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Coupling to a glioblastoma-directed antibody potentiates antitumor activity of curcumin

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Current therapies for glioblastoma are largely palliative, involving surgical resection followed by chemotherapy and radiation therapy, which yield serious side effects and very rarely produce complete recovery. Curcumin, a food component, blocked brain tumor formation but failed to eliminate established brain tumors *in vivo*, probably because of its poor bioavailability. In the glioblastoma GL261 cells, it suppressed the tumor-promoting proteins NF-κB, P-Akt1, vascular endothelial growth factor, cyclin D1 and BCl_{XL} and triggered cell death. Expression of exogenous p50 and p65 subunits of NF-κB conferred partial protection on transfected GL261 cells against curcumin insult, indicating that NF-κB played a key role in protecting glioblastoma cells. To enhance delivery, we coupled curcumin to the glioblastoma-specific CD68 antibody in a releasable form. This resulted in a 120-fold increase in its efficacy to eliminate GL261 cells. A very similar dose response was also obtained with human glioblastoma lines T98G and U87MG. GL261-implanted mice receiving intratumor infusions of the curcumin-CD68 adduct followed by tail-vein injections of solubilized curcumin displayed a fourfold to fivefold reduction in brain tumor load, survived longer, and about 10% of them lived beyond 100 days. Hematoxylin–eosin staining of brain sections revealed a small scar tissue mass in the rescued mice, indicating adduct-mediated elimination of glioblastoma tumor. The tumor cells were strongly CD68+ and some cells in the tumor periphery were strongly positive for microglial Iba1, but weakly positive for CD68. This strategy of antibody targeting of curcumin to tumor comes with the promise of yielding a highly effective therapy for glioblastoma brain tumors.

Glioblastoma is one of the most aggressive and deadly forms of cancers affecting the nervous system. Prognosis remains very poor, as anticancer agents have difficulty crossing the blood–brain barrier and tumor cells are resistant to standard therapies. Even with surgery, radiation and chemotherapies, treatment remains only palliative. Surprisingly, the highest incidence rate of this type of cancers is observed in the industrially developed countries of the world.[1,2] Well-designed epidemiological studies, especially one performed in China, have strongly related this observation to lifestyle and intake of large quantities of processed meat and animal protein.[2,3]

Although the recent focus on personalized therapy has led to massive, technically sophisticated and expensive analyses of the genetic profiles of cancer patients, the fact that most cancer cells display some common features and molecular characteristics that can be targeted by harmless natural agents has been largely ignored. For example, epidemiological studies have clearly shown that colon cancer is virtually absent in the Southeast Asian countries where the powerful anticancer agent turmeric is consumed in large quantities in a regular diet as a culinary spice.[4–7] Furthermore, it has also been demonstrated that the coloring component of turmeric curcumin is highly potent in eliminating a large variety of cancer cell types without damaging normal tissue.[4,7,8] Although very promising in preclinical studies, the efficacy of curcumin in human trials has been limited. This in part is due to curcumin's low solubility in water causing poor systemic bioavailability and rapid clearance. We have shown that this difficulty can be overcome by synthetic linkage of curcumin

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What's new?
Curcumin, the most active ingredient of the yellow spice turmeric traditionally used in Indian cuisine, has known antitumor activities. However, its low bioavailability is a major obstacle to its use in cancer therapy. Here the authors tested the efficacy of curcumin in cell culture and mouse models of glioblastoma, a highly treatment-resistant brain cancer. Curcumin was reversibly coupled to the glioblastoma-specific CD68 antibody, which resulted in a markedly increased efficacy to eliminate glioblastoma cells in vivo. The study raises hope that with the appropriate targeting curcumin may rise to a new anti-brain cancer agent in the future.

to an antibody for enhanced tissue delivery.[9] Antibody drug conjugates traditionally attempt to selectively deliver cytotoxic drugs to tumors. However, significant adverse effects are encountered from off-target release of the toxin. Gemtuzumab ozogamicin, an FDA-approved anti-CD33 drug conjugate (Mylotarg^{TM}) for the treatment of acute myeloid leukemia, was withdrawn from the market because of its toxicity.[10]

Attempts to increase efficacy by concentrating compounds derived from dietary or medicinal plants into tumor tissue have been received with concern and deemed similar to antibody-targeted toxic agents like Mylotarg. We emphasize that antibody-mediated targeting does not have to only carry toxic chemotherapeutic agents to specific cancer cells. Rather, it can be used to enhance the delivery of potent natural anticancer agents like curcumin, which cannot be otherwise used to combat established cancers because of issues related to poor systemic bioavailability.

Using this concept and a carefully crafted scheme of targeting curcumin to melanoma cells, we have already shown that our strategy is highly effective in rescuing mice with established, melanoma-evoked brain tumors.[9] Our study uses a similar scheme in which we create a CD68 antibody–curcumin conjugate (CDC) that is highly specific and effective in eliminating two human glioblastoma cell lines *in vitro*. Our *in vivo* model, created by intracranially implanting mouse glioblastoma cancer cells (GL261), shows CDC-mediated shrinkage of established glioblastoma brain tumors. This increases the longevity of the mice and completely rescues some of the animals. Like in the melanoma cells, the primary target of curcumin in the glioblastoma GL261 cells appears to be NF-κB, which is dramatically upregulated in a large number of cancer cells.[11]

Material and Methods

See Supporting Information for further details.

Animals

Adult C57BL/6 male mice (2–6 months old) were used for the experiments. Animals were bred in the College of Staten Island (CSI) Animal Care Facility and maintained on a 12-hr light/dark cycle with *ad libitum* access to food and water. All animals were handled and used for surgery following an experimental protocol approved by the Institutional Animal Care Committee (IACUC) of the College of Staten Island (CUNY).

Reagents, antibodies and cell lines

See Supporting Information for details.

Synthesis of curcumin carboxylate NHS ester; 2,5-dioxycyclopentyl 4-((1E,6E)−7-(4-hydroxy-3-methoxyphenyl)−3,5-dioxohepta-1,6-dienyl)−2-methoxyphenyl glutarate. The curcumin NHS ester was synthesized as described in detail in our earlier publications.[9,12,13]

Cell culture

GL261 mouse glioblastoma cells and the human glioblastoma U87MG and T98G cells were cultured in RPMI 1640 containing 10% (v/v) FBS (fetal bovine serum) and 1% (v/v) penicillin–streptomycin (Pen-Strep) with 2 mM glutamine and 2 mM glutamax. Before and during drug treatment, the cells were placed in neurobasal medium containing 2% (v/v) B-27 supplement and 1% (v/v) Pen-Strep.

