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1 **Mucoadhesive thermosensitive hydrogel for the intra-tumoral delivery**
2 **of immunomodulatory agents, in vivo evidence of adhesion by means**
3 **of non-invasive imaging techniques**

4
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20

21 **Key words**

22 Thermosensitive gel, bioadhesivity, Dendritic cells, optical imaging, local immunomodulation,
23 antitumor immune response.

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25

26 **ABSTRACT**

27

28 Intratumoral injection of biocompatible gels is increasingly used for the sustained delivery of
29 drugs and vaccines to enhance the anti-cancer immune response. Granulocyte-macrophage
30 colony stimulating factor (GM-CSF) has become an attractive adjuvant thanks to its ability to
31 boost the antitumor immune response by inducing proliferation, maturation and migration of
32 the dendritic-cells (DCs) and the differentiation of lymphocytes. Killed Mycobacteria, such as
33 Heat-killed Mycobacterium tuberculosis (HKMT) have been used in several studies as TLR-2
34 agonist to increase maturation of DCs. In this study, we designed a mucoadhesive
35 thermosensitive formulation for the local delivery of GM-CSF and HKMT in order to enhance
36 DCs activation and improve the local antitumor immune response. This formulation was
37 selected based on its elastic and mucoadhesive properties obtained thanks to rheological
38 studies. More importantly, intratumoral residence time of the labelled gel and protein were
39 evidenced by means of MRI and non invasive *in vivo* optical imaging. Then, the efficacy of the
40 combination of immunomodulators loaded thermogel was demonstrated *in vitro* and *in vivo*. The
41 selected thermogel exhibits rheological properties which confer a good elasticity and increased
42 residence time of the immunostimulatory agents in the tumor, thus increasing the recruitment
43 of DCs and T cytotoxic CD8⁺ lymphocytes.

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50 **1. Introduction**

51 Dendritic cells (DCs) based immunotherapy is a promising strategy in the treatment of cancer.
52 DCs are the most important antigen presenting cells (APCs) involved in the regulation of innate
53 and adaptive immunity and play a central role in the antitumor immune response (Sabado et al.,
54 2017). Indeed, DCs mature quickly after exposure to stimuli and get the ability to migrate and
55 activate naive lymphocytes into cytotoxic T cells (Banchereau et al., 2000).

56 In the past two decades, there has been a growing attempt to enhance immune responses by
57 immunizing cancer patients with their own DCs that have been isolated and activated *ex vivo*
58 (Gilboa, 2007). Several studies used *ex vivo* activated DCs of patients to treat various cancers
59 such as kidney cancer, breast cancer, prostate cancer and colorectal carcinoma (Barth et al.,
60 2010; Jähnisch et al., 2010). However, these clinical trials lost their interest because of the low
61 effectiveness of DCs therapy related to their short viability and the suppressive immune
62 environment of the tumor (Palucka et al., 2010). It was also shown that generated cells have a
63 low *in vivo* migration capacity (Justus et al., 2014).

64 To overcome these limits, studies focused on direct *in vivo* targeting of DCs adjuvants such as
65 TLR agonists, Cytokines or Interleukins (Tacke et al., 2007). Toll-like-receptors agonists, such as
66 TLR2, TLR4, TLR7 or TLR9 were used to mature DCs *in vivo*. For example, CpG-DNA is a TLR 9
67 agonist which is used as an effective adjuvant to enhance immune response (Häcker et al.,
68 2002). BCG (Bacilli of Calmette-Guerin) is a TLR-2/4 agonist approved as an adjuvant for non-
69 invasive bladder cancer treatment (Alexandroff et al., 1999; Orihuela et al., 1987). TLR agonists
70 administered by systemic pathways, were however, not well tolerated because they induce the
71 release of high levels of cytokines, including IFNs, which are believed to cause most of the
72 observed adverse reactions. In order to avoid these side effects, some studies demonstrated the
73 interest of Heat killed mycobacteria as an adjuvant in cancer immunotherapy of multiple
74 cancers (Stebbing et al., 2012).

75 Heat-killed (HK) Mycobacterium preparation has demonstrated immunotherapeutic effect for
76 the treatment of advanced pancreatic cancer confirmed by a significant increase on the overall
77 and progression-free survival of these patients (Dailey et al., 2006). Otherwise, heat-killed (HK)

78 Mycobacterium preparation has been shown to be safe and well tolerated among patients with
79 stage III/IV melanoma (Stebbing et al., 2012). The immunological effect of these compounds
80 was related to their abilities to induce innate immune reaction. Indeed, HK mycobacterium was
81 reported to promote the development of CD11c⁺ APCs in mice (Adams et al., 2004). *In vitro*
82 stimulation of human blood cells with HK mycobacteria are able to modulate the expression of
83 cell surface receptors on APCs and to stimulate Th-1 antitumor immune response (Fowler et al.,
84 2012). A recent study showed that HK mycobacterium contribute to up-regulation of CD11c,
85 CD80 and MHC class II expression on DCs via interaction with TLR2 and TLR1 receptors (Bazzi et
86 al., 2017).

87 GM-CSF is critical to the regulation of anti-tumor immune responses by the activation of both
88 innate and adaptive immunity through the activation of its heteromeric receptor CD116,
89 present on multiple cell types (Gillessen et al., 2003; Mach et al., 2000; Qin et al., 1997). The
90 cytoplasmic domains of the GM-CSF receptor beta chain are associated with the Janus kinase 2
91 (JAK2) whose phosphorylation triggers multiple intracellular signaling pathways, including STAT5
92 and MAPK.1. Then multiple GM-CSF target genes are constitutively activated, including the
93 transcription factor PU.1, which regulates differentiation and maturation of macrophages
94 (Hansen et al., 2008; Suh et al., 2005). Nevertheless, through *in situ* interactions in the tumors
95 micro-environment, GM-CSF was described as an immune-independent tumor-promoting
96 factor, increasing tumor cell growth, in multiple cancer types (Braun et al., 2004; Gutschalk et
97 al., 2012; Mueller and Fusenig, 1999; Obermueller et al., 2004; Orłowski and Baldwin, 2002; Pei
98 et al., 1999). High concentrations of GM-CSF can also result in immune suppression, explained
99 through the mobilization of Gr-1⁺ CD11b⁺ Myeloid Derived Suppressor Cells (MDSC), inhibition
100 of DCs migration and also down-regulation of DCs expression of the CCL19 receptor CCR7 and
101 MHCII (Serafini et al., 2004).

102 Considering cytokines and TLR agonists adverse effects, *in situ* controlled release may be the
103 most efficient way to create a microenvironment where host DCs can migrate, proliferate and
104 differentiate (Kizana and Alexander, 2003). Encapsulation device is then a limiting step in the
105 design of such strategy.

106 Intratumoral injection of biocompatible gels is increasingly used for the enhancement of drug
107 delivery. Local delivery of immunomodulatory agents using biomaterials such as, microspheres
108 or gel, has been proposed to enhance vaccine efficacy, avoid the side effects of the systemic
109 delivery, and also protect encapsulated degradable molecules from denaturation. The first
110 example showing the potential of this approach was given by Drissens et al. with pluronic
111 entrapped GM-CSF (Wu et al., 2017). Poloxamers are FDA approved copolymers. Upon
112 temperature and concentration increase, they undergo a sol-gel transition phase (Dumortier et
113 al., 2006). This transition can be modified by addition of salts and viscous agents. Bioadhesives
114 polymers have also been added to increase adhesion of the gel and its viscosity (Bhowmik et al.,
115 2013). We have shown previously that addition of sataxiane in poloxamer based hydrogel
116 allowed the release of salbutamol sublingually (Zeng et al., 2014). More recently, we found that
117 *in situ* delivery of anticancer drugs could slightly reduce the size of the tumors allowing their
118 removal via surgery (Mignet et al., 2015). In this study, we ought to deliver immunomodulators
119 *in situ* within the tumor and evaluate the effect of the immunotherapy. As referred to the
120 literature, the combination of immunomodulators chosen are expected to provide both DCs
121 activation and maturation. The originality of the study lies in the demonstration of the residence
122 time of the gel with or without bioadhesive agent by means of non-invasive MRI and *in vivo*
123 optical imaging. The therapeutical possible effect was also pre-evaluated by following by *in vivo*
124 optical imaging the release of a protein model imbedded within the gel. Finally, we evaluated if
125 the improved viscosity and bioadhesivity properties of the immunostimulating thermogel
126 resulted in an *in situ* immunomodulation effect evidenced by tumor volume regression and
127 tumor infiltrating lymphocytes.

128

129 **2. Material and methods**

130 *2.1. Materials, cell culture and animals*

131 Poloxamer 407 (Kolliphor® P407) and xanthan gum (SATIAXANE CX 930) were purchased from
132 BASF, France. Mucin from porcine stomach Type II (M2378-100G) and 4-nitrophenyl
133 chloroformate were bought from Sigma Aldrich, France. Triethylamine, dichloromethane,
134 methanol and DMSO were purchased from Carlo-Erba, France. GM-CSF (130-095-735) was
135 bought from Miltenyi, France. Heat-killed Mycobacterium tuberculosis HKMT (tIrl-hkmt-1) was
136 purchased from Invivogen, France. Antibodies against CD11c, CD86, CD80, were obtained from
137 Becton Dickinson, France. Anti-CD3 was bought from Dako, France. Anti-CD4 was purchased
138 from e-Bioscience, France. Anti-CD8 was bought from Neomarkers LabVision, US, and anti-Fox
139 P3 from Abcam, France. Peroxidase/diaminobenzidine Rabbit PowerVision kit was purchased
140 from ImmunoVision Technologies, US and Polink 2 plus HRP detection kit from GBI Labs, US. The
141 transwell plates with 0.45 µm pore filters were bought from Sigma Aldrich, France.

142 CT26 colon adenocarcinoma cell line (CRL-2638) was purchased from American Type Culture
143 Collection LGC Standards, France. Cells were cultured in Dulbecco's Modified Eagle Medium
144 (DMEM, Gibco Life Technologies) containing 10% fetal bovine serum (FBS, Gibco Life
145 Technologies, France), 100 µM of streptomycin, 100 U/mL of penicillin (Gibco Life Technologies,
146 France).

147 8 week old female BALB/cJrj mice were obtained from Janvier laboratories, Le Genest de l'île,
148 France. Studies were conducted following the recommendations of the European Convention
149 for the Protection of Vertebrate Animals Use for Experimentation and the local Ethics
150 Committee on Animal Care and Experimentation, Paris Descartes university. (APAFIS # 11352).

