Triggering anti-GBM immune response with EGFR-mediated photoimmunotherapy

Justyna Mączyńska1†, Florian Raes1†, Chiara Da Pieve1, Stephen Turnock1, Jessica K. R. Boult1, Julia Hoebart1, Marcin Niedbala2, Simon P. Robinson1, Kevin J. Harrington1, Wojciech Kaspera2* and Gabriela Kramer-Marek1*

Abstract

Background: Surgical resection followed by chemo-radiation postpones glioblastoma (GBM) progression and extends patient survival, but these tumours eventually recur. Multimodal treatment plans combining intraoperative techniques that maximise tumour excision with therapies aiming to remodel the immunologically cold GBM microenvironment could improve patients’ outcomes. Herein, we report that targeted photoimmunotherapy (PIT) not only helps to define tumour location and margins but additionally promotes activation of anti-GBM T cell response.

Methods: EGFR-specific affibody molecule (ZEGFR03115) was conjugated to IR700. The response to ZEGFR03115-IR700-PIT was investigated in vitro and in vivo in GBM cell lines and xenograft model. To determine the tumour-specific immune response post-PIT, a syngeneic GBM model was used.

Results: In vitro findings confirmed the ability of ZEGFR03115-IR700 to produce reactive oxygen species upon light irradiation. ZEGFR03115-IR700-PIT promoted immunogenic cell death that triggered the release of damage-associated molecular patterns (DAMPs) (calreticulin, ATP, HSP70/90, and HMGB1) into the medium, leading to dendritic cell maturation. In vivo, therapeutic response to light-activated conjugate was observed in brain tumours as early as 1 h post-irradiation. Staining of the brain sections showed reduced cell proliferation, tumour necrosis, and microhaemorrhage within PIT-treated tumours that corroborated MRI T2*w acquisitions. Additionally, enhanced immunological response post-PIT resulted in the attraction and activation of T cells in mice bearing murine GBM brain tumours.

Conclusions: Our data underline the potential of ZEGFR03115-IR700 to accurately visualise EGFR-positive brain tumours and to destroy tumour cells post-conjugate irradiation turning an immunosuppressive tumour environment into an immune-vulnerable one.

Keywords: Photoimmunotherapy, Glioblastoma, Affibody molecules, IR700
Background

Glioblastoma (GBM) is the most common primary malignant brain tumour in adults and is associated with an extremely aggressive clinical course and poor prognosis [1]. The median progression-free survival in primary GBM is 6.9 months, and the median overall survival is 14.6 months with standard-of-care surgery, radiation therapy, and temozolomide [2, 3]. Consequently, there is a high unmet clinical need for new treatment paradigms yielding more durable remissions.

The current neurosurgical management of GBM aims for maximal resection while avoiding additional neurological damage. Numerous methods have been developed to facilitate surgery, including 5-aminolevulinic acid (5-ALA) fluorescence-guided surgery, intraoperative neuro-navigation, and neurophysiological monitoring [4, 5]. However, GBM recurrence is almost inevitable due to residual areas of diffuse microscopic infiltration of tumour cells into the surrounding brain parenchyma and intratumoural heterogeneity at the cellular and molecular levels.

Approximately 57% of GBMs contain a mutation, rearrangement, splicing alteration, and/or amplification of the epidermal growth factor receptor (EGFR). The most common EGFR variant is a deletion of exons 2–7, EGFRvIII, which often co-occurs with focal EGFR amplification, which together are associated with a more aggressive, immuno-evasive tumour phenotype and worse prognosis [6]. Despite the well-known role of EGFR in GBM, the potential of targeting the receptor with tyrosine kinase inhibitors (TKIs) as well as monoclonal antibodies (mAbs) have been unfulfilled so far. Furthermore, a phase III study (ACT IV), for newly diagnosed patients with GBM treated with Rindopepimut, an EGFRvIII-targeted vaccine, also failed to demonstrate a survival benefit [7].

Interestingly, recent studies have shown that inhibiting EGFR signalling may reduce tumour cell-intrinsic EGFR-induced programmed death-ligand 1 (PD-L1) upregulation, as well as extrinsic IFNγ-induced signals associated with CD8+ T cell infiltration into the tumour microenvironment (TME) [1, 8]. However, attempts to incorporate immune checkpoint inhibitors (ICPIs) into GBM treatment regimens have demonstrated only modest and unpredictable responses [9, 10]. This is most likely due to low burdens of somatic mutations and a relatively immune-depleted ("cold") GBM microenvironment characterised by a high level of immunosuppressive cytokines (e.g. TGFβ, IL-10) which inhibit immune effector cell activity [11]. Excitingly, several research groups have reported that high-level infiltration of immune effector cell populations, including CD8+ cytotoxic T-lymphocytes (CTLs), into the TME can improve response to ICPIs in GBM [12, 13]. Therefore, in a clinical context, it would be desirable to restore intratumoural infiltration of CD8+ T cells to create an immunologically “hot” TME and, thus, promote the responsiveness of GBM to ICPIs.

One way to activate the TME immunologically would be through the use of photoimmunotherapy (PIT) and conventional photodynamic therapy (PDT).

PIT is a light-mediated therapeutic approach, where a photosensitiser (PS) is conjugated to a highly specific monoclonal antibody (mAb), antibody fragment, or affibody molecule that has the ability to engage the selected target of interest. Near-infrared (NIR) light irradiation of the conjugate lead to ligand release reaction of IR700 and under normoxic conditions to the production of heat and reactive oxygen species (ROS) that, consequently, initiate target-selective cell death and stimulate inflammation, followed by vascular shutdown and tissue ischaemia [14–16]. For example, Nagaya et al. have shown that anti-CD44-IR700-mediated PIT can significantly delay tumour growth following a single treatment in three CD44-expressing syngeneic mouse models of oral squamous cell carcinoma [17]. In addition, NIR-PIT targeting EGFR with anti-can225-IR700 resulted in rapid cell death in vitro and tumour growth inhibition in vivo, improving mouse survival [18]. More importantly, EGFR-targeting IR700-cetuximab (ASP-1929, Akalux™, Rakuten Medical, Inc.) is currently being investigated in a global phase III clinical trial in head and neck cancer (NCT03769506) [19] and was registered for clinical use in Japan [20]. Furthermore, it has been shown that both PIT and PDT can trigger immunogenic cell death (ICD), as exemplified by the release of damage-associated molecular patterns (DAMPs), including calreticulin (CRT), heat shock proteins HSP70/90, ATP, and high-mobility group box-1 (HMGB1) nuclear protein that subsequently activate immune cells upon binding to pattern recognition receptors [21].