Preparation of curcumin-CD68 Ab and Dylight 800-CD68 Ab adducts

Curcumin was coupled to the CD68 antibody according to reported procedures by adding tenfold molar excess of succinimidyl curcumin carboxylate (curcumin-NHS ester) dissolved in dimethylsulfoxide (DMSO, 10% v/v) to CD68 antibody (20 μg) in 50 mM sodium bicarbonate buffer (pH 8.3).[14] After the coupling reaction between the curcumin-NHS ester and the CD68 antibody, the adduct (CDC) was purified using a spin column. The curcumin:Ab ratio was determined by spectroscopy and also by MALDI-TOF (Supporting Information Fig. 1).[9,15] (Further details have been included in Supporting Information.)

Determination of IC50

GL261 cells (2,000 per well) were grown for 24 hr in a 96-well plate and then curcumin or curcumin-CD68 Ab treatment at indicated concentrations was performed in triplicate wells for 24–48 hr in serum-free neurobasal medium (with 2% B27 and 1% Pen-Strep).[8] Live cells were counted in six or more randomly chosen fields per well to obtain mean count per field of view.

In some experiments, subsequent to free curcumin or curcumin-Ab adduct treatment in triplicate wells, 10% by volume of WST-1 (Clontech, Mountain View, CA) was added to each well followed by incubation at 37°C for 2 hr and measurement of absorbance at 440 nm. Results obtained from two experiments performed with triplicate samples were

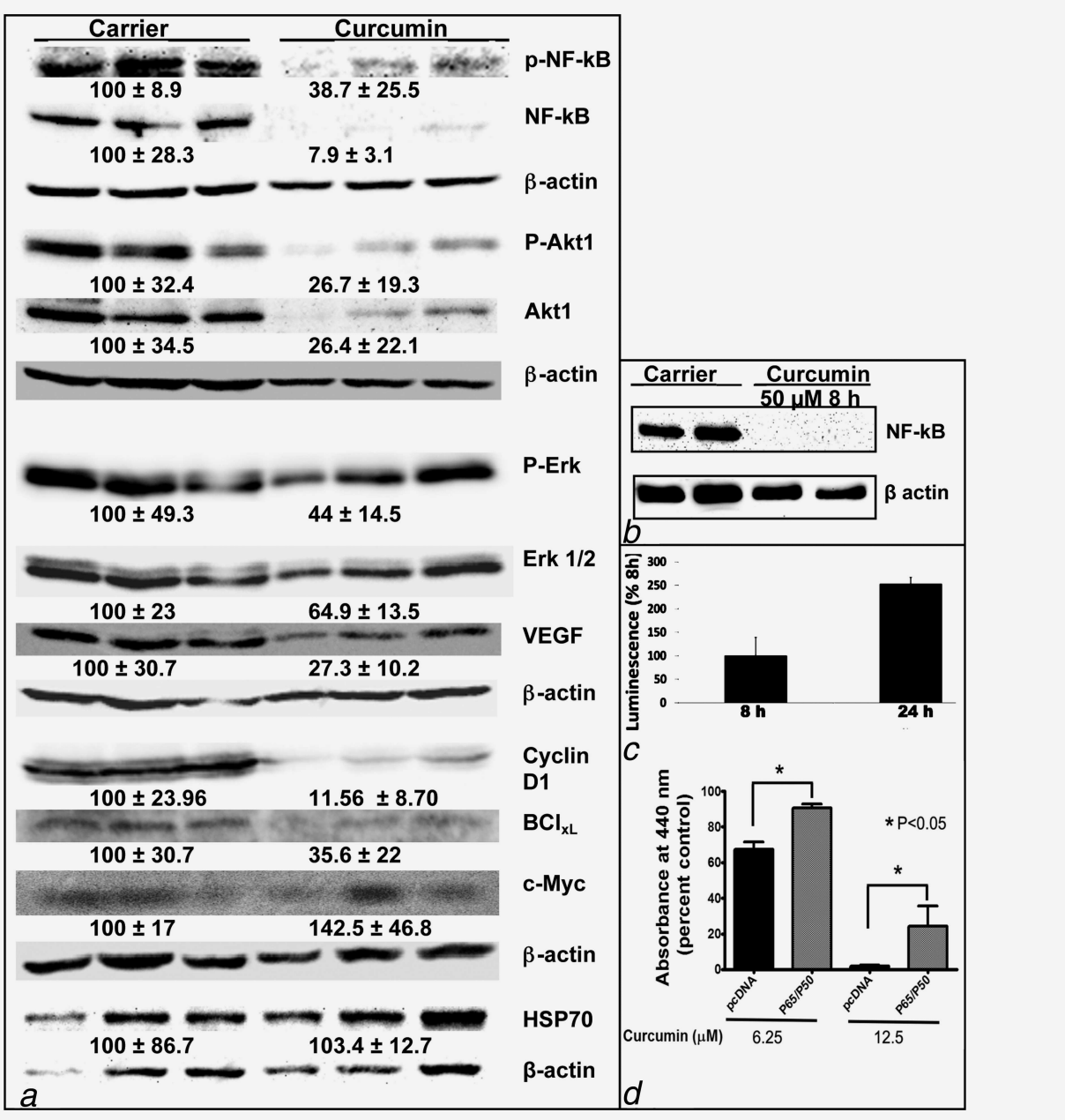

Figure 1. NF-κB could be the primary target of curcumin in the GL261 cells. (*a*) The expression levels of NF-κB, Akt-1, VEGF, cyclin D1 and BCL_{XL} normalized to the corresponding β-actin levels were significantly reduced after curcumin (50 μM) treatment for 24 hr. Student's *t*-test *p* values: 0.028778 (NF-κB), 0.041 (pNF-κB), 0.048 (Bcl_{XL}), 0.044654 (Akt), 0.038 (pAkt), 0.043 (VEGF), 0.100 (Erk 1/2), 0.181 (pErk 1/2), 0.252 (c-Myc), 0.015 (cyclin D1) and 0.953 (HSP70). Curcumin (50 μM) treatment for 8 hr does not cause morphological degeneration (Supporting Information Fig. 2a), but elicits rapid inhibition of NF-κB expression (*b*). (*c*) Expression of exogenous NF-κB subunits p50 and p65 in GL261 cells causes induced expression of luciferase from a NF-κB-luciferase reporter construct. (*d*) Exogenous NF-κB partially rescues the cells from death caused by 96 hr of curcumin (6.25 and 12.5 μM) treatment (measured by WST-1 assay) ($p < 0.5$; *t*-test).

analyzed using *t*-test (two-tailed distribution, two-sample unequal variance).