151

152 *2.2. Thermogels preparation*

153 Thermogels were prepared according to the 'cold method' inspired from Smolka et al (Zeng et
154 al., 2014). P407 21 % w/v (T-gel) was prepared by dispersing 2.1 g of P407 in 7ml of water
155 (water for injection WFI, CDM Lavoisier, France). The preparation was left overnight at 4 °C until

156 clear solutions was obtained, and then the volume was completed to 10 mL. For the P407 /
 157 Satiaxane 21 / 0.1 % w/v (MT-gel) preparation, the Satiaxane 0.1 % solution was first prepared
 158 by dispersing 10 mg in 7 mL of sterile water, stirring continuously 30 min. at room temperature
 159 until complete dissolution. Then 2.1 g of P407 were added to the already swollen Satiaxane
 160 solution. The immunomodulatory thermogels (MTI-gel) were prepared at the day of experiment
 161 from the following composition P407 / Satiaxane 25 / 0.12 % w/v. GM-CSF (5 μ L, 1mg/ml water,
 162 5 μ g) and/or HKMT (5 μ L, 2mg/ml NaCl 0.9%, 10 μ g) were added in 50 μ L of the MT-gel and
 163 incubated at 4°C for 1 hour to get a complete solubilisation.

164

	P407	Satiaxane	GM-CSF +HKMT
T-gel	+	-	-
MT-gel	+	+	-
MTI-gel	+	+	+

165

166 **Table. 1.** Thermogels composition.

167

168 *2.3. Rheology studies*

169 Gelling temperature (T_g) of thermogel formulations was measured with a rheometer (Anton
 170 Paar model MCR 102, Courtaboeuf, France), fitted with a cone-plane mobile geometry
 171 (diameter = 50 mm; angle = 1) and a humidity cover to prevent influence of evaporation. A
 172 volume of 750 μ L of thermogel was loaded on the support and then the mobile was descended
 173 to a distance equal to 0.1 mm from the support. Non-destructive oscillatory measurements at 1
 174 Hz, performed in this study, allow one to obtain the elastic modulus (G'), the viscous modulus
 175 (G'') and the phase angle ($\tan \sigma = G''/G'$). The plate was heated at a rate of 1 °C/min from 20°C to
 176 40°C. The sol-gel transition temperature was defined as the point where G₀ began to shift to
 177 higher temperatures (Sandri et al., 2011). Each formulation was analyzed three times.

178

179

180 *2.4. In vitro muco-adhesion test*

181 Adhesion test was carried out using the comparison of the result obtained with a cone-plane
182 mobile coated or not by mucin (rheometer Anton Paar model MCR 102). A solution of Porcine
183 gastric mucin of type II (MUC2) (M2378, Sigma Aldrich) was prepared at 2 % in water . The
184 mobile was placed in this mucin solution and incubated at 37°C for 10 minutes. When the film
185 was dried, the mobile was settled and 550 µL of the sample loaded on the support of the
186 apparatus. The mobile was then brought down to a distance of 0.1 mm from the support. The
187 strength required to tear the gel was measured at 37°C (Detachment Force).

188

189 *2.5. P407 labelling with Cy5*

190 Hydroxyl groups of **poloxamer P407 (1)** were activated by reacting P407 (0.4 g) with 4-
191 nitrophenyl chloroformate (40 mg), triethylamine (30 µL) in dichloromethane (1.2 mL) (Fig. 4A).
192 The reaction was allowed to stir under argon overnight at room temperature. The **activated**
193 **polymer (2)** was precipitated in anhydrous diethyl ether and redissolved in methylene chloride.
194 This purification procedure was repeated three times and the activated P407 was then dried
195 under vacuum. To get **aminated P407 (3)**, activated P407 (0.2 g) dissolved in methanol (4 mL)
196 was reacted with ethylenediamine (0.1 g) overnight at room temperature and then precipitated
197 with anhydrous diethyl ether. Dried aminated P407 (10 mg) was finally reacted with Cy5-NHS (2
198 mg) in DMSO (1 mL) for 12 hours at room temperature. Byproducts were removed by dialysis
199 (MWCO 3500) against distilled water. Finally, **Cy5-P407 polymer (4)** (5 mg) was recovered by
200 lyophilization. To obtain Cy5-T-gel or Cy5-MT-gel, 1% of Cy5-P407 polymer was mixed with non-
201 labeled polymer in the formulation process.

202

203 *2.6. In vivo monitoring of the unlabelled thermogel by MRI*

204 Experiments were carried out at 7 T on a vertical imaging spectrometer fitted with an ultra
205 shielded refrigerated 300WB magnet (Bruker, Avance II, Wissembourg, France) equipped with a
206 whole-body 40 mm birdcage RF coil (Bruker) for mouse and Paravision 5.1 acquisition software.

207 For *in vivo* experiments, the mouse was positioned in a specific cradle maintaining the head via
208 a face mask, which provided volatile anesthesia. The mice were anesthetized with 1.3%
209 isoflurane (TEM, Bordeaux) in a mixture of oxygen-air (1:2, 1 L/min). The animal's temperature
210 was monitored and controlled. Breath triggering was performed using a plastic sensor located
211 on the mouse's thorax and the physiological monitoring for respiratory gating (SA Instruments
212 Inc.). A 2D multislice GE sequence (TE = 5 ms) was first used for anatomical location of the
213 tumor. The experimental parameters were as follows: hermitian pulse 3 ms, 30°, TR/TE
214 0.5s/3ms, with 20 slices of thickness of 1 mm, FOV = 3 x 3 cm. Images of 256 x 192 acquisition
215 points were acquired, corresponding to an in-plane resolution of 120 μm x 157 μm . Then T2
216 weighted multiecho SE (RARE, 4 echoes) images with fat suppression were recorded
217 (TR=3000ms, TE = 40ms). The experimental parameters were as follows: hermitian pulse 2 ms,
218 TR/TE 1,5 s/40 ms, RARE factor 4, with a slice thickness of 1 mm, FOV = 3 x 3 cm. Images of 256
219 x 256 acquisition points were acquired, corresponding to an in-plane resolution of 117 μm x 117
220 μm . To understand *in vivo* residence time behaviour, CT26 tumor were bilaterally implanted in
221 the flank of 8 weeks old BALB/cJrj females mice. Seven days after tumor implantation, T-gel or
222 MT-gel (60 μL) was injected intratumorally with the help of a syringe (into the right and left
223 tumor) of the mice (n=3). T2-weighted images were recorded, as described above, sequentially
224 30 min. and 6 h post thermogel injection to evidence the detection of the gel in hypersignal. The
225 area of the gel was obtained after manual segmentation with image J software in the axial slice
226 with the upper level of thermogel into the tumor. The percentage of gel area was calculated as
227 function of the tumor area in the same axial slices.

228

229 *2.7. In vivo monitoring of the labelled thermogel by optical imaging*

230 Mice bearing CT26 tumor were anesthetized by i.p injection of a mixture 10 mg/kg Xylazine and
231 80 mg/kg Ketamine. Cy5-T-gel (60 μL) was injected on the left tumor whereas Cy5-MT-gel (60 μL)
232 was injected on the right tumor. Fluorescence signal was acquired with an epifluorescence
233 system (Fluobeam camera, Fluoptics, France) at various time points post-injection. The signal
234 was quantified over a region of interest (ROI) applied to the tumors using Image J software.

235 Results were expressed as the percentage (%) of the signal taken at t0 as regard to the
236 percentage (%) of the signal at at time t, according to the formula:

$$\text{Percent of signal (\% of } S) = \frac{t}{t_0} \times 100$$

237
238 *2.8. In vivo monitoring of the protein release from the thermogel by optical imaging*
239 Cy5-NHS was coupled to albumin, used as a model protein in PBS pH 7.4 for 30 min at room
240 temperature. Free cyanine 5 was eliminated by ultracentrifugation at 12000 rpm, 4 °C during 10
241 min (6 times). Fluorescence and mass spectrometry confirmed the obtention of the labelled
242 protein (Cyal) (M=67398 Da) (Lemdani et al., 2018).

243 Mice bearing CT26 tumor were anesthetized by i.p.injection of a mixture 10 mg/kg Xylazine and
244 100 mg/kg Ketamine. Cyal (0.5 mg/mL) embedded in the thermosensitive polymeric MT-gel
245 (Cyal-MT-gel) was injected In the left tumor, whereas a solution of Cyal was injected In the right
246 tumor. Fluorescence signal was acquired with a CDDi camera (Biospace Lab, France) at different
247 time points post-injection. The quantification was performed over a region of interest (ROI)
248 applied to the tumors using the M3 vision software. The release data were expressed as the
249 percentage (%) of the signal taken at t0 as regard to the percent of the signal at at time t,
250 according to the formula described in 2.7. part.

251
252 *2.9. Cytotoxicity study*
253 3LL Lewis lung carcinoma cell line (ATCC® CRL-1642™), B16 Melanoma cell line (ATCC® CRL-
254 6323™), CT26 colorectal carcinoma cell line (ATCC® CRL-2638™) and murine NIH/3T3 (ATCC®
255 CRL1658™) fibroblast cells were grown in DMEM medium containing 2 mL-glutamine, 10 % fetal
256 bovine serum (3LL, B16 and CT26) or bovine serum (NIH/3T3), 100 U/mL penicillin, and
257 100 mg/mL streptomycin (37 °C, 5% CO₂). Cells in exponential growth were plated into 96-well
258 plates at 20000 cells per well in 100 µL of culture medium. Twenty-four hours after plating, 60µL
259 of T-gel, MT-gel or water were added on the cells, and incubated for 24 h at 37 °C and 5% CO₂.

260 After this exposure period, cell viability was assayed using the MTT test. The 3-(4, 5-
261 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reagent (0.5 mg/mL) was added in each
262 well and the plates were incubated 2 h at 37 °C. Then, the supernatant was carefully removed,
263 and the formazan precipitate was solubilized in 100 µL of DMSO after 10 to 15 min agitation.
264 Finally the absorbance was read at 562 nm with spectrophotometer (Plate reader Infinite 200
265 PRO - Life Sciences - Tecan) and the cell viability percent was calculated as regard to the value of
266 the control (water for injectable preparation, 0.2 µm filtration).

267

268 *2.10. In vitro Bone marrow dendritic cells generation*

269 The bone marrow-derived dendritic cells (BMDCs) generation protocol was adapted from the
270 procedure described by Lutz et al (Lutz et al., 1999). In brief, bone marrow from femurs and
271 tibias of BALB/cJrj mice were taken under sterile conditions. The red blood cells were lysed with
272 ammonium chloride buffer and the cells were cultured in RPMI medium supplemented with
273 10% FBS, 1 % PS, 1 % L-Glutamine and 20 ng/mL GM-CSF. Cultures were initiated by incubating
274 2×10^6 bone marrow cells in 10 mL of medium on 100 mm × 15 mm petri dishes (P5606-400EA,
275 Sigma Aldrich. France). At day 3, an additional 7 mL of fresh complete medium was added. At
276 day 6, the suspended BDMC cells were collected for further analysis.