In view of the high expression rate and oncogenic nature of EGFR, we have postulated that PIT targeting this receptor could promote CD8+ T cell attraction and activation and overcome the immunologically “cold” status of GBM.

As an alternative to full-size antibodies, we have previously investigated the smaller, IR700-labelled EGFR-specific affibody molecule (ZEGFR03115-IR700), aiming for more effective tumour penetration, faster delivery, and clearance from non-targeted tissues [22]. After demonstrating that ZEGFR03115-IR700 cell uptake enables imaging of EGFR expression in an orthotopic brain tumour model (U87-MGvIII), our proof-of-concept in vivo PIT study also showed the conjugate’s therapeutic efficacy in subcutaneous glioma xenografts [22].

In the current study, we report that ZEGFR03115-IR700-PIT promotes the production of DAMPs from cancer
cells, also leading to dendritic cell (DC) maturation in vitro. In addition, when applied in a syngeneic mouse model, the treatment induces T cell responses that might overcome the “immunologically cold” status of GBM. Therefore, we believe that this therapeutic approach, following complete or cytoreductive resection of GBM, could lead to (i) elimination of residual or surgically inaccessible EGFR+ve cancer cells and (ii) subsequent stimulation of anti-tumour immunity.

Methods
Preparation of ZEGFR03115-IR700
The conjugation of IRDye700DX-maleimide (IR700, ex. 689 nm, em. 700 nm; LI-COR® Bioscience, USA) to the ZEGFR03115-Cys affibody molecules (Affibody, Sweden) is described in detail in the supporting information (Additional File 1).

Cell lines and cell culture
Human GBM cell line DKMG and murine GBM cell line GL261 were purchased from the Cellther Polska (Poland) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany), respectively. U87-MG and U87-MGvIII were kindly provided by Dr. Frank Furnari (Ludwig Cancer Research, USA) [23]. The primary, patient-derived cell lines WSz4, WSz50, and WSz57 have been recently established in our lab [22]. The cells were grown as described in the supporting information. BL6-NPE-GFP-Luc murine GBM cell line was kindly provided by Dr. Steven Pollard (University of Edinburgh, UK) and cultured as previously reported [24]. The genetic origin of all the cell lines was tested and authenticated by short tandem repeat (STR) DNA profiling analysis (Eurofins Medigenomix, Germany). The cells were also routinely tested and found to be negative for Mycoplasma contamination (PCR detection kit, Surrey Diagnostics Ltd., UK).

Singlet oxygen production assay
Singlet oxygen (1O₂) production was determined using the Singlet Oxygen Sensor Green reagent (SOSG, Thermo Fisher Scientific, UK) according to the protocol provided by the manufacturer. More details about the assay are described in the supporting information.

Cellular binding of ZEGFR03115-IR700
Human and murine GBM cells were harvested and incubated in a medium with ZEGFR03115-IR700 (30 nM) for 1 h at 4 °C, and samples were analysed using flow cytometry (BD™ LSRII). To test the targeting specificity and internalisation of the conjugate, cells were plated on confocal glass-bottomed dishes (Thermo Fisher Scientific, USA) in complete medium with ZEGFR03115-IR700 (1 μM) for 1 h at 4 °C or 1, 3, and 6 h at 37 °C and analysed using a Zeiss LSM700 confocal microscope (Carl Zeiss Inc., Germany). A detailed description of the procedures is given in the supporting information.

In vitro PIT studies
U87-MGvIII cells were seeded on petri dishes 24 h before experiments. Afterwards, cells were incubated with ZEGFR03115-IR700 (0.1 to 1 μM) for 1, 3, or 6 h at 37 °C. The media were then changed for phenol red-free DMEM medium and cells irradiated (8 or 16 J/cm²) using a LED light source (L690–66–60, Marubeni America Co., USA). Cell viability was determined using the CellTiter-Glo® (Promega, USA) luminescent assay 24 h post-light exposure. To assess ROS production, 5 μM 2’,7’-dichlorofluorescein diacetate (DCFDA; Sigma, UK) was added to phenol-red free medium during irradiation. The cell death at 1, 4, and 24 h post-irradiation was assessed using the Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific, UK) according to the manufacturer’s instruction. To determine the post-PIT ATP and HMGB1 release, the ENLITEN® ATP assay (Promega, USA) and an HMGB1 ELISA kit (Tecan, IBL International, Germany) were used. Calreticulin exposure on the membrane was measured by flow cytometry (BD™ LSRII). All the methods are described in detail in the supporting information.

Co-culture with dendritic cells
The experimental details about co-culturing the immature dendritic cells (iDCs) with PIT-treated U87-MGvIII or DKMG cells are given in the supporting information.

Western blot
Western blotting was performed as previously described [22]. Proteins released into the medium were extracted using an acetone precipitation protocol (Thermo Fisher Scientific, USA). The list of antibodies used and densitometric analysis are provided in the supporting information.

18F-AlF-NOTA-ZEGFR03115 preparation
The preparation of NOTA-ZEGFR03115 and its radiolabelling with the 18F-AI complex was performed as previously described [25].

In vivo studies
All experiments were performed in compliance with licences issued under the UK Animals (Scientific Procedures) Act of 1986 and following local ethical review. Studies were compliant with the UK National Cancer Research Institute Guidelines for Animal Welfare in Cancer Research [26] and the ARRIVE (animal research: reporting in vivo experiments) guidelines [27].
Mouse models
The detailed methods are described in the supporting information. Briefly, NCr athymic female mice (5–6 weeks) were bred in-house. C57BL/6j female mice (6–7 weeks) used for the syngeneic model, were purchased from Charles River, UK. The orthotopic GBM U87-MGvIII or BL6-NPE-GFP-Luc mouse models were established as previously described [22, 24]. For the subcutaneous GBM xenografts, U87-MGvIII cells were injected over the right shoulder. Once tumours reached approximately 60 mm³, mice were randomly distributed into the experimental groups.

PIT in vivo
For PIT treatment studies, subcutaneous and intracranial GBM U87-MGvIII xenografts were randomised into the following treatment groups: (i) light exposure only (100 J/cm²) and (ii) 18 μg ZEGFR:03115-IR700 with light exposure (100 J/cm², 0.0886 W/cm²). For immunocompetent mice bearing intracranial tumours, 50 J/cm² light dose was used. The tumours were irradiated with a LED light source (L690–66–60, peak 690 ± 20 nm) 1 h post-conjugate i.v. injection. More details are provided in the supporting information.