Immunostaining of GL261, U87MG and T98G cells

Attached cells in the wells were rinsed with 10 mM phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, rinsed thrice with PBS, blocked in 10% goat serum in PBS plus 0.1% Triton X-100 and then immunostained using a CD68 Ab (sc-9139, which recognizes both mouse and human CD68) (1:200) and a secondary antibody (Alexa Fluor 568 goat anti-rabbit) (1:1,000). CD68 Ab-stained images were acquired from random fields with a Zeiss Axio Observer Z1 microscope and an AxioVision 4.6.3-AP1 camera at excitation wavelength of 488 nm.

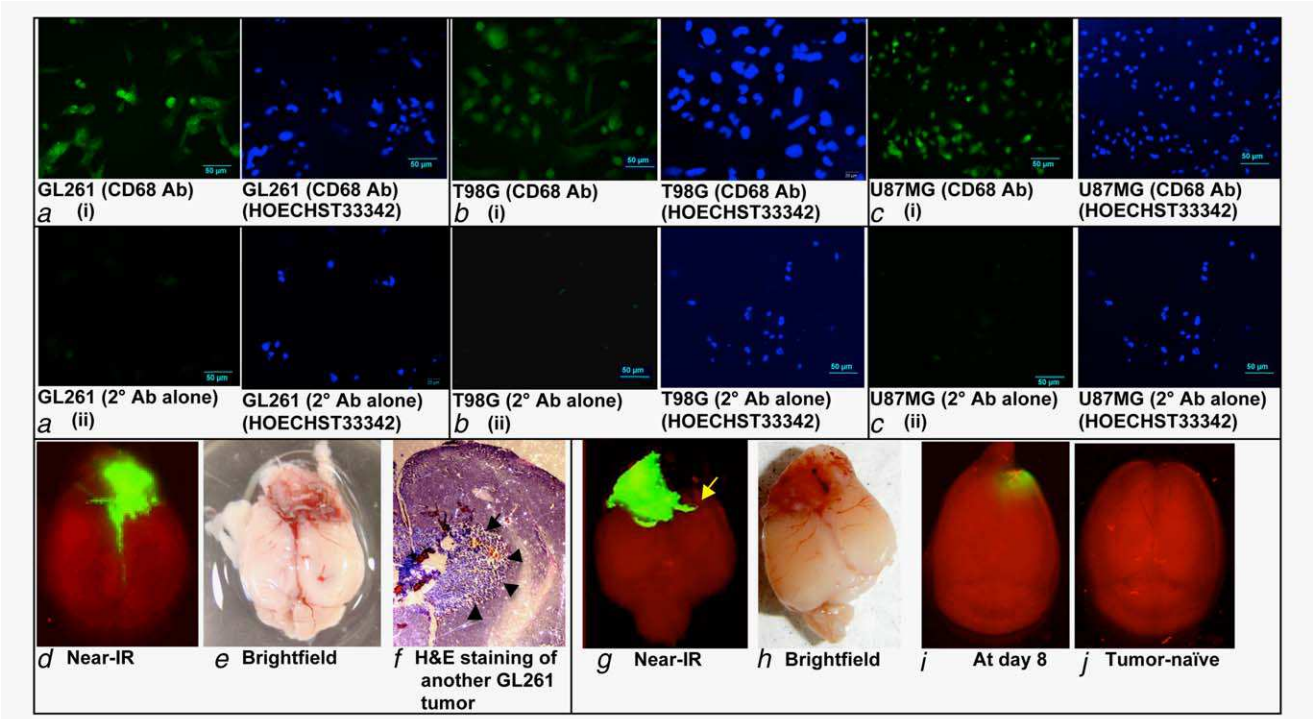

Figure 2. CD68 is expressed by both human and mouse glioblastoma cells and a CD68 Ab shows dramatically lower binding to normal brain tissue. (*a–c*) In each case an Alexafluor488-linked (green) anti-mouse 2° antibody was used. Nuclei were stained using DAPI (blue). The green frame (left) and blue frame (right) have been juxtaposed to show the presence or absence of staining with the CD68 Ab or the 2° Ab alone. (*d* and *g*) GL261 (10^5) cells were implanted in the right forebrain on Day 1 and CD68 Ab-Dylight800 (CD68Dy) infused into the same site on Day 21, and after 24 hr, the mice were sacrificed and brains extricated for imaging using near-IR scanning. (*e*) Brightfield image of the brain shown in (*d*) demonstrating colocalization of the tumor with the CD68Dy stain. (*f*) H&E staining of brain section from another brain confirms the presence of tumor (black arrows). (*g*) CD68Dy infused at the original site of BL261 implantation on right (yellow arrow) diffuses and binds to tumor on left. (*h*) Brightfield image of brain shown in (*g*) confirms colocalization of tumor with CD68Dy labeling (green) (black arrow). (*i*) The status of the tumor at Day 8 after GL261 implantation. (*j*) Similar infusion of CD68Dy into the right forebrain of a tumor-naïve mouse followed by imaging after 24 hr shows no CD68Dy labeling. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Caspase3/7 assay

This assay was performed as described earlier.[8] Statistical analysis was performed using Student's *t*-test or ANOVA with Bonferroni *post hoc* tests.

Implantation of cancer cells in mice and intracranial drug infusion[16]

See Supporting Information for details.

Hematoxylin–eosin staining of tissue sections

The fixed brains were cryosectioned and hematoxylin–eosin (H&E) stained according to our earlier report.[8]

Immunohistochemistry

Coronal sections (30 μm) were made from 4% paraformaldehyde-fixed and 30% sucrose-soaked mouse brains harboring glioblastoma tumors. Sections containing tumor tissue were chosen and subjected to immunohistochemistry using CD68, Iba1 (microglia/macrophage)[17] and RM0029-11H3 (macrophage)[18] antibodies, each at 1:50 dilution. The secondary antibodies were goat anti-rabbit Alexaflour 488 for CD68 (H-255) (green), goat anti-rat phycoerythrin (sc3740) for RM0029-11H3 (red) and rabbit anti-goat Alexafluor 633 for Iba1 (far red). Confocal images were acquired using a Leica SP2 AOBS confocal microscope (Leica Microsystems, Heidelberg, Germany). Further details have been included in Supporting Information.

