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Chains of magnetosomes with controlled endotoxin release and partial tumor occupation induce full destruction of intracranial U87-Luc glioma in mice under the application of an alternating magnetic field.

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23 **ABSTRACT**

24 Previous studies have shown that magnetic hyperthermia could efficiently destroy tumors both
25 preclinically and clinically, especially glioma. However, antitumor efficacy remained suboptimal and
26 therefore required further improvements. Here, we introduce a new type of nanoparticle synthesized by
27 magnetotactic bacteria called magnetosomes with improved properties compared with commonly used
28 chemically synthesized nanoparticles. Indeed, mice bearing intracranial U87-Luc glioma tumors injected
29 with 13 μg of nanoparticles per mm^3 of tumor followed by 12 to 15 of 30 minutes alternating magnetic
30 field application displayed either full tumor disappearance (40% of mice) or no tumor regression using
31 magnetosomes or chemically synthesized nanoparticles, respectively. Magnetosome superior antitumor
32 activity could be explained both by the increased production of heat and by endotoxin release under the
33 alternating magnetic field application. Most interestingly, this behavior was observed when
34 magnetosomes occupied only 10% of the tumor volume, which suggests that an indirect mechanism,
35 such as an immune activity, is involved in tumor regression. This is desired for the treatment of
36 infiltrating tumors, such as glioma, for which whole tumor coverage by nanoparticles can hardly be
37 achieved.

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39

40 **KEYWORDS**

41 Magnetosomes, magnetotactic bacteria, magnetic hyperthermia, alternating magnetic field, glioblastoma.

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47 1. INTRODUCTION

48 A series of different nanotechnologies, such as lipid-based nanoformulations, polymer-drug conjugates,
49 recombinant viruses, metallic nanoparticles, drug-targeted antibodies, and herbal nanoparticles, have
50 been successfully developed for cancer treatment both at fundamental and industrial levels (1). Among
51 them, technologies in which nanoparticle anti-tumor activity can be controlled by applying an external
52 source of energy, such as a laser (2), (3), radiation (4), (5), ultrasound (6), (7), or magnetic field (8), (9)
53 appear promising because they can potentially enhance both treatment safety and efficacy. Strategies to
54 increase nanoparticle SAR by combining magnetic hyperthermia with photothermia using magneto-
55 plasmonic nanohybrids have also been suggested (10), (11). There is interest in developing this type
56 of nanotechnology for glioblastoma (GBM) treatment, especially for the most severe GBM cases, which
57 are non-operable. With the current standard of care, *i.e.*, maximal safe surgery, concurrent
58 radiochemotherapy and adjuvant chemotherapy, GBM, which affects 25,000 patients per year in the
59 United States and Europe (12), (13), remains incurable with a poor prognosis. The five-year survival
60 time of patients with GBM is low at 10% (14), (15), demonstrating the need for new treatment
61 options (16), (17), (18), (19), (20). Among the potential treatments for GBM are different types
62 of nanothermotherapies (21), such as photodynamic therapy (22) and magnetic hyperthermia, which
63 have been tested to treat GBM (9). Compared with other thermotherapies, magnetic hyperthermia (23),
64 (24), (25), (26), (27), in which tumors are typically heated to 43-50 °C by intratumor
65 administration of iron oxide nanoparticles followed by alternating magnetic field (AMF) application,
66 appears promising. This is due to more localized heat produced by the nanoparticles, which results in
67 enhanced efficacy, and to tumor destruction achieved at lower temperatures, which strengthens
68 treatment safety. In a clinical trial that enrolled 14 patients with GBM, magnetic hyperthermia treatment
69 performed using small chemically synthesized iron oxide nanoparticles (IONP) was well-tolerated by

70 patients, did not produce major side effects, and led to an increased survival time of 7 months compared
71 with conventional treatments (28), (29).

72 Treatment efficacy could potentially be further improved by using chains of magnetosomes (CM), which
73 are iron oxide nanoparticles synthesized by magnetotactic bacteria, instead of IONP. In previous studies,
74 CMs have been shown to destroy MDA-MB-231 breast tumors subcutaneously xenografted under the
75 skin of mice more efficiently than IONPs (30), (31). Magnetosome efficacy was attributed to the
76 large magnetosome size, leading to stable ferrimagnetic properties and to a large amount of heat
77 produced under AMF application, as well as to an arrangement of chains that prevented aggregation and
78 promoted homogenous magnetosome distribution in the tumor (32), (33), (34), (35).

79 In the present study, anti-tumor activity against U-87-Luc GBM was studied both *in vitro* and *in vivo*
80 with a suspension containing CMs. We first demonstrated that the amount of endotoxins released from
81 the CMs can be controlled under AMF applications. CM cytotoxicity and internalization properties in
82 the presence of U87-Luc cells were also studied *in vitro*. Mice bearing intracranial U87-Luc tumors
83 were then treated under conditions that could be followed clinically, *i.e.*, using an AMF with 30 mT and
84 198 kHz ($H.f = 8 \cdot 10^8 \text{ A.m}^{-1}.\text{s}^{-1}$, below the threshold of $5 \cdot 10^9 \text{ A.m}^{-1}.\text{s}^{-1}$, above which Eddy currents may
85 occur) (26), (36). Treatment parameters, such as the number of magnetic sessions and quantity of
86 CMs administered, that lead to complete disappearance of the intracranial U87-Luc tumors were
87 determined and compared with IONP treatment, which has been previously used to perform preclinical
88 magnetic hyperthermia tumor treatments (37), (38). During the various treatments, nanoparticle
89 distribution in the tumors and the presence of different cell types in the brain were also examined by
90 histology. This study also suggests a mechanism for tumor destruction and verified that the brain tumor
91 disappeared fully following magnetic hyperthermia treatment with CMs.

92 **2. MATERIALS AND METHODS**

93 ***2.1. Preparation of the injectable CM suspension***

Magnetospirillum magneticum AMB-1 magnetotactic bacteria at biosafety level 1 were purchased from ATCC (79024 strain). Five milliliters of a suspension containing 4.10^6 of these bacteria per milliliter were introduced into one liter of sterile 1653 ATCC culture medium containing nutrients and additives, such as minerals, vitamins, and an iron source, as listed in the 1653 ATCC medium, which is required for the proliferation of these bacteria and for magnetosome production. The bottles were completely filled with sterile medium to prevent contamination by other bacterial species and to avoid a high oxygen concentration in the growth medium, which would prevent magnetosome production. The media containing the bacteria were then placed in an incubator at 30 °C for 7 days to enable bacterial growth and magnetosome production. After 7 days, the media was centrifuged at 4000 g for 45 minutes. The bacterial pellet was washed using 1 ml of sterile water (Millipore®). Magnetotactic bacteria were concentrated using a strong Neodinium magnet (0.6 Tesla), re-suspended in 0.05 M TRIS and sonicated with a sonicating finger at 0 °C for 2 hours at 30 W. The suspension containing the chains of magnetosomes extracted from the magnetotactic bacteria was washed several times with sterile water using a magnet to isolate the CMs from the supernatant containing cellular debris and residual bacteria until the cellular debris disappeared from the supernatant. Sonication was performed at 30 W by a series of three 2-second pulses between each wash. The CMs were then re-suspended in 1 ml of sterile water. For the intracranial injections, the CMs were re-suspended in a sterile injectable solution containing 5% glucose and exposed to ultraviolet lamp (UV) irradiation for 12 h for partial sterilization.

