Curcumin changes the polarity of tumor-associated microglia and eliminates glioblastoma

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Glioblastoma (GBM) is one of the most pernicious forms of cancer and currently chances of survival from this malady are extremely low. We have used the noninvasive strategy of intranasal (IN) delivery of a glioblastoma-directed adduct of curcumin (CC), CC-CD68Ab, into the brain of mouse GBM GL261-implanted mice to study the effect of CC on tumor remission and on the phenotype of the tumor-associated microglial cells (TAMs). The treatment caused tumor remission in 50% of GL261-implanted GBM mice. A similar rescue rate was also achieved through intraperitoneal infusion of a lipid-encapsulated formulation of CC, Curcumin Phytosome, into the GL261-implanted GBM mice. Most strikingly, both forms of CC elicited a dramatic change in the tumor-associated Iba1+ TAMs, suppressing the tumor-promoting Arginase1high, iNOSlow M2-type TAM population while inducing the Arginase1low, iNOShigh M1-type tumoricidal microglia. Concomitantly, we observed a marked induction and activation of microglial NF-κB and STAT1, which are known to function in coordination to cause induction of iNOS. Therefore, our novel findings indicate that appropriately delivered CC can directly kill GBM cells and also repolarize the TAMs to the tumoricidal M1 state.

In recent years, curcumin (CC), a plant-derived polyphenol and the coloring component of the culinary spice turmeric has generated considerable interest by proving to be a strong anti-cancer agent against a wide range of cancer cells while being nontoxic to normal cells.1–3 Yet, presumably due to its low bioavailability in vivo, curcumin has found little clinical use in treating cancer. Among multiple strategies designed by us to boost the in vivo efficacy of CC, antibody targeting of CC to cancer cells in the brain has shown great promise.4,5 In our prior studies, we targeted the food-derived anti-cancer agent CC to the mouse GBM cells GL261 and orthotopic GBMs by linking it through a cleavable ester bond to an antibody (Ab) against CD68, which is expressed highly by GBM cells and at a lower level by microglia.5 Although this strategy increased the potency of CC by two to three orders of magnitude, delivering the antibody-CC adduct into the brain remained as a formidable challenge. Here, we show that intranasal (IN) delivery of the Ab-CC adduct overcomes this hurdle, thereby rescuing mouse glioblastoma GL261-implanted mice. Additionally, a commercially available lipid-complexed form of curcumin (Curcumin Phytosome-Meriva) also proved to be highly effective even when delivered intra-peritoneally (i.p.). However, it is not clear if the efficacy of appropriately delivered curcumin is only due to its ability to kill cancer cells. The current study addresses this question and identifies an activity of CC that may contribute greatly to its ability to eliminate established tumors in the brain.4,5

Earlier reports indicate that many plant-derived food items stimulate our immune system.2,6 It is also known that several gene mutations can trigger carcinogenesis,7 and cancer is prevented if such aberrantly generated cells are eliminated by a vigilant immune system. Thus it is likely that plant-derived foods play an important role in stimulating our immune system to eliminate cancer cells. Curcumin regulates the activity of T-cells in the peripheral immune system and also the
What’s new?
Curcumin is widely known for its culinary uses, though it is also gaining recognition for its anticancer activity, demonstrated primarily in cells with limited success in vivo, due to low bioavailability. To overcome this obstacle, the authors of the present study coupled curcumin to a glioblastoma (GBM)-specific antibody and administered it intranasally in mice. The treatment caused a dramatic switch in tumor-associated microglia, with repolarization from a population of tumor-promoting M2-type cells to a milieu of tumoricidal M1-type cells. Treatment also led to tumor remission in 50–60% of GBM-harboring mice.

Materials and Methods

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 animal protocol approved by the Institutional Animal Care Committee (IACUC) of the College of Staten Island (CUNY).

Creation of glioblastoma lines GBM6, GBM46, GBM994 from three different glioblastoma patients
The glioblastoma lines GBM6 and GBM46 were created by Sarkaria and coworkers at the Mayo Clinic, Rochester, Minnesota.15,16 These GBM samples were obtained with the consent of the patients and authorization of the Institutional Review Board of the Mayo Clinic. The patient-derived GBM994 cells were similarly created by Boockvar and coworkers (unpublished data). The tumor sample for GBM994 was derived from a patient with a left temporal lobe recurrence after radiation and temozolomide treatment. It was obtained with the consent of the patient and authorization of the Weill Cornell Institutional Review Board.

