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

4 *Edouard Alphandéry*^{a,b,*}, *Ahmed Idbah*^c, *Clovis Adam*^d, *Jean-Yves Delattre*^c, *Charlotte Schmitt*^c,
5 *François Guyot*^a, *Imène Chebbi*^b.

6

7 ^a Institut de minéralogie, de physique des matériaux et de cosmochimie, UMR 7590 CNRS, Sorbonne
8 Universités, UPMC, University Paris 06, Muséum National d'Histoire Naturelle, 4 Place Jussieu, 75005,
9 Paris, France.

10 ^b Nanobacterie SARL, 36 boulevard Flandrin, 75016, Paris, France.

11 ^c Inserm U 1127, CNRS UMR 7225, Sorbonne Universités, UPMC, University Paris 06, UMR S 1127,
12 Institut du Cerveau et de la Moelle épinière, ICM, F-75013, Paris, France.
13 AP-HP, Hôpitaux Universitaires Pitié Salpêtrière - Charles Foix, Service de Neurologie 2-Mazarin, F-
14 75013, Paris, France.

15 ^d Laboratoire de neuropathologie, GHU Paris-Sud-Hôpital Bicêtre, 78 rue du Général Leclerc, 94270 Le
16 Kremlin Bicêtre, France.

17

18 *Corresponding author Email address:

19 edouardalphandery@hotmail.com

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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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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 ($H.f = 8 \cdot 10^8 \text{ A}\cdot\text{m}^{-1}\cdot\text{s}^{-1}$, below the threshold of $5 \cdot 10^9 \text{ A}\cdot\text{m}^{-1}\cdot\text{s}^{-1}$, 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***

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

112 **2.2. Preparation of IONPs:**

113 IONPs, which are starch-coated iron oxide nanoparticles, were purchased from Micromod
114 Partikeltechnologie GmbH company, Friedrich-Barnewitz-Str. 4, D-18119, Rostock, Germany (ref: 10-
115 00-102).

116 **2.3. Nanoparticle Characterization:**

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

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

130 ***2.4. Set-up for generating the alternating magnetic field and for measuring the temperature***

131 We used an induction system operating at 198 kHz to generate the alternating magnetic field. Mice,
132 Eppendorf tubes, petri dishes containing nanoparticles and possibly cells were inserted into a coil with a
133 7-cm diameter with four spires to generate a magnetic field with an average strength of 30 mT. We used
134 an infrared camera (EasIR-2, Optophase, France) positioned 20 cm above the coil to measure the
135 temperature both *in vitro* and *in vivo*. We verified the temperature with a thermocouple positioned at the
136 tumor center and that the infrared camera measured the temperature at the tumor center. This was
137 possible due to the shallowness of the tumor.

138 ***2.5. Amount of endotoxins released under AMF application***

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

145 ***2.6. Materials and cells culture***

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

153 ***2.7. U87-Luc cytotoxicity in the absence of AMF using an MTT assay***

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

172 **2.8. *In vitro* efficacy of hyperthermia**

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

195 **2.9. *Internalization of nanoparticles***

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

198 mixtures were then centrifuged, and the supernatant was removed and replaced with 286 μl of HNO_3
199 (70%); the resulting treated mixtures were maintained at 4 $^\circ\text{C}$ for 24 h to lyse cells and dissolve the
200 nanoparticles into free iron. Finally, 10 ml of filtered water was added to all the treated mixtures and the
201 iron concentration was then determined using ICP-AES measurements. We deduced the average
202 quantity of iron coming from the nanoparticles, which was internalized in each cell.

203 **2.10. *In vivo experiments***

204 *In vivo* experiments were performed following ethical guidelines and surgery was performed following
205 the guidelines of the Institutional Animal Care and Use Committee (“Ethic committee Charles Darwin
206 N°5”, project ethic number: 00764.03). Mice were fed and watered according to these guidelines and
207 euthanized by cervical dislocation when their weights had decreased by more than 20% or when signs of
208 pain, unusual posture or prostration were observed. At day 0 (D0), a cell suspension containing 10^5 U87-
209 Luc cells per microliter was first inoculated into the brains of 7-week-old CD-1 female nude mice with a
210 mean weight of 20 g from Charles River. To achieve the surgical procedure leading to cell implantation,
211 the mice were first anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (8 mg/kg). The
212 mouse head was then fixed in a stereotactic frame, a craniotomy was performed at the fixed coordinates
213 (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).
214 Tumors grew for 8 days between D0 and D8. At D8, 7 different groups containing 10 mice each were
215 treated as follows (Table S1(a)):

216 - Group 1 received a 30 mm^3 GBM at coordinates (0.2.2) mm and 2 μl of an isotonic solution
217 containing 5% glucose without any further treatment;

218 - Group 2 received a 5 mm^3 GBM at coordinates (0.2.2) mm and the same glucose solution as
219 Group 1, followed by magnetic treatment consisting in 12 magnetic sessions (S) on D8 (S1), D9 (S2),
220 D10 (S3), D15 (S4), D16 (S5), D17 (S6), D22 (S7), D23 (S8), D24 (S9), D29 (S10), D30 (S11), and
221 D31 (S12);

222 - Group 3 received a 3 mm^3 GBM at coordinates (0.2.2) mm and a suspension containing 40 μg of
223 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

250 horizontal histological sections positioned at various tumor heights, which were collected from mice
251 euthanized on the same day at the BLI measurements. A linear relationship was observed between the
252 logarithm of tumor bioluminescence intensity (BLI), $\log(\text{BLI})$, and the logarithm of the tumor volume
253 expressed in mm^3 , $\log(\text{volume}(\text{mm}^3))$ (Fig. S3). A linear coefficient of 1.4 was deduced by fitting the
254 plot in Fig. S3 with a linear function, a value that agrees with previously reported values (40), (42),
255 (43), (44).

256 **2.12. Representation of tumor volume variations and estimates of survival time**

257 The variations in tumor volume in time were represented in Tukey diagrams using the Kaleida Graph
258 4.03 software. Mouse survival times are plotted using the Kaplan-Meier model method (45), (46).
259 The statistical significance of the survival time between the different groups was evaluated using the log
260 rank test. Parameters are expressed as the median with p-values (9).

