with N-2 Supplement (Thermo Fisher Scientific, Waltham, MA, USA), 20 ng/ml FGF-2 (Preprotech, Rocky Hill, NJ, USA), 20 ng/ml EGF (Promega, Madison, WI, USA), 50 µg/ml heparin (Sigma-Aldrich, St Louis, MO, USA), 2 mM L-glutamine and 100 units/ml penicillin/100 µg/ml streptomycin. Intracranial murine tumours were generated by implanting  $3 \times 10^5$  NSCG cells in 2.5 µl PBS into 4-5 week-old male and female FvBN Rag-deficient mice, or by injecting  $2 \times 10^5$  NFpp10-GBM cells into 6-8 week-old male and female C57BL/6 mice as described [33]. All animal studies were approved by the University of California, San Francisco Institutional Animal Care and Use Committee. Fresh human WHO grade IV astrocytomas and grade I meningiomas were collected with patient consent at the time of surgical resection and frozen in OCT compound. All human studies were approved by the Sir Charles Gairdner Group and St John of God Health Care Human Research Ethics Committees, Western Australia, Australia (2015-084).

### *Peptide synthesis*

Linear peptides were synthesized as described [34] and labelled with a 5(6)-carboxyfluorescein (FAM) separated by a 6-aminohexanoic acid spacer.

#### *Micelle synthesis*

Peptide-tagged micelles were prepared as described [35]. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). DSPE-PEG<sub>2,000</sub> was either labelled with fluorescein (FAM) or tagged to FAM-labelled peptides including RGR (FAM-X-CRGRST), CGKRRK (FAM-X-CGKRRK) or NGR (FAM-X-CNGRCG) by coupling the FAM-cysteine or cysteine on the N-terminus of the peptide to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-maleimide(polyethylene glycol)<sub>2,000</sub> (DSPE-PEG<sub>2,000</sub>-maleimide) at a 2:1 molar ratio. The coupling reaction was performed at room temperature for a minimum of 4 h under gentle rotation, and was then dialyzed in 3 changes of degassed water and lyophilized. 18:0 PEG2000 PE and DSPE-PEG<sub>2,000</sub>-FAM or FAM-peptides were dissolved in chloroform/methanol (3:1, v/v) in a glass vial at 0.7:0.3 molar ratios. Lipid film was created manually by gentle rotation of the lipid mixture under flow of nitrogen gas to evaporate the solvent. The dried lipid film was re-dissolved in degassed PBS at 50 °C, vortexed and sonicated in a waterbath sonicator (Elmasonic SH40, ELMA Ultrasonic, Singen, Germany) for 20 min at 50 °C. A final product of micelle solution approximately 12–15 nm in diameter was generated, as measured by dynamic laser light scattering (refractive index, 1.59; viscosity, 0.89) on a Malvern Zetasizer Nano (Malvern Paralytical, Almelo, The Netherlands). The micelle solution was sequentially filtered through 0.2 µm and 0.1 µm filters and stored for up to 2 wk at 4 °C until further use. Micelles were sonicated for 1 min prior to use.

#### *Production of LIGHT-VTP*

Sequences for recombinant murine LIGHT (aa 58 to 220, 17 kDa) with C-terminal CGKRRK were cloned into a modified pET-44a plasmid which contains a tobacco etch virus (TEV) cleavage site (kindly provided by E. Ingley, Harry Perkins Institute, WA, Australia). Biologically active LIGHT-CGKRRK protein was produced as described previously [25]. In brief, LIGHT- CGKRRK expressing pET-44a/TEV plasmids were transfected in *E-coli* (Rosetta) for optimal protein expression. After isopropyl- $\beta$ -d-galactopyranoside (IPTG) induction for 6 h at 22 °C in the presence of 5 mM EGTA, cultures were pelleted by centrifugation, resuspended in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, 1 mM DTT, 1mM PMSF, 1 mM EDTA/EGTA, 1% triton-X100, protease inhibitor cocktail (Sigma-Aldrich, Castle Hill, NSW, Australia), 1 $\mu$ g/ml pepstatin (Calbiochem, Merck, Bayswater, Vic, Australia), pH 8.0), followed by sonication, and subsequent purification using Ni-NTA beads (Qiagen, Chadstone, Vic, Australia). Recombinant fusion protein was dialysed overnight at 4 °C in Tris buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, pH 8.0). The Nus•Tag/His•Tag was cleaved using TEV protease (Thermo Fisher Scientific Australia, Scoresby, Vic, Australia), for 90 min at 30 °C. After cleavage, LIGHT-CGKRRK was re-purified using Ni-NTA beads in the presence of protease inhibitors (1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 $\mu$ g/ml pepstatin and protease inhibitor cocktail), salts (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole) and 0.005% BSA. Purity was assessed on Coomassie Brilliant Blue stained protein gels and the concentration was determined by measuring protein intensity in comparison to a band of similar size and known concentration. Endotoxin levels were determined as <0.01 EU per 1  $\mu$ g protein using the Limulus Amebocyte Lysate (LAL) method (LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific Australia).

#### *Treatment of tumour-bearing mice*

For peptide homing studies, mice were i.v. injected 2 wk after tumour implantation with one dose of 1 mM FAM-peptide-tagged micelles in 100  $\mu$ l or PBS vehicle. Mice were sacrificed after 2 h and tissue frozen in OCT compound. For LIGHT-VTP therapy, mice were treated from day 9 after tumour implantation with bi-weekly injections of 20 ng/kg recombinant LIGHT-CGKRK i.v. for 2 wk. In combination therapies mice were treated with LIGHT-CGKRK and bi-weekly i.p. injections of 15 mg/kg of the anti-mouse VEGF B20 biosimilar (B20S) [29] and 10 mg/kg anti-PD-L1 (BE0101, BioXcell, West Lebanon, NH, USA) for two wk. At endstage, mice were anesthetized and heart-perfused with 2% formalin. Some mice were injected with pimonidazole (60 mg/kg, circulated for 50-60 min, Hypoxyprobe<sup>TM</sup>-1 Kit, Hypoxyprobe, Inc., Burlington, MA, USA). Tissues were submerged in 10% sucrose for 2 h, incubated in 30% sucrose overnight and frozen in OCT compound.