Cell culture
GL261 mouse glioblastoma cells were cultured in RPMI 1640 containing 10% (v/v) FBS (fetal bovine serum) and 1% (v/v) Gentamicin. Before and during drug treatment, the cells were placed in RPMI medium containing 1% (v/v) ITS (insulin-transferrin-selenium- Gibco BRL) and 1% (v/v) Gentamicin. For culturing the human patient-derived GBM994, GBM46 and GBM6 (non-adherent) cell lines, we coated the T75 flasks with 6 ml poly-lysine (0.1 mg/ml in H2O) for 30 min at room temperature, followed by a rinse with sterile H2O. Next, 6 ml of Laminin (Sigma L2020) (75 μl of stock in 10 ml sterile PBS) was used to coat the flask followed by incubation for 2 hrs at 37°C. The cells were grown in Stem-Pro media (Invitrogen–Life Technologies A10509-01).

Immunocytochemistry
( Please see Supporting Information).

Drug treatments and determination of IC50
Aliquots of CC-CD68 Ab and curcumin phytosome (CCP) were suspended in the required volume of medium (serum-free RPMI with 1% ITS supplement for GL261 and stem-pro medium for GBM994, GBM46, and GBM6 cells), serially diluted in the respective medium to generate a series of working concentrations, which were tested in vitro on GL261, GBM994, GBM46 and GBM6 cells to determine the cytotoxic potency of the two curcumin derivatives. The cells were treated with drugs when the wells reached 30–40% confluence. Cells were visually monitored for degeneration (membrane blebbing and degradation) 48 hrs after treatment by light microscopy. After 96 hrs of treatment, the medium in each well was aspirated, and the cells were gently rinsed three times with PBS. Next, the cells were incubated at 37°C for 45 min with 10% WST-1 (v/v) (Clontech, Mountain View, CA) diluted in serum-free DMEM (v/v) as described in our earlier report,3 and the absorbance of the resultant solution monitored at 440 nm using a plate reader. Dose dependent response curves were generated from the absorbance to determine the IC50 of the drugs for all the brain tumor cell lines. Microsoft Excel was used for graphical analysis.

Preparation of Dylight 800-CD68 Ab and curcumin-CD68 Ab adducts
(Please see Supporting Information). The curcumin:Ab ratio in the CC-CD68Ab adduct was determined by spectroscopy and also MALDI-TOF as 1:1 (Supporting Information Fig. 1).

Implantation of cancer cells in mice
( Please see Supporting Information).

Intranasal delivery of the curcumin-CD68 Ab adduct and intraperitoneal delivery of curcumin phytosome on GL261 implanted mice
In a pilot experiment, we used a mouse implanted with 80,000 GL261 cells. From Day 11 after the implantation,
intransal application of vehicle (30 µl sterile 10 mM PBS) was conducted every 72 hrs intranasally as described for CC-CD68 Ab application in the larger experiments. For the larger experiments using mice implanted with $10^5$ GL261 cells, on the tenth day after implantation, the mice were anesthetized, placed in the supine position, and 120 pmol of CC-CD68 Ab adduct (CC:Ab 1:1) (containing 18 µg of CD68 Ab) dissolved in 30 µl of sterile 10 mM PBS was applied as 3-µl drops using a micropipette, alternately, every two minutes, to each nostril and allowed to be snorted in.

We also used a commercially available product, Curcumin Phytosome featuring Meriva (CCP),17 sold as a supplement for joint health containing about 96 mg of curcuminoids complexed with soy lipids and excipients in 500 mg of an amorphous solid in each capsule. For intraperitoneal (i.p.) administration of CCP, 10 days after implantation of $10^5$ GL261 cells, each 30-g mouse received i.p. injections of vehicle (PBS) or a CCP emulsion containing 2 mg of CCP in 200 µl of PBS every 72 hrs. CCP was dispersed vortexing vigorously in 200 µl of sterile PBS, the insoluble solids were allowed to settle for 2 min, and then the translucent supernatant was used for i.p. injection into each mouse.

Post-mortem brain examination by ex vivo near-IR scanning

When the mouse in the pilot experiment appeared morbid, it was anesthetized and then 30 µl of the DyLight-800-CD68 Ab adduct in 3-µl aliquots was administered, every 2 min, to alternate nostrils while holding the mouse in the supine position. Six hours after administration, the mouse was sacrificed, the brain removed and fixed in 4% paraformaldehyde. Near-IR images were acquired using the Odyssey® Imaging System (LI-COR Biosciences, Nebraska). Brightfield images were acquired using a digital camera. The Dylight-800 fluorescence is pseudocolored green and the enhanced autofluorescence of the brain tissue (≈700 nm, autofluorescence) is pseudocolored red.