MR imaging
To monitor the orthotopic tumour growth, mice were imaged using the 1 T M3™ MRI system (Aspect Imaging, Israel) with a T2-weighted imaging sequence and a dedicated head coil. To perform high-resolution acquisitions, mice were scanned using the 7 T Biospec® horizontal micro-imaging system (Biospec®, Bruker, Germany). The imaging protocols are described in the supporting information.

PET imaging
Mice (n = 5) with MRI-confirmed brain tumours received an i.v. injection of 18F-AIF-NOTA-ZEGFR:03115 (12 μg; 2.4 ± 0.15 MBq/mouse), and PET/CT scans were acquired 1, 3, and 5 h post-injection of the radiotracer using an Albira PET/SPECT/CT imaging system. The detailed imaging and data analysis protocols are given in the supporting information.

Fluorescent imaging
In vivo and ex vivo fluorescence images were acquired as stated in the supporting information using an IVIS Spectrum/CT system (Perkin Elmer, USA).

Immunohistochemistry
Formalin-fixed brain and tumour tissues were embedded in paraffin, sectioned (5-μm-thick slices), and mounted on microscope slides. Frozen embedded tissues were sectioned into 10-μm-thick slices and mounted on microscope slides before being fixed in ice-cold acetone. The detailed staining procedures with the various antibodies are described in the supporting information.

Tumour and T cell isolation
Tumour and surrounding brain tissue were harvested and dissociated via enzymatic digestion (Liberase TL, Roche, Switzerland). Single-cell suspension was prepared by straining the digested tissue through a 70-μm mesh. Further experimental details are given in the supporting information.

Serum cytokine analysis
The serum was separated from the whole blood collected from the mice at the 24 h endpoint, snap-frozen, and stored at −80 °C until further analysis. Concentrations of various cytokines were analysed using a Mouse Cytokine Proinflammatory Focused 10-plex Array (Eve Technologies, Canada).

Statistical analysis
Unless otherwise stated, data were expressed as the mean ± SD. Statistical significance, sample size calculations, and correlation analysis are described in detail in the supporting information.

Results
ZEGFR:03115-IR700-PIT leads to an EGFR expression-dependent response in vitro
The affibody molecule (ZEGFR:03115), which recognises the murine and human extracellular epitope of EGFR, was conjugated to IR700. Specific and receptor expression-dependent ZEGFR:03115-IR700 binding (Fig. S1A), as measured by flow cytometry, was in line with the total EGFR level assessed via Western blot (Fig. S1B) in a panel of human and mouse GBM cell lines. In order to confirm that ZEGFR:03115-IR700 PIT induces target-specific cell death, U87-MGvIII (EGFR high), DKMG (EGFR high), WSz57 (EGFR medium), and U87-MG (EGFR low) cells were incubated with increasing concentrations of the conjugate (0–0.5 μM; 1 h) and exposed to dose of NIR light selected based on our previous studies [22]. A significant decrease in cell viability in a conjugate concentration-dependent manner was seen in both U87-
Fig. 1 (See legend on next page.)
MGvIII and DKMG cells 24 h post-irradiation with 16 J/cm² (Fig. 1A). However, DKMG appeared to be more resistant to the treatment in the presence of low concentrations of the conjugate (survival 75% and 59% at 0.1 and 0.25 μM, respectively) compared to U87-MGvIII cells (survival 36% and 28%, respectively). At the highest concentration of ZEGFR:03115-IR700 (0.5 μM), both cell lines demonstrated a dramatic loss in cell viability (less than 10% survival). A small but significant reduction in cell viability (81% survival) was observed in U87-MG cells when the highest concentration of the conjugate was tested (0.5 μM). The patient-derived WSz57 cell line was less sensitive to the treatment, most likely due to highly heterogeneous EGFR expression (Fig. S1C). A longer incubation (6 h) of U87-MG, DKMG, and WSz57 cells with the ZEGFR:03115-IR700 (Fig. S1D) led to an enhanced PIT-mediated cell death that was in line with an increased internalisation of the conjugate as confirmed via confocal microscopy (Fig. S1E).

Further, to determine the mechanism of cell death following the treatment, we used the Annexin V/PI assay. Flow cytometric analysis showed that U87-MGvIII cells were dying rapidly, and within 1 h post-irradiation (0.25 μM conjugate, 8 or 16 J/cm²), there were three distinct cell populations: viable (Annexin−/PI−; 45-56%), apoptotic (Annexin−/PI+; 8-12%), and necrotic (Annexin+/PI+; 35-43%). Importantly, by 24 h post-irradiation, the population of viable cells was 17.5% and 4% (ZEGFR:03115-IR700 + 8 J/cm² or 16 J/cm² delivered light, respectively) compared to the control groups (91%) (Fig. 1B).

It is well recognised that an essential component of the intracellular pathways that enables ICD and the release of DAMPs is ROS production [21]. Therefore, we subsequently investigated whether ZEGFR:03115-IR700 PIT-mediated generation of ROS will trigger the activation and trafficking of DAMPs to the extracellular space in vitro. The capability of ZEGFR:03115-IR700 to induce singlet oxygen (¹O₂) after NIR light activation was initially measured in cell-free conditions. The studies confirmed a significant light dose-dependent SOSG fluorescence enhancement post-irradiation (Fig. S1F). Moreover, U87-MGvIII cells subjected to ZEGFR:03115-IR700-based PIT (0.25 μM; 16 J/cm²) showed a prominent increase of intracellular ROS production that was significantly suppressed by the ROS scavenger NAC (Fig. 1C). This quenching effect consequently resulted in an inhibition of PIT-induced cell death (Fig. 1D). After PIT with just 8 J/cm², only a slight enhancement in ROS generation was measured in U87-MGvIII cells (Fig. 1C). However, NAC successfully inhibited PIT-induced cell death under each conjugate concentration (Fig. 1D).