261 **2.13. Temperature measurements**

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

264 **2.14. Histological analysis**

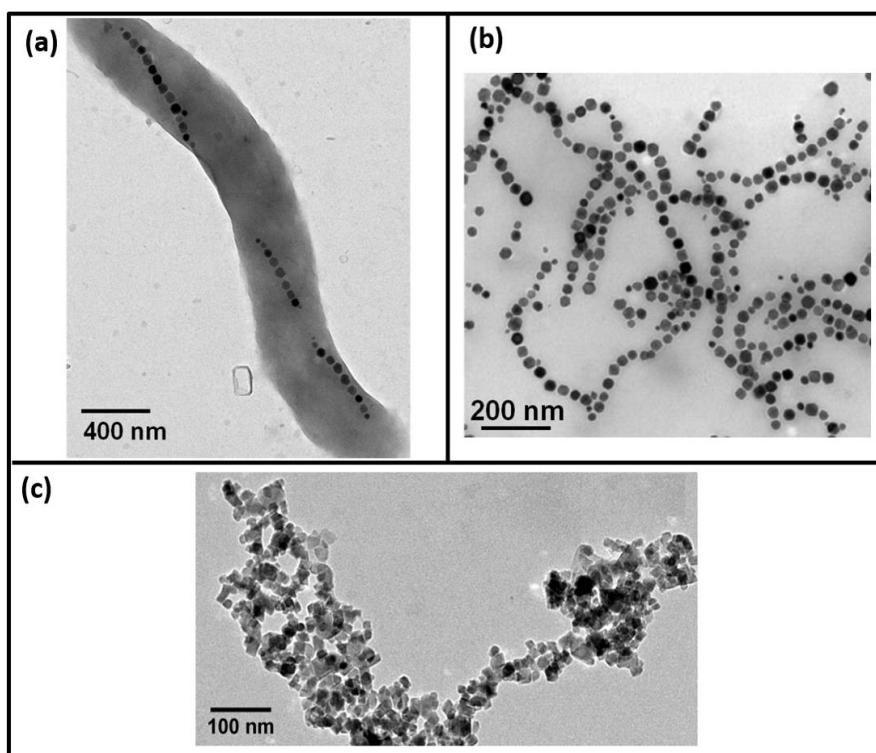
265 Histological studies were performed on extracted brains that were fixed with a 4% solution of
266 formaldehyde for 24 hours, cut into 2-mm thickness transverse slices, washed in an ethanol (70%) bath
267 for 12 hours and included in paraffin. Sections of paraffin blocks with a thickness of 4 μm were
268 deposited on glass slides and stained with hematoxylin-eosin (H&E) and/or Prussian blue to distinguish
269 between healthy and tumor areas and to determine the location of the magnetosomes, polynuclear
270 neutrophils (PNN), and tumor and healthy cells. Histological analysis was also performed on the mice
271 that received glucose on day 8 and were euthanized 6 or 24 hours following glucose injection (Figs.
272 S5(a) and S5(c)). Six mice were analyzed at various times, including after 1 magnetic session
273 (Figs. S5(b)) or 24 hours after 3 magnetic sessions (Fig. S5(d)), in which they received CM on day 8 and
274 were euthanized 4 hours (Figs. 9(a) and 9(c)) or 72 hours (Figs. 9(b) and 9(d)) after the CM injection;
275 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).



290

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)		

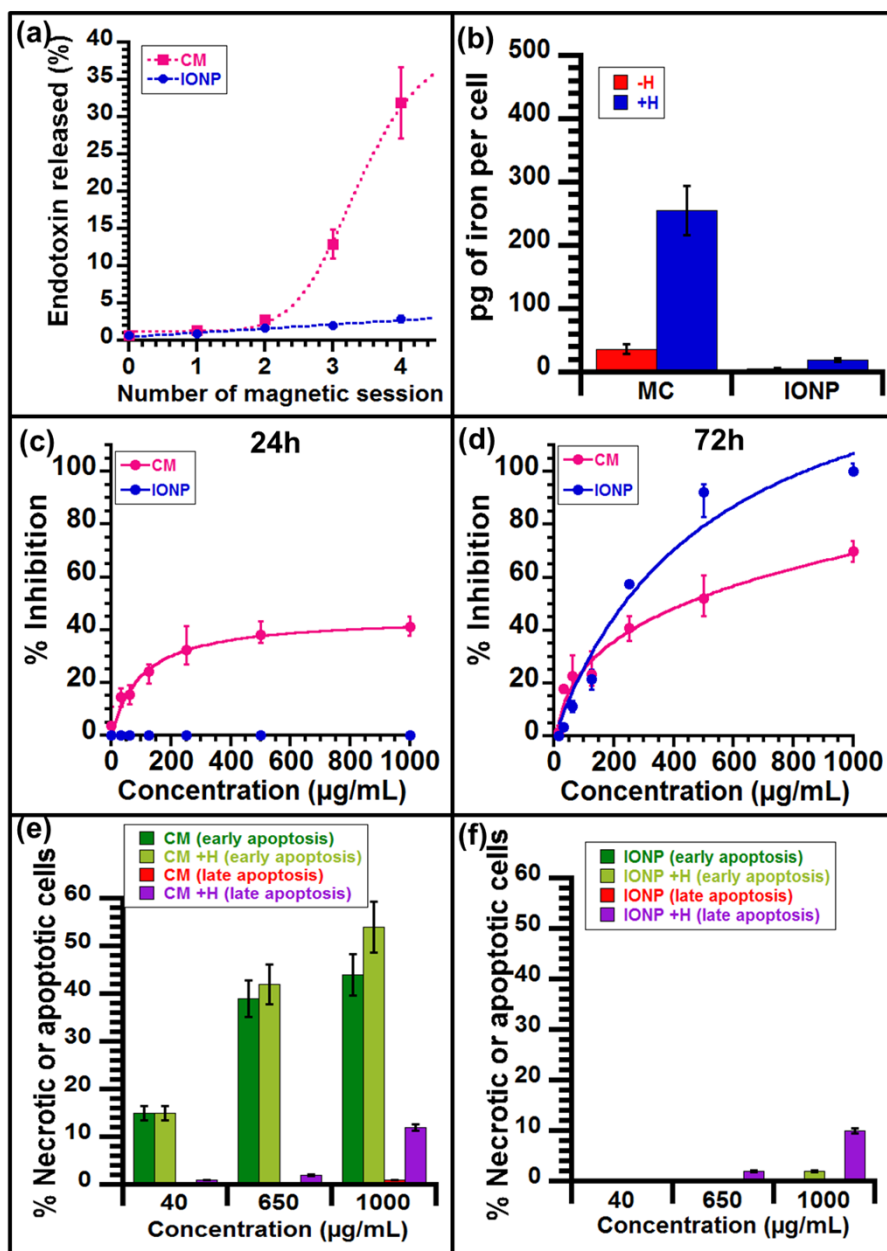
306
 307 **Table 1:** The endotoxin content estimated in Endotoxin units (EU per mg in iron per ml), percent decreased absorption
 308 measured at 480 nm in suspensions containing CM and IONP at a concentration of 1 mg in iron per ml after twenty minutes,
 309 and estimates of the isoelectric point and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