#### *Immunohistochemistry (IHC)*

Frozen 7  $\mu$ m thick sections were fixed in ice-cold acetone prior to IHC. FAM-labelled micelles were detected directly on sections using fluorescence microscopy without amplification. Where indicated, FAM-signals were amplified with anti-fluorescein isothiocyanate (FITC) antibody (goat polyclonal ab6655, 1:300, Abcam, Cambridge, UK) followed by anti-goat IgG (H+L)-cyanin 3 (Cy3, 305-165-003, 1:3000, Jackson ImmunoResearch, West Grove, PA, USA) or anti-goat IgG (H+L)-AF488 (donkey polyclonal, ab150129, 1:2000, Abcam). The following primary antibodies were used: Caldesmon (E89, 1:100, Abcam), Calponin (EP7998Y, 1:100, Abcam), CD3 (rabbit polyclonal ab5690, 1:100, Abcam), CD4 (GK1.5, 1:50, Becton Dickinson (BD), North Ryde, NSW, Australia), CD8 (Ly-2, 1:50, BD), CD13 (R3-63, 1:100, BioRad, Gladesville, NSW, Australia), CD31 or CD31-biotin (MEC13.3, 1:100, BD), collagen I (rabbit polyclonal, pAB13488, 1:100, Abnova, Taipei City, Taiwan), FoxP3 (FJK-16s, 1:100, eBioscience,



Scoresby, VIC, Australia), GrzB (rabbit polyclonal, P1-26616, 1:100, Invitrogen, Scoresby, VIC, Australia; amplified using the TSA<sup>TM</sup> Cy3 System, Perkin Elmer, Melbourne, VIC, Australia), ICAM-1 (3E2B, 1:100, Invitrogen), PNA<sup>d</sup> (MECA79, 1:50, BD),  $\alpha$ SMA-FITC (1A4, 1:50, Sigma-Aldrich Australia, amplified using the mouse on mouse kit, M.O.M, Vector, Burlingame, CA, USA), VCAM (429, 1:100, eBioscience) and VE-cadherin/CD144 (11D4.1, 1:100, BD). Primary antibodies were detected using anti-rat IgG (H+L)-Cy3 (112-165-003, 1:3000, Jackson ImmunoResearch), anti-rabbit IgG (H+L)-Cy3 (111-166-003, 1:3000, Jackson ImmunoResearch), Streptavidin-Cy3 (016-160-084, 1:3000, Jackson ImmunoResearch), or anti-rat IgG (H+L)-7-Amino-4-methylcoumarin-3-acetic acid (AMCA, 112-155-003, 1:200, Jackson ImmunoResearch). 4',6-diamidino-2-phenylindole (DAPI) was used in some tumours to visualise cell nuclei. Hypoxia was quantified in mice treated with pimonidazole using anti-pimonidazole antibodies following the manufacturer's instructions (Hypoxyprobe<sup>TM</sup>-1 Kit, Hypoxyprobe, Inc. Burlington, Massachusetts, USA). Assessment of perfusion using lectin (Vector) was performed as previously described [36]. Human fresh frozen tumours were sectioned (7  $\mu$ m), fixed in ice-cold acetone and incubated at room temperature with 100  $\mu$ l of 2  $\mu$ M FAM-labelled peptide solution in PBS for 60 min, followed by 3 $\times$ 5 min washing in PBS. Sections were stained for the vascular markers CD105 (SN6h, 1:100, Dako, Mulgrave, VIC, Australia) or CD13 (WM15, 1:100, Abcam). FAM-labelled peptides were either directly detected or amplified as described above for FAM-peptide-tagged micelles. A Nikon C2si confocal microscope and NIS software (Nikon, Rhodes, NSW, Australia, version 4.5) were used to image lectin. A Nikon Ti-E microscope and NIS software (Nikon, version 4.0) were used for all other imaging. At least three mice or tumours were analysed per treatment group unless indicated otherwise; 5-15 images per tumour were analysed. Vessel diameter was calculated by dividing vessel area by vessel length, as described previously [10].

### *Statistical Analysis*

Data are presented as mean  $\pm$  SEM. Numbers (n) of mice or tumours per group and *P* values are shown in figure legends. For comparison of cell numbers/percentages between histological groups one-way ANOVA or two-tailed unpaired Student's *t*-test were used as indicated in figure legends. *P* values  $<0.05$  were considered significant. GraphPad Prism software (version 5; GraphPad Software, La Jolla, CA, USA) was used for statistical analyses.

### **Results**

#### *Vascular targeting peptides bind to angiogenic blood vessels in murine GBM models*

To assess the binding capacity of selected VTPs (RGR, CGKRK and NGR) to angiogenic blood vessels in brain tumours, we employed two clinically relevant orthotopic GBM models. NSCG tumour cells express a constitutively active mutant human epidermal growth factor receptor (EGFRVIII) in INK4/Arf-deficient neural stem cells and were implanted into immunocompromised mice [31]. NFpp10-GBM tumours, derived from C57BL/6 p53/PTEN-deficient neural stem cells, were grown orthotopically in syngeneic C57BL/6 hosts [29]. Two weeks after intracranial implantation, mice were injected i.v. with lipid micelles (size: 12–15 nm) containing 5-carboxyfluorescein (FAM) or FAM-labelled peptides (Figure 1A). *In vivo* binding of micelles to tumour blood vessels was analysed by immunohistochemistry after 2 h circulation. Our results demonstrated that peptide-loaded micelles are able to pass through the circulation into brain tumours and bind specifically to the angiogenic vasculature but not normal vessels (Figure 1B, C, D). In both brain tumour models (NSCG- and NFpp10-GBM)  $>80\%$  of CD31-positive tumour blood vessels were covered with CGKRK-FAM loaded

micelles. RGR-micelles bound to 30-40% of tumour vessels whereas NGR micelle binding was undetectable by directly visualizing fluorescent signals or after signal amplification using an anti-FITC antibody (Figure 1 B, C; supplementary material, Figure S1A). This is consistent with the presence of the NGR receptor aminopeptidase N/CD13 on normal brain vessels [37] but absence in mouse brain tumours (supplementary material, Figure S1B). Thus, CGKRK was the best performing peptide for payload delivery into highly angiogenic GBM models which closely resemble human GBM.

*LIGHT-CGKRK treatment normalizes angiogenic blood vessels and induces pericyte phenotype switching*

Given the strong and specific CGKRK coverage of angiogenic GBM blood vessels compared to RGR or NGR peptides, we used recombinant DNA technology to produce LIGHT-CGKRK consisting of murine LIGHT<sub>58-220</sub> fused with the C-terminus of CGKRK. Mice harbouring established NFpp10-GBM tumours were treated with i.v. injections of LIGHT-CGKRK fusion compound twice a week for 2 weeks (Figure 2A) followed by histological analysis of tumour stroma. LIGHT-VTP therapy at a dose of 20 ng per mouse (approximately 600 ng/kg body weight) significantly reduced tumour vessel length and diameter compared to untreated GBM, thus resembling the calibre of normal brain capillaries (Figure 2B, C, D). Importantly, overall vascularity was not reduced, indicating that single LIGHT-CGKRK treatment did not destroy tumour vessels (Figure 2E). To further assess the potential of LIGHT-CGKRK to “normalize” or “tighten” the leaky brain tumour vasculature, we first focused on pericytes, the outer layer of blood vessels. LIGHT-VTP treatment did not change the number of pericytes that were attached to endothelial cells compared to untreated tumours as shown by quantification of desmin-positive pericytes surrounding CD31<sup>+</sup> tumour blood vessels (Figure 3A). We recently discovered in a mouse model of neuroendocrine pancreatic

cancer that pericytes, much like their close relatives the vascular smooth muscle cells (vSMC), can switch from an angiogenic, proliferative or synthetic phenotype, to a more quiescent, contractile and mature state [10]. To assess the maturation state of brain tumour pericytes, we quantified the contractile markers  $\alpha$  smooth muscle actin ( $\alpha$ SMA), calponin and caldesmon as well as the synthetic marker collagen I in tumour blood vessels before and after therapy. Under LIGHT-VTP treatment, all contractile markers were significantly upregulated whereas synthesis of the ECM marker collagen I was reduced (Figure 3B–E). This indicates that pericytes become more mature during LIGHT-CGKRRK treatment as part of brain tumour vessel normalization.