**PIT induces the production of DAMPs in GBM cell lines and maturation of iDCs in vitro**

Next, we measured the efficacy of ZEGFR:03115-IR700-based PIT (0.25 μM; 16 J/cm²) to lead to DAMPs (CRT, HMGB1, HSP70, and HSP90) release in U87-MGvIII cells. As shown in Fig. S2A, there was a transient but significant increase in CRT expression level as early as 5 min post-NIR irradiation. We also observed a rapid (5 min) secretion of ATP into the cell culture media post-irradiation (Fig. 2A). Western blot densitometric analysis (Fig. S2) revealed a rapid release of HMGB1, HSP90, and HSP70 into the culture medium after PIT. These strong immunogenic signals were in line with the pronounced U87-MGvIII PIT-induced cell death (Fig. 1A, 0.25 μM; 16 J/cm²) and most likely high sensitivity of these cells to oxidative stress. No other DAMP upregulation was detected in any of the control groups (Fig. 2B; Fig. S2). Additionally, ELISA results (corroborated by Western blot data) showed that cells irradiated 1 h after ZEGFR:03115-IR700 treatment released HMGB1 in a time-dependent manner when compared to the control cells (Fig. 2C). We then investigated whether the enhanced levels of ICD markers, induced by ZEGFR:03115-IR700 PIT
Fig. 2 (See legend on next page.)
in U87-MGvIII cells, would trigger phenotypic maturation of DCs in a similar manner to conjugates reported by others [28]. We co-cultured ZEGFR:03115-IR700-PIT-treated U87-MGvIII cells with iDCs for 48 h. Subsequent flow cytometry analysis showed a significant increase in the expression of CD86 (Fig. 2D) and MHC class II (HLA-DR) (Fig. 2E) molecules on the surface of DCs exposed to PIT-treated U87-MGvIII cells compared to controls. However, there was no change in the CD40 expression level (Fig. 2F).

**Imaging EGFR expression in orthotopic GBM tumours**

The capability of ZEGFR:03115 to target EGFR-expressing cells in vivo was evaluated using an orthotopic U87-MGvIII tumour model (Fig. 3). Five days post-cell implantation, the progression of intracranial malignancies was assessed by T₂-weighted MRI (Fig. 3A). To confirm the targeting abilities of ZEGFR:03115, PET/CT studies were performed. Images were acquired at 1, 3, and 5 h post-administration using the well-established EGFR-targeting imaging agent ^18^F-AIF-NOTA-ZEGFR:03115 (Fig. 3A) (n = 3) [25]. The images recorded 3 h post-injection demonstrated preferential and focal accumulation of the radioconjugate in the tumour mass (Fig. 3A and Fig. S3A-B). Negligible activity was observed in the normal cerebral tissue that provided sharp delineation of the tumours with high tumour/parenchyma contrast. The ROI quantitative analysis showed a time-dependent increase of tumour radioactivity uptake with the highest value observed 5 h p.i. (%ID/g_S0 = 5.14 ± 1.17) (Fig. 3B). H&E staining of axial brain sections confirmed the presence of well-defined tumour masses, which were in line with PET/CT and MRI signals in vivo as well as radioactivity signals measured ex vivo (Fig. S3B). When ZEGFR:03115-IR700 was administered i.v., the strong fluorescence signal of the conjugate was detected ex vivo within the brain EGFR-positive lesions (2–3 mm in diameter) as early as 1 h post-injection in mice bearing MRI-confirmed tumours (Fig. 3C) (n = 3).

**Monitoring tumour response to ZEGFR:03115-IR700-PIT in vivo**

To assess whether ZEGFR:03115-IR700-PIT shows an anti-tumour effect against GBM in vivo, subcutaneous and intracranial U87-MGvIII tumours were established in nude mice. In contrast to our previous studies using three ZEGFR:03115-IR700-PIT doses over three consecutive days [22], herein, we tested whether administration of just one dose would inhibit tumour growth. The conjugate (18 μg) was injected intravenously (Fig. S4A), and the subcutaneous tumours were irradiated 1 h later with 100 J/cm^2_. A significant delay in tumour growth was observed during the initial 7 days in the group receiving ZEGFR:03115-IR700-PIT compared to the control (light only 100 J/cm^2) (Fig. S4B). However, due to the regrowth of measurable tumours at this point, a second PIT dose was delivered. Unfortunately, no further growth inhibition was observed (Fig. S4B). Next, we studied the early anti-tumour response to PIT using the orthotopic U87-MGvIII model by MRI. Following tumour establishment, T₂- and T₂*-weighted images were acquired at baseline and 1 or 4 h post-ZEGFR:03115-IR700-PIT (18 μg; 100 J/cm^2) (Fig. 4A). In the group that received ZEGFR:03115-IR700-PIT as compared to the control group (Fig. 4A), the T₂*-weighted images showed an intratumoural signal decrease corresponding to haemorrhage and hemosiderin deposition 1 h post-NIR irradiation in mice treated with ZEGFR:03115-IR700-PIT as compared to the control group (Fig. 4A). We also observed an enlargement of the lesions on T₂*-weighted images caused by direct cytotoxic effects on tumour cells, damage to the tumour vasculature, and induction of an inflammatory reaction post-PIT, which resulted in the swelling of the surrounding brain tissue and a mass effect (Fig. 4A). Furthermore, increased signal intensity on T₂*-weighted images of parenchyma surrounding the tumour growing from the top of the skull along the pathway of cell implantation most likely corresponds to the cerebral oedema formation following PIT.
The difference between areas of increased T$_2$-weighted signal intensity in the surrounding tumour brain parenchyma (Fig. 4A, yellow vs blue arrows) was probably due to the changes in light distribution. The intratumoral quantification of R$_2^*$ (Fig. 4B, C) indicated that mice exposed to ZEGFR:03115-IR700-PIT had a higher percentage of changes in R$_2^*$ values compared to the controls (3.1% ($n = 3$) for the control group vs 11.3% ($n = 3$) and 41.1% ($n = 2$) for 1 h and 4 h groups post-PIT, respectively), which effectively confirmed an acute tumour response to PIT. Hypointense cores highlighted after PIT are compliant with early necrosis. These changes were consistent with H&E staining of the brain sections showing tumour