Immunohistochemistry of brain tumor and scar tissue region

Coronal sections (30 µm) were made from 4% paraformaldehyde-fixed and 30% sucrose-soaked mouse brains harboring glioblastoma tumors or scar tissue as described by us earlier.3 Randomly chosen sections containing tumor tissue (Supporting Information Fig. S4A) or scar tissue area (Supporting Information Fig. S4B) were processed as follows for antigen retrieval: incubation with formamide:2xSSC (1:1), 55°C for 2 hrs, and then 5 min in 2xSSC (30 mM sodium citrate, 30 mM sodium chloride) at room temperature. After blocking overnight at 4°C in 0.1% Triton X-100, 3% goat serum and 10% rabbit serum in 100 mM PBS, the sections were treated overnight with primary antibodies: anti-Iba1 (goat IgG)(C20) (sc285830) (1:50), anti-iNOS (rabbit IgG) (NOS2 sc-651) (1:100), anti-arginase1 (rabbit IgG) (sc-20150) (1:100), anti-p65 (NF-kB) (mouse IgG) (sc-8008) anti-P-Ser276-p65 (NF-kB) (rabbit IgG) (sc-101749) (1:100), anti-STAT1 (rabbit IgG) (Sc-592) (1:100), and anti-P-Tyr701-STAT1 (mouse IgG) (sc-8394) (1:100). All antibodies were diluted in 2% goat serum and 2% rabbit serum and 0.1% Triton X-100 in PBS (GRT-PBS). Control sections were incubated overnight at 4°C in the blocking solution. After washing three times with PBS, the respective secondary antibodies (Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 633 goat anti-rabbit, Alexa Fluor 568 goat anti-rabbit, and Alexa Fluor 633 goat anti-mouse) (1:500 dilutions in GRT-PBS) were added to wells treated with the respective primary antibodies as well as those not treated with primary antibodies (2° Ab controls). Following overnight incubation at 4°C and three washes with PBS, the sections were treated with HOECHST33342 (10 µg/ml) for 30 min at room temperature. The sections were then washed three times with PBS and mounted on microscope slides with Gold anti-fade mounting fluid. Confocal Imaging was conducted using a Leica SP2 microscope from multiple randomly chosen fields encompassing regions in and around the tumor and scar tissue region (Supporting Information Fig. S4). ImageJ was used to obtain the iNOS, arginase1, p65 (NF-kB), P-Ser276-p65, STAT1, P-Tyr701-STAT1 and HOECHST33342 fluorescence intensity. The fluorescent intensity of all the above-mentioned antibodies were normalized to HOECHST33342 intensity (blue). Since p65 NF-kB and STAT1 displayed both induction as well as phosphorylation-mediated activation, the HOECHST-normalized staining intensities were expressed both as P-p65 NF-kB/p65 NF-kB and P-STAT1/STAT1 as well as P-p65 NF-kB/HOECHST and P-STAT1/HOECHST.

Flow cytometry of immunostained brain tumor and scar tissue cells

The brains from the GL261-implanted, morbid mice (vehicle-treated control mice) and the rescued mice (GL261 implanted, curcumin derivative treated mice living >120 days) were extricated without fixing (after anesthesia), and rinsed twice with PBS. Next, only the cells from the tumor area (vehicle-treated) (the tumor core, periphery and most area around it) and scar tissue area (curcumin treated, rescued) (scar tissue core, periphery and most area around it) from these unfixed brains were dissociated by mild trypsinization using 0.25% trypsin-EDTA and repeated gentle trituration using a fire-polished Pasteur pipette to generate single cell suspensions.18-20 The cells were filtered through a sieve to remove clumps and fixed using freshly made 4% paraformaldehyde (PFA). The fixed cells were counted and stored in PFA at 4°C. Around 2 million fixed cells from each animal were used for immunostaining as described under immunocytochemistry. After each antibody treatment, the cells were pelleted down and resuspended in washing buffers. Antibodies against Iba1 (C20) (1:200), iNOS (1:200) and Arginase1 (1:200) were used for staining. Secondary antibody-treated cells were used to set the threshold.

Flow cytometry was performed using Accuri C6 flow cytometer (BD). For each sample, events above 10^4 forward
and 10^3 side scatter were gated, and 100,000 gated events were analyzed. After compensation of fluorescent events to minimize the spillover from each channel, subpopulations of cells appeared in different quadrants based on their fluorescence intensities. Events from autofluorescence and non-specific fluorescence from the "28 antibody only" samples appeared as a distinct population in the lower-left quadrant (LL quadrant). The double-stained fluorescent events from ARG1+, Iba1+ or iNOS+, Iba1+ cells appearing as subpopulations in the upper right (UR) quadrant within the coordinates 520 nm (green for ARG1 and iNOS) (FL1-A) and 580 nm (red for Iba1) (FL2-A) were considered and mean fluorescence intensity was measured using the mean method of statistical analysis per double-stained cell.

**Hematoxylin and eosin staining of tissue sections**
(Please see Supporting Information).

**Statistical analysis**
Two-tailed t-tests with unequal variance were used while comparing the two groups, vehicle-treated and CC-treated and rescued, and p ≤ 0.05 was considered as significant.