*LIGHT-CGKRRK treatment improves vessel function and activates endothelia*

Normalization of the angiogenic tumour vasculature comprises qualitative changes in pericytes and endothelial cells [9]. In particular, vascular endothelial (VE)-cadherin, a component of endothelial cell-to-cell adherens junctions contributes to the re-establishment of vessel integrity and is upregulated during endothelial normalization in a variety of tumour models [38-40]. We found that in NFpp10-GBM treated with LIGHT-CGKRRK for 2 wk, VE-cadherin expression was upregulated and signals were less disjointed compared to untreated controls (Figure 4A). This is also consistent with more continuous and tighter adherens junctions induced by LIGHT in endothelial cells *in vitro* [25]. Importantly, enhanced pericyte contractility (Figure 3) and endothelial barrier function correlated with improved tumour perfusion as shown by reduced intratumoral hypoxia (Figure 4B) and enhanced lectin-FITC binding to tumour vessels (supplementary material, Figure S2) in LIGHT-CGKRRK treated compared to untreated mice. Moreover, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), markers for activated endothelium, were upregulated in LIGHT-CGKRRK-treated murine GBM (Figure 4C,D). Re-expression of

adhesion molecules on angiogenic blood vessels together with improved intratumoral blood flow may in turn facilitate leukocyte transmigration into brain tumours [36,41–43].

*LIGHT-CGKRRK induces HEV formation and lymphocyte accumulation in murine GBM*

To assess whether LIGHT-CGKRRK treatment increases spontaneous T cell infiltration, NFpp10-GBM bearing mice were treated with 20 ng LIGHT-CGKRRK for 2 wk and tumours analysed for expression of the HEV/PNAd marker MECA79 (Figure 5A). While naïve NFpp10-GBM brain tumours did not harbour HEVs (Figure 5B), 20% of CD31<sup>+</sup> blood vessels in LIGHT-CGKRRK treated brain tumours were positive for MECA79 (Figure 5B) indicating substantial angiogenic vessel reprogramming but no reduction in overall vascularity (Figure 5B). Furthermore, untreated NFpp10-GBM were sparsely infiltrated by CD3<sup>+</sup> T cells (Figure 5C) which did not cluster around CD31<sup>+</sup> blood vessels (supplementary material, Figure S3, left). Following LIGHT-CGKRRK treatment, significantly more CD3<sup>+</sup> immune cells had infiltrated the brain tumours ( $P=0.023$ , Figure 5C) and specifically accumulated around HEVs (supplementary material, Figure S3, right). We have previously shown that a combination of anti-VEGF therapy and immune checkpoint blockade using a specific antibody against program cell death protein ligand 1 (PD-L1) is highly effective in stimulating HEVs and anti-tumour immunity in preclinical models of neuroendocrine pancreatic and breast cancer, but not NFpp10-GBM [29]. Here, we tested whether triple therapy consisting of LIGHT-CGKRRK, anti-VEGF and anti-PD-L1 (Figure 5A) would potentiate LIGHT-CGKRRK effects. Indeed, treatment of mice bearing GBM with triple therapy for 2 wk increased the abundance of MECA79<sup>+</sup> CD31<sup>+</sup> double positive vessels to 40% compared to 20% in LIGHT-CGKRRK single treatment groups (Figure 5B). This correlated with a 6–7 fold increase in CD3<sup>+</sup> tumour infiltrating T cells (Figure 5C). Both, CD8<sup>+</sup> and CD4<sup>+</sup> T cells were increased in abundance (Figure 5D; supplementary material,

Figure S4A), and T cell infiltration was accompanied by upregulation of granzyme B (GrzB) expression and downregulation of FoxP3 (Figure 5E; supplementary material, Figure S4B). These findings were taken to imply that LIGHT-CGKRK synergizes with combination treatments to attract cytotoxic effector T cells into brain cancer. This was supported by a significantly reduced tumour burden, as quantified by GFP<sup>+</sup> NFpp10 tumour cells in brain, following 2 wk of treatment with LIGHT-VTP ( $P=0.04$ ) or triple therapy ( $P=0.002$ ) compared to untreated GBM (Figure 5F).

#### *VTPs efficiently bind to human GBM*

VTPs have been extensively tested for vascular targeting in murine syngeneic and xenograft cancer models [14]. To investigate VTP binding activity in human brain malignancies, we analysed freshly resected patient biopsies from primary vascularised brain tumours of different, namely WHO grade IV astrocytoma and grade I meningioma origin (supplementary material, Table S1). Meningiomas arise from the membranous layer of the central nervous system and are the second most common type of adult primary intracranial neoplasms. Although grade I meningiomas are angiogenic, they are normally slow-growing and employed in this study as angiogenic, “benign” tumours [44]. We first assessed CD105<sup>+</sup> blood vessel number and calibre in both tumour types (Figure 6A). The frequency of blood vessels, their lengths and diameters were found significantly increased in human GBM compared to meningioma specimens ( $P\leq 0.02$ , Figure 6 B, C, D). Next, we examined binding specificities of RGR, CGKRK or NGR to both, GBM and meningioma. FAM-labelled peptides were incubated with fresh frozen tumour sections and directly visualized by fluorescent microscopy in conjunction with the vascular marker CD105. Figure 6E (upper) shows that CGKRK and RGR bound to >80% of GBM blood vessels but not tumour cells; NGR was associated with <20% of GBM vessels, demonstrating lower binding activity.

Further amplification of NGR-FAM signals with anti-FITC antibody was required to detect NGR signals on CD105<sup>+</sup> tumour blood vessels, consistent with CD13 expression on all GBM vessels (supplementary material, Figure S5A, B). Only 20% of blood vessels were found covered by VTPs in meningiomas (Figure 6E, lower). Overall, these results provide proof of the concept that VTP targeting of highly angiogenic human GBM blood vessels, in particular with RGR and CGKRK peptides, is a viable approach that may be explored further to normalize brain tumour blood vessels and boost anti-cancer immunity.