![Fig. 3 Characterisation of the orthotopic U87-MGvIII model. A In vivo axial T$_2$-weighted MRI image and corresponding axial, coronal, and sagittal PET/CT images of the orthotopic U87-MGvIII tumour 3 h post-injection of the $^{18}$F-AIF-NOTA-ZEGFR:03115 compared to the haematoxylin/eosin staining. B In vivo uptake values 1, 3, and 5 h after i.v. injection of the radiotracer (mean ± SEM), measured as % ID/g$_{50}$ and % ID/g$_{max}$. C In vivo axial T$_2$-weighted MRI image and corresponding ex vivo photography and fluorescence image 5 days after tumour cell engraftment (tumour diameter, 2.7 mm). Brain collection and fluorescence imaging were performed 1 h after i.v. injection of 18 μg of ZEGFR:03115-IR700. Haematoxylin/eosin staining and near-infrared image of ZEGFR:03115-IR700 were performed on the consecutive brain sections. The EGFR immunostaining confirmed the high level of EGFR.](image-url)
necrosis and micro-haemorrhage patterns associated with pyknosis and apoptotic bodies on the margins of the PIT-treated tumours (Fig. 5, Fig. S4C). The tumour necrosis became extensive 24 h post-PIT, showing the strong effect of PIT especially on the core of U87-MGvIII tumours. Moreover, visual assessment of IHC staining for Ki67 indicated reduced cell proliferation 24 h post-conjugate irradiation. Our data also indicate that ZEGFR:03115-IR700-PIT triggered a substantial release of HSP70 within 1 h after treatment (Fig. 5).

**ZEGFR:03115-IR700 PIT triggers immune response in vivo**

We used the BL6-NPE-GFP-Luc syngeneic model to investigate whether a T cell-focused immune response can be elicited by light-activated ZEGFR:03115-IR700 in the brain setting (18 μg/50 J/cm²; Fig. 6A). In this case, we decided to lower the light intensity as we observed in some mice bearing intracranial U87-MGvIII tumours that the mass effect led to a significant deterioration of their condition. Flow cytometry analysis of tumour samples at 24 h post-PIT (18 μg/50 J/cm²) showed higher
levels of CD4+ and CD8+ immune cells in tumours exposed to PIT compared to the control groups (Fig. 6B). The detailed gating strategy is implemented in Fig. S5A. A pronounced number of CD8+ cells was also detected on IHC slices of treated tumour (Fig. S5B). A similar but less pronounced trend was observed in CD69 expression level on T cells, an early activation antigen involved in the transmission of costimulatory signals (Fig. 6B).

Thereafter, we measured the level of selected pro-inflammatory cytokines which have the ability to enhance the immune response towards tumours by activating NK cells, CD8+ T cells, and macrophages. We found a significant increase of IL-6 and an upward trend of IL-1β, TNF-α, and IL-12 levels from the control groups to the Z EGFR:03115-IR700-PIT group, while the level of IL-10, indicating immunosuppression revealed no change regardless of the treatment group (Fig. 6C).

Finally, to check the effect of PIT on the PD-1/PD-L1 axis, we assessed whether ZEGFR:03115-IR700-PIT induces changes in the PD-L1 expression on tumour cells by flow cytometry. We initially confirmed that IFN-γ stimulation leads to a considerable increase in PD-L1 expression in multiple GBM cell lines (Fig. S5C). Subsequently, we detected a significant decrease of PD-L1 in U87-MGvIII cells in response to ZEGFR:03115-IR700-PIT with and without IFN-γ stimulation compared to the controls in vitro (Fig. S5D-E). These results were next corroborated by in vivo findings showing a downregulation of PD-L1 also on the surface of BL6-NPE-GFP-Luc cells post-PIT (Fig. 6D).

**Discussion**

Extensive GBM cell invasion into the normal brain parenchyma makes complete tumour removal practically impossible and disease recurrence inevitable. Besides, the GBM TME is recognised as highly immunosuppressive, posing a major hurdle for inducing immune-mediated destruction of remaining cancer cells. As a...
Fig. 6 (See legend on next page.)
However, the large molecular size of mAbs factor cells locally in the GBM TME. So far, mAbs-based induce direct GBM cell killing via ICD and attracts T effector neurotoxicity [32].duced inflammatory responses in GBM patients turning CNS, and oncolytic viruses increase anti-GBM immunity focal radiation therapy, cancer cell-directed immunotoxicity approaches can alert and trigger the immune response [9, 10]. Recently, it became clear that some treatment GBM patients have failed to demonstrate clear efficacy result, clinical trials evaluating checkpoint blockade in GBM patients have failed to demonstrate clear efficacy [9, 10]. Recently, it became clear that some treatment approaches can alert and trigger the immune response within the immunosuppressive GBM TME. For example, studies in preclinical models have shown that the combination of ICPIs with a concurrent administration of focal radiation therapy, cancer cell-directed immunotins, and oncolytic viruses increase anti-GBM immunity [29–31]. Moreover, EGFRvIII CAR-T cell therapy induced inflammatory responses in GBM patients turning “cold” GBM microenvironment into “hotter” without inducing neurotoxicity [32].

In the present study, we demonstrate that NIR-PIT may induce direct GBM cell killing via ICD and attracts T effector cells locally in the GBM TME. So far, mAbs-based conjugates have been most frequently utilised for PIT purposes [33, 34]. However, the large molecular size of mAbs and their extended blood circulation may slow penetration of the proteins into the tumour parenchyma. Consequently, it may hamper the response to PIT and result in long-lasting systemic photosensitivity [35].

To overcome such limitations, van Driela et al. have recently demonstrated that the use of small EGFR-targeted nanobody-IRDye700DX conjugates (15 or 30 kDa) leads to higher tumour:background contrast and enhanced tumour necrosis when compared with full-size mAb-based IRDye700DX conjugate [36]. Along the same line, our previous studies suggested that affibody molecules (~7 kDa) conjugated to IR700 due to their rapid tumour accumulation and blood clearance are promising candidates for PIT purposes [22, 37].

Herein, we further demonstrated that ZEGFR:03115-IR700-PIT can trigger a local immune response in the brain tumour microenvironment. The conjugate binding to EGFR on the membrane of GBM cells induced receptor expression-dependent cell death upon NIR light exposure which was, in part, due to ROS production. Interestingly, Kato et al. have recently provided a theoretical mechanism by which photoactivated hydrolysis reaction following irradiation of mAb-based IR700 conjugates cause changes in the silicon-oxygen bond and silanol formation, which converts the dye from very hydrophilic to very hydrophobic [16]. Whether similar effects occur in response to irradiation of ZEGFR:03115-IR700 will need to be investigated. Additionally, the efficacy of ZEGFR:03115-IR700 in vitro increased in a conjugate concentration-dependent manner, and significant phototoxicity was observed within 1 h post-light exposure of conjugate-treated cells.