310 Estimates of the coating thickness measured by transmission electron microscopy and percentage of carbon (% C) measured
311 with CHNS and assignments from the different FT-IR peaks measured by FT-IR for the CM and IONP dried suspensions.
312 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
313 oxide mixed in water or introduced inside the mouse brains.
314 Compared with CMs, IONPs are also ferromagnetic iron oxide nanoparticles but characterized by the
315 following distinct features. Their geometric structure is parallelepipedic, they are not organized in chains
316 and tend to form small aggregates as observed in the TEM image in Figure 1(c). IONPs are made of a
317 core measuring \sim 19 nm by TEM (49), and whose composition is iron oxide as determined by FT-IR
318 measurements that display an Fe-O peak at 610 cm^{-1} (Table 1). The IONP core is surrounded by
319 synthetic hydroxyethyl starch, as suggested by FT-IR and CHNS measurements performed on the
320 IONPs, which shows FT-IR peaks at 1150 cm^{-1} and 1050 cm^{-1} that are attributed to hydroxyethyl starch
321 ether and alcohol functional groups, and has a carbon percent of 8.7%, revealing the presence of an
322 organic coating surrounding the inorganic iron oxide core (Table 1). Compared with CMs, the IONP
323 surface contains a much lower endotoxin concentration of 50 EU per ml per mg of iron oxide, as
324 measured with the LAL assay. Furthermore, IONPs are characterized by ferrimagnetic properties at
325 physiological temperatures but at the following lower values: (1) $H_c \sim 120$ Oe and (2) $M_r/M_s \sim 0.15$
326 (49), (Fig. S6 and Table 1), which should yield lower SAR values. Furthermore, given that they are not
327 less stable than CMs (Table 1) and that their anti-tumor efficacy has been demonstrated on tumor-
328 bearing mice using magnetic hyperthermia (50), IONPs appear to be good standard for comparison to
329 assess CM efficacy.

330 ***3.2. Under AMF applications, CM can release heat and an immune-stimulating substance more*** 331 ***efficiently than IONPs***

332 In an attempt to mimic the conditions in the *in vivo* treatments and to study the release of endotoxins
333 from the CM and IONP surfaces in these conditions, we introduced 2 μ l of suspensions containing 40
334 μ g of these nanoparticles in iron oxide at the bottom of a tube that we exposed to 1, 2, 3, and 4 magnetic
335 sessions during which an AMF of 198 kHz and average strength 30 mT was applied for 30 minutes.
336 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 $SAR=C_{water}(\Delta T/\delta t)/C_m$, 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], $C_{water}= 4.2$ 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.



344

345 **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
346 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
347 iron released from MCs and IONPs, measured in pg and internalized per cell from U87-Luc cells incubated for 24 hours with
348 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
349 percentage inhibition of cell proliferation as a function of nanoparticle concentration for CMs and IONPs in maghemite in
350 U87-Luc cells incubated for (c) 24 hours or (d) 72 hours with nanoparticle concentrations varied between 8 μ g/mL and 1
351 mg/mL. (e and f) The percentages of late apoptotic (+ necrotic) and early apoptotic cell death as a function of nanoparticle
352 concentration with(H)/without(-H) AMF where the AMF is the same as in (a) in U87-Luc cells incubated for 24 hours with
353 (e) CM and (f) IONP at concentrations of 40 μ g/mL, 650 μ g/mL, and 1 mg/mL.

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

366 ***3.3. CMs induce enhanced U87-Luc early apoptotic cell death and internalization in vitro***

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

372 0 to 60%, than for IONPs, *i.e.*, from 0 to 100% (Figure 2(d)). While CMs were observed to be more
373 cytotoxic than IONPs at 24 hours, the opposite behavior was observed at 72 hours.

374 U-87-Luc cells were also incubated for 24 hours with CMs and IONPs at three different concentrations
375 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
376 seconds and 30 mT for the remaining 29.5 minutes in an attempt to reach 41 $^{\circ}\text{C}$, a temperature above
377 which mild hyperthermia conditions are expected to occur (30). The 40 and 650 $\mu\text{g/ml}$ concentrations
378 led to temperature increases of less than 2 $^{\circ}\text{C}$, which were insufficient to reach the hyperthermia
379 conditions. These concentrations resulted in necrosis and late apoptosis percentages that remained below
380 $\sim 4\%$ with/without AFM for the CMs and IONPs and early apoptosis percentages that were similar
381 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
382 0% for IONPs (Figs. 2(e) and 2(f)). In contrast, at a concentration of 1 mg/ml, both CMs and IONPs
383 yielded a more significant temperature increase of 7 $^{\circ}\text{C}$ up to 41 $^{\circ}\text{C}$ (Fig. S2(b)), which resulted in an
384 increase in the necrotic and late apoptotic cell death percentages by 10%. These *in vitro* heating
385 behaviors are In contrast, with those observed in solution, where CMs were observed to heat much more
386 than IONPs.