**Results**

**CD68 expression in patient-derived GBM explant cells and IC50 for targeted curcumin in the CC-CD68Ab adduct**

In our prior reports we had shown that CD68, an antigen expressed at a low level by microglia is highly expressed by the mouse glioblastoma GL261 cells, and targeting of CC in a releasable form using a CD68 antibody causes a 125-fold decrease in the IC50 of CC in eliminating GL261 cells. Here we investigated the cellular localization and the levels of expression of CD68 in three patient-derived GBM cell lines. We wanted to investigate if the levels of cell-surface expression of CD68 did in any way influence the IC50 value for CC-CD68 Ab. As shown in (Fig. 1a), the GBM6 cells expressed lower levels of cell surface and higher levels of nuclear CD68. This was also associated with a higher IC50 for CC-CD68 Ab (still in the nanomolar range—300 nM—indicating much higher potency than free CC), suggesting that less cell-surface antigen molecules were available for antibody binding. Our earlier studies have also shown that the targeting antibody without the attached CC molecules does not kill targeted cells and also that the CD68Ab-CC is taken up by the GL261 cells, thereby causing CC release.
inside (marked by an increase in fluorescence due to CC) and destruction of the targeted cell. Among the three patient-derived GBM cells, GBM994 displayed the highest sensitivity to CC-CD68 Ab. Whereas free curcumin eliminated the GBM994 cells with an IC50 of 22.5 μM, the IC50 of targeted-CC (CC-CD68 Ab) was 800-fold lower (25 nM) (Figs. 1b and 1c). Our MALDI-TOF analysis showed that one molecule of CC was linked to one Ab molecule in the adduct (Supporting Information Figs. S1A and S1B), which enabled us to calculate the concentration of Ab-linked curcumin for all treatments. Lack of staining with the secondary antibody alone (Supporting Information Fig. S2) confirms that the CD68 staining was specific.

Based on the curcumin content of CCP, this lipid-complexed preparation of CC was able to eliminate GL261 cells with an IC50 of 15 μM, which was similar to the IC50 observed for free curcumin for the GL261 cells in culture (Supporting Information Fig. S3).

**Intranasal delivery of CC-CD68Ab conjugate and CCP both cause elimination GL261 GBM brain tumor and rescue of mice**

In a pilot experiment, a GL261-implanted mouse was allowed to proceed to morbidity (Fig. 2a) and then anesthetized for intranasal (IN) delivery of the CD68 Ab linked to a near-infrared (Near-IR) dye Dylight800 as illustrated in Figure 2b. The antibody-dye conjugate reached the brain and accumulated in the GBM, the location of which was detected by both near-IR scanning and also bright-field imaging (Figs. 2c and 2d). This confirmed that the CD68Ab could be delivered to the brain via the IN route. In our earlier studies, free curcumin, administered through the tail vein, was able to reach the brain within fifteen minutes, but the transient level of curcumin achieved in the brain through this route was not sufficient to bring about a rescue of the tumor cell-implanted mice. To test if the lipid-complexed preparation, CCP, was capable of producing an effect on GBM, we suspended 2 mg of this solid (CCP) in 200 μl sterile PBS and injected this translucent emulsion per mouse (i.p.) from Day 10 after implantation of 100,000 GL261 cells per mouse on day 1. In parallel, GL261-implanted mice were also i.p. injected with PBS (vehicle-treated). Such i.p injection of CCP but not vehicle every 72 hrs for one month was able to rescue 60% of the GL261-implanted mice (Fig. 2e). In parallel, CC-CD68Ab adduct was administered IN every 72 hrs from Day 10 after GL261 implantation. Adduct treatment (120 pmole per mouse, per treatment) every 72 hrs (four times) and then once every seven days (four times) caused remission of GBM in 50% of the GL261-implanted mice (Fig. 2e). Thus, both strategies for curcumin delivery seem to be highly effective for GBM therapy.

We identified the tumor and scar tissue areas along with the surrounding areas by Hematoxylin and eosin (H&E) staining in parallel brain sections (Supporting Information Fig. S4) and then used the above-mentioned areas from adjacent sections for our subsequent analyses. Being cognizant of CC’s tumoricidal ability from our previous study, we investigated the presence of tumor (CD68high) cells at the tumor core of the vehicle-treated mice (Supporting Information Fig. S4A) and the scar tissue area (Supporting Information Fig. S4B) of the CC-treated and rescued mice. As expected, the tumor core had a plethora of CD68high tumor cells, but the scar tissue area of the CC-treated and rescued mice harbored no CD68high GBM tumor cells (Supporting Information Fig. S5).

Around Day 30, 50% of the vehicle-treated mice (control) were already dead whereas 100% of the IN-CC-CD68Ab-treated and 90% of the CCP-treated mice were still alive (Fig. 2e). Similarly, by Day 90, all of the vehicle-treated mice were dead, whereas 70% of the IN-CC-CD68Ab-treated and 60% of the CCP-treated mice were still alive. Thus, our results demonstrate that appropriately delivered CC not only rescues 50–60% of the GBM mice, but also prolongs the life of the treated mice.