2.2. Preparation of IONPs:

IONPs, which are starch-coated iron oxide nanoparticles, were purchased from Micromod Partikeltechnologie GmbH company, Friedrich-Barnewitz-Str. 4, D-18119, Rostock, Germany (ref: 10-00-102).

2.3. Nanoparticle Characterization:

Nanoparticle sizes, shapes, and organization were determined using a transmission electron microscope (JEM-2100, JEOL, Japan). The iron concentrations of the different nanoparticle suspensions were measured by mixing these suspensions with hydrochloric acid and hydrogen peroxide to produce Fe^{3+}

ions complexed with potassium thiocyanate, and total iron was then determined via an absorbance measurement at 476 nm. The concentrations were expressed as mg of maghemite contained in the CMs and IONPs per ml of suspension or per mm³ of tumor. The zeta potentials of the different nanoparticles in suspension were measured by dynamic light scattering (DLS, ZEN 3600, Malvern Instruments, UK). The stability of the nanoparticle suspensions was estimated by measuring the changes in the optical densities of these suspensions, which were measured at 476 nm for 15 min. Nanoparticle FTIR spectra were recorded with a FTIR spectrometer (Vertex 70, Bruker, USA). The amount of organic material at the nanoparticle surface was measured using an elemental CHNS analyzer (Flash EA 1112, Thermo Fisher Scientific, USA). More details about each of these techniques are provided in the supplementary materials section.

2.4. Set-up for generating the alternating magnetic field and for measuring the temperature

We used an induction system operating at 198 kHz to generate the alternating magnetic field. Mice, Eppendorf tubes, petri dishes containing nanoparticles and possibly cells were inserted into a coil with a 7-cm diameter with four spires to generate a magnetic field with an average strength of 30 mT. We used an infrared camera (EasIR-2, Optophase, France) positioned 20 cm above the coil to measure the temperature both *in vitro* and *in vivo*. We verified the temperature with a thermocouple positioned at the tumor center and that the infrared camera measured the temperature at the tumor center. This was possible due to the shallowness of the tumor.

2.5. Amount of endotoxins released under AMF application

Suspensions containing 2 µl of CMs or IONPs at a concentration of 20 mg/ml in iron oxide were treated as follows. Nanoparticle suspensions were exposed to 0, 1, 2, 3, and 4 magnetic sessions, during which an AMF of 198 kHz and average strength of 30 mT was applied for 30 minutes. After AMF applications, 100 µl of nonpyrogenic water was added to the suspensions, and the suspensions were mixed for one minute and centrifuged at 13 000 rotations per minute for 15 minutes. The endotoxin concentration of the supernatant was measured using the Limulus ameobocyte lysate (LAL) assay.

2.6. Materials and cells culture

U87-MG Luc human GBM cell lines, which were transduced with a luciferase gene, were obtained from Keith Ligon's lab and used in this study. After thawing, the U87-MG Luc adherent cells were cultivated at 37 °C in the presence of 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Once the cells reached confluence, the culture dishes were rinsed with Hank's Balanced Salt Solution (HBSS). The cells were then detached with trypsinization for 5 minutes at 37 °C in the presence of 5% CO₂. The trypsin activity was stopped by the addition of medium containing FBS. The cellular concentration was then determined using a Malassez cell.

2.7. U87-Luc cytotoxicity in the absence of AMF using an MTT assay

U87-Luc cytotoxicity studies were evaluated using an MTT assay. This technique measures the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple formazan crystals. Cells were seeded at a density of 10⁴ cells per well in 96-well flat-bottom plates and incubated in completed culture medium for 24 h. Then, the U87-Luc cell medium was removed and replaced with 10% FCS-medium containing CMs or IONPs at varying concentrations ranging from 7.8 µg/ml to 1000 µg/ml in maghemite. After 24 h of incubation, the cells were washed with HBSS and incubated with 0.1 ml of MTT (2 mg/ml) for an additional 4 h at 37 °C. The insoluble product was then dissolved by the addition of 100 µl of isopropanol. The absorbance corresponding to the solubilized formazan pellets, which reflect the relative viable cell numbers, was measured at 540 nm using a microplate reader. We measured the absorbance at 540 nm for the cells with the nanoparticles without formazan blue and we have subtracted this signal from the signal for the cells with nanoparticles with formazan blue. The percent inhibition of cell proliferation, inhibition (%), was then estimated using the following formula: % inhibition = $[1 - (OD_{TC}/OD_{UC})] \times 100$, where OD_{UC} is the optical density of the suspension of untreated cell, *i.e.*, cells that have been washed with PBS and have not been in the presence of nanoparticles, measured at 540 nm, and OD_{TC} is the optical density of the suspension of treated cells, *i.e.*, nanoparticle suspensions that have been added to cells, measured at 540 nm. The percent inhibition of cell proliferation was plotted as a function of the nanoparticle concentration. The experiments were performed in triplicate.

2.8. *In vitro* efficacy of hyperthermia

A total of 500,000 U87-Luc cells were seeded in a Petri dish (35 mm) for 24 hours. Then, 2 ml of IONP and CM suspensions were added to the cells at different concentrations varying between 1 mg/ml, which is sufficient to induce an increase in temperature increase, and 40 $\mu\text{g/ml}$, which corresponds to the maximum magnetosome concentration in the tumor, *i.e.*, 40 μg of magnetosomes per mm^3 of tumor. When the same amount of nanoparticles used, we observed an increase in the temperature in solution but not *in vitro*, possibly due to the lower concentrations *in vitro* (2 ml at 20 $\mu\text{g/ml}$) than in solution (2 μl at 20 mg/ml). Next, these assemblies were exposed (or not) to a 30 mT and 198 kHz AMF for 30 minutes. The cells were then incubated with the nanoparticles for 24 h. The nanoparticle media was removed, and the cells were washed twice with cold PBS. We verified that there were no observable nanoparticle aggregates at the cell surface and that the nanoparticles were mostly internalized in cells by optical microcopy. It is possible that nanoparticles that were not observed with the optical microscope remained at cell surface, meaning that the measurement provides an estimate of the maximum quantity of internalized nanoparticles or the amount of nanoparticles internalized in cells or at the cell surface. Next, 250 μl of trypsin were added to detach the cells, and then the culture media was mixed with cells. Following this *in vitro* treatment, the mixtures were centrifuged at 800 rpm for 7 minutes. The supernatant was removed and replaced with 100 μl of annexin-binding buffer, 5 μl of Alexa Fluor 488 Annexin V for early apoptosis detection, and 1 μl of propidium iodide at 100 $\mu\text{g/ml}$ for late apoptosis and necrosis detection. The mixtures were incubated at room temperature for 15 minutes. Then, 400 μl of annexin-binding buffer was added to this cell suspension and mixed gently. Ten microliters of this suspension were loaded into the sample slide and were inserted completely into a Countess™ II FL Automated Cell Counter (Thermo Fisher scientific), which was able to detect the Annexin and Propidium Iodide emissions. The percentages of necrotic and apoptotic cells were thus obtained.