Results

Curcumin treatment causes a dramatic inhibition in the levels of NF-κB, Akt-1, vascular endothelial growth factor, cyclin D1 and BCl_{XL}

The mouse GL261 glioblastoma cells were treated with carrier or curcumin (50 μM) for 24 hr. Western blot analysis of lysates prepared from the post-treatment cells revealed that both NF-κB as well as its activated version, phospho-NF-κB, were suppressed in a dramatic manner (Fig. 1). In our earlier studies, activated NF-κB stimulated Akt-1 in the B16F10 cells.[9] In the GL261 cells, both expression and activation levels of Akt-1 (shown by the levels of phospho-Akt-1) were inhibited in a dramatic manner by curcumin (Fig. 1*a*). In addition, we also observed that the levels of BCl_{XL}, an

inhibitor of apoptosis, cyclin D1, a cell cycle promoter, and vascular endothelial growth factor (VEGF), a promoter of angiogenesis, were suppressed significantly. However, curcumin did not significantly affect the expression of c-Myc, HSP70, Erk1/2 and pErk1/2 (Fig. 1*a*).

We expected that the primary target of curcumin would be most rapidly regulated even before the cells show any sign of degeneration. We observed no sign of degeneration of GL261 cells upon treatment with 50 μM of curcumin for 8 hr (Supporting Information Fig. 2a), but this treatment was sufficient to cause a dramatic suppression of NF-κB synthesis (Fig. 1*b*). To test the involvement of NF-κB in curcumin-evoked GL261 death, we first overexpressed the p50 and p65 subunits of NF-κB along with a luciferase reporter vector driven by an NF-κB-inducible promoter to demonstrate increased expression of NF-κB in the transfected cells 24 hr post-transfection (Fig. 1*c*). Twenty hours after transfection, these cells were treated with curcumin at indicated concentrations to observe partial reversal of curcumin-evoked viability in GL261 cells expressing exogenous p50 and p65 subunits of NF-κB (Fig. 1*d*).

Mouse glioblastoma (GL261) as well as human glioblastoma (T98G and U87MG) cells express high levels of CD68

Although curcumin has a strong cytotoxic activity toward a wide range of cancer cells,[8] treatment of established brain tumors with implanted melanoma cells is not eliminated by curcumin treatment alone.[8] Subsequently, we had shown that covalent linking of curcumin to a cancer cell-specific antibody dramatically increased its anticancer potency against melanoma cells.[9] To use the same strategy, we looked for an antigen that showed dramatically higher expression on the surface of glioblastoma cells than the other brain cells. CD68 is a 110-kDa transmembrane glycoprotein that is expressed by monocyte/macrophage lineages and reactive microglia also express CD68.[19] However, in malignant astrocytoma cells CD68 positivity is known to be very high.[20] As presented below, we observed strong CD68 staining in the GL261 tumors and weak CD68 staining in Iba1(+) cells (microglia) in the tumor periphery (Fig. 3). Furthermore, survival analysis has shown that CD68 tumor staining has prognostic value for glioma patients, as high antigen expression is associated with shorter survival.[21] Therefore, differentially expressed CD68 in brain tissue was an ideal target for our CDC.

As expected, immunocytochemical analysis revealed that CD68 was highly expressed on GL261 as well as the human glioblastoma cells U87MG and T98G in culture (Figs. 2*a*–2*c*).

To test the efficacy of CD68 antibody-mediated targeting to glioblastoma brain tumors, we covalently linked this antibody to the near-infrared dye Dylight800 (CD68Dy) and intracranially infused CD68Dy into mice harboring glioblastoma generated by implanting 10^5 GL261 cells into the right forebrain of mice. CD68Dy concentrated in the tumor after 24 hr regardless of its final location in the right or left forebrain (Figs. 2*d* and 2*g*). Even at Day 8 after implantation of GL261 tumor cells, CD68Dy labels a small tumor in the brain (Fig. 2*i*). In sharp contrast, similar infusion of CD68Dy into the brain of tumor-naïve mice did not produce any detectable labeling of normal brain cells (Fig. 2*j*). The location of the glioblastoma tumor was also detectable by bright-field imaging (Figs. 2*e* and 2*h*) and histopathologic examination using H&E staining (Fig. 2*f*).

The cells in the tumor show strong immunohistochemical staining with CD68 antibody, and peripheral cells show staining with Iba1 (microglia) antibody but not with RM0029-11H3 (macrophage) antibody

Coronal sections of GL261 tumor-containing brain were prepared and consecutive sections containing the tumor were analyzed for immunohistochemical staining with antibodies against CD68 (Figs. 3*a* and 3*b*), Iba1 (microglia marker) (Figs. 3*c* and 3*d*) and RM0029-11H3 (macrophage marker) (Fig. 3*e*). Results shown in Figure 3 demonstrate high levels of CD68 expression (green) of the tumor cells. Staining using the microglia/macrophage-specific Iba1 antibody (red) showed the presence of microglial cells in the tumor periphery (Figs. 3*d* and 3*e*), which were weakly CD68+ (Fig. 3*f*, left panel, green), but were not stained with the macrophage-specific RM0029-11H3 antibody (red) (Fig. 3*c*).

Attachment to CD68 antibody causes a 120-fold decrease in the IC50 of curcumin

We covalently linked curcumin through a cleavable linkage to the CD68 and verified that the antibody:curcumin ratio was 1:1 (Supporting Information Fig. 1). Next, the GL261 cells were treated with carrier or various concentrations of curcumin in one experiment and with CD68 antibody alone (control) or various concentrations of CD68 antibody-linked curcumin (CDC) in a second experiment. Cell viability was measured by counting healthy cells and also by WST-1 assays. Although curcumin treatment alone yielded an IC50 of 15 μM (Figs. 4*a*–4*d*), treatment with the CD68 antibody-conjugated curcumin indexed an IC50 of 125 nM (Figs. 4*f*−4*h*). Very little inhibition of the viability of normal human fibroblasts was observed even at much higher concentrations of curcumin (Fig. 4*i*). CDC also eliminated human glioblastoma cells U87MG and T98G in the nanomolar range of IC50 (400 and 225 nM, respectively) (Figs. 4*k* and 4*m*), whereas the IC50 values for curcumin alone were 25 and 8 μM, respectively.