**Curcumin treatment and rescue is associated with a dramatic shift in the relative expression profiles of AR1 and iNOS in Iba1+ microglia**

Cognizant of the influence of CC on the peripheral immune cells, we asked if, similarly, the major immune cells of the CNS, the microglia, were influenced by CC. Using Iba1 staining to identify the activated microglia within the GBM mass (TAMs), we counterstained with an antibody against Arginase 1 (ARG1), which is highly expressed by the immuno-suppressive and tumor-promoting M2-type microglia. Immunohistochemistry (IHC) analysis of GBM sections revealed that the GBM tumors and the surrounding areas (Supporting Information Fig. S4A) contained a large number of Iba1+ microglia, which also expressed high levels of ARG1 (Fig. 3a). In sharp contrast, these sections expressed very low levels of inducible nitric oxide synthase (iNOS), which is highly expressed by the M1-type microglia (Fig. 3a).

Next, we analyzed the relative expression of ARG1 and iNOS by Iba1+ cells present in the scar tissue and the surrounding areas (shown within a circle in Supporting Information Fig. S4B) in the brains of the rescued mice. A dramatic decrease in ARG1 along with a striking increase in iNOS expression was observed in Iba1+ cells contained within the scar tissue region (Figs. 3b and 3c). Normalization of the staining intensity of ARG1 or iNOS to that for HOECHST (nuclear staining) and quantification from random fields of multiple mouse brains confirmed that CC treatment and rescue was associated with an 85.1% (6.71-fold) decrease in ARG1 along with a 206.3% (3.06-fold) increase in iNOS in the Iba1+ microglia (Figs. 3d and 3e).

The specificity of the primary antibodies was confirmed by the lack of staining with the secondary antibodies alone (Supporting Information Fig. S7).
Flow cytometry analysis in GBM or scar tissue indicates that discrete populations of Iba1+ microglia are affected during CC treatment and rescue

Based on the observed immunohistochemistry results, flow cytometry analysis was performed on cells dissociated from vehicle-treated tumor areas (Supporting Information Fig. S4A) and CC-treated and rescued scar tissue areas marked with a circle in Figure S4B. The dissociated cells were fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100, divided into multiple aliquots, and then individual aliquots of cells were immunostained using antibodies against ARG1 and Iba1 or iNOS and Iba1. For each sample, events above $10^4$ forward and $10^3$ side scatter were gated, and 100,000 gated events were analyzed (Figs. 4a and 4f). Fluorescence from the 2° Ab-treated cells was used to mark the threshold ($10^3$ for FL1-A at 520 nm (green) and $10^2$ for FL2-A at 580 nm (red)) confining the 2° Ab fluorescence to the lower left (LL) quadrant (Fig. 4k). The fluorescence events from ARG1+, Iba1+ or iNOS+, Iba1+ cells occurred above the secondary fluorescence in the upper right (UR) quadrant of FL1-A (iNOS or ARG1) and FL2-A (Iba1) (Figs. 4b, 4c, 4g and 4h). From these double-positive populations, the total fluorescence

Figure 2. Effective delivery strategies for CC-CD68Ab and CCP. In a pilot experiment, GL261 cells (80,000) were implanted in the right brain of a 3-month old male C57BL/6 mice on Day 1 by stereotaxic injection.4,5 (a) The mouse received PBS (i.p.) from Day 11, and appeared immobile and morbid on day 29. (b) The mouse was anesthetized, placed in the supine position, and CD68Ab-Dylight conjugate containing 60 µg (400 pmole) of antibody in 30 µl of PBS was applied in 3-µl drops alternately, every two minutes, to each nostril, and allowed to be snorted in (IN) (illustration obtained with the kind permission of William Frey II).21 The mouse was allowed to recover and then sacrificed after 6 hrs. (c) Its brain was extricated without fixing and the soft, gel-like tumor-containing brain, was placed with its dorsal face up on the scanner and near-IR scanned from the ventral side. Subsequently, the brain was formaldehyde-fixed, placed with ventral face up and imaged using a bright-field camera from the ventral side. In the bright-field image, extensive hemorrhage was observed mainly in the right brain, and near-IR (800 nm; pseudo-colored green) labeled, CD68+ tumor cells were observed mainly in the right brain and also in other regions due to metastasis. Autofluorescence from the remaining brain tissue was pseudo-colored red. (d) The brain was then paraformaldehyde fixed and imaged from the ventral face using a digital camera. (e) In survival experiments, 100,000 GL261 cells were intracranially implanted on Day 1 in each mouse. On Day 10, one group was treated with PBS (i.p) (ten mice), a second group was treated with CCP (i.p. delivered, 2 mg per mouse in 200 µl of PBS) (ten mice) and a third group was treated (IN) with CC-CD68Ab (120 mole per treatment in 30 µl of PBS) (eight mice). Results show that treatment with each of CCP (n = 10) and CC-CD68Ab (n = 8) caused 50% or higher rescue of GL261-implanted mice, whereas all PBS-treated mice succumbed to GBM (n = 10). [Color figure can be viewed at wileyonlinelibrary.com]
intensity from all the cells was recorded (Figs. 4d and 4i) and mean fluorescence per cell was used for quantification (Figs. 4e and 4j). While the Iba1+ cells in the tumor area were ARG1<sup>high</sup>, iNOS<sup>low</sup> in the vehicle-treated mice, in the scar tissue area of the rescued mice the Iba1+ cells were ARG1<sup>low</sup>, iNOS<sup>high</sup> (Figs. 4e and 4j). These changes were 69.58% (a 3.28-fold decrease) for ARG1 and 154% (2.54-fold increase) for iNOS (Figs. 4e and 4j).