2.9. *Internalization of nanoparticles*

Following the *in vitro* treatments described in 2.7, the different mixtures were loaded into the sample slide and completely inserted into a Countess™ II FL Automated Cell Counter to count the cells. The

mixtures were then centrifuged, and the supernatant was removed and replaced with 286 μl of HNO_3 (70%); the resulting treated mixtures were maintained at 4 °C for 24 h to lyse cells and dissolve the nanoparticles into free iron. Finally, 10 ml of filtered water was added to all the treated mixtures and the iron concentration was then determined using ICP-AES measurements. We deduced the average quantity of iron coming from the nanoparticles, which was internalized in each cell.

2.10. *In vivo experiments*

In vivo experiments were performed following ethical guidelines and surgery was performed following the guidelines of the Institutional Animal Care and Use Committee (“Ethic committee Charles Darwin N°5”, project ethic number: 00764.03). Mice were fed and watered according to these guidelines and euthanized by cervical dislocation when their weights had decreased by more than 20% or when signs of pain, unusual posture or prostration were observed. At day 0 (D0), a cell suspension containing 10^5 U87-Luc cells per microliter was first inoculated into the brains of 7-week-old CD-1 female nude mice with a mean weight of 20 g from Charles River. To achieve the surgical procedure leading to cell implantation, the mice were first anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (8 mg/kg). The mouse head was then fixed in a stereotactic frame, a craniotomy was performed at the fixed coordinates (0.2.0) and 2 μl of a suspension containing $2 \cdot 10^5$ U87-Luc cells were injected at the coordinates (0.2.2). Tumors grew for 8 days between D0 and D8. At D8, 7 different groups containing 10 mice each were treated as follows (Table S1(a)):

- Group 1 received a 30 mm^3 GBM at coordinates (0.2.2) mm and 2 μl of an isotonic solution containing 5% glucose without any further treatment;
- Group 2 received a 5 mm^3 GBM at coordinates (0.2.2) mm and the same glucose solution as Group 1, followed by magnetic treatment consisting in 12 magnetic sessions (S) on D8 (S1), D9 (S2), D10 (S3), D15 (S4), D16 (S5), D17 (S6), D22 (S7), D23 (S8), D24 (S9), D29 (S10), D30 (S11), and D31 (S12);
- Group 3 received a 3 mm^3 GBM at coordinates (0.2.2) mm and a suspension containing 40 μg of magnetosome chains in maghemite without any further treatment;

224 - Group 4 received a 3 mm³ GBM at coordinates (0.2.2) mm and the same suspension as Group 3,
 225 followed by magnetic treatment consisting in 15 magnetic sessions on D8 (S1), D9 (S2), D10 (S3), D15
 226 (S4), D16 (S5), D17 (S6), D22 (S7), D23 (S8), D24 (S9), D29 (S10), D30 (S11), D31 (S12), D36 (S13),
 227 D37 (S14), and D38 (S15);

228 - Group 5 received a 25 mm³ GBM at coordinates (0.2.2) mm and the same suspension as group 3,
 229 followed by 15 magnetic sessions on D8 (S1), D9 (S2), D10 (S3), D15 (S4), D16 (S5), D17 (S6), D22
 230 (S7), D23 (S8), D24 (S9), D29 (S10), D30 (S11), D31 (S12), D36 (S13), D37 (S14), and D38 (S15);

231 - Group 6 received a 5 mm³ GBM at coordinates (0.2.2) mm and 40 µg of IONPs in maghemite
 232 without any further treatment; and

233 - Group 7 received in a 3 mm³ GBM at coordinates (0.2.2) mm and the same suspension as Group
 234 6, followed by 12 magnetic sessions on D8 (S1), D9 (S2), D10 (S3), D15 (S4), D16 (S5), D17 (S6), D22
 235 (S7), D23 (S8), D24 (S9), D29 (S10), D30 (S11), and D31 (S12).

236 For the different groups of mice, the bioluminescence intensity emitted by the living tumor cells was
 237 measured on D7, D14, D21, D28, D35, D42, D49, D56 and D150.

238 During each magnetic session (S1 to S15), the mice were exposed to an AMF at an average strength of
 239 30 mT and a frequency of 198 kHz that was applied for 30 minutes.

240 **2.11. Bioluminescence intensity and tumor volume measurements**

241 The mice were first anesthetized using isoflurane. The bioluminescence intensity was then measured
 242 using an IVIS Spectrum “*In Vivo Imaging System*” (PerkinElmer, Inc.) 10 minutes after intraperitoneal
 243 injection of 100 mg/kg of beetle luciferin potassium salt (Promega: E1605) in PBS, which reacts with
 244 luciferase to produce luminescent oxo-luciferin, (39), (40), (41). Images were analyzed with the
 245 living Image 4.0 software from Caliper, Life Sciences. The bioluminescence intensity at the tumor site
 246 was divided by the bioluminescence intensity measured in the flanks to remove the mouse auto-
 247 luminescence signal. Then, the relationship between tumor bioluminescence intensity and tumor volume
 248 was determined. For that, bioluminescence intensities were measured at D7, D14, D21, D28 and D35 in
 249 a series of living mice. The tumor volumes were deduced from surface measurements of several

horizontal histological sections positioned at various tumor heights, which were collected from mice euthanized on the same day at the BLI measurements. A linear relationship was observed between the logarithm of tumor bioluminescence intensity (BLI), $\log(\text{BLI})$, and the logarithm of the tumor volume expressed in mm^3 , $\log(\text{volume}(\text{mm}^3))$ (Fig. S3). A linear coefficient of 1.4 was deduced by fitting the plot in Fig. S3 with a linear function, a value that agrees with previously reported values (40), (42), (43), (44).

2.12. Representation of tumor volume variations and estimates of survival time

The variations in tumor volume in time were represented in Tukey diagrams using the Kaleida Graph 4.03 software. Mouse survival times are plotted using the Kaplan-Meier model method (45), (46). The statistical significance of the survival time between the different groups was evaluated using the log rank test. Parameters are expressed as the median with p-values (9).