277

278 *2.11. In vitro activation of BMDCs*

279 Empty gel (MT-gel) or MTI-gel containing either GM-CSF (5 µg) or HKMT (10 µg) or the
280 combination of GM-CSF (5 µg) and HKMT (10 µg) were prepared. The transwell plates with
281 0.45 µm pore filters were used to study BMDCs migration and maturation. The thermogels were
282 placed in the lower compartment of the transwell, whereas 2×10^5 BMDCs were seeded in the
283 upper compartment. The cells which migrated into the lower compartment were collected 24h
284 after incubation and labelled with rat anti-mouse PE CD11c (clone HL3), rat anti-mouse APC
285 CD86 (clone GL1) and Hamster Anti-Mouse PerCP-Cy™5.5 CD80. The cells were analyzed with
286 flow cytometry (FACS CANTO II, Becton Dickinson, France).

287 *2.12. Intratumoral injection of the MTI-gel*

288 BALB/cJRj 8 weeks old mice were injected in the right flank with 10^5 CT26 cells as previously
289 described (Seguin et al., 2013). After 11 days of inoculation, the mice were anesthetized under
290 Isoflurane mask. I-solution (GM-CSF-HKMT-Solution), MT-gel or MTI-gel were kept at 4°C and
291 were injected into the tumor using a 23G needle. The maximum volume to be administered
292 intratumorally was defined at 100 μ L by workman et al (Workman et al., 2010). Injection of
293 various volume of hydrogel was tested from 30 -100 μ L. The volume 60 μ L was the most
294 adapted to include immunomodulators and prevent the reflux from the tumor site. The tumors
295 were measured every three days and their volume estimated with the formula:

$$Tumor\ Volume\ (mm^3) = (W^2 \times L)/2$$

296 The tumor growth is graphically represented as a function of time. When one of the mice was
297 sacrificed because it had reached an ethical limit point, the graphical representation of the
298 entire group was stopped. The median tumor volume of the control group (C) was determined
299 and when it reached approximately 250 mm^3 , the median tumor volume of each treated group
300 (T) were measured, including zeros. The T/C value in percentage is calculated as follows:

$$T/C\ (\%) = \frac{Median\ tumor\ volume\ of\ the\ treated}{Median\ tumor\ volume\ of\ the\ control} \times 100$$

301

302 *2.13. Immunohistochemical study*

303 *2.13.1 Cell staining*

304 Freshly collected tumors were fixed in Zinc and embedded in paraffin, and sections (4 μ m) were
305 realized. Paraffin sections were processed for heat-induced antigen retrieval, incubated with
306 rabbit anti-mouse CD3 antibody, monoclonal rat anti-CD4 and rabbit anti-mouse Fox P3
307 antibody. Staining was visualized by using the peroxidase/diaminobenzidine Rabbit PowerVision
308 kit (ImmunoVision Technologies). For CD8 immunohistochemistry, paraffin sections were
309 incubated with a rat monoclonal anti-CD8 (Neomarkers, LabVision). The membrane signal was

310 revealed with the Polink 2 plus HRP detection kit (GIBCO Labs). All slides were immunostained in
311 cover plates the same day, to guarantee a standardized intensity of staining.

312

313 *2.13.2. Quantification of T cells density*

314 Each slide was examined using a microscope. The lymphocyte density was quantified on
315 ten representative images realized with Leica Digital Microscopes (Leica DM 4000 B, Wetzlar,
316 Germany) at x 20 magnification, with the help of image J software (Schneider et al., 2012). An
317 original macro was created, which was dedicated to segment histologic parameters on the basis
318 of contrast and color. Thus from original picture, a color deconvolution with DAB vectors was
319 applied and the next step of analysis was performed on the red channel obtained. This image was
320 converted in 8-bit and the brightness and contrast was setting from 60 to 100. After binarization
321 of the successive images 3 process was applied "Dilate, Fill Holes and Watershed" then the
322 analysis of particles from 15-200 μm^2 (FOXP3) or 20-200 μm^2 (other cell type) was made.

323

324 *2.14. Statistical analysis*

325 Graph Pad Software was used to analyze data and determine statistical significance between
326 groups. Data are shown as the means \pm SEM. Mann-Whitney test was used to compare the
327 difference between two groups. One way ANOVA test with Bonferroni posttest was used for
328 multiple comparisons P values < 0.05 were considered significant. *The symbol meaning : ns non*
329 *significant P > 0.05, * P \leq 0.05, ** P \leq 0.01, *** P \leq 0.001.*

330

331 **3. Results**

332 **3.1. Formulation, mucoadhesion and cytotoxicity**

333 *3.1.1. Formulation*

334 P407 concentration was adjusted to reach a gelation temperature (T_g) ranging between 18 and
 335 25°C and a constant of elasticity G' above 10,000 Pa. The appropriate P407 concentration was
 336 fixed at 21 % w/v according to our previous study (Zeng et al., 2014). In order to endow the
 337 P407-based gel with bioadhesive properties, various amounts of Satiaxane gum (Sx) were added
 338 ranging from 0.05 up to 0.3 % w/v. The maximum proportion of Sx that can be added while
 339 remaining within the viscosity specification in the sol state was found to be 0.1 % w/v.

340 In table 2 are presented the measured parameters T_g , G' , G'' , and $\tan\delta$ of P407 (T-gel) and
 341 P407-Sx (MT-gel) gels at 37°C. For both formulations, the T_g value was determined for $\tan\delta = 1$
 342 and found to be very close to 21°C, interestingly about 16°C below the physiological
 343 temperature. At 37°C, i.e., in the gel state, G' was found higher than 10,000 Pa, reflecting strong
 344 elastic properties, moreover $\tan\delta$ is close to 0, highlighting a strong solid behavior. Therefore,
 345 both formulations met the appropriate rheological specifications, without noticeable impact
 346 caused by the satiaxane gum in terms of gelling temperature or viscoelastic properties.

Composition (%w/v)	T_g (°C)	G' at 37°C (Pa)	G'' at 37°C	$\tan\delta$ at 37°C
T-gel P407 21%	21.0 ± 0.010	21400 ± 500	1100 ± 250	0.05 ± 0.001
MT-gel P407 21% Sx 0.1%	21.2 ± 0.005	21900 ± 150	600 ± 200	0.03 ± 0.001

347
 348 **Table. 2.** Values of the temperature of gelification (T_g), the elastic modulus (G'), the viscous modulus
 349 (G'') and $\tan\delta$ of the gel formulations. (n=3, mean ± SEM).

350
 351 *3.1.2. In vitro mucoadhesion*

352 The main objective in adding Sx was to obtain a mucoadhesion *in vivo* and thus ensure longer
 353 residence time of the gel once injected in the tissue to be treated. To evaluate this property, a
 354 rheological method has been adapted from Ponchel et al (Ponchel et al., 1987). In order to
 355 evaluate the adhesiveness and the mucoadhesion of the thermogels, the mobile of the
 356 rheometer covered with a mucin film was put in contact with the support containing the gel
 357 (Fig. 1A). The obtained curves had a profile type IV characterized by four stages: cavitation,
 358 foam formation, elongation, and failure (Schmolka, 1972) (Fig. 1B). It appears in all conditions

359 that the normal force of cavitation reached 100 N and the normal force of elongation amounted
360 to 55 N. Only the duration of the elongation stage depended on the presence of Sx or mucin.
361 When the mobile was coated with mucin, the time to break the interaction increased by a factor
362 5 for T-gel (2.7 ± 0.6 s) and a factor 10 for the MT-gel (6.0 ± 1.6 s). As expected, the gel based on
363 P407 alone had a mucoadhesive character (Dumortier et al., 2006). The addition of Satiaxane
364 increased the *in vitro* mucoadherence by a factor 2 (Fig. 1C).

365 3.1.3. *In vitro* cytotoxicity

366 Prior evaluating the mucoadhesion of the thermogel *in vivo*, we ought to look for the toxicity of
367 the gel *in vitro* (Fig. 2). The effect of T-gel and MT-gel was evaluated on 3 tumor cell lines and 1
368 fibroblast cell line as regard to water, which is the solvent contained in the gel. No significant
369 difference was found between the formulations and water, indicating an absence of toxicity
370 related to the gel components.

371

372 3.1.4 *In vivo* mucoadhesion

373 In order to evaluate if mucoadhesion data obtained by rheometry could reflect a mucoadhesion
374 *in vivo*. We performed non-invasive longitudinal monitoring of the gel by MRI. T-gel and MT-gel
375 were injected in CT26 tumors grafted in the flank of mice. We showed in Fig. 3 that the gel could
376 be followed thanks to an hypersignal obtained by T2-MRI. The signal related to the water
377 confined within the gel was therefore taken as a signature of the thermogel. By this method, we
378 could evidence the effect of the mucoadhesive agent on the gel. Indeed, MT-gel could be
379 detected longer within the tumor area than T-gel. Two time-points illustrate this observation
380 (Fig. 3).

381 The difficulty in this experiment is that the signal of water is followed and multifactors might
382 occur, the release of water but also the swelling of the gel. A covalent label of the polymer
383 would lead to more reliable data to follow the gel. Therefore, to confirm that the residence time
384 of MT-gel was longer than the one of T-gel, we labelled poloxamer P407 with a fluorescent dye,
385 cyanine 5 (Cy5) in order to follow the gel by optical imaging. The labeling was realized in three

386 steps from commercially available dihydroxypoloxamer (P407) (Fig. 4A). The labeled polymer
387 has the fluorescence characteristics of the Cy5 dye (λ emission = 640 nm in PBS) . To prepare
388 fluorescent hydrogel (T-gel or MT-gel), one percent of the labeled polymer (4) was mixed with
389 non-labeled polymer during the formulation process and the obtained sol was injected
390 intratumorally. After intratumoral injection, we observed a similar level of fluorescence for both
391 T and MT gel, however 24 h after the injection, a strong difference was noticeable with 54 % of
392 the initial signal for T-gel and 65 % for the MT-gel. The calculated half-life indicated a value of
393 14.20 h for T-gel and 23.91 h for MT-gel, revealing a residence time increased by 1.7 fold when
394 the gel was mucoadhesive. Based on rheological properties, *in vitro* mucoadherence results and
395 *in vivo* residence in the tumor, the MT-gel formulation was selected for the evaluation of *in vitro*
396 and *in vivo* bioactivity studies.

397

398 3.2. Bioactivity

399 3.2.1. *In vivo* release study of Cyal.

400 Cyanin-5 labelled albumin, also called Cyal and described in (Lemdani et al., 2018), was used as a
401 model protein. The *in vivo* release of Cyal was performed by optical imaging. Cyal loaded MT-gel
402 was injected into the left tumor and Cyal solution in the right tumor of CT26 bearing mice. In
403 Fig. 5, a decrease of the fluorescence signal of Cyal solution was observed few hours after
404 injection with a total elimination at 24h. Fifty percent of the signal issued from the Cyal-MT-gel
405 persisted long hours after injection. The half life and the area under the curve of Cyal-MT-gel
406 was 19.2 h and 1565 % x h respectively as regard to 5.1 h and 1078 % x h for Cyal solution
407 confirming a sustained released of protein *in vivo* using the thermogel.