### **Discussion**

Treatment of aggressive, highly angiogenic primary brain cancers remains a major challenge. Our study evaluated peptide-mediated targeting of brain tumour vessels, and the capacity of a newly developed reagent to improve vessel function and T cell infiltration into GBM.

We found, in two different murine orthotopic GBM models, that the CGKRK peptide shows superior binding activity when compared to peptides containing the RGR or NGR binding motifs. This is consistent with previous data showing that CGKRK effectively binds blood vessels in brain tumour xenograft models and is therapeutically active when delivered in a nanoparticle formulation together with a pro-apoptotic peptide [19].

In fresh human GBM specimens, both CGKRK and RGR targeted the majority of highly angiogenic vessels thus demonstrating that peptide-mediated payload delivery into grade IV GBM is highly feasible. Peptide binding to grade I meningiomas was significantly lower, potentially correlating with less aggressive growth and angiogenic activity. Strong RGR binding to human GBM is a new finding, because RGR targeting studies have until now been limited to murine pancreatic neuroendocrine and breast cancers [15,25,36,42,45]. Our result therefore warrants further investigations to explore its full potential for clinical applications.

NGR which binds to a tumour-specific isoform of aminopeptidase N/CD13 has been used to target a variety of murine and human cancers, including GBM [17,46]. For instance, NGR-tagged quantum dots specifically accumulated in a rat C6 glioma model as demonstrated by *in vivo* imaging [46]. However, since CD13 is also expressed on C6 cancer cells, a lack of vascular homing may be masked by direct NGR binding to tumour cells. In contrast to human GBM which shows strong vascular CD13 expression [47], blood vessels in our mouse GBM models are CD13 negative by immunohistochemistry, indicating low expression levels. Similarly, in a model of neuroendocrine pancreatic cancer, CD13 is downregulated during tumour progression, thus making NGR binding to tumour vessels less effective [48]. Since cyclic NGR when conjugated to TNF $\alpha$  is therapeutically more active than its linear counterpart, it is also possible that linear NGR, as employed throughout this study, has inferior tumour vessel binding activity [49].

Having identified CGKRK as the best performing vessel homing peptide for GBM, our subsequent functional studies were performed with a murine LIGHT-CGKRK fusion compound. Our data demonstrate that the brain tumour microenvironment is susceptible to LIGHT-CGKRK-mediated vessel normalization which increases pericyte maturity. This supports that the concept of pericyte phenotype switching in tumours, first described in pancreatic cancer [10], is also an integral part of brain tumour vessel normalization. Induction of a more mature pericyte state confers long lasting effects [10], and thus may address limitations of transient vessel normalization using anti-VEGF/VEGFR therapy.

Tighter, less leaky and activated tumour vessels in murine GBM reduce hypoxia and also enable enhanced lymphocyte infiltration into tumours. In fact, vessel normalization and T cell influx correlate with and may even be causally involved in HEVs induction in LIGHT-CGKRK-treated GBM; an interesting concept which has also been observed in models of pancreatic neuroendocrine and breast tumours using anti-VEGF and anti-PD-L1 combination



treatment [29] and human cancer [50]. In contrast to pancreatic or breast cancer models, mice bearing NFpp10-GBM are, however, refractory to combined anti-angiogenic and anti-PD-L1 therapy [29]. We show here that LIGHT-CGKRK treatment alone facilitates HEV induction and cytotoxic T cell infiltration which can be significantly enhanced with triple therapy of LIGHT-CGKRK with anti-VEGF/anti-PD-L1. This effect is most likely mediated by intratumoral LIGHT-LT $\beta$ R engagement and, consistent with reduction in tumour burden in a 2 week treatment regimen, is expected to convey a survival advantage; treatment with agonistic LT $\beta$ R antibody also stimulates HEV formation and significantly reduces tumour burden in NFpp10-GBM bearing mice [29]. Therefore, vascular targeting of LIGHT in GBM holds great promise to improve anti-tumour immunity locally when combined with immune-enhancing modalities. There is growing evidence that the brain is less immunologically privileged than previously thought and brain cancers can profit from systemic immunostimulation [51]. Whilst immunotherapy faces challenges in brain cancer patients, there are subsets of patients who respond to treatment. Our findings are thus significant in the context of ongoing clinical trials which combine anti-VEGF treatment with checkpoint blockade [52-53].

The data presented here encourage further exploration of LIGHT-VTP therapy in angiogenic tumours such as GBM with potential multiple benefits ranging from blood vessel normalization, oedema reduction and increase in immune cell infiltration and function via HEV induction.

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**Author contributions statement**

Study concept and design RG, GB, GL and AKN. Performed experiments and/or collected specimens BH, AJ, VS, AJP, RJ, JH, and GL. Generated reagents IPM, VRK, AJP, JH. Analysed and interpreted data BH, JH, RG. wrote manuscript RG, BH. All authors reviewed the manuscript.