2.13. Temperature measurements

Intratumor temperatures were measured as a function of time during the various treatments in living mice using an infrared camera (EasIRTM², Optophase) placed 20 cm above the coil.

2.14. Histological analysis

Histological studies were performed on extracted brains that were fixed with a 4% solution of formaldehyde for 24 hours, cut into 2-mm thickness transverse slices, washed in an ethanol (70%) bath for 12 hours and included in paraffin. Sections of paraffin blocks with a thickness of 4 μm were deposited on glass slides and stained with hematoxylin-eosin (H&E) and/or Prussian blue to distinguish between healthy and tumor areas and to determine the location of the magnetosomes, polynuclear neutrophils (PNN), and tumor and healthy cells. Histological analysis was also performed on the mice that received glucose on day 8 and were euthanized 6 or 24 hours following glucose injection (Figs. S5(a) and S5(c)). Six mice were analyzed at various times, including after 1 magnetic session (Figs. S5(b)) or 24 hours after 3 magnetic sessions (Fig. S5(d)), in which they received CM on day 8 and were euthanized 4 hours (Figs. 9(a) and 9(c)) or 72 hours (Figs. 9(b) and 9(d)) after the CM injection; just after or 4 hours after 1 magnetic session (Figs. 8(a), 8(b) and 8(c)); and 24 hours after 3 magnetic

276 sessions (=72 hours, Figs. 8(d) to 8(g)), in which they received IONPs on day 8 and were euthanized 6
277 or 72 hours after IONP administration (Figs. S4(a) and S4(c)) or 24 hours after 3 magnetic sessions (72
278 hours (Figs. S4(b) and S4(d)).

279 **3. RESULTS AND DISCUSSION**

280 ***3.1. Properties of CMs and IONPs used for the tumor magnetic hyperthermia treatments***

281 CMs were extracted from AMB-1 whole magnetotactic bacteria, a species of bacteria that produces
282 chains of nanoparticles called magnetosomes, as shown in the TEM image of a typical magnetotactic
283 bacterium presented in Figure 1(a). After extraction from the magnetotactic bacteria, the magnetosomes
284 maintain their organization in chains (Figure 1(b)), a type of organization that is advantageous for the
285 treatment because it prevents aggregation and promotes uniform distribution and homogenous heat
286 production. Magnetosomes appear to possess a cubooctahedric geometry, more uniform sizes and shapes
287 than IONPs (Figs. S7(a) and S7(b)), a size that is larger than 20 nm for the majority of them and a mean
288 size of ~45 nm (Fig. S1(a)), which leads to a coercivity H_c of ~200-300 Oe and a ratio between remnants
289 and saturating magnetization of $M_r/M_s \sim 0.35$ at physiological temperature (Fig. S6), (47).

291 **Figure 1:** Transmission electron microscopy images of (a) an AMB-1 *Magnetospirillum magnetotacticum* magnetotactic
 292 bacterium, (b), chains of magnetosomes extracted from magnetotactic bacteria and (c) an IONP. Seven microliter suspensions
 293 containing magnetotactic bacteria, chains of magnetosomes, and IONPs were deposited and dried on top of a carbon grid
 294 before TEM observation.

295 Magnetosomes are composed of a core, which is made of iron oxide, as revealed by the FT-IR Fe-O
 296 peak at 580 cm⁻¹ (table 1) and further determined to be maghemite by saturating isothermal remnant
 297 magnetic measurements (47). The core is surrounded by a coating of organic material, including
 298 phospholipids and lipopolysaccharides, as revealed by a series of FT-IR peaks [C=O at 1740 cm⁻¹,
 299 amide at 1650 cm⁻¹, amine at 1530 cm⁻¹, P=O at 1250 cm⁻¹, and PO at 1050 cm⁻¹ (Table 1)]. The
 300 coating, whose function is to bind magnetosomes together in chains (48), is estimated to be 1 to 5 nm
 301 thick (Figure 1(b)) and to contain 13.9% carbon, as estimated by CHNS analysis. LAL measurements
 302 further indicated that the organic material at the magnetosome surface is partially derived from
 303 endotoxins whose concentration in CM suspensions is estimated as 1400-8400 EU per ml per mg of iron
 304 oxide. Moreover, CM suspensions appear sufficiently stable to enable their *in vivo* administration, as
 305 their absorption measured at 480 nm does not decrease by more than 30% within 20 minutes (Table 1).

		CM						IONP					
Endotoxin level (EU/mg/mL)		1400-8400						< 50					
% stability on water (iron concentration = 1mg/mL)		70						100					
Coating thickness (nm)		1 - 5						1					
Isoelectric Point (a.u)		4.2						9.5					
	pH	2	4	6	8	10	12	2	4	6	8	10	12
	ζ-Potential (mV)	20	2.5	-18	- 26	-34	-38	7	6	6	5	3	- 20
% C		13.9						8.7					
SAR (2μL, 40 μg of iron) (W/gFe)		Water			Brain			Water			Brain		
		57			4			10			0		
FT-IR (cm ⁻¹)		580	1050	1250	1530	1650	1740	610	1025		1150		
		Fe-O	P-O	P=O	N-H (amine)	N-H (amide)	C=O	Fe-O	C-O (alcohol)		C-O (ether)		

306
 307 **Table 1:** The endotoxin content estimated in Endotoxin units (EU per mg in iron per ml), percent decreased absorption
 308 measured at 480 nm in suspensions containing CM and IONP at a concentration of 1 mg in iron per ml after twenty minutes,
 309 and estimates of the isoelectric point and zeta potential as a function of pH for suspensions containing CMs or IONPs.

Estimates of the coating thickness measured by transmission electron microscopy and percentage of carbon (% C) measured with CHNS and assignments from the different FT-IR peaks measured by FT-IR for the CM and IONP dried suspensions. Estimates of the specific absorption rates (SAR) in W per gram in iron for 2 μ l containing 40 μ g of CMs or IONPs in iron oxide mixed in water or introduced inside the mouse brains. Compared with CMs, IONPs are also ferromagnetic iron oxide nanoparticles but characterized by the following distinct features. Their geometric structure is parallelepipedic, they are not organized in chains and tend to form small aggregates as observed in the TEM image in Figure 1(c). IONPs are made of a core measuring $\sim$ 19 nm by TEM (49), and whose composition is iron oxide as determined by FT-IR measurements that display an Fe-O peak at 610 cm^{-1} (Table 1). The IONP core is surrounded by synthetic hydroxyethyl starch, as suggested by FT-IR and CHNS measurements performed on the IONPs, which shows FT-IR peaks at 1150 cm^{-1} and 1050 cm^{-1} that are attributed to hydroxyethyl starch ether and alcohol functional groups, and has a carbon percent of 8.7%, revealing the presence of an organic coating surrounding the inorganic iron oxide core (Table 1). Compared with CMs, the IONP surface contains a much lower endotoxin concentration of 50 EU per ml per mg of iron oxide, as measured with the LAL assay. Furthermore, IONPs are characterized by ferrimagnetic properties at physiological temperatures but at the following lower values: (1) $H_c \sim 120\text{ Oe}$ and (2) $M_r/M_s \sim 0.15$ (49), (Fig. S6 and Table 1), which should yield lower SAR values. Furthermore, given that they are not less stable than CMs (Table 1) and that their anti-tumor efficacy has been demonstrated on tumor-bearing mice using magnetic hyperthermia (50), IONPs appear to be good standard for comparison to assess CM efficacy.