408

409 3.2.2 *In vitro* DCs activation

410 To activate DCs, the immunostimulatory protein GM-CSF and the killed bacteria HKMT were
411 entrapped within the MT-gel in order to form an immune stimulating thermogel (MTI-gel). The
412 inclusion of these molecules within the gel did not alter the transition phase of the gel using

413 differential scanning calorimetry (DSC). No effect was found at the onset of micellization
414 (Supplementary information Fig. S1). The capacity of non-entrapped HKMT to activate bone
415 marrow-derived dendritic cells (BMDCs) as compared with a positive control LPS
416 (lipopolysaccharide) was first tested. We found a similar level of CD80⁺ (61.7 vs 50.8 %) and of
417 CD86⁺ (58.9 % vs 50.6 %) expression in HKMT and LPS conditions, respectively (Supplementary
418 information Fig. S2).

419 After these preliminary tests, MT-gel, GM-CSF-MT-gel, HKMT-MT-gel or MTI-gel were deposited
420 on the lower compartment of transwells. The DCs generated from bone marrow were seeded
421 on the upper compartment (Fig. 6A). The cells which crossed the membrane of the transwell
422 were collected and analyzed by flow cytometry for CD80⁺ and CD86⁺ activation markers. The
423 fluorescence intensity quantified in Fig. 6B, showed a high expression of CD86⁺ with the GM-
424 CSF-MT-gel and MTI-gel conditions. The MT-gel and the HKMT-MT-gel did not induce any DCs
425 activation. Interestingly, the expression of CD80⁺ on DCs was higher for the combination of
426 immune stimulating molecules. The double stained populations CD11c⁺ CD80⁺ and CD11c⁺
427 CD86⁺ are presented in Fig. 6C. The co-expression of both CD80⁺ and CD86⁺ on dendritic cells is
428 necessary for T lymphocytes activation. Therefore, we found here that MTI-gel revealed a
429 superior ability to induce dendritic cells maturation.

430

431 *3.2.3. Local effect of intratumoral administration of the MTI-gel*

432 To evaluate the effect of the immunostimulatory gel after intratumoral administration, we
433 injected CT26 tumor bearing mice with MT-gel, a gel containing one or both immunomodulatory
434 molecules (GM-CSF-MT-gel, HKMT-MT-gel and MTI-gel) or the two immunomodulatory
435 molecules in solution (I-solution) (Fig. 7). The various treatments did not show a significant
436 influence regarding the body weight apart from the control group which showed a decrease of
437 **weight** 20 days after tumor implantation, due to the tumor toxicity (Fig. 7A). Regarding the
438 tumor growth represented Fig. 7B, solely the complete treatment with both immunomodulatory
439 molecules was able to decrease the tumor growth. In fact, a median tumor volume of 58.5 mm³
440 for I-solution and 19.4 mm³ for MTI-gel compared with 235,1 mm³ for the control group were

441 measured at day 15 (Table 3). These data led to a growth delay of 5 days for the MT-gel
442 embedded one immunomodulatory molecules, 7 days for those two in solution and greater
443 than 31 days for the MTI-gel.

444

	Median volume tumor (mm ³ , d15)	T/C % d15	Time for median tumor to reach 250 mm ³ (day)	T-C (day)
Control	235,1		15	
I-solution	58,8	25,01	22	7
MT-gel	82,5	35,09	20	5
GM-CSF-MT-gel	212	90,18	17	2
HKMT-MT-gel	65,9	28,01	20	5
MTI-gel	19,4	8,25	>31	>16

	MT-gel vs					GM-CSF-gel vs				HKMT-gel vs			MTI-gel vs	
Days	control	GM-CSF-gel	HKMT-gel	MTI-gel	I-solution	control	HKMT-gel	MTI-gel	I-solution	control	MTI-gel	I-solution	control	I-solution
8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
11	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
13	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
15	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns
17	ns	ns	ns	ns	ns	***	ns	ns	**	ns	ns	ns	ns	ns
20	***	ns	ns	ns	ns	***	ns	ns	ns	***	ns	ns	***	ns

445 **Table 3.** Effect of thermogel intratumoral injection

446 **Table 4.** Statistical analysis of the effect of thermogel intratumoral injection. Two-way ANOVA with
447 Bonferroni posttests was performed, n=6, ns non significant, * P<0.05, ** P <0.01, ***P <0.01

448

449 3.2.4. Local immune infiltration after MTI-gel intratumoral administration

450 In order to analyze the immune environment within intratumoral administration of the MTI-gel,
451 CD3 cells, effector T cells (CD4+), cytotoxic T cells (CD8+), regulatory T cells (FoxP3) were
452 analyzed with immunohistochemistry (Fig. 8A). We observed a significant increase of CD3 and

453 CD8 cell density on the tumor with injection of MTI-gel as compared to MT-gel. There was no
454 difference on CD4, FoxP3 cell density on the two conditions (Fig. 8B).

455 **4. Discussion**

456 In this study, we proposed a mucoadhesive thermogel for the intratumoral delivery of GM-CSF
457 and HKMT. These immunomodulatory agents are known to induce the recruitment and
458 maturation of DCs, which are necessary to activate T cells and induce an effective immune
459 response (Palucka and Coussens, 2016). Local immunomodulation has shown extensive
460 interests these last years (Marabelle et al., 2017; Mazzolini et al., 2005; Narita et al., 2015; Park
461 et al., 2018; Sangro et al., 2004; W. Tong et al., 2012). The interest of poly (ethylene oxide)–poly
462 (propylene oxide)–poly (ethylene oxide) (PEO-PPO-PEO) triblock copolymers based gels for
463 human use is now clearly established (Jeong et al., 2012). Among these, the poloxamer P407 is a
464 FDA approved water-soluble polymer (Matthew et al., 2002). When dissolved in water at a
465 specific range of concentration, poloxamer P407 exhibits thermosensitive properties attributed
466 to its chemical structure which explain its widespread use as a drug delivery system for
467 controlled release applications (Hoare and Kohane, 2008; Jeong et al., 2012).

468 Properties of bioadhesion can be provided to the gel by mucoadhesive agents for the purpose of
469 extending the residence time at the injection site (Khutoryanskiy, 2011; Madsen et al., 1998).
470 Based on our previous local delivery studies using P407 (Zeng et al., 2014), we chose to
471 strengthen the residence time of the thermogel by the addition of mucoadhesive agents within
472 the gel (Zeng et al., 2014). Satiaxane was chosen for the viscosity it will bring to the gel as well
473 as its capacity to interact with mucins (Ceulemans et al., 2002). Adapted rheological studies
474 were performed to quantify the bioadhesion by means of the gel failure upon destroying its
475 interaction with mucin. Mucin was chosen as an adequate matrix overexpressed in colorectal
476 tumors (Niv and Rokkas, 2018). Based on the rheological and mucoadhesion properties, the MT-
477 gel was chosen, owning a fitting cohesiveness, hardness and mucoadhesion. In order to insure
478 that these *in vitro* results would reflect a mucoadhesion *in vivo*, the residence time of the gel
479 was evaluated by means of two non-invasive imaging techniques, MRI and optical imaging, post
480 intratumoral injection. By both methods, we observed a persistence of the mucoadhesive gel.

481 Interestingly, MRI allowed the detection of confined water within the gel without any labelling.
482 With this method, we could follow the early time after injection to evaluate the behavior of the
483 gel and in particular its spilling. However, after few hours, other mechanisms interfere such as gel
484 swelling and volume reduction. This internalisation and release of water of the gel impacts the
485 MRI signal and would require more development to explain the signal related mechanism. Then,
486 P407 was labelled with Cy5 for optical imaging studies. The migration of the labelled P407 from
487 the gel could induce false data interpretation but it seems, that it remained within the gel as we
488 could evidence that the MT-gel was still present 5 days after injection in the tumor.

489 Release studies and kinetic of gel stability are usually evaluated *in vitro* using flow-through
490 method operated in open-loop mode capable of maintaining a continuous flow of fresh
491 dissolution medium (Gao, 2009). However, data obtained are relative results based on the
492 experimental conditions. In order to evaluate the gel elimination and protein release in relevant
493 conditions, we performed these experiments *in vivo* using labelled protein. As regard to Cyal,
494 only one Cy5 was grafted to avoid any aggregation of the protein and modification of its
495 structure. This was controlled by means of flow field flow fractionation coupled with multi-angle
496 light scattering (Salmon et al., 2019). Using optical imaging, we observed that the signal of Cy5
497 labelled protein incorporated in the mucoadhesive gel persisted 7 days after intratumoral
498 injection.

499 Based on these results, we could therefore argue that the mucoadhesive thermogel studied
500 present required qualities for local immunomodulation. Still, some parameters remain to be
501 optimized such as the process of DCs maturation which is currently greater than 100 hours and
502 a life expectancy of only few days for matured DCs that migrate to lymph nodes (Sallusto and
503 Lanzavecchia, 2002). Thus, a prolonged release of immunomodulatory agents on the tumor
504 environment over 100 hours could improve the maturation of DCs and increase the proportion
505 of migrated cells to the lymph nodes.

506 The effectiveness of DCs maturation is dependent on the bioactivity of the immunomodulatory
507 agents after gel incorporation. We demonstrated the ability of the gel containing GM-CSF and
508 HKMT to increase the maturation of DCs. Indeed, the expression of CD86⁺/CD80⁺ costimulatory

509 factors on matured DCs was higher after incubation with the complete gel formulation as
510 compared to gel containing GM-CSF or HKMT alone. The expression of co-stimulatory factors is
511 necessary to activate naïve T cells (Madsen et al., 1998). It was demonstrated that CD86⁺/CD80⁺
512 expression by DCs is reduced in GM-CSF-deficient mice (Madsen et al., 1998). DCs interaction
513 with mycobacteria family is known to increase the surface density of molecules such as MHC II,
514 CD80⁺, CD86⁺, and IL-12 production that are involved in interaction with T cells (Sinha et al.,
515 2007).

516 The intratumoral administration of the immunomodulators in the gel led to an inhibition of
517 tumor growth, while the administration of a solution of these immunomodulatory agents
518 demonstrated minimal effects. Indeed, the injection of the solution in the tumor increased the
519 concentration of GM-CSF on the tumor microenvironment and probably caused the protein
520 leak into the circulatory system, which induced immunosuppressive effects as described by
521 several studies (Clive et al., 2010; Parmiani et al., 2007; Serafini et al., 2004). We also showed
522 that the local delivery of GMCSF alone was not sufficient to induce an immune response, the
523 combination of the immunomodulators was required to obtain a complete response. The
524 rationale of GMCSF and HKMT combination is not demonstrated. In preclinical studies,
525 vaccination with a BCG-secreting GM-CSF showed an increased DCs maturation through CD11c
526 MHCII_ and CD11c CD11b F480 expressing cells and IFN γ secreting T cells compared with the
527 use of the BCG alone. This vaccination resulted in the enhanced expression of co-stimulatory
528 molecules on DCs in the draining lymph nodes. The strategy combining BCG and GMCSF resulted
529 in a 10-fold increase in protection against disseminated *M. tuberculosis* (Ryan et al., 2007).
530 GM-CSF is expressed following infection with *Mycobacterium tuberculosis* and is necessary to
531 restrict *M. tuberculosis* growth in experimental models (Rothchild et al., 2017). Mycobacteria
532 increase GM-CSF mRNA and protein expression in a dose dependent manner. Secretion of GM-
533 CSF, mediated via the PI3-K, MEK1 and p38 MAPK-associated signaling pathway. Induction of
534 GM-CSF mRNA expression peaked 6 h after infection, and returning to its basal levels at 72 h
535 (Cho et al., 2013). This short constitutive expression kinetics of low level of GMCSF may explain
536 the failure of HKMT alone to induce an effective immune response in our study. In our recent
537 published study, we showed that association of radiofrequency with *in situ* modulation using

538 BCG and GM-CSF resulted in the complete cure of distal metastases (Lemdani et al., 2019).
539 According to the obtained results in this study and in order to avoid BCG side effects, HKMT
540 could be proposed to replace BCG in preclinical studies.