The mean Iba1 fluorescence per double-stained cell (ARG1+ or iNOS+) was also significantly different between the two groups. While the Iba1 fluorescence intensity per cell in the ARG1+ cells in the scar tissue area of the rescued mice was 73.92% (3.83-fold) less than in the tumor area of the vehicle-treated mice (Fig. 4i). Thus, CC treatment caused a parallel decrease in both ARG1 and Iba1 fluorescence and a parallel increase in iNOS and Iba1 fluorescence in the double-stained TAMs. However, the mean Iba1 intensity did not significantly differ between the two groups (iNOS+, Iba1+ and ARG1+, Iba1+ cells) (Fig. 4m).

This supported the possibility that CC treatment was responsible for the silencing of one group of ARG1+ TAMs and the parallel activation of a separate set of iNOS+ TAMs.

CC-treated and rescued mice show a sharp induction and activation of NF-κB (p65) and STAT1 in the Iba1+ microglia

Earlier studies performed in macrophages posit that co-stimulated NF-κB (p65) and STAT1 cooperate by binding to
appropriate enhancer elements to activate the iNOS gene.\textsuperscript{26,27} To effect such a change, CC treatment should cause activation of both of these two transcription factors in the tumor-associated microglia.

As expected, the Iba1\textsuperscript{1} cells in the scar tissue of the CC-treated and rescued mice showed an 85\% (1.85-fold) increase in activated p65 NF-κB (P-Ser276-p65 normalized to total p65 NF-κB) compared to that in the tumor sections (Figs. 5\textsuperscript{c}). In parallel, a 300\% (4-fold) increase in p65 expression was observed in the Iba1\textsuperscript{1} scar tissue TAMs (Fig. 5\textsuperscript{d}), which resulted in a net increase in 642\% (7.42-fold) in P-p65 NF-κB in the scar tissue TAMs over that in the tumor TAMs in the vehicle-treated mice (Fig. 5\textsuperscript{e}).

Similarly, the Iba1\textsuperscript{1} cells in the scar tissue from the CC-treated and rescued mice showed a 591\% (6.9-fold) increase in activated STAT1 (P-Tyr701-STAT1 normalized to total STAT1) (Figs. 6\textsuperscript{a}–6\textsuperscript{c}). In parallel, a 652\% (7.52-fold) induction in STAT1 was observed (Figs. 6\textsuperscript{a}, 6\textsuperscript{b} and 6\textsuperscript{d}), which resulted in a net increase of 5134\% (52.34-fold) in activated (P-STAT1) in the CC-treated and rescued mice (Figs. 6\textsuperscript{a}, 6\textsuperscript{b} and 6\textsuperscript{e}).

The activated p65 (NF-κB) and STAT1 were co-localized in the same Iba1\textsuperscript{1} microglia in both tumor-associated and scar tissue-associated TAMs (Supporting Information Fig. S6). The specificity of the primary antibodies was confirmed by the lack of staining with the secondary antibodies alone (Supporting Information Fig. S7).