3.2. Under AMF applications, CM can release heat and an immune-stimulating substance more efficiently than IONPs

In an attempt to mimic the conditions in the *in vivo* treatments and to study the release of endotoxins from the CM and IONP surfaces in these conditions, we introduced 2 μ l of suspensions containing 40 μ g of these nanoparticles in iron oxide at the bottom of a tube that we exposed to 1, 2, 3, and 4 magnetic sessions during which an AMF of 198 kHz and average strength 30 mT was applied for 30 minutes. During each of these 4 magnetic sessions, CM and IONP suspensions led to maximal temperatures

337 ($T_{\max}$) of 57 °C and 28 °C, respectively, after 3 minutes of AMF application (Fig. S2(a)). Specific
 338 absorption rates (SAR) were deduced from the formula $SAR = C_{\text{water}}(\Delta T/\delta t)/C_m$, where $\Delta T/\delta t$ are the
 339 initial slopes of the plot in Fig. S2(a) [$\Delta T/\delta t = 0.27$ °C/sec and 0.05 °C/sec for CM and IONP,
 340 respectively], $C_{\text{water}} = 4.2$ J/g.K is the specific heat capacity of water, and $C_m = 0.02$ g/mL is the
 341 concentration in iron oxide of the different nanoparticle suspensions. The SARs were estimated as $57 \pm$
 342 6 W/g_{Fe} and 10 ± 3 W/g_{Fe} for CMs and IONPs, respectively. Therefore, the heating properties of CMs
 343 appear far superior to those measured for IONPs.

Figure 2: (a) Percentage of endotoxin release from 2 μ l suspensions containing 40 μ g of CM or IONP in iron oxide that were exposed to 1, 2, 3 or 4 S with an AMF of 198 kHz and average strength of 30 mT applied for 30 minutes. (b) The quantity of iron released from MCs and IONPs, measured in pg and internalized per cell from U87-Luc cells incubated for 24 hours with 1 mg/mL of CM or IONP in maghemite with(+H)/without(-H) AMF, where the AMF is the same as in (a). (c and d) The percentage inhibition of cell proliferation as a function of nanoparticle concentration for CMs and IONPs in maghemite in U87-Luc cells incubated for (c) 24 hours or (d) 72 hours with nanoparticle concentrations varied between 8 μ g/mL and 1 mg/mL. (e and f) The percentages of late apoptotic (+ necrotic) and early apoptotic cell death as a function of nanoparticle concentration with(H)/without(-H) AMF where the AMF is the same as in (a) in U87-Luc cells incubated for 24 hours with (e) CM and (f) IONP at concentrations of 40 μ g/mL, 650 μ g/mL, and 1 mg/mL.

Moreover, the percentage of endotoxin release, which remains relatively similar at 1 to 3% during the first 2 magnetic sessions, strongly increased to 11% and 32% following the third and fourth magnetic sessions, respectively (Fig. 2(a)). This indicates that endotoxins, which are initially at the magnetosome surface, are progressively released from CMs under AMF application. Using CMs exposed to AMF, it therefore appears possible to simultaneously produce heat and release an immune-stimulating substance such as an endotoxin. Although endotoxins cannot be used in medicinal preparations, they may be replaced by a nontoxic equivalent, such as M-PLA, (51), (52). Combining the production of localized heat with the release of such a substance may possibly enhance antitumor efficacy. Compared with CMs, IONPs released a lower quantity of endotoxins and their percentage of endotoxin release did not exceed 5% between 1 and 4 S (Fig. 2(a)). Taken together, these heating and endotoxin release behaviors suggest that IONPs are less promising than CMs for the magnetic hyperthermia and endotoxin treatment in tumors.

3.3. CMs induce enhanced U87-Luc early apoptotic cell death and internalization in vitro

In the absence of the AMF, *the* cytotoxicity of CMs towards U87-Luc cells was compared with that of IONPs using nanoparticle suspensions that were added to the cells and incubated for 24 and 72 hours at concentrations between 40 μ g/mL and 1 mg/mL. At 24 hours, the percent inhibition of cell proliferation increased with increasing nanoparticle concentrations from 0 to 40% for CM and remained at 0% for the IONPs (Figure 2(c)). At 72 hours, the percent inhibition increased more significantly for CMs, *i.e.*, from

0 to 60%, than for IONPs, *i.e.*, from 0 to 100% (Figure 2(d)). While CMs were observed to be more cytotoxic than IONPs at 24 hours, the opposite behavior was observed at 72 hours.

U-87-Luc cells were also incubated for 24 hours with CMs and IONPs at three different concentrations of 40, 650, and 1000 $\mu\text{g/ml}$ and then exposed to an AMF of 198 kHz and strength 47 mT for the first 30 seconds and 30 mT for the remaining 29.5 minutes in an attempt to reach 41 $^{\circ}\text{C}$, a temperature above which mild hyperthermia conditions are expected to occur (30). The 40 and 650 $\mu\text{g/ml}$ concentrations led to temperature increases of less than 2 $^{\circ}\text{C}$, which were insufficient to reach the hyperthermia conditions. These concentrations resulted in necrosis and late apoptosis percentages that remained below $\sim 4\%$ with/without AFM for the CMs and IONPs and early apoptosis percentages that were similar with/without AFM at $\sim 15\%$ and $\sim 40\%$ for CMs at 40 and 650 $\mu\text{g/ml}$ in iron oxide, respectively, and at 0% for IONPs (Figs. 2(e) and 2(f)). In contrast, at a concentration of 1 mg/ml, both CMs and IONPs yielded a more significant temperature increase of 7 $^{\circ}\text{C}$ up to 41 $^{\circ}\text{C}$ (Fig. S2(b)), which resulted in an increase in the necrotic and late apoptotic cell death percentages by 10%. These *in vitro* heating behaviors are In contrast, with those observed in solution, where CMs were observed to heat much more than IONPs.

The mechanisms of cellular death induced by CMs and IONPs were further examined. CMs appear to induce cellular death mainly through early apoptosis, with a percentage of early apoptotic cell death that increased with increasing CM concentrations from 15% at 40 $\mu\text{g/ml}$ to 50% at 1 mg/ml. Similar percentages were observed for CMs with/without AMF (Fig. 2(e)); this cell death mechanism was not observed with IONPs (Fig. 2(f)).