541

542 **5. Conclusions**

543 This study finally shows that *in situ* sustained delivery of HKMT and GMCSF related to the use of
544 mucoadhesive poloxamer allows readjustment of the doses and dynamics of the two adjuvants
545 needed for DCs recruitment and maturation. These results confirmed the need of a sustained
546 delivery form to control the delivery of GM-CSF and avoid systemic side effects. We have shown
547 a control of tumor volume mediated by cytotoxic T cells infiltration on the tumor with MTI-gel
548 treatment. In human, involvement of tumor infiltration of T cells to obtain an antitumor
549 immune response is considered to be predictive factor, associated with long-term survival of
550 patients (Emile et al., 2017). Thus, the MTI-gel designed in this study could be a vector for the
551 sustained release of immunomodulatory molecules in the tumor microenvironment to improve
552 the antitumor immune response.

553

554 **Author contributions**

555 NM VB and RM designed the thermogel and managed the project. CL and CAS performed
556 rheological studies. CR labelled the poloxamer with Cy5. BTD CL and JS did MRI experiments. KL
557 and JS did *in vivo* experiments and IHC analyses. KL did the cellular *in vitro* tests. CC participated
558 to flow cytometry analysis. KL RM JS and NM wrote and the manuscript. CL and VB participated
559 to manuscript edition.

560

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569

570 **Conflict of interest**

571 Authors declare that they have no conflict of interests.

572

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Figures caption

Fig. 1. *In vitro* mucoadhesion test. (A) Mucoadhesion tests were carried out using a Rheometer (Anton Paar rheometer, model MCR 102, fitted with a 50 mm plane-plane mobile geometry) as described in methods (B) Detachment curves profile (C) Detachment curves of P407 (T-gel) and P407-Sx (MT-gel) formulations without (-) or in the presence of mucin (+) as function of time (n=3).

Fig. 2. Cell viability after exposure to H₂O (Control), T-gel or MT-gel of 3LL, B16, CT26 and NIH3T3 Cell lines. Data was expressed by Mean \pm SEM (n = 6), $P > 0.05$ ns, Two-way ANOVA with Bonferroni posttests was realized with GraphPad prism software.

Fig. 3. (A) *In vivo* T2-weighted MRI images of CT26 tumors 0.5h. (up) and 6 h (down) after injection of T-gel (left) and MT-gel (right). (B) Quantification of the thermogel area inside the tumor (%), Mean \pm SEM, n=4). $P > 0.05$ ns, Two-way ANOVA with Bonferroni posttests was realized with GraphPad prism software.

Fig. 4. Synthesis of the labelled polymer and kinetic of elimination. (A) Synthetic procedure to label the poloxamer 4. i) Poloxamer P407 is first activated to dinitrophenyl carbonate polymer 2. ii) The activated polymer is then converted to diaminocarbamate 3. iii) Finally cyanine labeled poloxamer 4 is recovered after reaction with Cy5-NHS. (B) *In vivo* fluorescence imaging of injected mice, the right tumor received Cy5-T-gel, the left tumor received Cy5-MT-gel. (C) Longitudinal monitoring of the labelled gels when mucoadhesive (black circles dots) or not (white circles dots) in CT26 tumor model (n=6, mean \pm SEM, nonlinear regression one phase exponential decay; $P > 0.05$ ns, Two-way ANOVA with Bonferroni posttests).

Fig. 5. Release kinetic of cyanine labelled protein from the mucoadhesive thermosensitive gel *in vivo*. (A) *In vivo* fluorescence imaging of injected mice at time 0, 2, 6 and 24h after injection. (R) The right tumor received Cyal solution and (L) the left tumor received Cyal-MT-gel. (B) Longitudinal monitoring of labelled protein release when injected as a mucoadhesive

thermosensitive gel (Black squares) or as an aqueous solution (white squares) in CT26 tumor model. The fluorescence signal quantification were expressed as the percentage (%) of the signal taken at t0 as regard to the the signal percent at time t (n=4, mean \pm SEM, non linear regression biphasic fit, $P > 0.05$ ns, Two-way ANOVA with Bonferroni posttests).

Fig. 6. *In vitro* activation of BMDC within the immunomodulatory gel. (A) Schematic representation of the experiment. (B) Flow cytometry quantification of fluorescence intensity of V450-CD80⁺ and APC-CD86⁺ expression in MT-gel (white bar), GM-CSF-MT-gel (clear grey bar), HKMT-MT-gel (dark grey bar) and MTI-gel (dark bar) conditions. (C) CD86⁺ and CD80⁺ populations gated on CD11c⁺ in the different conditions (n=5). Two-way ANOVA with Bonferroni posttests was done with GraphPad prism software.

Fig. 7. Effect of intratumoral administration of MTI-gel. (A) Difference in body weight after intratumoral injection for the Control (clear triangle), GM-CSF-MT-gel (clear diamond), HKMT-MT-gel (clear circle), I-solution (dark diamond), MT-gel (clear square) and MTI-gel (dark square) groups. Tumor growth after administration of those groups. Two-way ANOVA with Bonferroni posttests was performed, n=6, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$.

Fig. 8. (A) Representative images of the CD3, CD4, CD8 and FOXP3 immunostaining of CT26 tumor. Tumors from control, MT-gel and MTI-gel groups were removed 15 days after injection and fixed for further analysis. The black scale represents 50 μ m. (B) Quantification of lymphocytes density on the tumor using macro image J as described on materials and methods; clear grey box represents the MT-gel group and dark grey box represents MTI-gel group. Mann-Whitney test, n=4, # $P < 0.05$, *** $P < 0.01$.

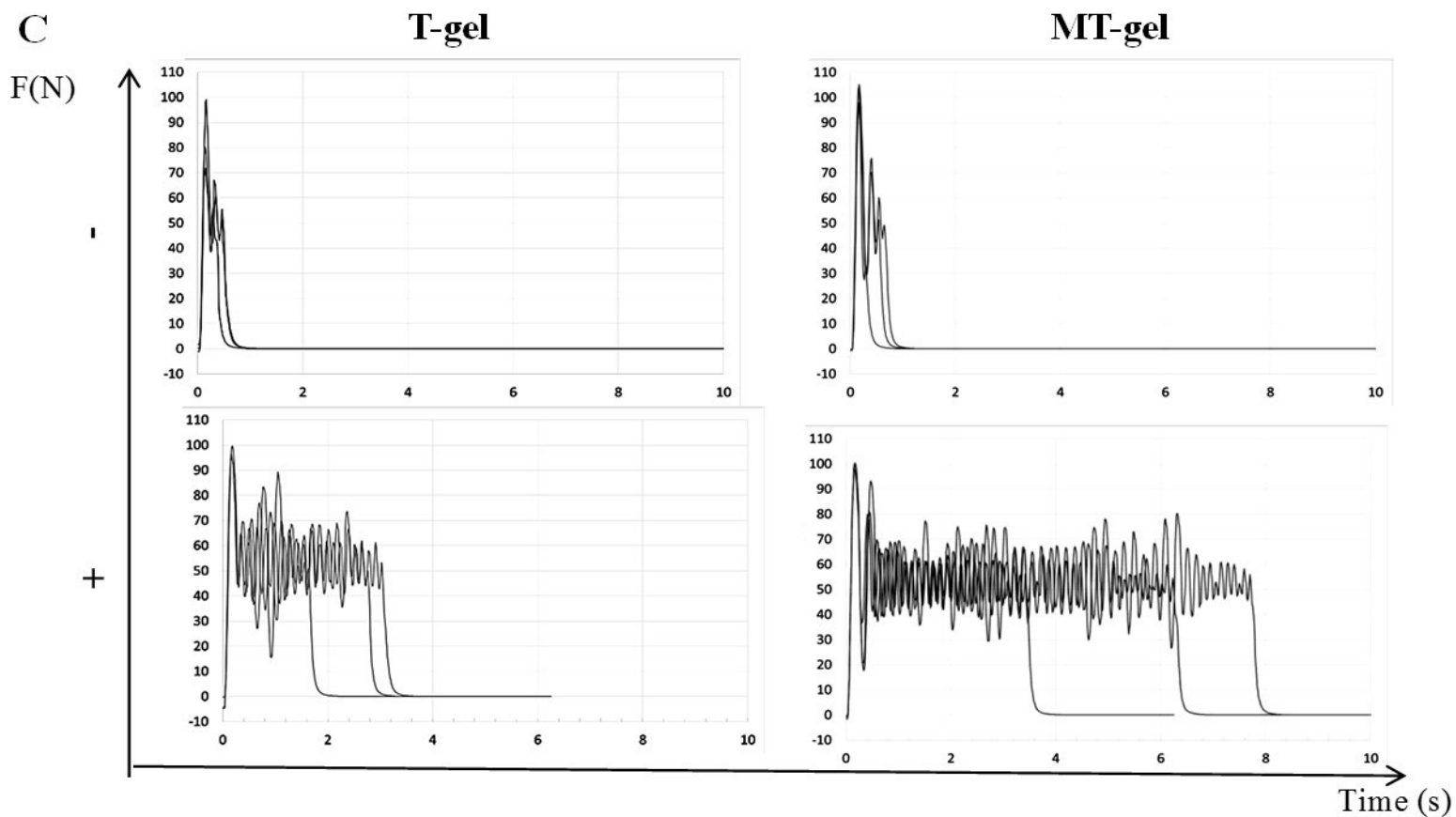
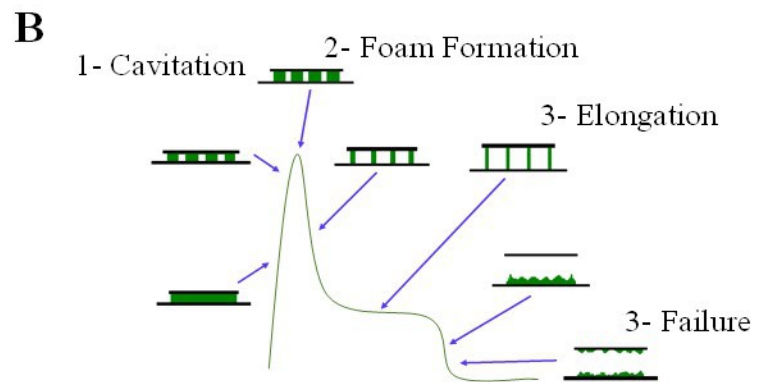
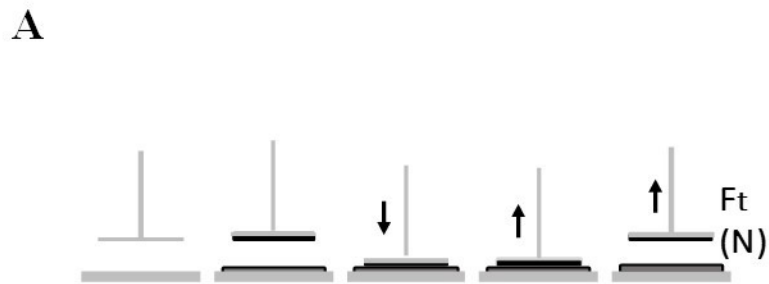