Curcumin fluorescence from CDC increases upon internalization and causes destruction of GL261 glioblastoma cells within 2 hr of treatment

GL261 cells, cultured in a 96-well plate, were placed in serum-free neurobasal medium with 1% B27 serum supplement and treated with 540 nM CDC and time-lapse photography was used both with and without fluorescence microscopy. The fluorescence increased from dull to intense

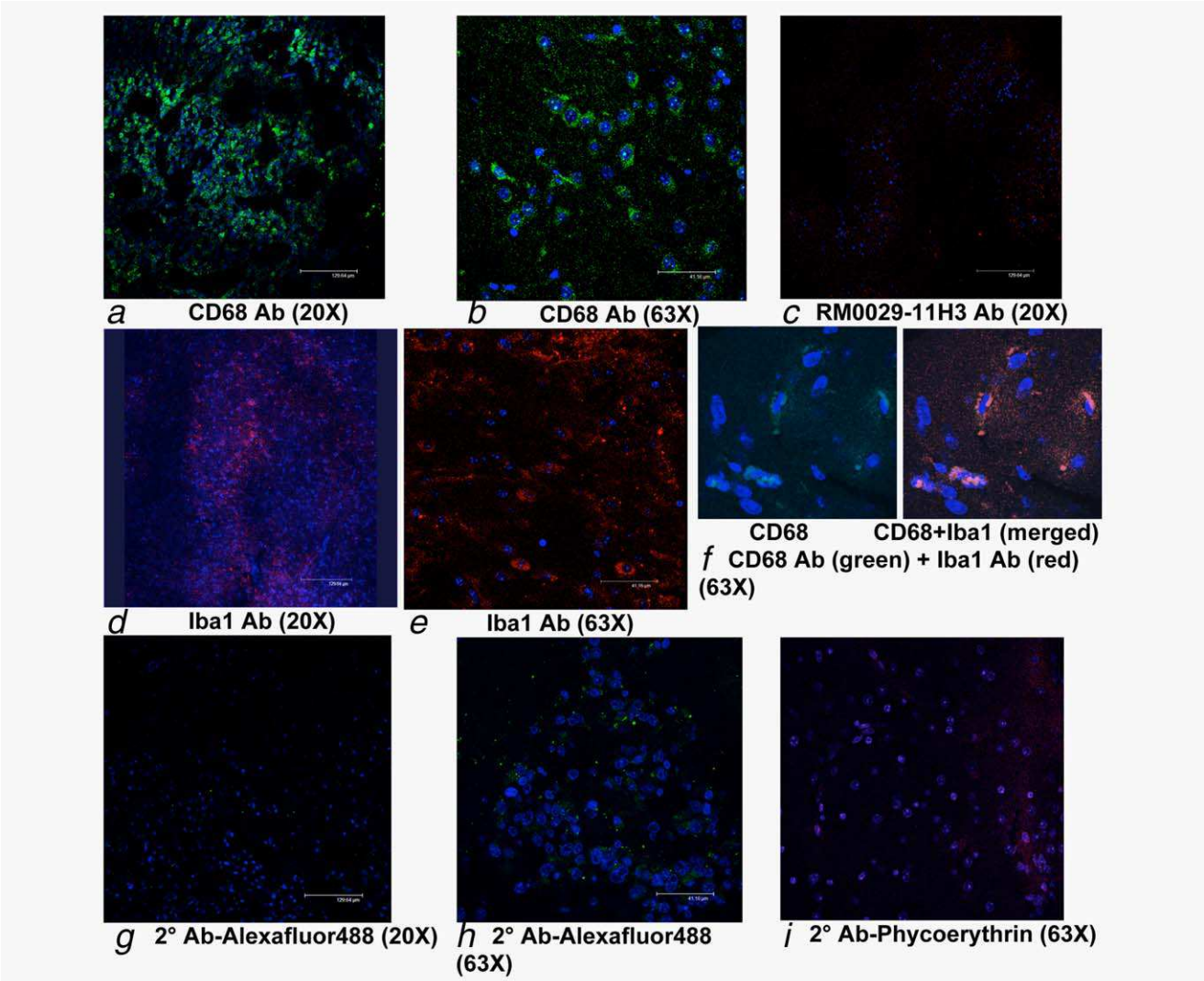

Figure 3. The cells in the tumor show strong staining with CD68 antibody, and peripheral cells show staining with Iba1 (microglia) antibody, and no staining with RM0029-11H3 (macrophage) antibody. (*a* and *b*) CD68 Ab (green) staining of tumor cells. (*c*) Tumor cells did not stain with RM0029-11H3 (macrophage) Ab (far red). (*d* and *e*) Some cells in the tumor periphery stained with Iba1 Ab (microglia and macrophage). (*f*) The peripheral cells were weakly stained with CD68 (green) and strongly stained with Iba1 (red). HOECHST33342 (blue) (nuclear staining). (*g–i*) No nonspecific staining was observed with any of the 2° antibodies. Scale bars: (×20) 129.64 μm; (×63) 41.16 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

when some membrane-attached CDC molecules fused to two cells, suggesting the release of free curcumin from the antibody owing to the action of intracellular esterases on the linker portion of the antibody–drug complex (Supporting Information Figs. 2b–2d). At this point brightfield imaging showed degradation of the cells (Supporting Information Fig. 2e). Complete morphological analysis with time-lapse imaging was also performed and at treatment, the cells appeared healthy (Supporting Information Fig. 2f), but blebbing and deformation of cells started within 45 min (Supporting Information Fig. 2g), and many cells released their internal contents (cell debris) within 2 hr (Supporting Information Fig. 2h). Upon treatment of GL261 cells with CDC for 24 hr a dramatic increase in caspase-3/7 activity was also observed (Supporting Information Fig. 2i).

C68 Ab-linked Dylight800 labels glioblastoma brain tumors and targeted curcumin causes a major reduction in tumor load and extends survival of glioblastoma-implanted mice

GL261 cells (10^5) were implanted in the right forebrain of each C57BL6 mouse on Day 1 and the mice were subjected to intracranial infusion of CD68 antibody alone or CDC (16 pmol per infusion) twice on Days 13 and 15. This was followed by tail-vein infusion of curcumin (667 μM in PBS containing 0.125% DMSO), six times, once every week. This treatment caused a major decrease in glioblastoma tumor burden (Figs. 5*a*–5*c*) and prolonged the life of the mice significantly (Fig. 5*d*). In a second set of experiments, eight mice were used in each of two groups. The control group received four intracranial infusions of 30 pmol of CD68 Ab