Figure 4. Flow cytometry analysis of Iba1\textsuperscript{1} microglia in GBM and scar tissue regions. Cells from GBM tumor (at death) or scar tissue areas (rescued mice, 150 days after GL261 implantation) were dispersed, fixed, permeabilized, and then immunostained using iNOS, Arginase1, and Iba1 (microglia marker) antibodies. (a and f) Events above 10\textsuperscript{4} forward (FSC) and 10\textsuperscript{3} side (SSC) scatter were gated. (b, c) Fluorescence intensity obtained above 10\textsuperscript{5} in horizontal (green, iNOS) and 10\textsuperscript{4} in vertical (red, Iba1) axes from Iba1\textsuperscript{1}, iNOS\textsuperscript{1} cells in each mouse was considered and the profiles of iNOS\textsuperscript{+} cells were obtained for the vehicle-treated GBM (red) and scar tissue (black) cells (d). (g, h) Similarly, fluorescence intensity obtained above 10\textsuperscript{5} in horizontal (green, ARG1) and 10\textsuperscript{4} in vertical (red, Iba1) axes from Iba1\textsuperscript{1}, ARG1\textsuperscript{1} cells dissociated from each mouse was considered and the profiles of ARG1\textsuperscript{+} cells were obtained for the vehicle-treated GBM (red) and scar tissue (black) cells (i, j, k, n). (e, l) Graphs showing a 69.58\% (3.28-fold) decrease in ARG1 staining and a 154\% (2.54-fold) increase in iNOS staining (with respect to vehicle-treated) using four vehicle-treated and three rescued (two CCP-treated and one CC-CD68 Ab-treated) mice (mean ± SEM). (k) Events from secondary antibody-stained cells appear below 10\textsuperscript{5} in horizontal axis and below 10\textsuperscript{3} in vertical axis. (l) Similar quantification of Iba1 fluorescence from the vertical axis for the Iba1\textsuperscript{1}, iNOS\textsuperscript{+} and Iba1\textsuperscript{1}, ARG1\textsuperscript{+} cells used in (a–e) and (f–j), show a parallel decrease in Iba1 fluorescence in the ARG1\textsuperscript{+} cells (73.92\%; 3.83-fold) and an increase in Iba1 fluorescence (184\%; 2.84-fold) in iNOS\textsuperscript{+} cells with respect to the vehicle-treated for each set (mean ± SEM). (m) No significant difference in total Iba1 fluorescent events between double-positive cells from the vehicle-treated tumor tissue and the CC-rescued scar tissue. [Color figure can be viewed at wileyonlinelibrary.com]
Discussion

As revealed by the sheer number of publications, the polyphenolic plant-derived food product CC has generated immense interest in the recent years. Its strong activity against a wide range of cancer cells and also its beneficial effects in mouse models for Alzheimer disease and cardiac hypertrophy have, at the very least, established its importance in health sciences. However, the lack of significant outcomes in human clinical trials has considerably dampened the interest in CC as a future therapeutic agent. Our group has persistently argued that such failures are due to its low bioavailability and that an improved design of delivery would resurrect CC as a promising therapeutic agent. As opposed to the delivery of toxic agents or radioisotopes specifically to the cancer cells, appropriate delivery of CC could overcome its poor bioavailability and also serve the function of stimulating the body to fight the residual cancer cells.

With such dual objective we used two strategies, one to deliver CC in a commercially available phytosome-complexed form that is already consumed as a supplement, and the other to deliver CC linked to a GBM-specific antibody. In the second strategy, we linked curcumin to a CD68 antibody through an ester bond that would be cleaved by intracellular esterases. This would target CC mainly to the CD68-loaded GBM cells and also direct it to the tumor-associated microglia that express lower levels of surface CD68. The central idea was that the large antibody molecule would solubilize, protect, and quickly deliver the CC molecules to GBM. Next, the CC released from the CC-CD68Ab molecules that are taken up by endocytosis by the GBM cells would kill these cells. Additionally, some CC-CD68Ab molecules that are targeted to and taken up by GBM-associated microglia would be expected to stimulate these cells to release cytokines to kill the GBM cells and then eliminate the dead cells through
However, this would require CC-mediated alteration of the tumor-associated microglia population from the tumor-promoting M2 to the tumoricidal M1 state.9–14 To analyze the cells of the microglial lineage, we first used IHC and then flow cytometry to identify and quantify the expression of ARG1 or iNOS in cells that coexpressed Iba1. This could not be achieved by Western blotting, because ARG1 and iNOS are also expressed by other cell types and tumor cells that do not express Iba1 and the whole tumor or scar tissue would yield ARG1 and iNOS profiles that may not be linked to the microglia. The overall repolarization was evident from both IHC and flow cytometry. Based on both immunohistochemistry and flow cytometry, in the CC-treated and rescued mice, we observed a 154–205% increase in iNOS fluorescence along with a concomitant 69.58–85% decrease in ARG1 fluorescence in the Iba1+ microglial cells (Figs. 3 and 4). Since Iba1 is the signature of the activated microglia,22,23 the results indicate that in the CC-treated and rescued mice, the tumor-associated ARG1high, Iba1+ M2 microglial populations are suppressed while a discrete population of iNOS high, Iba1+ M1-type microglia is activated. Alternatively, M1-type macrophages, which are also identified by Iba1 staining, could be recruited into the brain from the peripheral system.42 Interestingly, the overall Iba1 intensity for both iNOS high/Iba1+ and ARG1 high/Iba1+ cells between vehicle treated and CC-rescued mice remained unchanged, indicating that the number of M2-TAMs (vehicle group) was approximately equal to the number of M1 TAMs (CC-treated) (Fig. 4). Thus, CC-evoked tumor elimination (Supporting Information Figs. S4 and 5) may involve suppression of these tumor-promoting and immunosuppressive M2-TAMs along with concomitant activation and/or recruitment of tumoricidal M1 TAMs.