Of importance, several studies, including ours, provide evidence that PIT can induce mobilisation of DAMPs involved in ICD [28, 33, 37]. These molecules serve as an “eat-me” signal and mediate anti-tumour immune responses that are critical for the efficacy of the therapy and formation of long-term immunological memory [38, 39]. Therefore, we investigated whether irradiation of ZEGFR:03115-IR700 will result in the release of these danger signals. We observed high-level cell surface CRT exposure, rapid ATP secretion, and HMGB1 release only in PIT-treated cells, indicative of ICD. However, in cells treated with either ZEGFR:03115-IR700 or light alone, these signals were not enhanced compared to controls. Furthermore, significant release of DAMPs by PIT-treated GBM cells subsequently activated and promoted maturation of antigen-presenting iDCs, as indicated by a marked expression of CD86 and HLA-DR.

Thereafter, in order to determine whether the conjugate is capable of inducing selective tumour cell death in vivo, we treated mice bearing subcutaneous U87-MGvIII xenografts with ZEGFR:03115-IR700-PIT. Burley et al. have recently reported that EGFR targeting affibody molecule (ZEGFR:03115) with high specificity recognise EGFR in vivo. For example, the U87-MGvIII-bearing mice injected with ZEGFR:03115-IR700 displayed a strong fluorescent signal as compared to ZTAQ-IR700 (a non-specific affibody molecule). The tumour fluorescent intensity of ZEGFR:03115-IR700 was 6-fold higher than ZTAQ-IR700 already 1 h post-injection [22]. Furthermore, when ZEGFR:03115 was radiolabelled with zirconium-89, only very low accumulation of the radioconjugate was found in tumours with low EGFR expression levels [25].
Apart from a targeting vector, also the light dose delivered and the method by which it is delivered are crucial to the success of PIT. However, physical dosimetry during PIT is a complex process due to the nature of dynamic interactions between light, conjugate, oxygen, and biological response of different tissues, which clearly depends on the concentration of cytotoxic photoproducts and on the intrinsic photosensitivity. In the murine models of GBM, the explicit dosimetry to map the distribution of light delivery and direct measurement of the light fluence are technically challenging. Therefore, for the purposes of this manuscript, we individually selected the intensity of light for U87-MGvIII and BL6-NPE-GFP-Luc models based on the initial validation experiments. For the xenograft model, the therapeutic light fluence was chosen to be 100 J/cm² in order to maximise treatment efficacy considering the penetration of the NIR light and inevitable photobleaching of IR700 during the illumination. Of note, this light dose was reduced to 50 J/cm² in the syngeneic model to lessen oedema-related swelling caused by direct cytotoxic effects on tumour cells and subsequent inflammation post-PIT. The irradiation of ZEGFR:03115-IR700 restrained the growth of subcutaneous U87-MGvIII tumours in the PIT-treated mice in comparison with controls (light only), which validated the model and procedure we employed.

Encouraged by this potent anticancer activity in vitro and in vivo, we further evaluated this approach in the brain setting. It is well known that GBM progression leads to blood-brain barrier (BBB) structural changes including neuronal death, astrocyte endfeet displacement, and heterogeneous pericyte and astrocyte subpopulations, all of which can reduce the barrier functions through the formation of fenestrations and disruption of tight junctions [40]. Even though it makes the BBB leaky and more permeable for small and large molecules, the barrier is still considered as one of the predominant restricting factors for the efficacy of therapies intended for the clinic. Given the limitations of planar optical imaging of brain tumours and quantification of fluorescence intensity, instead of ZEGFR:03115-IR700, we initially used the radiolabelled conjugate 18F-AIF-NOTA-ZEGFR:03115 to assess the efficacy of the affibody molecule in targeting EGFR-positive tumours in the brain setting. The acquired PET/CT images showed discrete focal accumulation of the radiotracer in the brain lesions already 1 h post-injection. Considering the small difference in size between the two conjugates, we expected ZEGFR:03115-IR700 to exhibit similar in vivo behaviour to 18F-AIF-NOTA-ZEGFR:03115.

Indeed, fluorescence images of the entire brain captured ex vivo post-ZEGFR:03115-IR700 administration clearly indicated accumulation of the conjugate in the tumour and provided insights into its delivery. Despite a relatively equal distribution of ZEGFR:03115-IR700 in the tumours, we observed some variability in the response to PIT between the mice. This could be linked to a non-uniform irradiation through the burr hole in the mouse skull resulting in uneven NIR-light delivery and light-induced photochemical production of ROS. In spite of these issues, hypointense signals were depicted on T₂*W images of U87-MGvIII tumours within 1 h post-PIT that corresponded to microhaemorrhagic lesions. Moreover, histopathological examination of the brain sections revealed high levels of necrosis induced by irradiation of ZEGFR:03115-IR700 24 h post-treatment. Of importance, necrosis has been previously reported to be the characteristic form of cellular death post-PIT [41, 42]. Furthermore, cytoplasmic HSP70, a stress-inducible chaperone protein, was released from the cells as early as 1 h after ZEGFR:03115-IR700-PIT, as confirmed by IHC staining of tumour sections. As published earlier, the translocation of HSP70 depends on the NIR light dose and is related to either mitochondrial or direct surface stress disruption [43, 44]. Moreover, accumulating evidence suggests that HSP70 plays a role in DC maturation and activation of other antigen-presenting cells [45]. For example, it has been reported that HSP70 secreted from PDT-treated tumour cells promoted stimulation of DC and NK cells as well as the production of pro-inflammatory cytokines [46]. In addition, Korbelik et al. showed that HSP70 secreted post-PDT was captured by macrophages that triggered toll-like receptor-based signal transduction and production of TNFα [47]. Finally, we used the BL6-NPE-GFP-Luc syngeneic tumour model to look into the local immune response and activation of tumour-infiltrating lymphocytes post-ZEGFR:03115-IR700 PIT. Excitingly, we identified enhanced immunological response after conjugate irradiation which resulted in the attraction and activation of CD4+ and CD8+ T cells in PIT-treated tumours compared to the control group. Furthermore, the expression of both IL-1β and IL-6, which have the ability to enhance the immune response against tumours by activating CD8+ T cells was also markedly increased. Interestingly, we also observed that ZEGFR:03115-IR700-PIT reduced the level of compensatory immunosuppressive PD-L1 in U87-MGvIII and BL6-NPE-GFP-Luc cells in vitro. We speculate that the remaining PD-L1+ cells could still suppress the anti-tumour immune response and allow the tumour cells to survive immunologic cytotoxicity. Of note, Kleinovink et al. have recently shown in tumour models of colon carcinoma that the addition of CTLA-4 blockade prior to bremaclorin-PDT leads to a significant reduction in tumour burden compared to either treatment alone [48].
Conclusions
In conclusion, the surgical options for GBM patients have not changed significantly over the last three decades and performing a complete tumour excision often presents an insuperable challenge. Residual tumour cells located in close proximity to critical functional areas are often left in the margins of the resection, leading to disease relapse. The possibility of enhanced surgical precision together with intra-operative adjuvant treatment could improve the outcome of GBM patients. In fact, it has been recently shown in patients with recurrent high-grade glioma that a combination of 5-ALA fluorescence-guided resection and open PDT after tumour removal is a promising strategy for local tumour control and targeting non-resectable, visibly fluorescent tumours [49]. Consistent with this, our studies highlight that ZEGFR:03115-IR700 fluorescence could guide resection of the tumour mass, and ZEGFR:03115-IR700-PIT lead to the eradication of residual tumour GBM cells simultaneously turning an immunosuppressive TME into an immunovulnerable one.