The differences in cell death mechanisms observed for IONPs and CMs as well as the more pronounced decrease in heating efficacy for CMs than IONPs observed between the *in vitro* and in suspension heating conditions may be explained by the different internalization properties. Indeed, the quantity of iron released from the nanoparticles, which are internalized in each cell, increased from 36.8 and 5.5 pg per cell without AMF application to 255.7 and 19.8 pg per cell with AMF application for the CMs and IONPs, respectively. It therefore appears that CMs are internalized better than IONPs and that the

internalization is enhanced by the presence of the AMF for both types of nanoparticles. This enhancement is more pronounced for CMs than for IONP by factors of 7 and 4, respectively (Figure 2(b)). The different internalization properties for the CMs and IONPs may be explained by differences in their charges, *i.e.*, CM and IONP are negatively and positively charged at physiological pH, respectively (Table 1)); differences in organization, *i.e.*, CM and IONP are well-dispersed and tend to aggregate, respectively; and to larger values of coercivity for CMs (200-300 Oe) compared to IONPs (120 Oe) that may enhance CM magnetic field coupling. Furthermore, CM organization in chains may favor internalization by groups containing several magnetosomes (30), *i.e.*, potentially 6 for chains averaging 250 nm in length (Fig. S1(b), while IONPs may result in nanoparticles being internalized one by one or at a lower rate. The enhanced internalization of CMs may favor early apoptosis, which was previously observed with iron oxide nanoparticles, (53), and reduce its heating properties, possibly by preventing nanoparticle Brownian heating mechanisms (54).

3.4. CMs promote enhanced antitumor efficacy towards intracranial U87-Luc glioblastoma

In the *in vivo* studies, mice receive the following treatment at the center of intracranial U87-Luc tumors: (1) glucose followed by treatment or not with magnetic sessions (groups 1 and 2), (2) 40 µg of CMs or IONPs in maghemite without any further treatment (groups 3 and 6), (3) 40 µg of CMs in maghemite followed by 15 magnetic sessions (groups 4 and 5), or (4) 40 µg of IONPs in maghemite followed by 12 magnetic sessions (group 7). The average size of the treated tumors was 3-5 mm³, except in group 5 where it was 25 mm³. Following injection, the mice were exposed to 12 to 15 magnetic sessions, during which an AMF of 198 kHz and strength 30 mT was applied for 30 minutes (see Table S1(a) for a summary of the treatment conditions). In this study, U87 cells were labeled with the firefly luciferase gene to evaluate the changes in tumor volumes following the treatments via tumor bioluminescence intensity (BLI) measurements using the relationship between BLI and tumor volumes that we established (Fig. S3) (40), (42), (43), (44). For the mice belonging to the different groups without any measurable production of heat (groups 1, 2, 3, 6, and 7), Figures 3(a) to 3(e) show that the tumor volumes increased following tumor cell implantation without any signs of antitumor activity. Mice

424 belonging to these groups were rapidly euthanized between days 28 and 42, leading to a rather low
 425 median survival time of 42 to 46 days. In contrast, in the mice belonging to groups with heated tumors
 426 (groups 4 and 5), antitumor activity is clearly observed and is more pronounced in the small (group 4)
 427 than in the large (group 5) treated tumors.

428

429 **Figure 3:** Variations in tumor volumes as a function of time during the days following U87-Luc tumor cell implantation at
 430 D0 for (a) untreated mice (group 1), (b) mice exposed to 15 magnetic sessions (group 2, +H), mice receiving a suspension
 431 containing either (c) 40 µg of CMs in iron oxide at the site of tumor cell implantation (group 3) or 40 µg of IONPs in iron
 432 oxide (group 6, IONP), or (e) 40 µg of IONPs in iron oxide and exposed to 15 magnetic sessions. These variations are plotted
 433 using Tukey diagrams, where error bars designate the maximum and minimum tumor volume values, circles represent single
 434 tumor values, lower and upper horizontal rectangular sides designate lower and upper quartiles, respectively, and horizontal
 435 lines within each box plot represents median tumor volumes. The number of living mice used to draw each box plot (n) is
 436 also indicated above each box plot.

437 Figures 4(a) shows that tumor growth is delayed in group 5 with a median tumor volume that first
 438 decreases from 25 mm³ at day 7 to 5 mm³ at day 14 and then increases from 5 mm³ at day 14 to 70 mm³
 439 at day 42. The behavior of a typical mouse belonging to this group is shown in Figure 4(b), which shows
 440 three tumor volume oscillations (decrease followed by increase). This suggests that AMF application
 441 causes repetitive anti-tumor activity without preventing tumor regrowth after 15 magnetic sessions. This
 442 therapy led to a survival time of 49 days, which is only slightly increased compared to the unheated
 443 groups.

445 **Figure 4:** (a) Variations in tumor volumes as a function of time during the days following U87-Luc tumor cell implantation at
 446 D0 for mice receiving a suspension containing 40 μg of CMs in iron oxide. The tumors reach an average volume of 25 mm^3
 447 at D7 and were treated with 15 magnetic sessions (group 5, CM+H). (b) Variations in tumor volume and bioluminescence
 448 intensity as a function of time during the days following tumor cell implantation in a mouse in group 5 with a tumor volume at
 449 D7 of $\sim 7.5 \text{ mm}^3$ that received 40 μg of a suspension of CMs in iron oxide on D7 followed by 15 magnetic sessions (S1 to
 450 S15).
 451 Figure 5(a) shows more significant antitumor activity in group 4 with full tumor disappearance at day 35
 452 without tumor regrowth following 1 to 15 magnetic sessions. For a typical mouse belonging to this
 453 group, Figure 5(b) shows the gradual decrease in the tumor volume following 1 to 15 magnetic sessions
 454 until full tumor disappearance at day 40 without tumor regrowth. Compared with the unheated groups,
 455 group 4 had the highest survival at 90 days (p-value < 0.0001).

Figure 5: (a) Variations in tumor volumes as a function of time during the days following U87-Luc tumor cell implantation at D0 for mice receiving a suspension containing 40 μg of CMs in iron oxide when the tumors reach an average volume of 3 mm^3 at D7 followed by 15 magnetic sessions (group 4, CM+H). (b) Variations in tumor volume and bioluminescence intensity as a function of time during the days following tumor cell implantation for a mouse belonging to group 4 with a tumor volume at D7 of $\sim 3 \text{ mm}^3$ that receiving 40 μg of CMs in iron oxide on D7 followed by 15 magnetic sessions (S1 to S15).