Figure 6. Induced STAT1 expression and activation in Iba1+ cells in the scar tissue region of the rescued mice. Brain sections parallel to those used in Figure 5 were used to determine the levels of STAT1 and P-Tyr701-STAT1 (activated) in the tumor and scar tissue areas of the vehicle-treated and CC-treated and rescued mice. (a–c) Relative to that in the tumor tissue, a 59% (6.9-fold) increase in P-STAT1/STAT1 was observed in the scar tissue Iba1+ cells. (a, b and d) Simultaneously, a 77% (8.75-fold) induction in STAT1 was observed in the scar tissue Iba1+ cells. (a, b, and c) This resulted in an increase of 5.134% (52.34-fold) in P-STAT1 in the scar tissue Iba1+ cells. Five sections per mouse were used for imaging and data were graphically presented as mean ± SEM. Scale bar: 47.62 μm. The activated P-p65 NF-κB and P-STAT1 molecules were co-localized in the same Iba1+ microglia in the scar tissue-associated TAMs (Supporting Information Fig. S6). The specificity of the primary antibodies was confirmed by the lack of staining with the secondary antibodies (Supporting Information Fig. S7). Scale bar: 47.62 μm. [Color figure can be viewed at wileyonlinelibrary.com]
Although our results demonstrate that following appropriate delivery, CC in both CCP and CC-CD68Ab brings about tumor remission in 50–60% of mice harboring established brain tumors, it is not clear why the remaining 40–50% mice were not rescued (Fig. 3). A likely reason was that the immune system of each mouse was discretely responsive to CC treatment. Our current experiments are addressing this question.

The mechanistic underpinnings of this CC-evoked switch in brain microglia/macrophage population may invoke some debate. Previous in vitro studies performed mainly on peripheral macrophages have shown that the non-classical M2 mode of activation of tumor-associated macrophages involves the stimulation of the transcription factor STAT3, which triggers induced ARG1 expression and blocks STAT1 activation. Thus, it is expected that in the GBM-harboring mice, CC treatment would be linked to STAT3 inhibition and removal of the STAT3-IL-10-mediated block of STAT1 activation. Therefore, based on earlier observations in tumor-associated macrophages, it is expected that CC treatment of the GBM-harboring mice would cause an increase in the level of active STAT1 (P-Tyr701-STAT1) in the ‘resting’ scar tissue microglia in the rescued mice, causing activation, proliferation and/or recruitment of the tumoricidal M1 microglia/macrophages. Corroboratively, we observed a dramatic increase in activated STAT1 (P-Tyr701-STAT1) in the Iba1+ microglia/macrophages in the CC-treated and rescued mouse brains (Fig. 6).

Concomitantly, we also observed a striking increase in activated NF-kB (P-Ser276-p65) in the Iba1+ scar tissue microglia (Fig. 5). Heterodimeric p50/pp65 NF-kB activation has been linked to the activation of M1-type macrophages, and tumor-associated macrophages have been shown to display alternative macrophage activation to the M2 phenotype through nuclear localization of the NF-kb p50-homodimer. By analogy, the increased presence of P-Ser276-p65 in the CC-treated GBM-associated Iba1+ microglia (Fig. 5) suggests that CC-treatment suppresses this p50 homodimer signaling in the microglia and promotes the activation of the p50/p65 NF-kB, which is linked to the classically activated M1 phenotype. A prototypic STAT1-sensitive gene, (e.g., the iNOS gene) also harbors an NF-kb-specific enhancer sequence in its promoter and a cooperation between STAT1 and NF-kB in the regulation of genes linked to inflammation has been demonstrated earlier. Thus the CC-evoked, concomitant induction and activation of STAT1 and p65 NF-kB could be the likely reason for the heightened appearance of iNOShigh, Iba1+ microglia in the scar tissue of the rescued mice. Collectively, results presented here indicate that upon appropriate delivery of CC, it could function in vivo by directly killing cancer cells and also stimulating the brain’s immune cells to eliminate the remaining GBM cells and debris.

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Patent Information

Our overall approach has been included in one approved patent and one pending patent application: (1) “Novel Currumin-antibody Conjugates as Anti-Cancer Agents”. Banerjee, P. (PI) and Raja, K. 1038–58 PCT/US; and (2) “New Use of Currumin Phytosome: Treating Brain and Peripheral Cancers” Banerjee, P. 1038-156P (filed January 19, 2016).

Conflict of Interest

None of the authors have any conflict of interest.

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