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

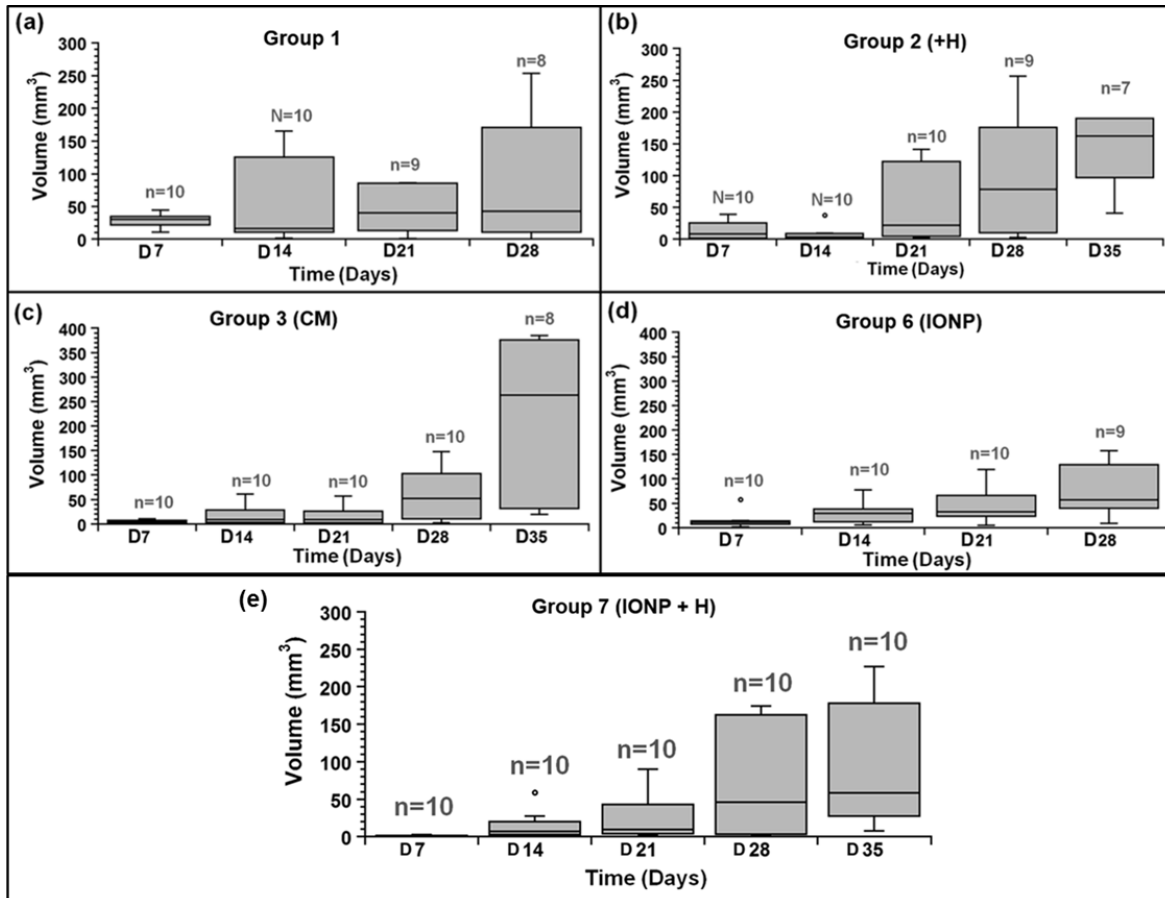
392 The differences in cell death mechanisms observed for IONPs and CMs as well as the more pronounced
393 decrease in heating efficacy for CMs than IONPs observed between the *in vitro* and in suspension
394 heating conditions may be explained by the different internalization properties. Indeed, the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

398 internalization is enhanced by the presence of the AMF for both types of nanoparticles. This
399 enhancement is more pronounced for CMs than for IONP by factors of 7 and 4, respectively (Figure
400 2(b)). The different internalization properties for the CMs and IONPs may be explained by differences
401 in their charges, *i.e.*, CM and IONP are negatively and positively charged at physiological pH,
402 respectively (Table 1)); differences in organization, *i.e.*, CM and IONP are well-dispersed and tend to
403 aggregate, respectively; and to larger values of coercivity for CMs (200-300 Oe) compared to IONPs
404 (120 Oe) that may enhance CM magnetic field coupling. Furthermore, CM organization in chains may
405 favor internalization by groups containing several magnetosomes (30), *i.e.*, potentially 6 for chains
406 averaging 250 nm in length (Fig. S1(b), while IONPs may result in nanoparticles being internalized one
407 by one or at a lower rate. The enhanced internalization of CMs may favor early apoptosis, which was
408 previously observed with iron oxide nanoparticles, (53), and reduce its heating properties, possibly by
409 preventing nanoparticle Brownian heating mechanisms (54).

410 **3.4. CMs promote enhanced antitumor efficacy towards intracranial U87-Luc glioblastoma**