Overall, more work is needed to fully unlock the potential of PIT as an effective treatment for GBM, especially concerning local and systemic immune responses and synergies with adjuvant treatments. In addition, there are practical aspects in the procedure that need further investigations including the assessment of light intensity, light delivery protocols, and dosimetry. In addition, as bleeding into the tumour may potentially result in oedema of brain parenchyma clinical application of PIT should be considered for GBM remnants within tumour resection cavity or for patients with small and deeply located tumours where stereotactic PIT could be attempted. Nevertheless, ZEGFR:03115-IR700-PIT holds a tremendous potential as a novel therapeutic approach against this aggressive type of brain tumour.

Abbreviations
S-ALA: 5-Aminolevulinic acid; ATP: Adenosine triphosphate; BBB: Blood-brain barrier; CRT: Calreticulin; DAMPs: Damage-associated molecular patterns; DC: Dendritic cell; DNA: Deoxyribonucleic acid; EGFR: Epithelial growth factor receptor; GBM: Glioblastoma; HMGB1: High-mobility group box-1; HSP70: Heat shock protein 70; IC: Immunogenic cell death; IDCs: Immature dendritic cells; MHC: Major histocompatibility complex; NAC: N-acetyl-l-cysteine; NIR-PIT: Near infrared-photoinmunotherapy; NK cells: Natural killer cells; PD-L1: Programmed death-ligand 1; PDT: Photodynamic therapy; PIT: Photoinmunotherapy; ROS: Reactive oxygen species; SOSG: Singlet oxygen sensor green reagent; TME: Tumour microenvironment

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12916-021-02213-z.

Acknowledgements
The authors gratefully thank Affibody AB (Stockholm, Sweden) for supplying the affibody molecule (ZEGFR:03115). We owe special thanks to Dr. Yann Jamin for the critical discussions of MRI findings. Also, we thank Daniela Ciobota for the technical support and Richard Symonds-Taylor for building the electronics box which provides the digital power control for the LED (The Institute of Cancer Research, London, UK). KJH acknowledges funding support from the ICR/RM CRUK RadNet Radiation Research Centre of Excellence (C7224/A28724). SPR acknowledges CRUK Programme Grant C16412/A27725.

Authors’ contributions
Conception and design: GKM, JM, FR, and WK. Development of the methodology: JM, FR, GKM, CD, JH, and JKR.B. Acquisition of the data: JM, FR, GKM, CD, ST, JKR.B, JH, MN, and S.P.R. Analysis and interpretation of the data: JM, FR, GKM, WK, and KJH. Resources: GKM, WK, and KJH. Writing—original draft preparation: GKM, WK, JM, and FR. Writing—review and editing: GKM, WK, JM, FR, JH, CD, ST, JH, MN, JKR.B, and S.P.R. Visualization: JM and FR. Study supervision: GKM and WK. All authors read and approved the final manuscript.

Funding
This work was funded by the CRUK Convergence Science Centre at The Institute of Cancer Research, London and Imperial College London (A26234), UK, and the National Science Centre, Poland (2017/25/B/NS2/00039).

Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding authors on reasonable request.

Declarations
Ethics approval and consent to participate
All experiments were performed in compliance with licences issued under the UK Animals (Scientific Procedures) Act of 1986 and following local ethical review. Studies were compliant with the UK National Cancer Research Institute Guidelines for Animal Welfare in Cancer Research [26] and the ARRIVE (animal research: reporting in vivo experiments) guidelines [27].

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Received: 29 September 2021 Accepted: 9 December 2021
Published online: 21 January 2022

References
1. Li CW, Lim SO, Xia W, Lee HH, Chan LC, Kuo CW, et al. Glycosylation and stabilization of programmed death-ligand-1 suppresses T-cell activity. Nat Commun. 2016;7(1):12632. https://doi.org/10.1038/ncomms12632.
2. Brown NF, Carter TJ, Ottaviani D, Mulholland P. Hamesing the immune system in glioblastoma. Br J Cancer. 2018;119(10):1171–81. https://doi.org/10.1038/s41416-018-0258-4.
3. Binder ZA, Thorne AH, Bakas S, Wileyto EP, Billello M, Akbari H, et al. Epidermal growth factor receptor extracellular domain mutations in glioblastoma present opportunities for clinical imaging and therapeutic development. Cancer cell. 2018;34(1):163–77 e7. https://doi.org/10.1016/j.ccell.2018.06.006.
4. Watts C, Price SJ, Santarius T. Current concepts in the surgical management of glioma patients. Clin Oncol (R Coll Radiol). 2014;26(7):385–94. https://doi.org/10.1016/j.clon.2014.04.001.
5. Majchrzak K, Kaspera W, Bobek-Billewicz B, Hebda A, Stanis-Pres G, Majchrzak H, et al. The assessment of prognostic factors in surgical treatment of low-grade gliomas: a prospective study. Clin Neurol Neurosurg. 2012;114(8): 1135–44. https://doi.org/10.1016/j.clineuro.2012.02.054.
6. Brennan OW, Verhaak RG, Mckenna A, Campos B, Noushmehr H, Salama SR, et al. The somatic genomic landscape of glioblastoma. Cell. 2013;155(2):38–77 e7. https://doi.org/10.1016/j.cell.2013.09.034.
7. Weller M, Butowsk N, Tran DD, Recht LD, Lim M, Hirst H, et al. Rindopepimut with temozolomide for patients with newly diagnosed,
EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. Lancet Oncol. 2017;18(10):1373–85. https://doi.org/10.1016/S1470-2045(17)30517-X.