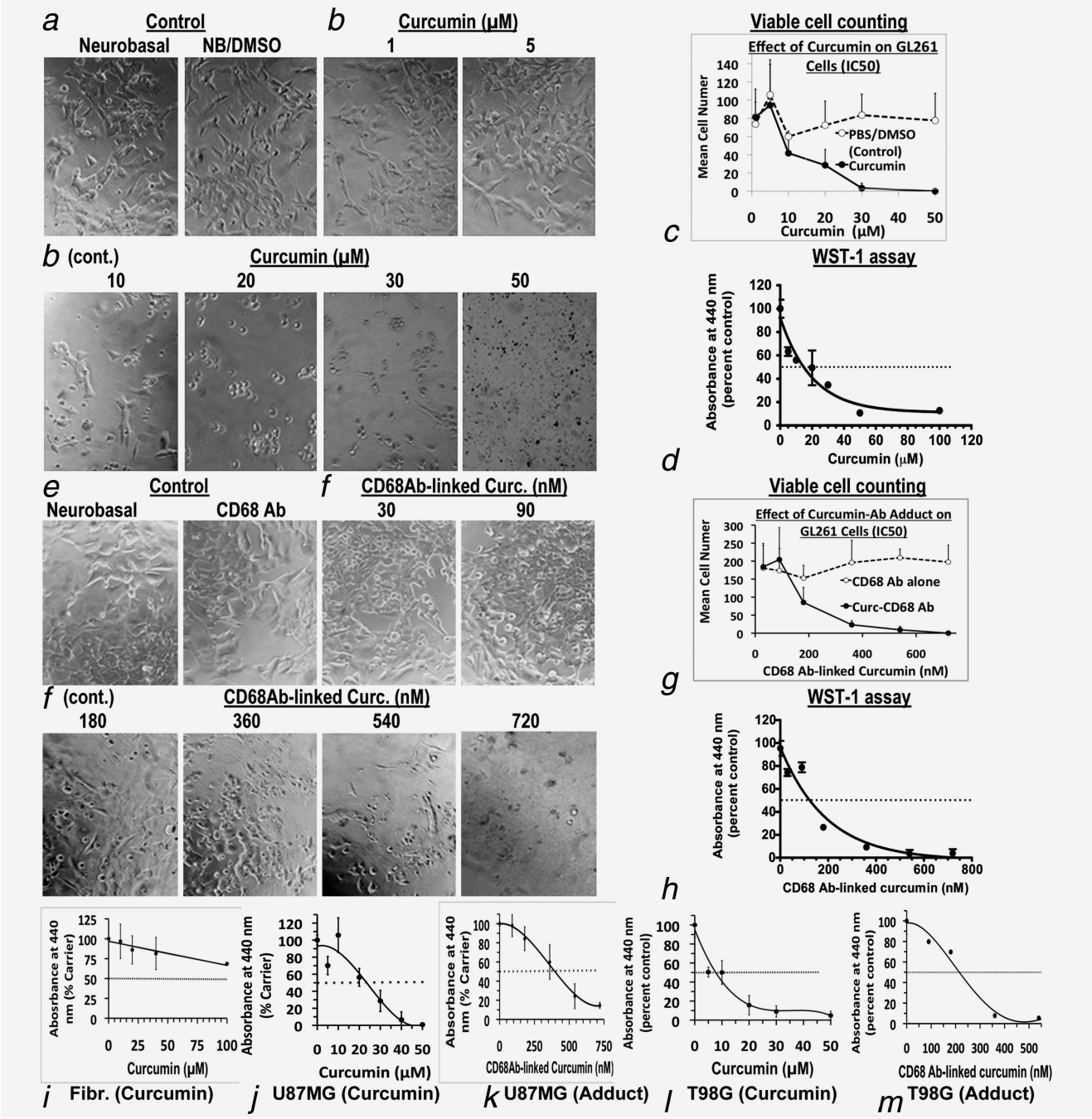

Figure 4. CD68 Ab-linked curcumin is 120 times more potent than curcumin alone in eliminating glioblastoma GL261 cells. (*a–d*) Effect of solubilized curcumin on GL261 cells (IC50 = 15 μM). GL261 cells subjected to treatment with DMSO-solubilized curcumin (neurobasal/DMSO < 0.2%) or neurobasal control for 24 hr. (*e–h*) Effect of curcumin adduct on GL261 cells (IC50 = 125 nM). Images of GL261 cells subjected to treatment with curcumin-CD68 adduct or CD68 control for 24 hr. Counting of viable cells and NADH oxidoreductase-based WST-1 assays[9] yielded very similar results. (*i*) Curcumin treatment of normal human fibroblasts causes very little inhibition of cell viability at much higher concentrations. (*k* and *m*) The CD68-linked curcumin eliminates human glioblastoma cells U87MG and T98G also in the nanomolar range of IC50 (400 and 225 nM, respectively).

alone and the second group received four intratumor infusions of 30 pmol CDC per infusion per mouse from Day 8, every 72 hr. Next, tail-vein infusion of curcumin (667 μM) was performed first every 72 hr (five times) and then once per week (two times). This yielded a major increase in longevity of the CDC-treated mice (Fig. 5*g*) and about 10% of the treated mice survived beyond 90 days in each experiment. Although H&E staining of a control brain not receiving the adduct showed widespread degeneration due to glioblastoma (Fig. 5*e*), of the mice that remained healthy and