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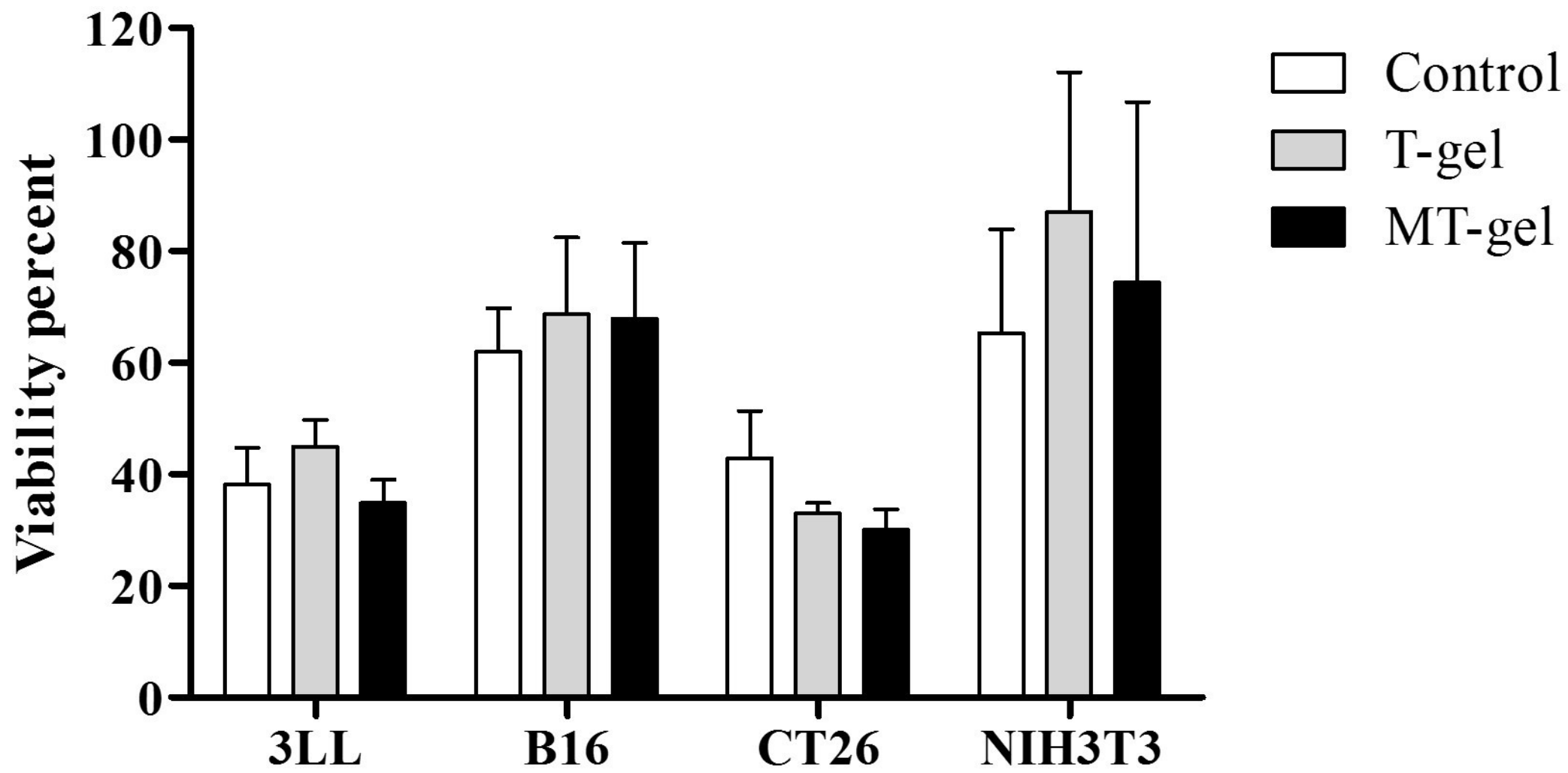


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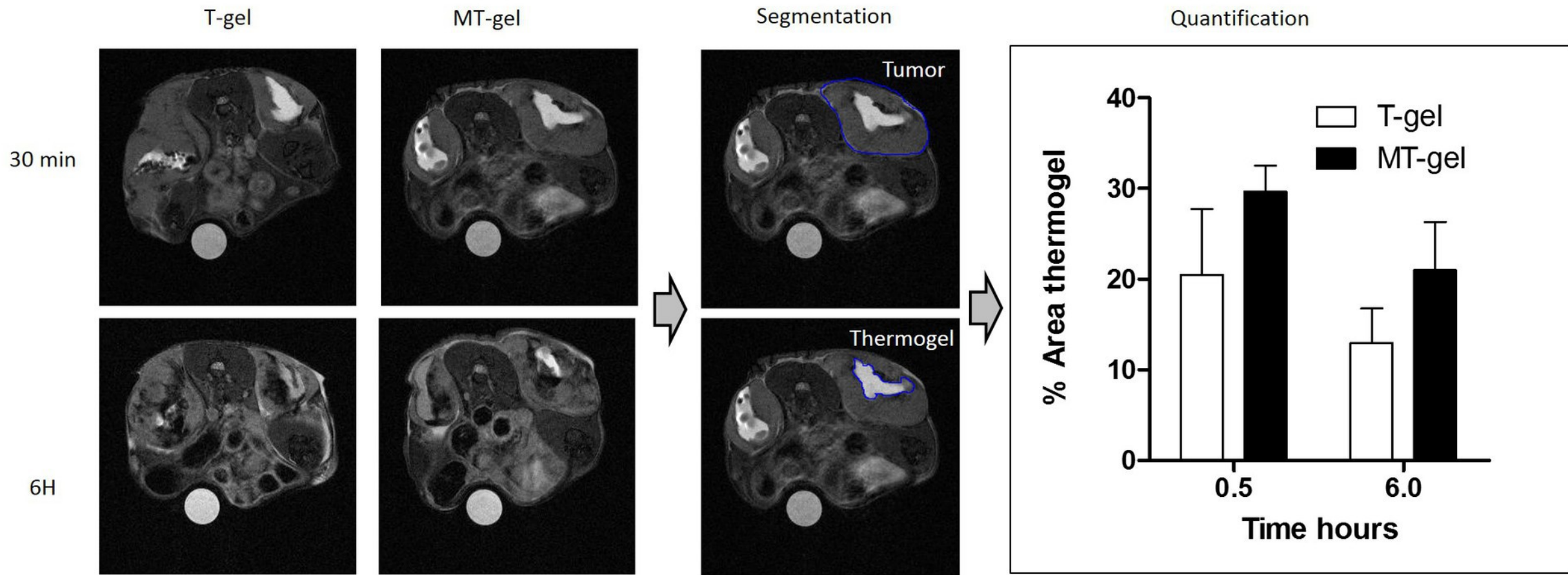


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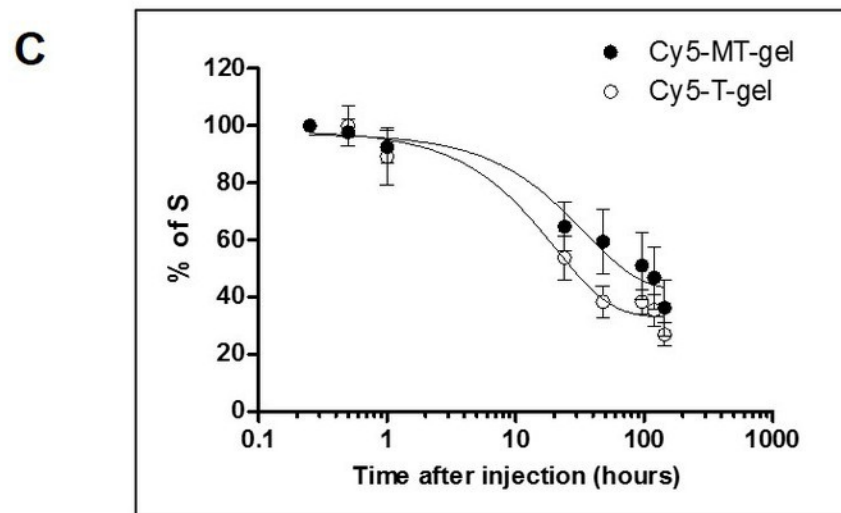
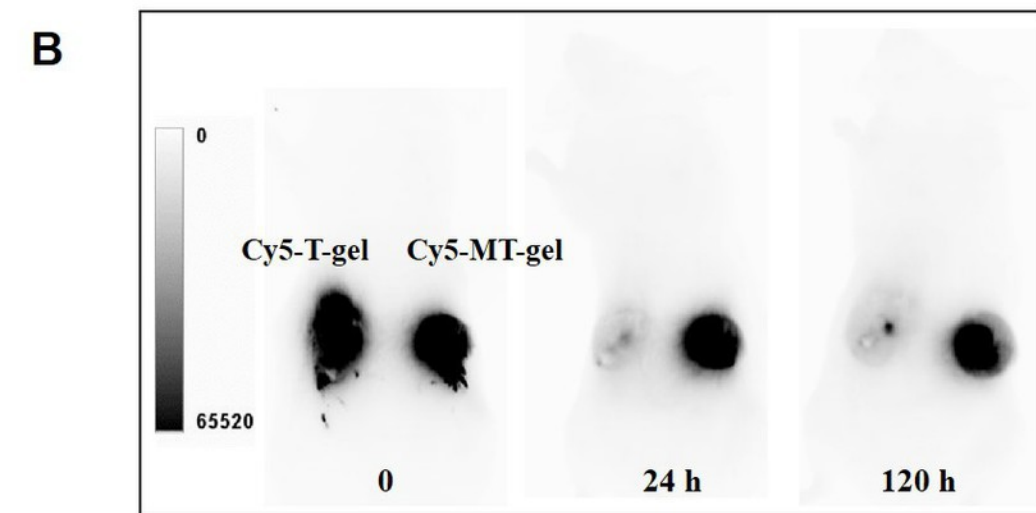
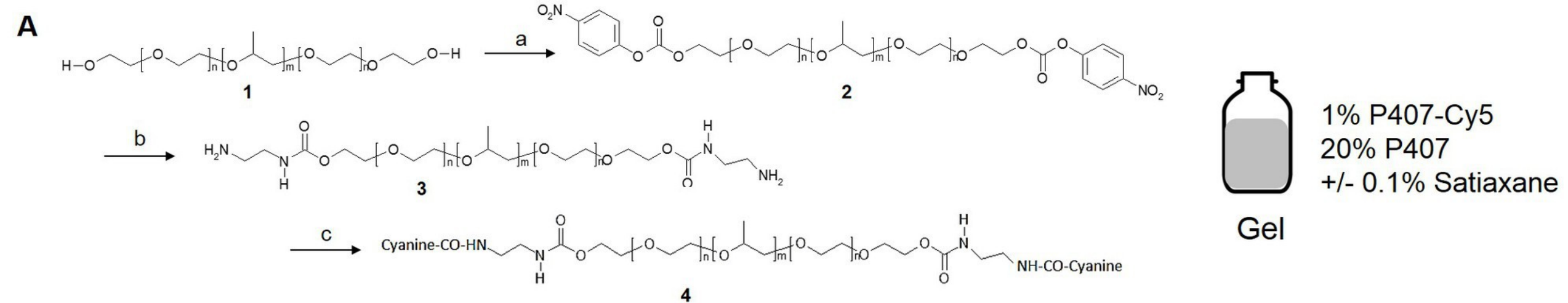


Fig. 4.