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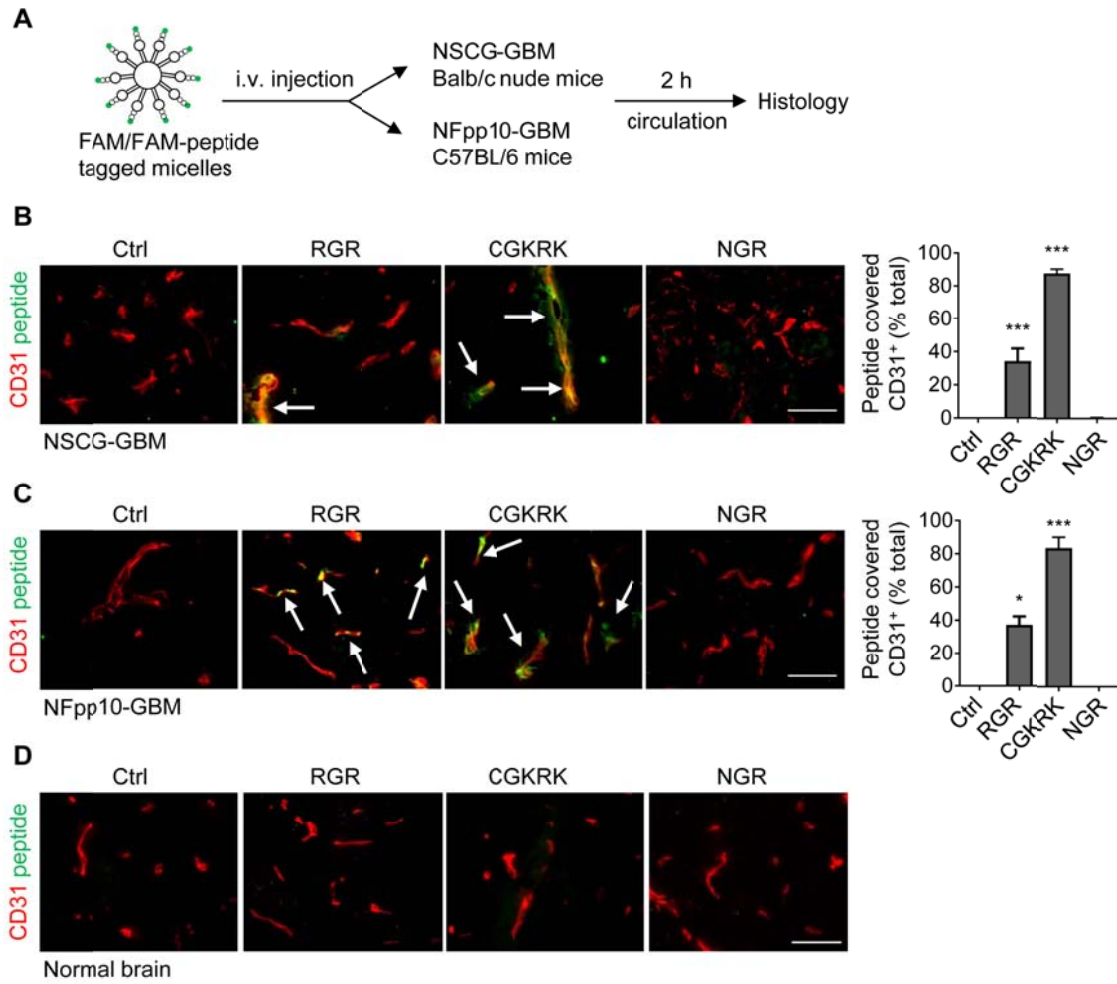
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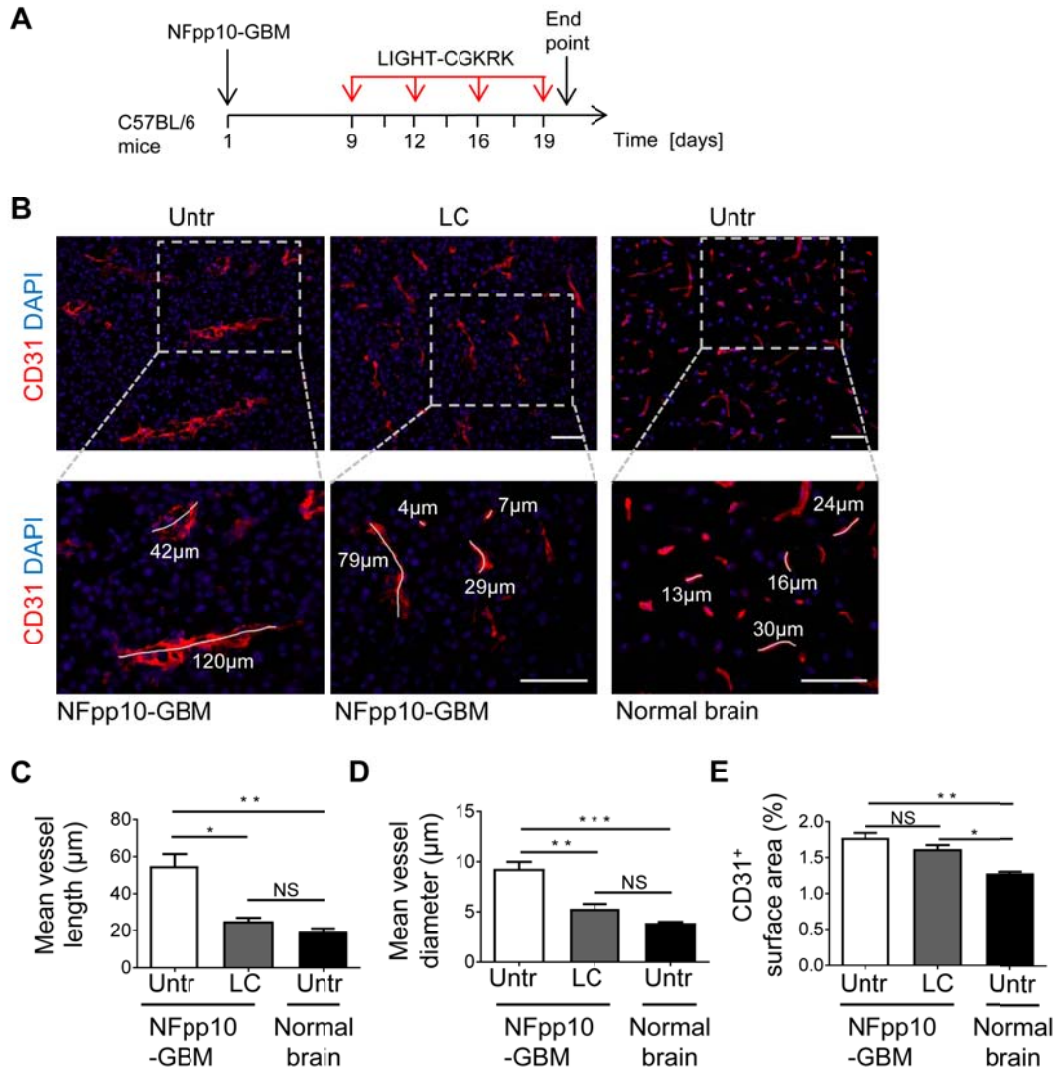