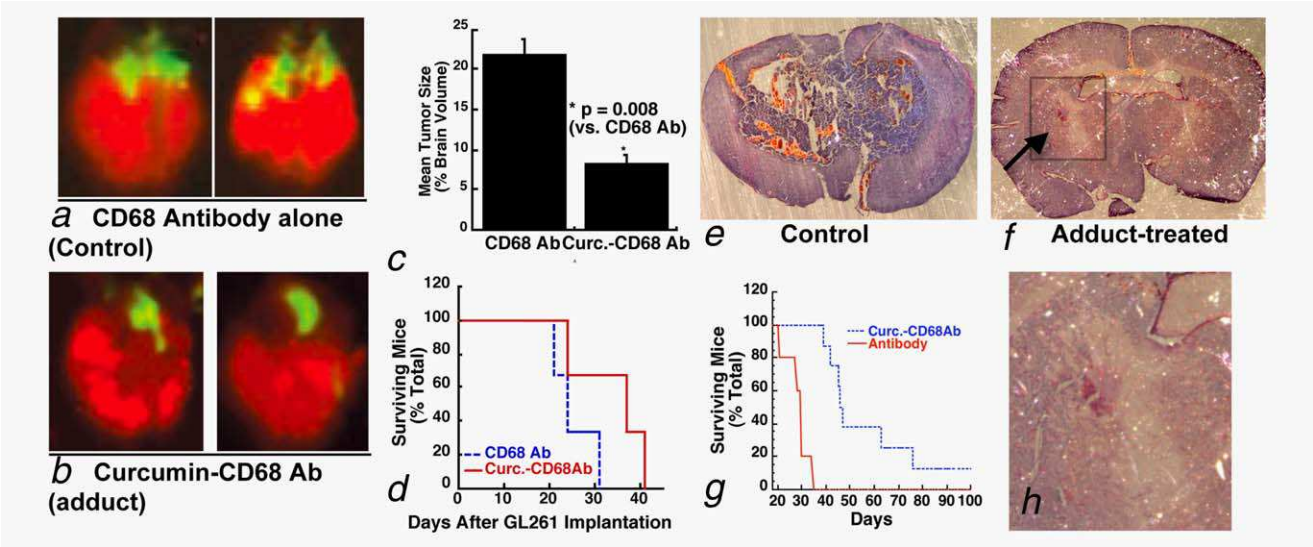

Figure 5. Intratumor adduct infusion followed by peripheral curcumin injection reduces tumor load, prolongs survival and rescues mice. GL261 cells (10^5) were implanted in each mouse on Day 1 to form the tumor. Sixteen picomoles of CD68 Ab-linked curcumin (CDC) in 5 μl PBS per injection (adduct) was infused on Days 13 and 15. (*a–d*) Three mice were used in each of two groups, one (control) receiving the CD68 antibody and the other receiving intratumor infusions of the adduct. Two intratumor injections of adduct every 72 hr followed by infusion of 200 μl of 667 μM in PBS plus 3% DMSO (curcumin) per tail-vein injection once every week until death. (*e–g*) Eight mice were used in each of two groups. Four intratumor infusions of 30 pmol CDC per mouse (from Day 8, every 72 hr), followed by tail-vein infusions of curcumin (667 μM) every 72 hr. (*e*) An H&E-stained control brain not receiving the adduct showing widespread degeneration due to glioblastoma. (*f*) Of the mice that remained healthy and normal (two) one was sacrificed 100 days after implantation of GL261 cells. The brain section shows the presence of scar tissue at the original site of tumor [enlarged view shown in (*h*)]. (*g*) Kaplan–Meier analysis shows a dramatic increase in survival in mice receiving adduct infusion into the tumor followed by curcumin infusion into the tail vein. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

normal, one was sacrificed 100 days after implantation of GL261 cells. H&E staining of brain sections from this mouse showed the presence of scar tissue at the original site of tumor (Fig. 5*f*; enlarged view shown in Fig. 5*h*), but no apparent sign of any inflammation in the tissue. Additionally, treatment with either curcumin[8] or CDC did not produce any inflammation or injury in the normal brain tissue of mice after 72 hr of treatment (Langone et al., unpublished data).

Discussion

Our report demonstrates that the glioblastoma-directed CD68 antibody binds to human glioblastoma cells, labels GL261-evoked mouse brain tumor, but does not label normal brain tissue. Thus, this report presents a possible strategy of potentiating the antitumor activity of a harmless food component curcumin by selectively targeting it to the glioblastoma brain tumor. Magnetic resonance imaging detection of human brain tumors can be achieved even when the tumor size is as little as 1.5 cm^3,[22] which constitutes about 0.1% of the total brain volume of an adult human being (1,400 cm^3). Using near-IR imaging, we had observed earlier that B16F10 melanoma-evoked brain tumors grow very rapidly from the time of intracranial implantation of tumor cells to assume almost 10–20% of mouse brain volume (400 μl) after 8 days.[9] In contrast, the GL261-evoked tumors grow slower, attaining about 5% (*i.e.*, about 20 μl) of total brain volume after 8 days. Based on such estimation, the concentration of antibody-linked curcumin achieved in the tumor after infusion of 30 pmol of CDC would be 1.5 μM. This was sufficient to cause a major increase in longevity and complete rescue in about 10% of the treated mice. However, our projected goal is to achieve a concentration of 4.5 μM or higher for our antibody-linked curcumin, this is expected to achieve much higher rates of remission. Our *in vitro* experiments have demonstrated that a 720 nM concentration of CDC is effective in eliminating glioblastoma cells. Allowing some loss of adduct after intracranial infusion, 4.5 μM should be optimal to achieve a dramatically higher percentage of complete recovery. The objective of our study was to validate the *in vivo* efficacy of our CDC as well as to test the specificity of our CD68 target. Our data show that even at very low doses, administration of our curcumin adduct has strong biologic efficacy. The concept of linking an antibody to a drug is not new; however, the majority of conjugates made have used toxins to eliminate cancer cells. In contrast, we use curcumin, which has been extensively shown in preclinical studies to be safe, demonstrating selectivity toward cancerous cells.[4,5] In our approach, we potentiate the anticancer efficacy of a compound from a dietary plant, and this strategy offers the possibility of having fewer side effects while maintaining potent anticancer activity of the agent.

The current mainstream of brain cancer therapy consists of tumor resection followed by radiation and chemotherapy.

The most common chemotherapeutic agent used for brain cancer treatment is an oral alkylating agent, temozolomide (Temodar), which crosses the blood–brain barrier but also produces major side effects.[23] Additionally, radiotherapy and chemotherapy can increase extracellular glutamate, which is converted into glutamine and consumed as fuel by brain tumor cells (glioblastoma).[24] Our approach of intracranial drug delivery is feasible as intracranial surgery is the first step taken in brain tumor therapy.

In a clinical setting, repeated access to the intracranial cavity could be accomplished *via* an intracranial shunt. Insertions of shunts to drain cerebrospinal fluid from the brain are done and can be maintained for long periods of time safely. This provides an opportunity for drug administration. At the outset, the intracranial delivery of targeted curcumin may raise many questions; however, clinical experience supporting this approach is possible. Intracranial delivery of proteins and peptides has been used as a therapy for neurodegenerative diseases.[25] Our study reports intracranial delivery of the trophic factor GDNF into the dorsal putamen, which resulted in improved motor function in advanced Parkinson's disease patients. In cancer therapy, bevacizumab (Avastin) is a humanized monoclonal antibody that inhibits vascular endothelial growth factor. It has been used *via* intra-arterial application to treat brain cancer patients.[26] Selective intra-arterial cerebral infusion techniques use a catheter, which is inserted into the femoral artery and passes to the carotid artery. A separate microcatheter inserted *via* the femoral catheter is then used to selectively explore the vessels of interest.[26] Bevacizumab has been delivered using microcatheters into the blood vessels inside the brain close to the site of the tumor in brain tumor patients.[27]

Such reports provide additional information on alternate delivery of proteins, which are often blocked from brain entry by the blood–brain barrier. These proteins could be made to enter the brain by the use of mannitol that transiently opens the blood–brain barrier (as used for the delivery of bevacizumab, when delivered peripherally). We also understand that antibodies developed in other animals cannot be used in humans. Thus, advancement into the clinic would require the use of humanized (chimeric) mouse monoclonal antibodies and also human antibodies developed using transgenic mice and phage display.[10,28–31]