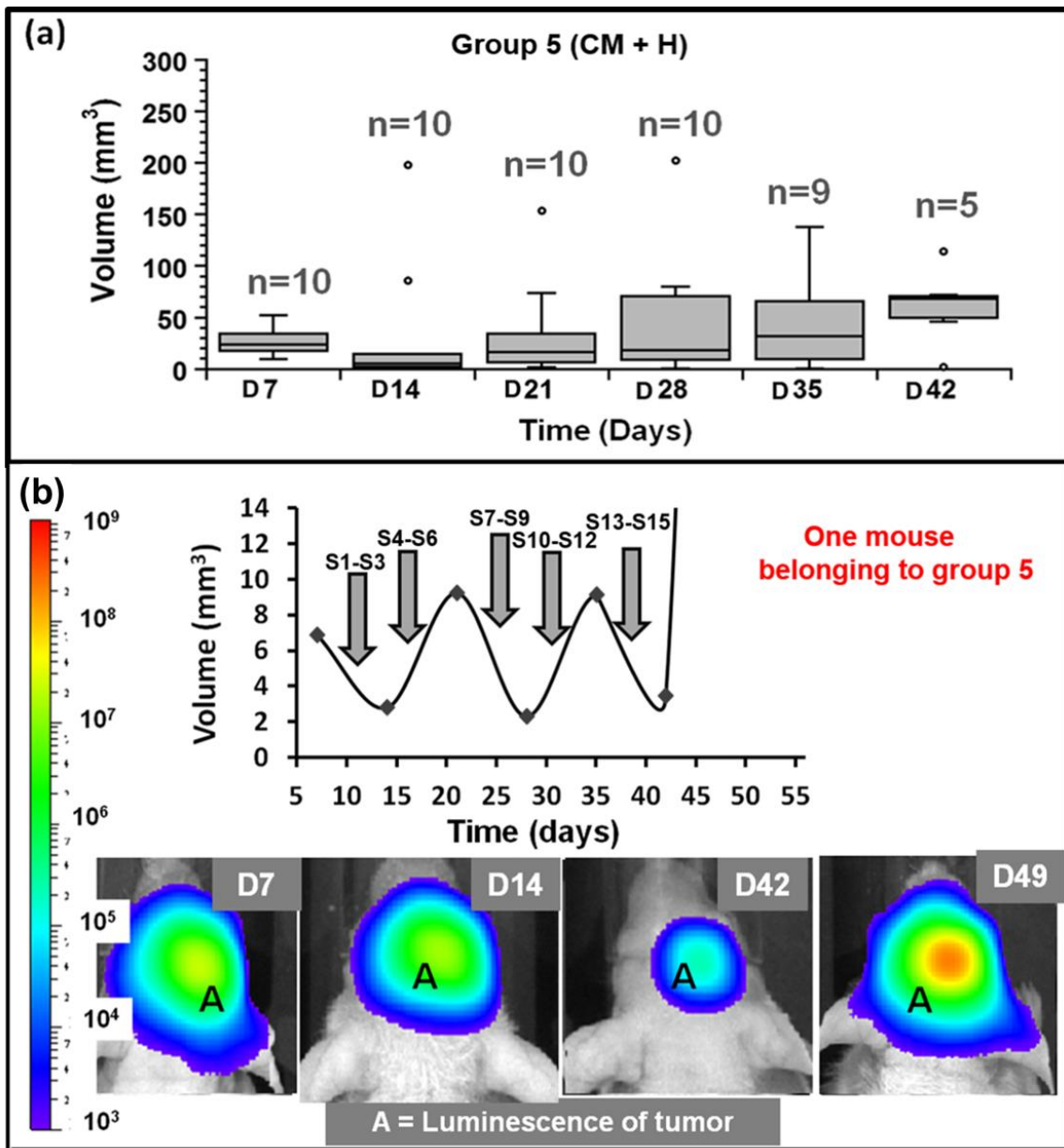
411 In the *in vivo* studies, mice receive the following treatment at the center of intracranial U87-Luc tumors:
412 (1) glucose followed by treatment or not with magnetic sessions (groups 1 and 2), (2) 40 μg of CMs or
413 IONPs in maghemite without any further treatment (groups 3 and 6), (3) 40 μg of CMs in maghemite
414 followed by 15 magnetic sessions (groups 4 and 5), or (4) 40 μg of IONPs in maghemite followed by 12
415 magnetic sessions (group 7). The average size of the treated tumors was 3-5 mm^3 , except in group 5
416 where it was 25 mm^3 . Following injection, the mice were exposed to 12 to 15 magnetic sessions, during
417 which an AMF of 198 kHz and strength 30 mT was applied for 30 minutes (see Table S1(a) for a
418 summary of the treatment conditions). In this study, U87 cells were labeled with the firefly luciferase
419 gene to evaluate the changes in tumor volumes following the treatments via tumor bioluminescence
420 intensity (BLI) measurements using the relationship between BLI and tumor volumes that we
421 established (Fig. S3) (40), (42), (43), (44). For the mice belonging to the different groups without
422 any measurable production of heat (groups 1, 2, 3, 6, and 7), Figures 3(a) to 3(e) show that the tumor
423 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.

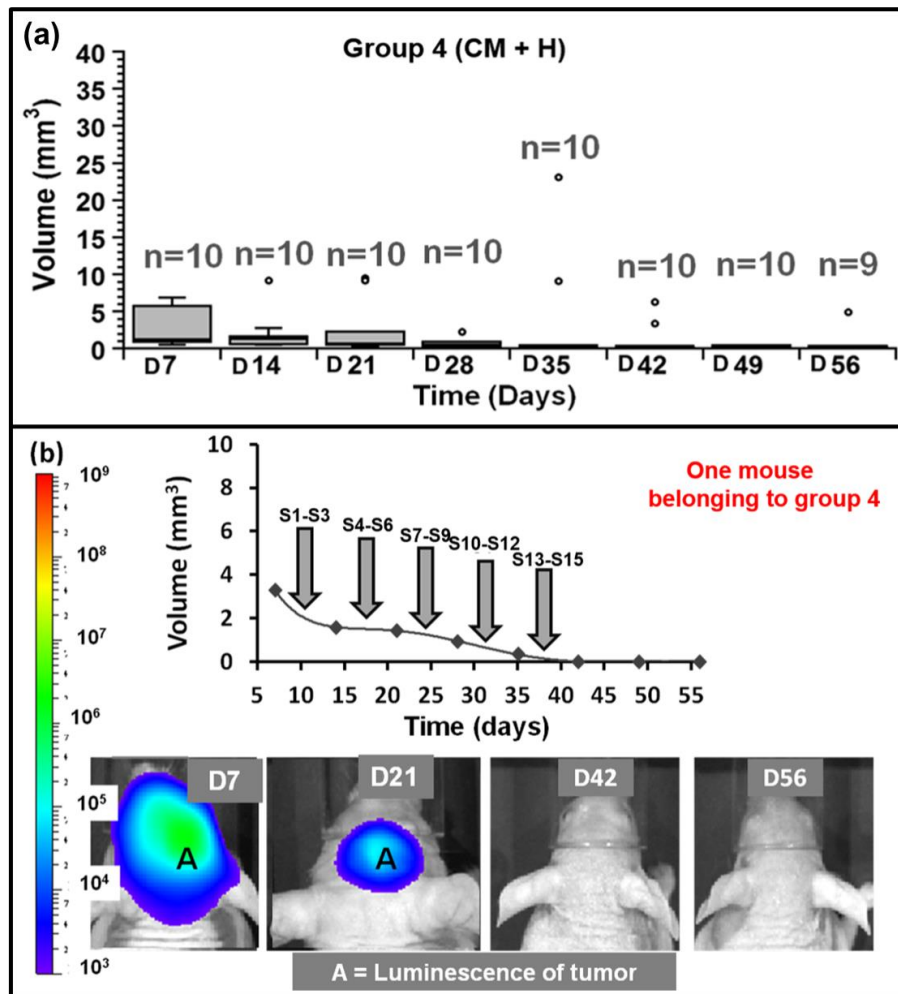
437 Figures 4(a) shows that tumor growth is delayed in group 5 with a median tumor volume that first
 438 decreases from 25 mm³ at day 7 to 5 mm³ at day 14 and then increases from 5 mm³ at day 14 to 70 mm³
 439 at day 42. The behavior of a typical mouse belonging to this group is shown in Figure 4(b), 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.