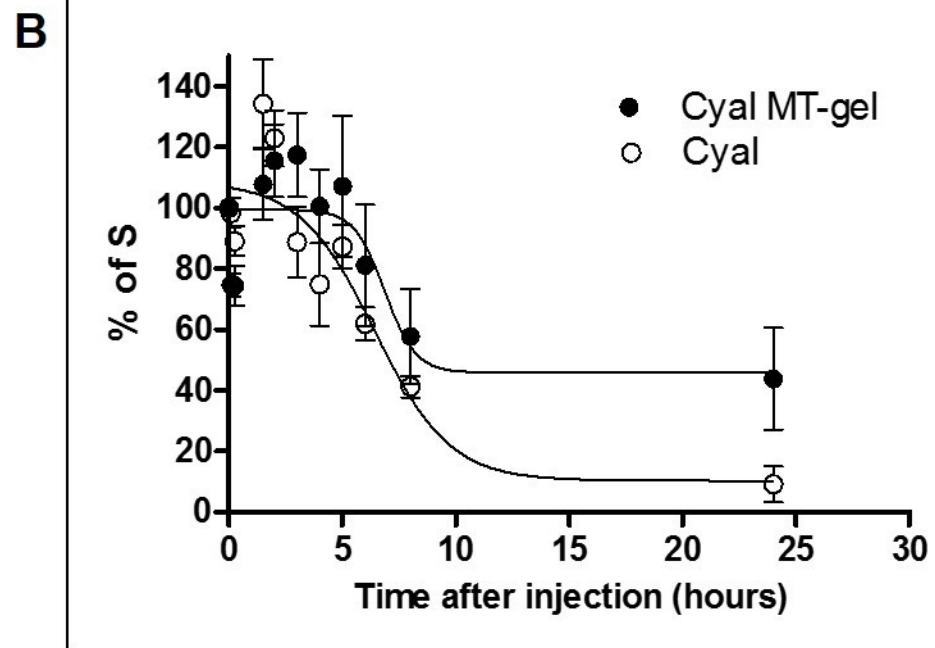
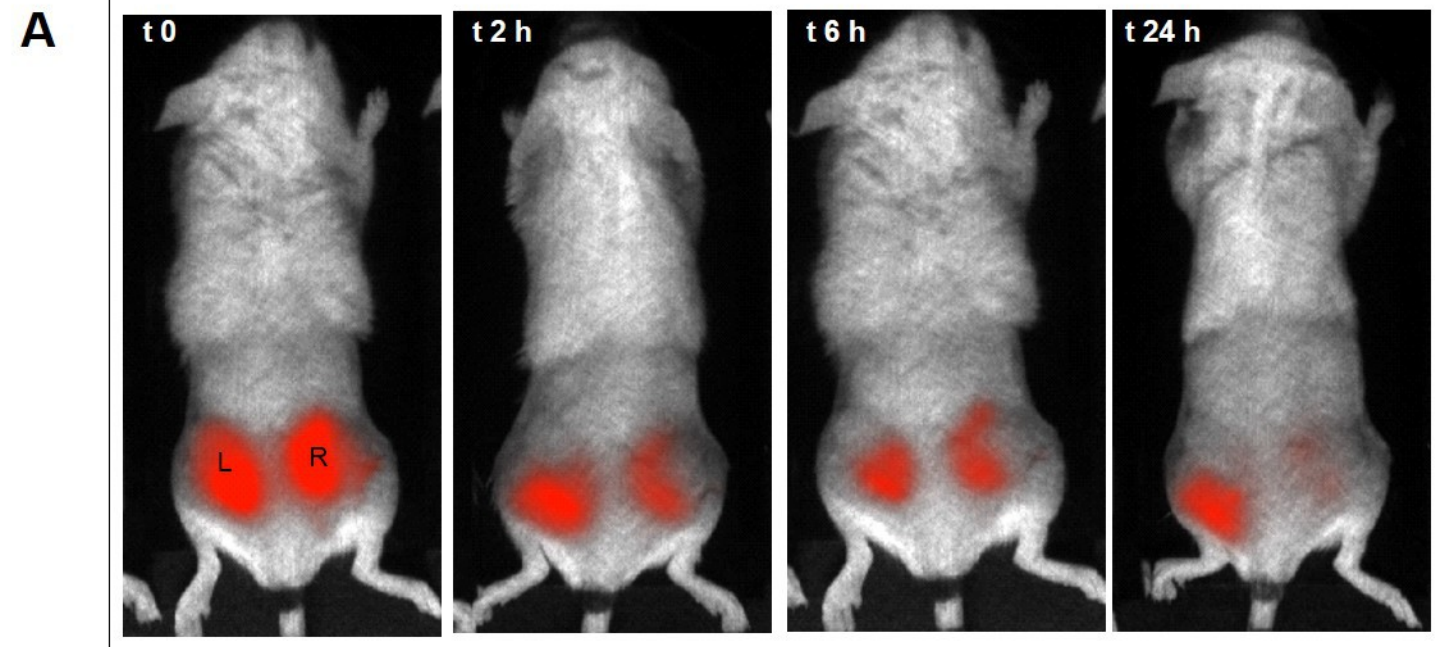


Fig. 5.