On day 150, 40% of the mice from this group were still alive without any unusual behaviors. Then, the mice were euthanized and slides of their brains from the tumor cell implantation region were imaged by optical microscopy, revealing the absence of tumors or tumor lesions and a brain neuronal structure identical to that of a healthy brain (Figure 6). These results suggest that the tumors had fully disappeared in these mice. Further analysis of mice from group 4 suggests that treatment does not result in any healthy tissue damage. Indeed, it can be deduced from the optical microscopy images of the brain sections from mice treated with CM with/without AMF collected at different times ranging from 4 hours to 150 days after CM administration (Figs. S4(a) to S4(d), S5(a) to S5(d) and Figs. 8(a) to 8(g), 9(a) to 9(d)) that healthy tissues in the hippocampus and brain do not contain lesions or edema. Such observations can be explained by an LPS-activated neuro-protective response by the microglia (55). Thus, the treatment of the mice in group 4 appears safe and fully efficient.

Figure 6: Optical microscopy image of a 4- μ m-thick brain section stained with hematoxylin and eosin from a mouse in group 4 that received 40 μ g of CMs in iron oxide followed by 15 magnetic sessions. This mouse was euthanized 7 months after magnetosome administration. No tumor was observed in this brain section.

3.5. Distribution of CMs in the tumor favors in vivo heating during the various S

CM led to temperature increases of 4 °C at D8 (S1), 1.7 °C at D9 (S2), 0.4 °C at D10, D15 and D16 (S3, S4, S5) and then constant temperatures after D17 (Figures. 7(a) and 7(b)).

Figure 7: (a), Temperature increases above 28.5°C observed during each magnetic session (ΔT) on D8, D9, D10, D15, D16 and D17 in the mice from groups 4 and 5 treated with CMs. (b) The same as in (a) for the mice in group 7 treated with IONPs. After D17, tumor temperature increases were no longer observed. (c) Variations in mouse survival times as a function of time during the days following U87-Luc tumor cell implantation for mice with the following treatments: (1) untreated (group 1), (2) exposed to 15 magnetic sessions (group 2), (3) treated with suspensions containing $40\text{ }\mu\text{g}$ of CMs in iron oxide without magnetic sessions (group 3), (4) treated with $40\text{ }\mu\text{g}$ of CMs in iron oxide in $\sim 3\text{ mm}^3$ tumors followed by 15 magnetic sessions (group 4), (5) administered $40\text{ }\mu\text{g}$ of CMs in iron oxide in $\sim 25\text{ mm}^3$ tumors followed by 15 magnetic sessions (group 5), (6) administered $40\text{ }\mu\text{g}$ of IONPs in iron oxide (group 6), and administered $40\text{ }\mu\text{g}$ of IONPs in iron oxide followed by 15 magnetic sessions (group 7).

The rather limited increases in temperature under AMF, which are less pronounced than in the capillaries (Fig. S2(a)), may be explained by the effects of AMF on CM distribution. Indeed, the tumor occupation percentage decreases from $\sim 50\%$ right after CM administration without AMF at D8 (Figs. 9(a) and 9(b)) down to 8-15% on D8 and D11 after 1 and 3 magnetic sessions, respectively, (Figs. 8(a),

8(b) and 8(d)). There percentages were estimated by delineating the regions containing the CMs and the tumors and by measuring the ratio between the surfaces of these two regions. Moreover, the decrease in CM heating properties over time may be attributed to internalization of CMs in U87 tumor cells, which was observed at 24 hours after 3 magnetic sessions (Fig 8(d) and 8(e)).

500

Figure 8: Optical microscopy images of 4 μ m thick brain sections stained with hematoeosin from mice receiving 40 μ g of CMs in iron oxide and exposed to (a-c) one or (d-g) three magnetic sessions. The mice were euthanized (a) 0 hours (0 h), (b and c) 4 hours (4 h), or (d-g) 72 hours (72 h) after MC administration with either (a-c) one or (d-g) three magnetic sessions. (c) An enlargement of the region in (b). The red arrows in (c) designate polynuclear neutrophils. (e), (f), and (g) are enlargements of three different regions from (d). PNN, M-PNN, U87-Luc, M-PNN & U87-Luc, and U87-Luc & magnetosomes designate polynuclear neutrophils, magnetosomes colocalized with polynuclear neutrophils, U87-Luc cells, magnetosomes colocalized with polynuclear neutrophils and U87-Luc cells, and U87-Luc cells colocalized with magnetosomes, respectively. The regions containing these different cells are surrounded in (d), while individual cells are indicated by arrows in (c), (e) and (f).

510 In contrast with CMs, IONPs do not produce any heat *in vivo* under the same excitation conditions. This
511 behavior can be explained by their lower concentration in the tumor under AMF applications, *i.e.*, they
512 are observed at the tumor surface as well as part of the tumor periphery at 6 hours (Figs. S4(a) and
513 S4(c)); their more rapid diffusion in the ventricles, *i.e.*, IONPs and CMs localize in the ventricles at 6
514 and 72 hours, respectively, (Figs. S4(a) and S4(b), Figs. 8(d)); and their earlier internalization in tumor
515 U87-Luc and healthy BETZ brain cells, *i.e.*, IONPs and CMs are internalized at 6 and 72 hours,
516 respectively, (Figs. S4(c) and S4(d), Fig. 8(e)). The combination of a low concentration in the tumor
517 together with rather low SAR may undermine the IONPs' heating power, as was observed *in vivo*.

518 ***3.6. CM exposed to AMF activate PNN, which are possibly involved in antitumor activities***

519 Previous studies have reported that immune cells, such as dendritic cells, natural killers, and B and T
520 cells, may participate in tumor destruction following hyperthermia treatment (56). Here, we examined
521 potential involvement of other types of immune cells, *i.e.*, polynuclear neutrophils (PNN), which have
522 only rarely been associated with antitumor activities (57), (58). While PNNs were not detected after
523 glucose (Fig. S5) or IONP (Fig. S4) administration with/without magnetic sessions, we observed that
524 these cells were localized at the same sites as the magnetosomes, forming a complex designated M-PNN
525 in the mouse euthanized 4 hours after CM administration with/without magnetic sessions (Figs. 8(b),
526 8(c), 8(d), 8(f), 8(g), 9(a) and 9(c)). More precisely, M-PNNs designate assemblies of magnetosomes
527 and PNNs where the magnetosomes are less than 1 μm away from the PNNs. The number of M-PNNs
528 was estimated as 14000-19000 per mm^2 4 hours after CM administration (Table S2). Because PNNs are
529 known to be recruited by endotoxins (59), this behavior could be attributed to the endotoxins
530 surrounding the magnetosome mineral core that attract PNN. Seventy-two hours later, the M-PNNs had
531 disappeared in the absence of AMF (Figs. 9(b) and 9(d) and Table S2) but were detected in large
532 quantities of 27,500 per mm^2 (Table S2) after 3 magnetic sessions either within the tumor periphery
533 (Fig. 8(d) and 8(f)) or in the tumor (Figs. 8(d) and 8(e)).