8. Concha-Benavente F, Ferris RL. Reversing EGFR mediated immunoescape by targeted monoclonal antibody therapy. Front pharmacol. 2017;8:332. https://doi.org/10.3389/fphar.2017.00332.

9. Persico P, Lorenz F, Dipasquale A, Pessina F, Navarra P, Politi LS, et al. Checkpoint inhibitors as high-grade gliomas treatment: state of the art and future perspectives. J Clin Med. 2021;10(7). https://doi.org/10.3390/jcm10071367.

10. Jackson CM, Choi J, Lim M. Mechanisms of immunotherapy resistance: lessons from glioblastoma. Nat Immunol. 2019;20(9):1100–9. https://doi.org/10.1038/s41590-019-0433-y.

11. Zhao J, Chen AX, Gartrell RD, Silverman AM, Aparicio L, Chu T, et al. Immune and genomic correlates of response to anti-PD-1 immunotherapy in glioblastoma. Nat Med. 2019;25(3):462–9. https://doi.org/10.1038/s41591-019-01349-y.

12. Wang HS, Wan J, Zhou HG, Xu JN, Lu YP, Ji XY, et al. Different T-cell subsets in tumors after near-infrared photodynamic immunotherapy. Nanophotonics-Berlin. 2019;8(10):1673–88. https://doi.org/10.1515/nanoph-2019-01816.

13. Rajendramakur SK, Uthaman S, Cho CS, Park K. Nanoparticle-based phototriggered cancer immunotherapy and its domino effect in the tumor microenvironment. Biomacromolecules. 2018;19(6):1889–87. https://doi.org/10.1021/acs.biomac.8b00406.

14. Kato T, Okada R, Goto Y, Furusawa A, Iragaki F, Wakiyama H, et al. Electron donors rather than reactive oxygen species needed for therapeutic photochemical reaction of near-infrared photodynamic immunotherapy. ACS Pharmacol Transl Sci. 2021;5(4):1689–701. https://doi.org/10.1021/acsptsci.1c00184.

15. Nagaya T, Nakamura Y, Oyama S, Ogata F, Maruoka Y, Choyke PL, et al. Synergistic mouse models of oral cancer are effectively targeted by anti-CD44-based NIR-PIT. Mol Cancer Res. 2017;15(12):1667–77. https://doi.org/10.1158/1541-5641.MCR-17-0333.

16. Nagaya T, Oyama S, Ogata F, Maruoka Y, Knapp DW, Karagiannis SN, et al. Near infrared photodynamic immunotherapy targeting bladder cancer with a canine anti-epidermal growth factor receptor (EGFR) antibody. Oncotarget. 2018;9(27):19026–38. https://doi.org/10.18632/oncotarget.24876.

17. Kato T, Wakiyama H, Furusawa A, Choyke PL, KobaYashi H. Near infrared photodynamic immunotherapy, an evidence of photosensitized tumor necrosis and hemodynamic changes. Cancer Lett. 2021;13(11):2535. https://doi.org/10.1002/1089-039X(202024)-00019.

18. Wakiyama H, Kato T, Furusawa A, Choyke PL, Kobayashi H. Near infrared photodynamic immunotherapy: a review of targets for cancer therapy. Cancers. 2021;13(11):2535. https://doi.org/10.3390/cancers13112535.

19. Wakiyama H, Kato T, Furusawa A, Choyke PL, Kobayashi H. Near infrared photodynamic immunotherapy; possible clinical applications. Nanophotonics. 2021;12(1):3135–51. https://doi.org/10.1515/nanoph-2021-00119.

20. Kysyko DV, Garg AD, Kaczmarek A, Kysyko O, Agостини P, Vandenberghe P. Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer. 2012;12(12):860–79. https://doi.org/10.1038/nrc3380.

21. Buttery TA, Maczynska J, Shah A, Szopa W, Harrington KJ, Boutet JR, et al. Near-infrared photodynamic immunotherapy targeting EGFR-shedding new light on glioblastoma treatment. Int J Cancer. 2018;142(4):2362–73. https://doi.org/10.1002/ijc.31246.

22. Huang PH, Mukasa A, Bonavia R, Flynn RA, Brewer ZE, Cavenee WK, et al. Quantitative analysis of EGFRvIII cellular signaling networks reveals a combinatorial therapeutic strategy for glioblastoma. Proc Natl Acad Sci USA. 2007;104(31):12867–72. https://doi.org/10.1073/pnas.0705158104.

23. Gangoso E, Southgate B, Bradley L, Rus S, Galvez-Cancino F, McGovern N, et al. Glioblastomas acquire myeloid-affiliated transcriptional programs via epigenetic immunoevading to elicit immune evasion. Cell. 2021;189(24):2470–2476.e10. https://doi.org/10.1016/j.cell.2021.03.023.

24. Buttery TA, Da Pieve C, Martins CD, Ciobota DM, Allott L, Lyon WI, et al. Affibody-based PET imaging to guide EGFR-targeted cancer therapy in head and neck squamous cell cancer models. J Nucl Med. 2019;60(3):353–61. https://doi.org/10.2967/jnumed.118.016699.
46. Wang XJ, Ji J, Zhang HY, Fan ZX, Zhang Li, Shi L, et al. Stimulation of dendritic cells by DAMPs in ALA-PDT treated SCC tumor cells. Oncotarget. 2015;6(42):44688–702. https://doi.org/10.18632/oncotarget.5975.

47. Korbelik M, Sun J, Cecic I. Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response. Cancer Res. 2005;65(3):1018–26.

48. Kleinovink JW, Fransen MF, Lowik CW, Ossendorp F. Photodynamic-immune checkpoint therapy eradicates local and distant tumors by CD8(+) T cells. Cancer Immunol Res. 2017;5(10):832–8. https://doi.org/10.1158/2326-6066.CIR-17-0055.

49. Schipmann S, Muther M, Stogbauer L, Zimmer S, Brokinkel B, Holling M, et al. Combination of ALA-induced fluorescence-guided resection and intraoperative open photodynamic therapy for recurrent glioblastoma: case series on a promising dual strategy for local tumor control. J Neurosurg. 2020;134(2):1–11. https://doi.org/10.3171/2019.11.JNS192443.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.