**Figure Legends**

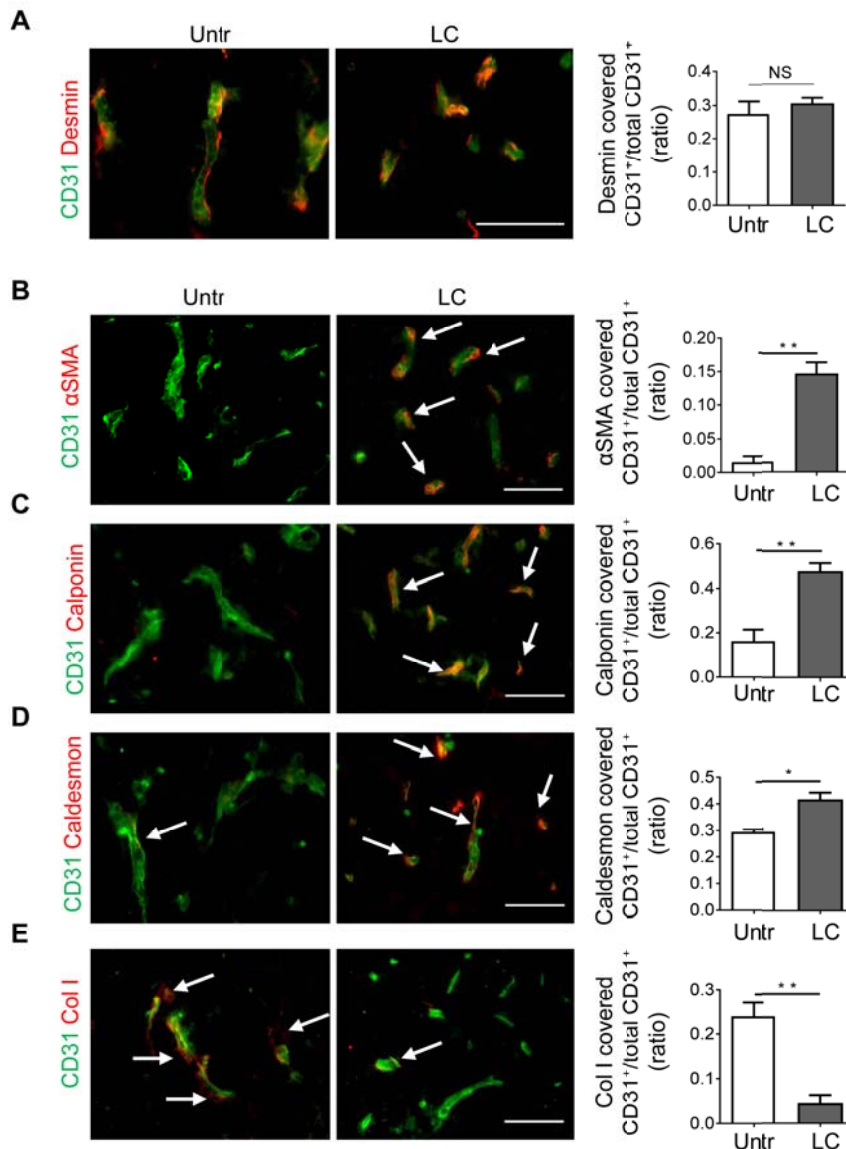
**Figure 1.** CGKRK peptide has high binding activity to angiogenic blood vessels in orthotopic GBM models. (A) Treatment scheme of mice bearing NSCG- or NFpp10-GBMs with peptide tagged micelles. (B) Immunohistochemical analysis of NSCG-GBM after micelle injection. Fam-labelled micelles were injected without peptide (control, Ctrl) or tagged with RGR-FAM, CGKRK-FAM or NGR-FAM peptides (green), and peptide covered (yellow) CD31<sup>+</sup> (red) tumour vessels were quantified, n=2, 200-400 blood vessels/mouse, \*\*\* $P < 0.0001$ . (C) Quantitative analysis of peptide loaded micelles in relation to blood vessels in NFpp10-GBM as in B), n=2 mice/group, 200-400 blood vessels/mouse. \* $P = 0.022$ , \*\*\* $P < 0.0001$ . ANOVA. Arrows point to some blood vessels with bound peptides. D) Representative images of FAM-peptide homing to normal brain (control). Scale bars, 50  $\mu\text{m}$ .



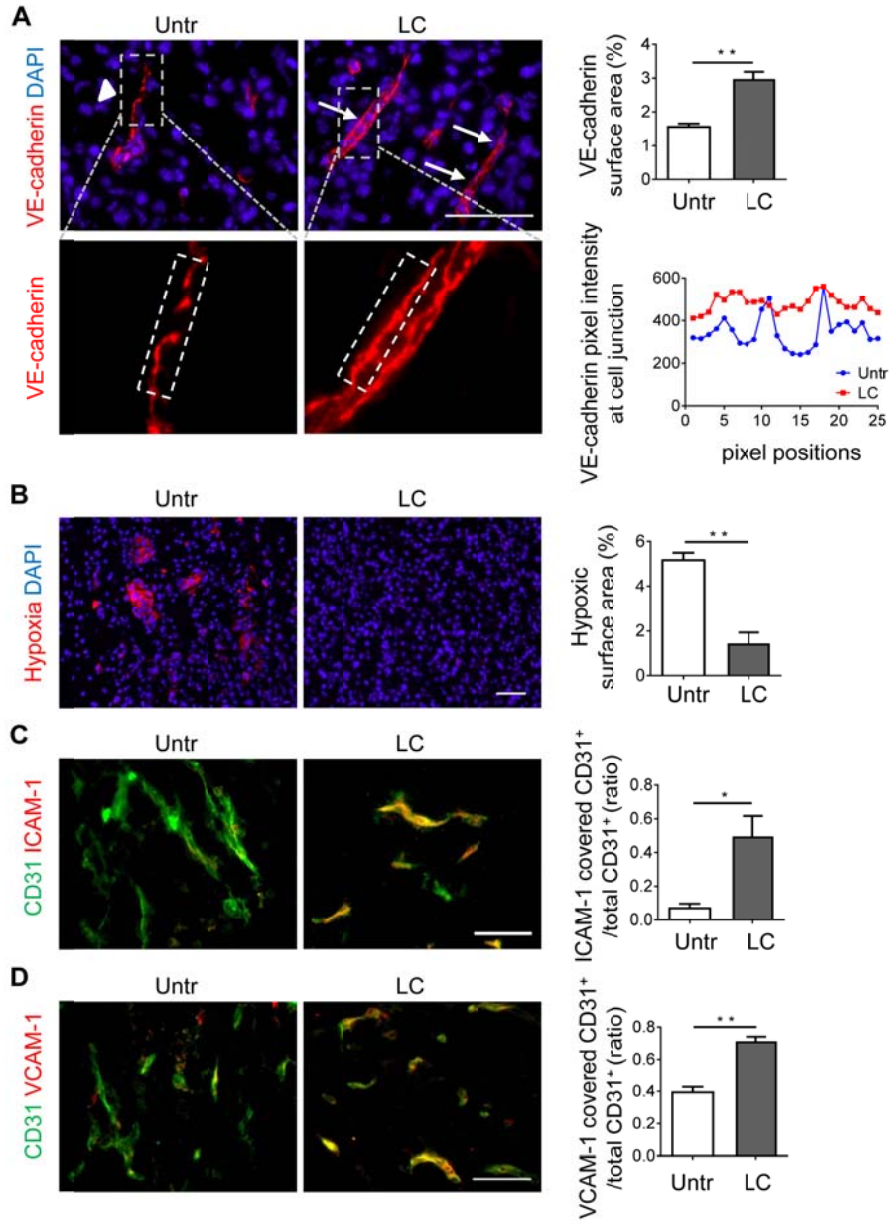
**Figure 2.** *In vivo* LIGHT-CGKRRK treatment normalizes GBM blood vessels. (A) LIGHT-CGKRRK treatment scheme in C56BL/6 mice harbouring established NFpp10-GBM. (B) Representative immunohistochemistry of blood vessels (CD31, red) in untreated brain tumours (NFpp10-GBM, Untr), NFpp10-GBM mice treated with four i.v. injections of 20 ng LIGHT-CGKRRK (LC), or normal brain from untreated mice. Dashed outlines indicate areas enlarged below. 2–4 representative vessels per group are demarcated to indicate vessel size. Scale bars, 50  $\mu$ m. Quantification of (C) mean vessel length,  $n=3$ ,  $*P=0.005$ ,  $**P=0.002$ , NS: not statistically significant, (D) mean vessel diameter,  $n=3$ ,  $**P=0.003$ ,  $***P=0.0006$ , and (E) overall vascularity (CD31<sup>+</sup> surface area),  $n=3$ ,  $*P=0.01$ ,  $**P=0.002$ , in treatment groups as described in B). ANOVA.