444

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).

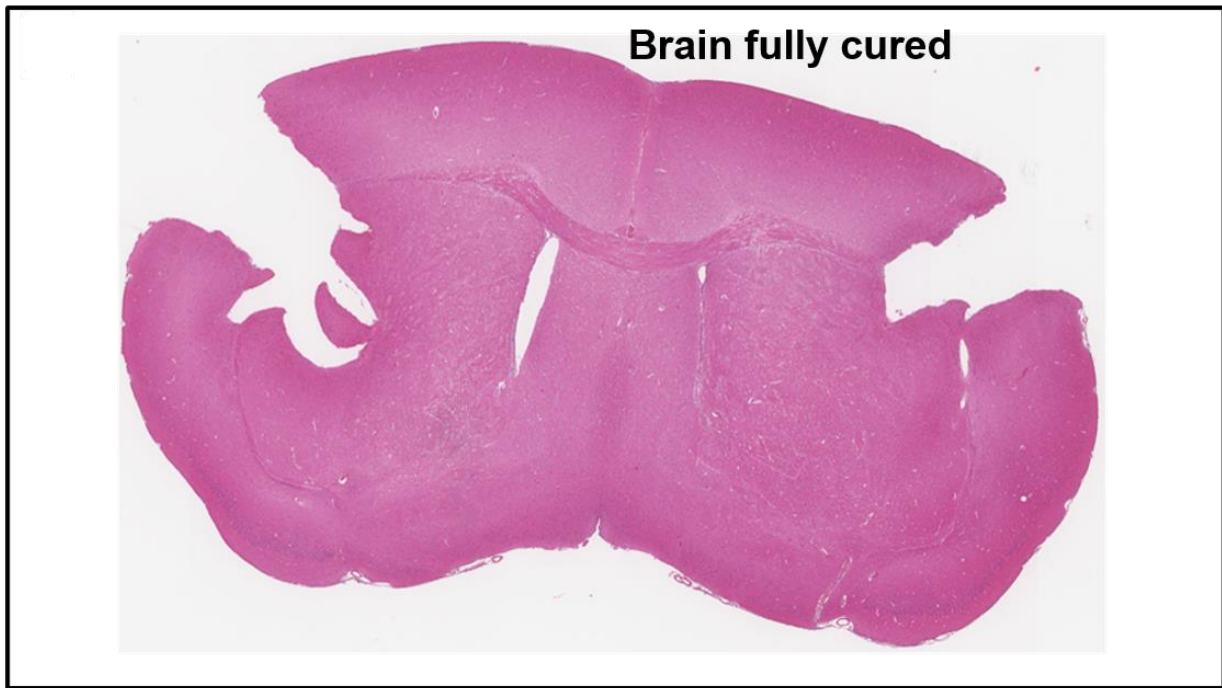
451 Figure 5(a) shows more significant antitumor activity in group 4 with full tumor disappearance at day 35
 452 without tumor regrowth following 1 to 15 magnetic sessions. For a typical mouse belonging to this
 453 group, Figure 5(b) shows the gradual decrease in the tumor volume following 1 to 15 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).



456

457 **Figure 5:** (a) Variations in tumor volumes as a function of time during the days following U87-Luc tumor cell implantation at
458 D0 for mice receiving a suspension containing 40 μg of CMs in iron oxide when the tumors reach an average volume of 3
459 mm^3 at D7 followed by 15 magnetic sessions (group 4, CM+H). (b) Variations in tumor volume and bioluminescence
460 intensity as a function of time during the days following tumor cell implantation for a mouse belonging to group 4 with a
461 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
462 S15).

463 On day 150, 40% of the mice from this group were still alive without any unusual behaviors. Then, the
464 mice were euthanized and slides of their brains from the tumor cell implantation region were imaged by
465 optical microscopy, revealing the absence of tumors or tumor lesions and a brain neuronal structure
466 identical to that of a healthy brain (Figure 6). These results suggest that the tumors had fully disappeared
467 in these mice. Further analysis of mice from group 4 suggests that treatment does not result in any
468 healthy tissue damage. Indeed, it can be deduced from the optical microscopy images of the brain
469 sections from mice treated with CM with/without AMF collected at different times ranging from 4 hours
470 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
471 9(d)) that healthy tissues in the hippocampus and brain do not contain lesions or edema. Such
472 observations can be explained by an LPS-activated neuro-protective response by the microglia (55).
473 Thus, the treatment of the mice in group 4 appears safe and fully efficient.