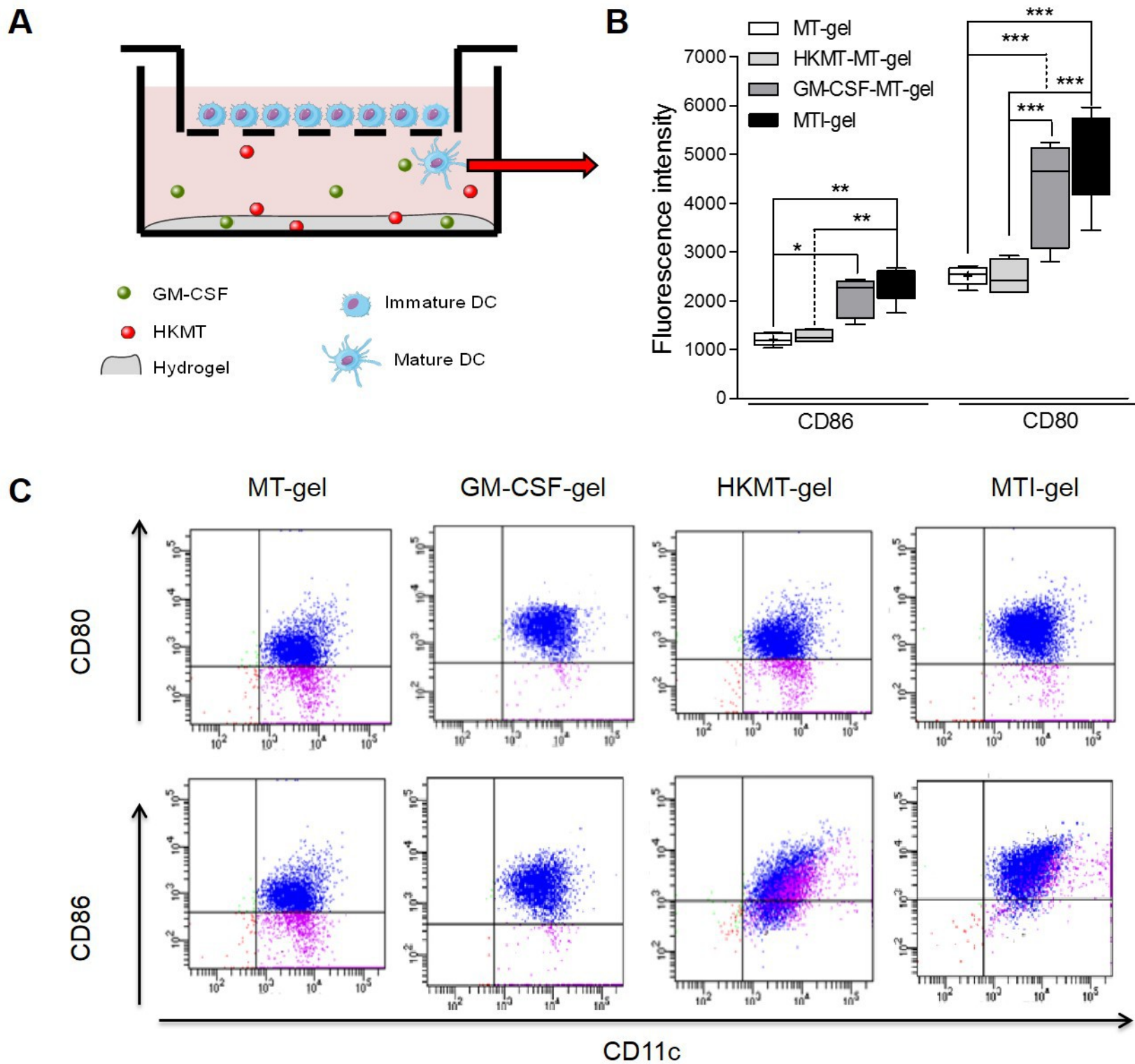


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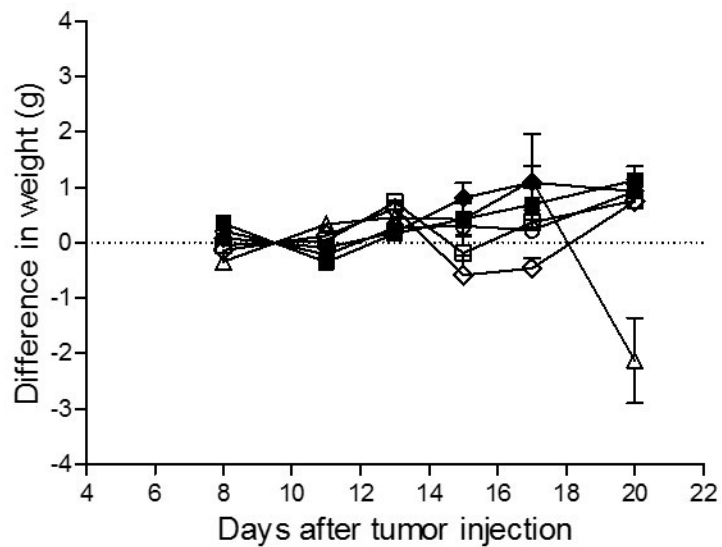
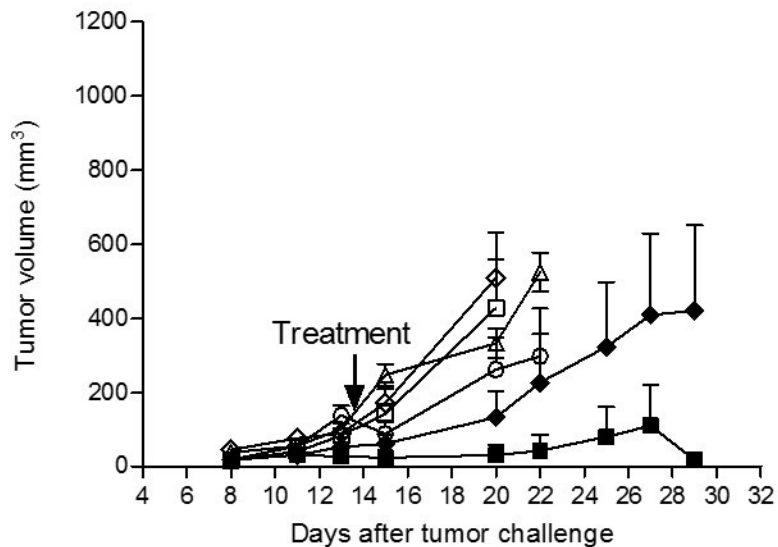
A**B**

Fig. 7.

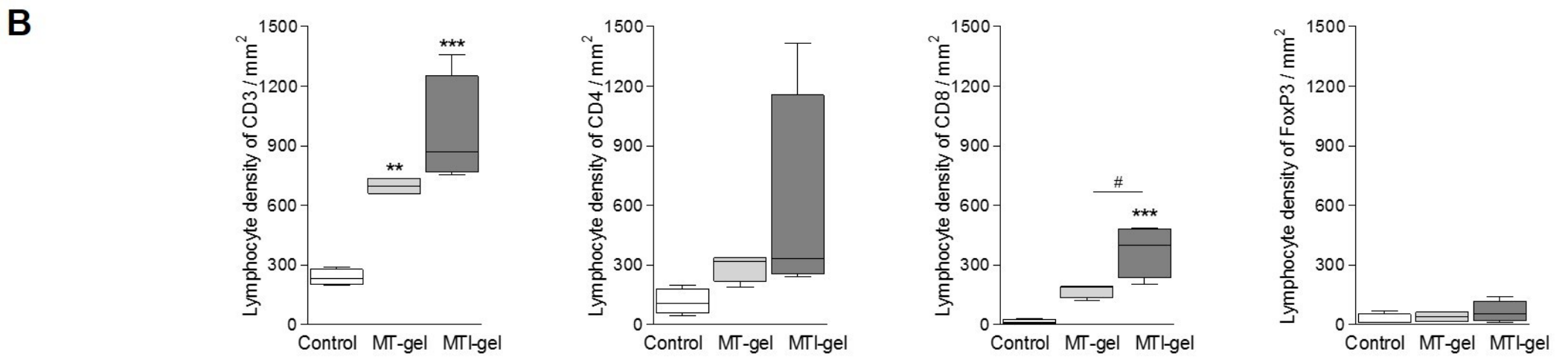
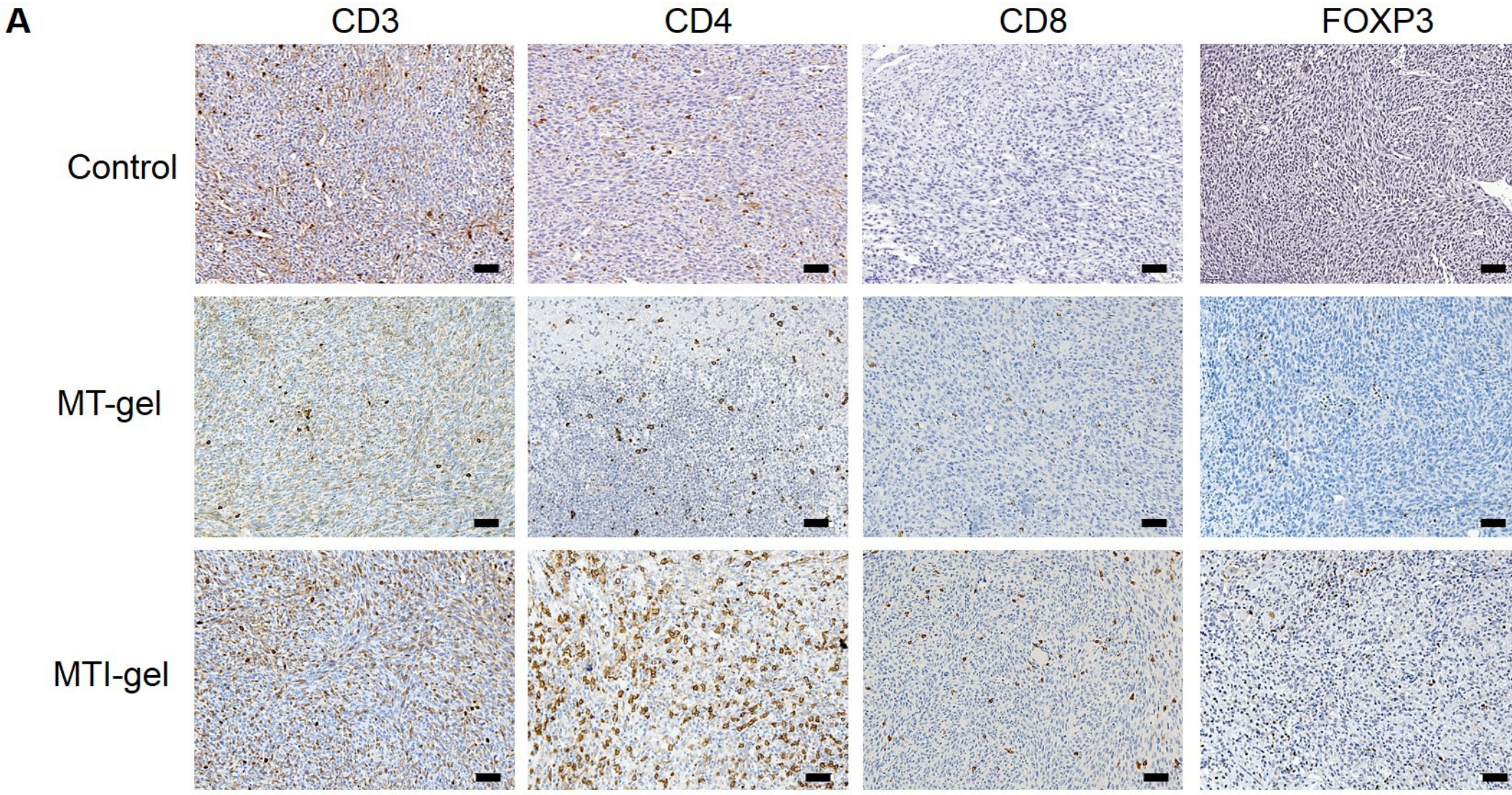
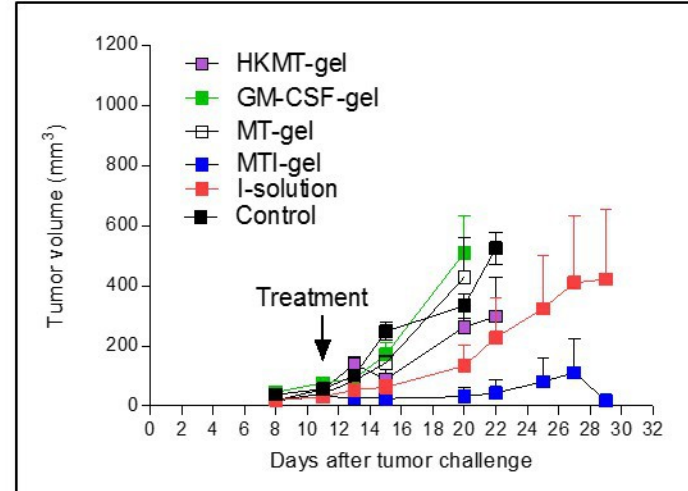
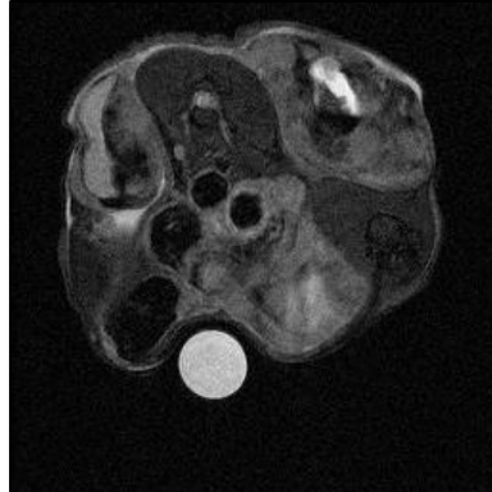
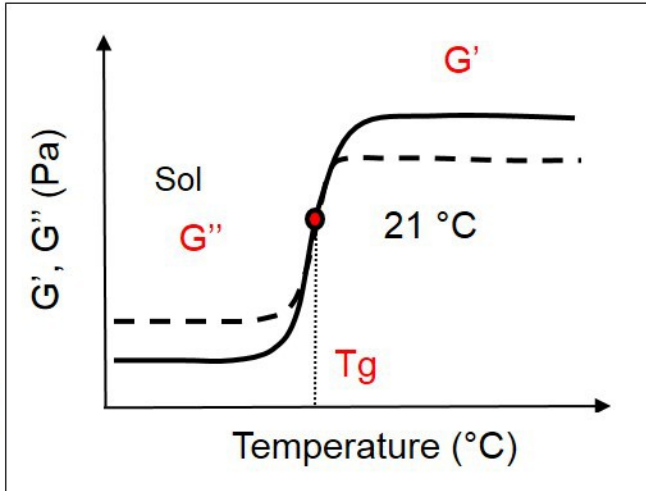


Fig. 8.



Gelification temperature

Gel residence

In vivo effect

Mucoadhesion

Protein residence time

Lymphocyte recruitment



Gel