**Figure 3.** LIGHT-CGKRK treatment changes pericyte phenotype without affecting pericyte numbers around GBM blood vessels. (A) NFpp10-GBM bearing mice were treated with 20 ng LIGHT-CGKRK (LC) for 2 wk or left untreated (Untr). Tumours were analysed by immunohistochemistry to quantify the coverage of endothelial cells (CD31, green) by pericytes expressing (A) the pericyte-specific marker desmin (red), or the contractile markers (B)  $\alpha$ SMA, n=3,  $**P=0.002$ , (C) calponin, n=3,  $**P=0.008$ , (D) caldesmon, n=3,  $*P=0.012$  or the synthetic marker collagen I (Col I), n=3,  $**P=0.006$ . Student's *t*-test. Arrows depict some blood vessels covered by overlapping markers (yellow). Scale bars, 50  $\mu$ m.

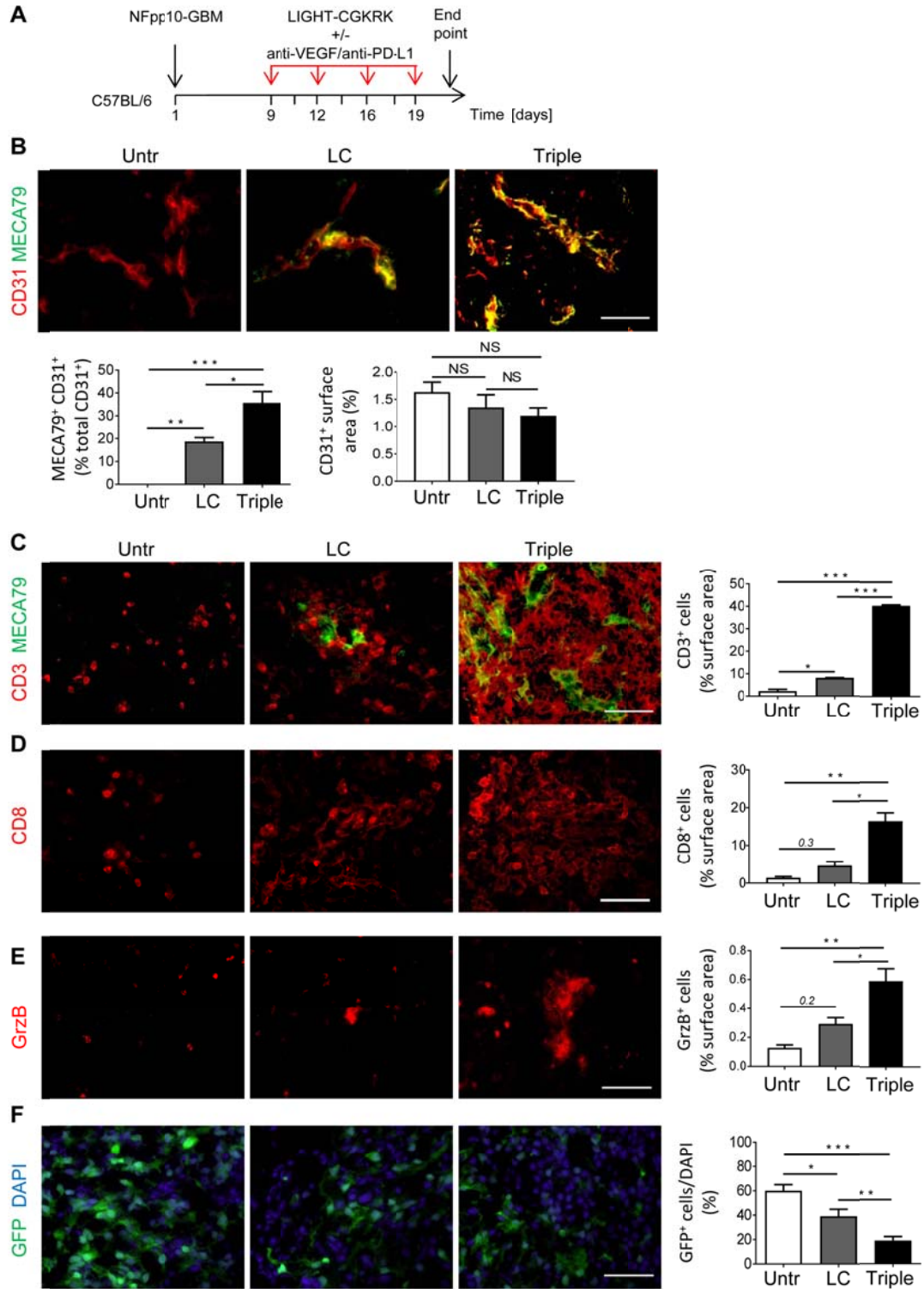


**Figure 4.** LIGHT-CGKRK treatment improves endothelial barrier integrity and vessel function. (A) NFpp10-GBM bearing mice were left untreated (Untr) or treated for 2 wk with LIGHT-CGKRK (LC). VE-cadherin expression in tumours was quantitatively (upper) and qualitatively (lower) assessed by immunohistochemistry. Arrowheads point to gaps in disjointed adherens junctions, arrows point to continuous VE-cadherin<sup>+</sup> adherens junctions, n=3, \*\**P*=0.0032. Dashed outlines (grey, upper) indicate areas enlarged below, and dashed white lines (below) indicate junctional areas quantified as VE-cadherin fluorescence (in pixels, same exposure for all groups) at various positions (horizontal axis) along the cell adherens junctions. (B) Assessment and quantification of intratumoral hypoxia by immunohistological detection of pimonidazole-positive areas (red) in treatment groups, n=3, \*\**P*=0.0028. (C) Expression of ICAM-1 or (D) VCAM on GBM tumour vessels with and without LC treatment, analysed by immunohistochemistry and quantified, n=3, \**P*=0.027, \*\**P*=0.0014. Student's *t*-test. Scale bars, 50 μm.