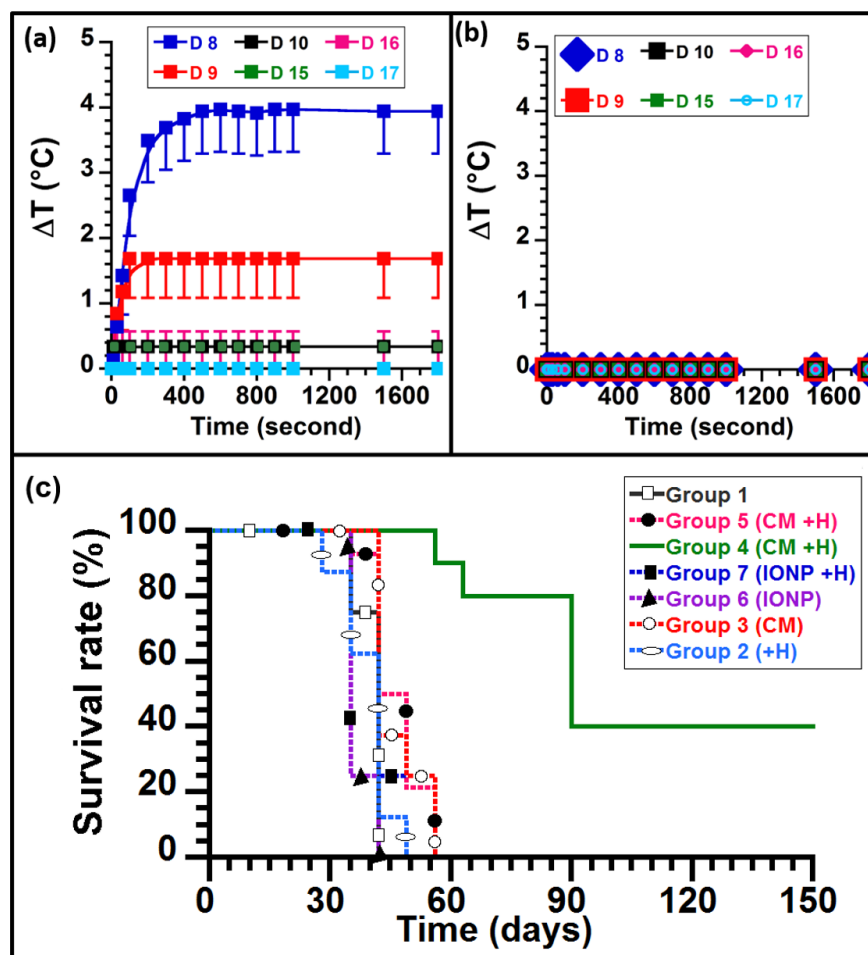
474

475 **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
476 received 40 μ g of CMs in iron oxide followed by 15 magnetic sessions. This mouse was euthanized 7 months after
477 magnetosome administration. No tumor was observed in this brain section.

478

479 ***3.5. Distribution of CMs in the tumor favors in vivo heating during the various S***

480 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,
481 S4, S5) and then constant temperatures after D17 (Figures. 7(a) and 7(b)).

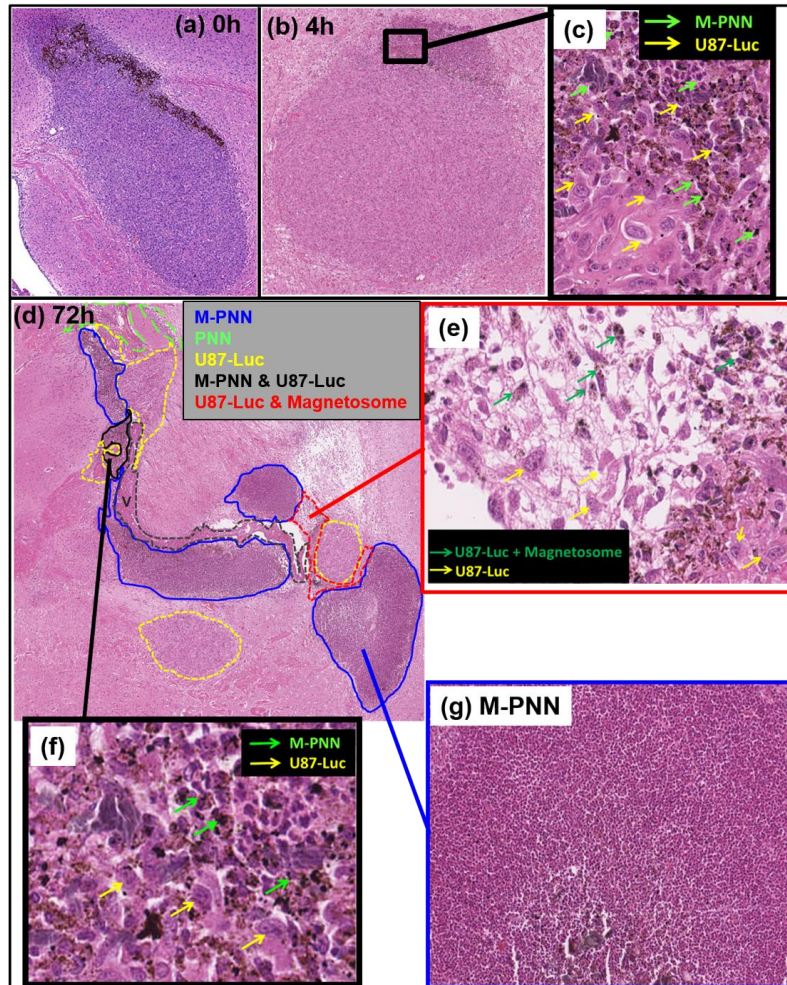


482

483 **Figure 7:** (a), Temperature increases above 28.5 °C observed during each magnetic session (ΔT) on D8, D9, D10, D15, D16
 484 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
 485 IONPs. After D17, tumor temperature increases were no longer observed. (c) Variations in mouse survival times as a function
 486 of time during the days following U87-Luc tumor cell implantation for mice with the following treatments: (1) untreated
 487 (group 1), (2) exposed to 15 magnetic sessions (group 2), (3) treated with suspensions containing 40 μg of CMs in iron oxide
 488 without magnetic sessions (group 3), (4) treated with 40 μg of CMs in iron oxide in $\sim 3 \text{ mm}^3$ tumors followed by 15 magnetic
 489 sessions (group 4), (5) administered 40 μg of CMs in iron oxide in $\sim 25 \text{ mm}^3$ tumors followed by 15 magnetic sessions (group
 490 5), (6) administered 40 μg of IONPs in iron oxide (group 6), and administered 40 μg of IONPs in iron oxide followed by 15
 491 magnetic sessions (group 7).

492 The rather limited increases in temperature under AMF, which are less pronounced than in the
 493 capillaries (Fig. S2(a)), may be explained by the effects of AMF on CM distribution. Indeed, the tumor
 494 occupation percentage decreases from $\sim 50\%$ right after CM administration without AMF at D8 (Figs.
 495 9(a) and 9(b)) down to 8-15% on D8 and D11 after 1 and 3 magnetic sessions, respectively, (Figs. 8(a),

496 8(b) and 8(d)). These percentages were estimated by delineating the regions containing the CMs and the
 497 tumors and by measuring the ratio between the surfaces of these two regions. Moreover, the decrease in
 498 CM heating properties over time may be attributed to internalization of CMs in U87 tumor cells, which
 499 was observed at 24 hours after 3 magnetic sessions (Fig 8(d) and 8(e)).