Although antibody drug conjugates are very promising and there are decades of preclinical and clinical studies that document their use, to date there are only four FDA-approved drugs. Of these, trastuzumab emtansine (T-DM1; Kadcycla™) is the most recently approved antibody. Trastuzumab (Herceptin™) targets HER2, which is overexpressed on cancer cells of various types such as breast and ovarian cancer. This monoclonal antibody is conjugated with a maytasanoid, a chemical derivative of a plant compound, maytansine. Like curcumin, this compound has been demonstrated to be safe and very potent in preclinical studies, but clinical trials were unsuccessful in demonstrating efficacy. This included 35 tumor types in more than 800 patients.[32] Nonetheless, these tests with the trastuzumab maytasanoid conjugate demonstrated that enhanced tissue delivery of targeted drugs could be achieved with current technology. Essential to this approach is the identification of a suitable target and the identification of a selective anticancer conjugate. Our study establishes CD68 as a promising marker to target glioblastoma. We have developed a CDC that is highly effective in eliminating human glioblastoma cells U87MG and T98G at a low nanomolar range of IC50. We show that this conjugate is also effective in our *in vivo* model and we present data that targeted curcumin, which is effective and can overcome the limitations of poor bioavailability of curcuminoids. Furthermore, we present a possible therapeutic approach to treat a deadly form of brain cancer by using targeted therapy.

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References

1. Ferlay J, Bray F, Pisani P, et al. Globocan 2000: cancer incidence, mortality and prevalence worldwideed. Lyon: IARC Press, 2000.
2. Ohgaki H, Kleihues P. Epidemiology and etiology of gliomas. *Acta Neuropathol* 2005;109:93–108.
3. Campbell TC, Campbell TM, II. The China study. *Dallas, TX: Benbella Books*, 2006.
4. Anand P, Sundaram C, Jhurani S, et al. Curcumin and cancer: an "old-age" disease with an "age-old" solution. *Cancer Lett* 2008;267:133–64.
5. Carter A. Curry compound fights cancer in the clinic. *J Natl Cancer Inst* 2008;100:616–17.
6. Chen A, Xu J, Johnson AC. Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1. *Oncogene* 2005;25: 278–87.
7. Kunnumakkara AB, Anand P, Aggarwal BB. Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. *Cancer Lett* 2008;269:199–225.
8. Purkayastha S, Berliner A, Fernando SS, et al. Curcumin blocks brain tumor formation. *Brain Res* 2009;1266C:130–8.
9. Langone P, Debata PR, Dolai S, et al. Coupling to a cancer cell-specific antibody potentiates tumoricidal properties of curcumin. *Int J Cancer* 2012;131:E569–E578.
10. Senter PD. Potent antibody drug conjugates for cancer therapy. *Curr Opin Chem Biol* 2009;13: 235–44.
11. Karin M, Cao Y, Greten FR, et al. NH-kB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2002;2:301–10.
12. Shi W, Dolai S, Rizk S, et al. Synthesis of monofunctional curcumin derivatives, clicked curcumin dimer, and a PAMAM dendrimer curcumin conjugate for therapeutic applications. *Org Lett* 2007; 9:5461–4.
13. Cline GW, Hanna SB. The aminolysis of N-hydroxysuccinimide esters. *A structure-reactivity study. J Am Chem Soc* 1987;109:3087–91.
14. Hermanson GT. Tags and probes bioconjugate techniques. San Diego, CA: Academic Press, 1996. 306.
15. Raja KS, Wang Q, Gonzalez J, et al. Hybrid virus-polymer materials. *1. Synthesis and properties of PEG-decorated cowpea mosaic virus. Biomacromolecules* 2003;4:472–6.
16. Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates, 2nd edn. New York: Academic Press, 2001.

17. Deininger MH, Seid K, Engel S, et al. Allograft inflammatory factor 1 defines a distinct subset of infiltrating macrophages/microglial cells in rat and human gliomas. *Acta Neuropathol* 2000;100:673–80.
18. Svensson M, Yadav M, Holmqvist B, et al. Acute pyelonephritis and renal scarring are caused by dysfunctional innate immunity in mCxcr2 heterozygous mice. *Kidney Int* 2011;80:1064–72.
19. Hulette CM, Downey BT, Burger PC. Macrophage markers in dagnostic neuropathology. *Am J Surg Pathol* 1992;16:493–9.
20. Leenstra S, Das PK, Troost D, et al. Human malignant astrocytes exress macrophage phenotype. *J Neuroimmunol* 1995;56:17–25.
21. Strojnik T, Kavalar R, Zajc I, et al. Prognostic impact of CD68 and kallikrein 6 in human glioma. *Anticancer Res* 2009;29:3269–80.
22. Haney SM, Thompson PM, Cloughesy TF, et al. Tracking tumor growth rates in patients with malignant gliomas: a test of two algorithms. *Am J Neuroradiol* 2001;22:73–82.
23. Drug Record: Temozolomide. In: NIDDK, ed. Clinical and Research Information on Drug-Induced Liver Injury: United States Library of Medicine, 2012.
24. Takano T, Lin JH, Arcuino G, et al. Glutamata release promotes growth of malignant gliomas. *Nat Med* 2001;7:1010–15.
25. Grondin R, Zhang Z, Ai Y, et al. Intracranial delivery of proteins and peptides as a therapy for neurodegenerative diseases. *Pro Drug Res* 2003;61:101–23.
26. Burkhardt JK, Riina HA, Shin BJ, et al. Intra-arterial chemotherapy for malignant gliomas: a critical analysis. *Interv Neuroradiol* 2011;17:286–95.
27. Grady D. A direct hit of drugs to treat brain cancer. The New York Times, 2010.
28. McCafferty J, Griffiths A, Winter G, et al. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 1990;348:552–4.
29. Lonberg N. Human antibodies from transgenic animals. *Nat Biotechnol* 2005;23:1117–25.
30. Reichert JM, Rosensweig CJ, Faden LB, et al. Monoclonal antibody successes in the clinic. *Nat Biotechnol* 2005;23:1073–8.
31. Reichert JM, Valge-Archer VE. Development trends for monoclonal antibody cancer therapeutics. *Nat Rev Drug Discov* 2007;6:349–56.
32. Cassady JM, Chan KK, Floss HG, et al. Recent developments in the maytansinoid antitumor agents. *Chem Pharm Bull (Tokyo)* 2004; 52:1–26.