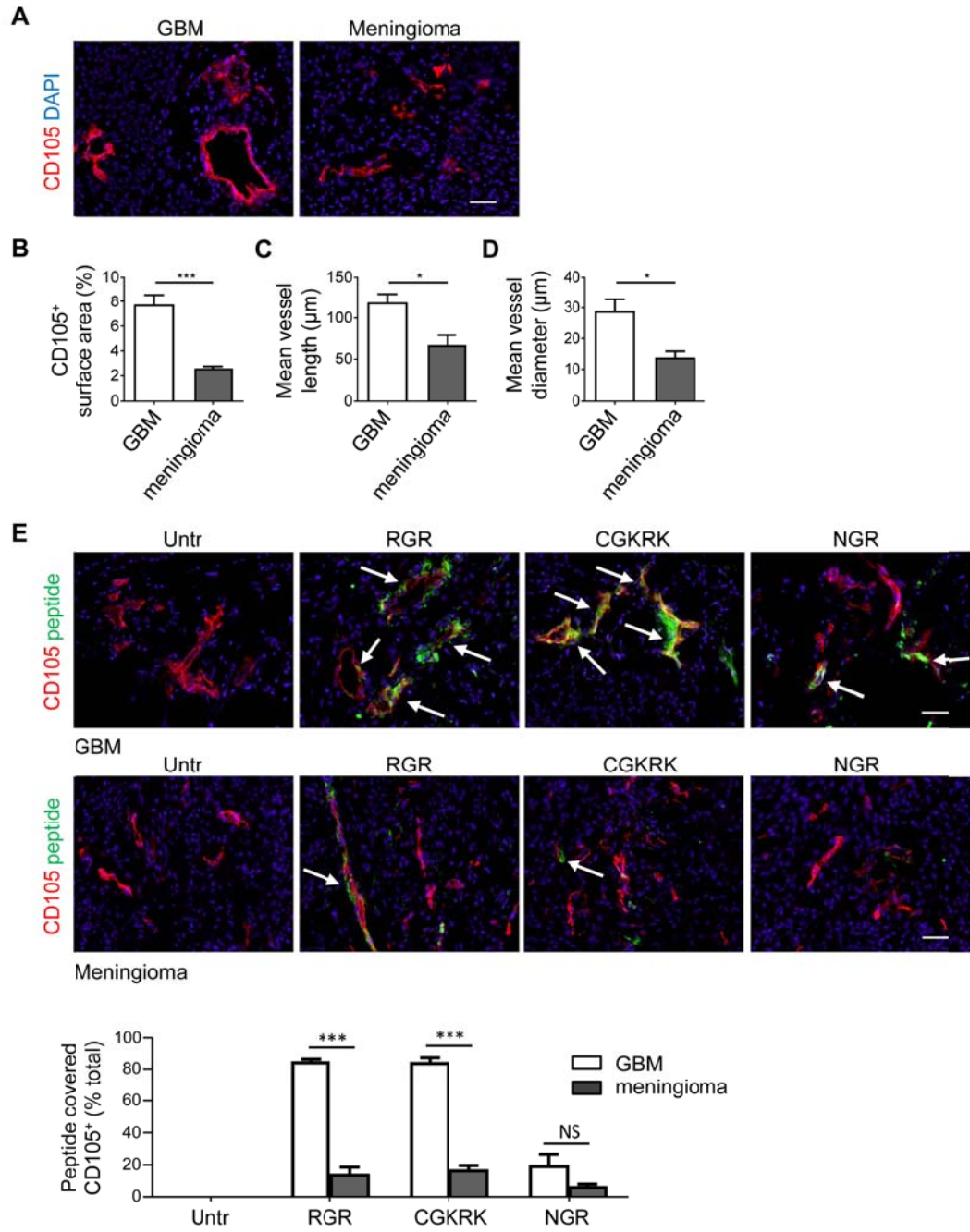


**Figure 5.** Induction of HEVs and T cell infiltration by LIGHT-CGKRK is further amplified with combination immunotherapy. (A) LIGHT-CGKRK or triple treatment (LIGHT-CGKRK + anti-VEGF + anti-PD-L1) scheme in C56BL/6 mice harbouring established NFpp10-GBM. (B) Mice bearing NFpp10-GBM were left untreated (Untr), treated for 2 wk with LIGHT-CGKRK (LC) as single reagent, or combined with anti-VEGF/anti-PD-L1 treatment (Triple). Tumours were analysed for the presence of the HEV marker MECA79 (green) and overlaid (yellow) with CD31<sup>+</sup> (red) endothelial cells and total CD31<sup>+</sup> vascularity, n=3, \**P*=0.0086, \*\**P*=0.0035, \*\*\**P*<0.0001, NS: not statistically significant. (C) Treatment groups as in (A) were assessed by immunohistochemistry for MECA79<sup>+</sup> HEVs (green) and CD3<sup>+</sup> T cells (red) and T cell infiltration into tumours quantified, n=3, \**P*=0.023, \*\*\**P*<0.0001. (D) Corresponding CD8<sup>+</sup> T cell infiltration and quantification, n=3, \**P*=0.003, \*\**P*=0.0007. (E) GrzB<sup>+</sup> immune cells and quantification, n=3, \**P*=0.02, \*\**P*=0.003. (F) Imaging and quantification of GFP<sup>+</sup> NFpp10 tumour cells in brain (DAPI), n=3, \**P*=0.05, \*\**P*=0.04, \*\*\**P*=0.002. ANOVA. Scale bars, 50 μm.





**Figure 6.** Peptide binding to human brain cancer blood vessels correlates with angiogenic activity. (A) Immunohistochemical analysis of CD105<sup>+</sup> tumour blood vessels (red) in grade IV GBM and grade I meningioma (for specimen details see Table 1). Quantification of (B) overall vascularity (CD105<sup>+</sup> surface area), \*\*\* $P=0.0009$ , (C) mean vessel length, \* $P=0.0133$ , and (D) mean vessel diameter, \* $P=0.0213$ , in GBM (n=6) and meningioma (n=4). (E) Histological assessment of FAM-labelled RGR, CGKRK and NGR peptide (green) binding to CD105<sup>+</sup> (red) blood vessels in GBM (upper, n=6) or meningioma (lower, n=4) and quantification of peptide covered vessels (yellow), \*\*\* $P<0.0001$ . Arrows depict some peptide covered blood vessels. Student's *t*-test. Scale bars, 50  $\mu\text{m}$ .



**SUPPLEMENTARY MATERIAL ONLINE**

**Supplementary materials and methods** NO

**Supplementary figure legends** NO, because the legends are embedded in a PDF file S1–S4

**Figure S1.** Murine GBM lack CD13 expression and NGR binding

**Figures S2.** LIGHT-CGKRK treatment increased tumour perfusion

**Figure S3.** Intratumoral T cells clustered around HEVs but not CD31<sup>+</sup> endothelia

**Figures S4.** Increased CD4<sup>+</sup> T cell infiltration correlated with reduced intratumoral FoxP3 signals

**Figures S5.** NGR-FAM binding to human GBM blood vessels correlated with CD13 expression

**Table S1.** Patient information and histopathology