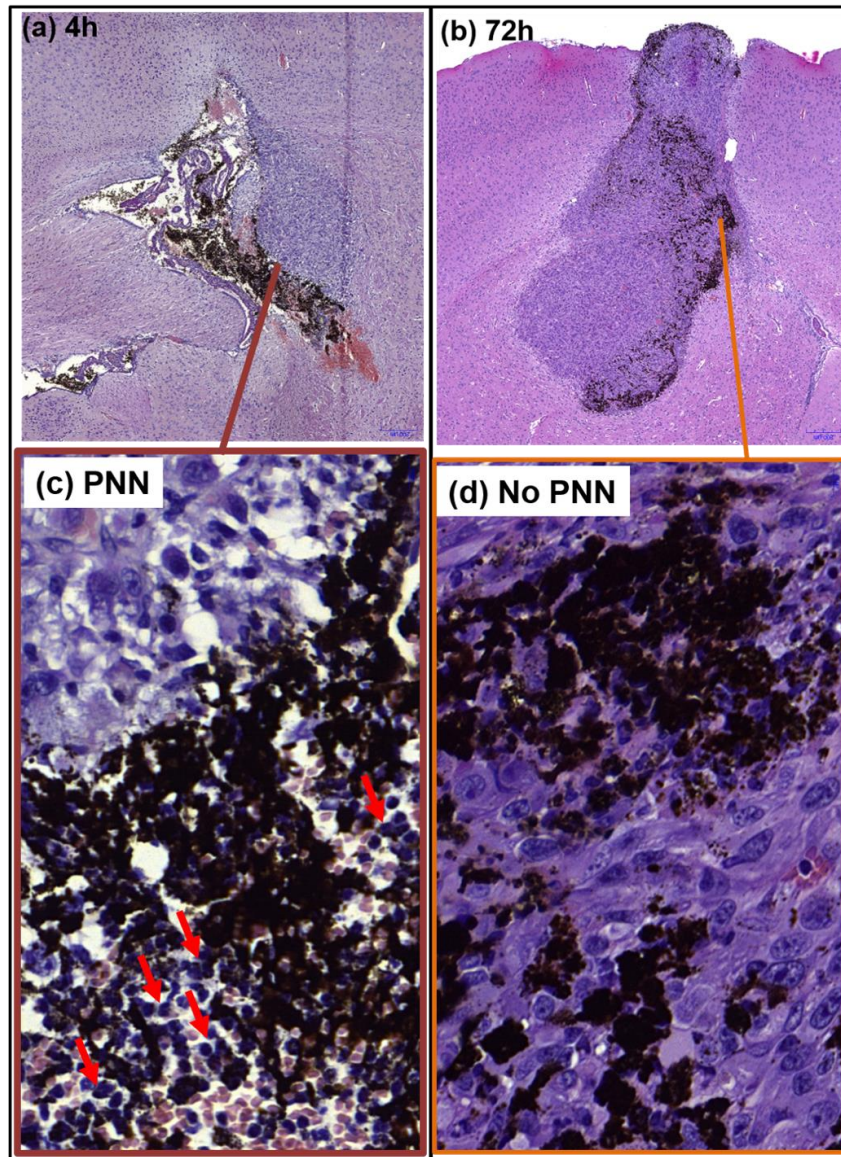
500

501 **Figure 8:** Optical microscopy images of 4 μm thick brain sections stained with hematoeosin from mice receiving 40 μg of
 502 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
 503 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.
 504 (c) An enlargement of the region in (b). The red arrows in (c) designate polynuclear neutrophils. (e), (f), and (g) are
 505 enlargements of three different regions from (d). PNN, M-PNN, U87-Luc, M-PNN & U87-Luc, and U87-Luc &
 506 magnetosomes designate polynuclear neutrophils, magnetosomes colocalized with polynuclear neutrophils, U87-Luc cells,
 507 magnetosomes colocalized with polynuclear neutrophils and U87-Luc cells, and U87-Luc cells colocalized with
 508 magnetosomes, respectively. The regions containing these different cells are surrounded in (d), while individual cells are
 509 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)).



534

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

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

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

563 ***3.7. CMs appear to destroy glioblastoma tumors more efficiently than chemically synthesized*** 564 ***nanoparticles under AMF applications***

565 Using the same quantity of nanoparticles administered, we have shown in this study that CMs lead to
566 40% of mice with full tumor disappearance, while IONPs do not yield any observable antitumor
567 activities. This result agrees with previous animal studies performed on mice and rats bearing GBM
568 using intratumor administration of chemically synthesized nanoparticles exposed to AMF that did not
569 improve survival (63) or increased survival by 15 days without resulting in a full cure (64), (26). In
570 addition to being more efficient, magnetosomes could also be used in a lower quantity, thus

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

575 **4. CONCLUSION**

576 We demonstrated that 40% of mice bearing intracranial U87-Luc tumors displayed full tumor
577 disappearance after intratumor administration of 40 μg of CMs followed by 12 to 15 magnetic sessions,
578 during which an AMF of average strength 30 mT and frequency 198 kHz was applied for 30 minutes. In
579 contrast, similar antitumor efficacy was not observed when mice were treated with the same conditions
580 and administered chemically synthesized nanoparticles instead of magnetosomes. Furthermore, the
581 absence of mouse brain damage during and following the various treatments appears to indicate that a
582 safe treatment could be performed with magnetosomes, provided that the endotoxins are replaced with
583 an equivalently active but nonpyrogenic substance such as M-PLA. Antitumor activity does not appear
584 to be dominated by nanoparticle cytotoxicity, as the CMs, which are more efficient than IONPs *in vivo*,
585 do not enhance cytotoxicity towards U87-Luc cells compared with IONPs. Moreover, nanoparticle
586 administration without AMF does not produce any observable anti-tumor effects. Instead, tumor
587 destruction appears to be due to the heat produced by the CMs under AMF application, as the only mice
588 that displayed clear anti-tumor effects had their tumors heated (groups 4 and 5). Under heating
589 conditions, tumor tissue destruction may be caused by irreversible cell damage; cytolysis; actin filament,
590 microtubule or mitochondrial dysfunction; DNA replication or RNA synthesis inhibition; or DNA
591 polymerase denaturation (65), but these mechanisms seem insufficient to fully explain the observed
592 antitumor activities. Indeed, tumor destruction does occur when the magnetosomes occupy a small
593 percentage of the total tumor volume (~10%), suggesting that magnetosomes occupying the tumor can
594 induce the destruction of the entire tumor. We have identified the following three possible mechanisms
595 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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613 Dervishi, as well as all other people and structures in France and Europe that have helped us and been
614 supportive and enthusiastic about our research activities.

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 ameocyte 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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