Figure 9: Optical microscopy images of 4 μm thick brain sections stained with hematoxylin from mice receiving 40 μg of CMs in iron oxide without magnetic sessions and euthanized (a) 4 hours (4 h) or (b) 72 hours (72 h) after magnetosome administration. (c) and (d) are enlargements of two regions in (a) and (b), respectively. PNN are observed in (c) and indicated by red arrows, but not in (d).

These interesting behaviors might suggest that in the absence of AMF, endotoxins are progressively deactivated, perhaps due to magnetosome internalization followed by magnetosome and endotoxin decomposition. However, in the presence of AMF, the endotoxins are re-activated, possibly through the release of endotoxins from the magnetosome surface, a behavior that perfectly fits with the observations in solution (Fig. 2(a)) and/or via the destruction of cells in which magnetosomes are initially internalized before AMF application. M-PNN complexes may have two main functions. First, they may be involved

in tumor destruction, as they are observed in the tumor region and Toll-like receptor (TLR) from the treated mice could be activated and trigger antitumor activity; this was previously reported for mice bearing B16 tumors (60). Second, they may also be involved in magnetosome capture and redistribution. Indeed, after one and three magnetic sessions, magnetosomes and PNNs alone are rarely observed in the entire brain, including the tumor regions (Figs. 7(b) and 7(d)), and M-PNNs appear to be located either in the tumor, near the tumor periphery or in the ventricles (Figs. 7(b) and 7(c)). These results suggest that PNNs progressively migrate in the region where the magnetosomes are located between 0 and 4 hours after CM administration to form M-PNN complexes and that these complexes carry the magnetosomes from the inside to the outside of the tumor, possibly through the ventricle blood vessels. This behavior further agrees with the absence of heat production after D17, which suggests that magnetosomes have left the tumor by this date and/or have been dissolved into free iron. This idea is also in line with the well-known function of PNNs, which is to remove bacterial residues (61), (62). Magnetosome progressive departure from the tumor, possibly followed by magnetosome redistribution within the mouse, could be appealing for the treatment by preventing crystallized magnetosomes from remain in the brain for too long a period of time. Optimum treatment conditions correspond to those where magnetosomes remain in the brain long enough to perform the treatment, but not for too long, to avoid unnecessary long-term magnetosome interactions with brain tissues. These conditions may be identified by controlling PNN activation and M-PNN complex formation under AMF application.

3.7. CMs appear to destroy glioblastoma tumors more efficiently than chemically synthesized nanoparticles under AMF applications

Using the same quantity of nanoparticles administered, we have shown in this study that CMs lead to 40% of mice with full tumor disappearance, while IONPs do not yield any observable antitumor activities. This result agrees with previous animal studies performed on mice and rats bearing GBM using intratumor administration of chemically synthesized nanoparticles exposed to AMF that did not improve survival (63) or increased survival by 15 days without resulting in a full cure (64), (26). In addition to being more efficient, magnetosomes could also be used in a lower quantity, thus

strengthening treatment safety. Indeed, the quantity of magnetosomes necessary to eradicate U87-Luc GBM in this study, which was 1 to 13 μg in maghemite per mm^3 of tumor volume, is much less than 60 μg per mm^3 of tumor volume, which produced less-efficient anti-tumor activities with chemically synthesized nanoparticles (9).

4. CONCLUSION

We demonstrated that 40% of mice bearing intracranial U87-Luc tumors displayed full tumor disappearance after intratumor administration of 40 μg of CMs followed by 12 to 15 magnetic sessions, during which an AMF of average strength 30 mT and frequency 198 kHz was applied for 30 minutes. In contrast, similar antitumor efficacy was not observed when mice were treated with the same conditions and administered chemically synthesized nanoparticles instead of magnetosomes. Furthermore, the absence of mouse brain damage during and following the various treatments appears to indicate that a safe treatment could be performed with magnetosomes, provided that the endotoxins are replaced with an equivalently active but nonpyrogenic substance such as M-PLA. Antitumor activity does not appear to be dominated by nanoparticle cytotoxicity, as the CMs, which are more efficient than IONPs *in vivo*, do not enhance cytotoxicity towards U87-Luc cells compared with IONPs. Moreover, nanoparticle administration without AMF does not produce any observable anti-tumor effects. Instead, tumor destruction appears to be due to the heat produced by the CMs under AMF application, as the only mice that displayed clear anti-tumor effects had their tumors heated (groups 4 and 5). Under heating conditions, tumor tissue destruction may be caused by irreversible cell damage; cytolysis; actin filament, microtubule or mitochondrial dysfunction; DNA replication or RNA synthesis inhibition; or DNA polymerase denaturation (65), but these mechanisms seem insufficient to fully explain the observed antitumor activities. Indeed, tumor destruction does occur when the magnetosomes occupy a small percentage of the total tumor volume ($\sim 10\%$), suggesting that magnetosomes occupying the tumor can induce the destruction of the entire tumor. We have identified the following three possible mechanisms that could be responsible for full tumor destruction:

596 i) Early apoptosis, as apoptotic cell death is observed to be dominant *in vitro* for CMs and heat was
 597 previously shown to favor apoptotic cell death (65), (66), (67);

598 ii) Microvascular damage due to the presence of magnetosomes in the ventricles that possibly leads
 599 to the destruction of the blood vessels supplying the tumor with oxygen, which was previously reported
 600 to occur during hyperthermia, (68), and could possibly occur because CMs were observed in the
 601 ventricles near the blood vessel; and

602 iii) the involvement of PNN, which were previously reported to be involved in antitumor activity,
 603 (58) and were attracted by the magnetosomes in the tumor region due to the presence of endotoxins at
 604 magnetosome surface.

605 Finally, our study also suggests a method to control magnetosome distribution by attracting PNNs to the
 606 magnetosome region, which promotes the formation of magnetosome/PNN complexes that may
 607 progressively remove magnetosomes from the tumor through the ventricle blood vessels and enable their
 608 elimination. This process appears to be governed by the application (or not) of an AMF.

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615 ***Abbreviations***

616	GBM	glioblastoma
617	AMF	alternating magnetic field
618	IONP	iron oxide nanoparticles
619	CM	magnetosome chains

620	UV	irradiation of an ultraviolet lamp
621	LAL	limulus amebocyte lysate
622	DMEM	Dulbecco's Modified Eagle Medium
623	FBS	fetal bovine serum
624	HBSS	Hank's Balanced Salt Solution
625	S	magnetic sessions
626	BLI	bioluminescence intensity
627	H&E	hematoxylin-eosin
628	PNN	polynuclear neutrophils
629	SAR	specific absorption rates
630	TLR	toll like receptor
631	M-PNN	PNN were localized at the same site as magnetosomes

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