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1 Efficient PD-L1 imaging of murine glioblastoma with FUS-aided
2 immunoPET by leveraging FcRn-antibody interaction.

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13 **ABSTRACT**

14 **Rationale:** The passage of antibodies through the blood-brain barrier (BBB) and the blood-
15 tumoral barrier (BTB) is determinant not only to increase the immune checkpoint inhibitors efficacy but
16 also to monitor prognostic and predictive biomarkers such as the programmed death ligand 1 (PD-L1) via
17 immunoPET. Although the involvement of neonatal Fc receptor (FcRn) in antibody distribution has been
18 demonstrated, its function at the BBB remains controversial, while it is unknown at the BTB. In this
19 context, we assessed FcRn's role by pharmacokinetic immunoPET imaging combined with focused
20 ultrasounds (FUS) using unmodified and FcRn low-affinity IgGs targeting PD-L1 in a preclinical
21 orthotopic glioblastoma model.

22 **Methods:** Transcranial FUS were applied over the whole brain in mice shortly before injecting the
23 anti-PD-L1 IgG ⁸⁹Zr-DFO-C4 or its FcRn low-affinity mutant ⁸⁹Zr-DFO-C4^{Fc-MUT} in a syngeneic
24 glioblastoma murine model (GL261-GFP). Brain uptake was measured from PET scans acquired up to 7
25 days post-injection. Kinetic modeling was performed to compare the brain kinetics of both C4 formats.

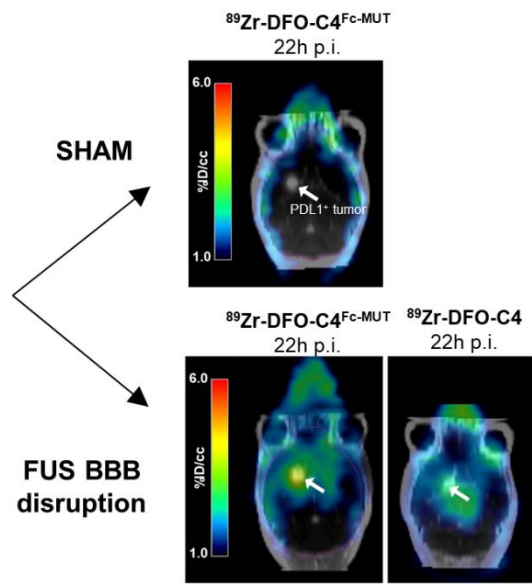
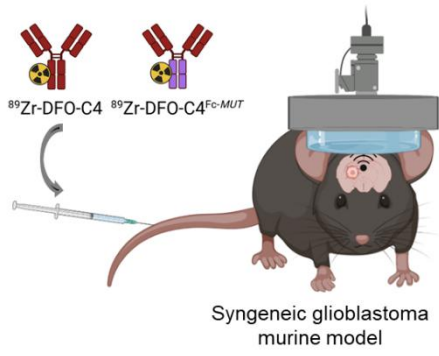
26 **Results:** FUS efficiently enhanced the delivery of both C4 radioligands in the brain with high
27 reproducibility. ⁸⁹Zr-DFO-C4^{Fc-MUT} mean concentrations in the brain reached a significant uptake of
28 3.75±0.41%ID/cc with FUS against 1.92±0.45%ID/cc without, at 1h post-injection. A substantial and
29 similar entry of both C4 radioligands was observed at a rate of 0.163±0.071 mL/h/g of tissue during
30 10.4±4.6min. The impaired interaction with FcRn of ⁸⁹Zr-DFO-C4^{Fc-MUT} significantly decreased the efflux
31 constant from the healthy brain tissue to plasma compared with non-mutated IgG. Abolishing FcRn
32 interaction allows determining the target engagement related to the specific binding as soon as 12h post-
33 injection.

34 **Conclusion:** Abolishing Fc-FcRn interaction confers improved kinetic properties to ⁸⁹Zr-DFO-
35 C4^{Fc-MUT} for immunoPET imaging. FUS-aided BBB/BTB disruption enables quantitative imaging of PD-L1
36 expression by glioblastoma tumors within the brain.

37
38 **Keywords:** ImmunoPET; therapeutic ultrasound; Fc receptor, neonatal; PD-L1, Immune Checkpoint
39 Inhibitors; Glioblastoma.

40 **Graphical abstract:**

41



42
43

44 **INTRODUCTION**

45 Patients diagnosed with glioblastoma (GBM) have a harrowing overall survival of 13 to 16 months
46 following the standard-of-care treatments [1]. GBM is the most common primary brain tumor and is highly
47 aggressive. Immunotherapies have the possibility to be a keystone in eradicating GBM as their
48 microenvironment is immunosuppressive. Ex vivo staining of biopsies have shown that the immune
49 checkpoint programmed death ligand 1 (PD-L1) is expressed by most GBM neoplastic tissues, which
50 suggests a promising role for immune checkpoints inhibitors (ICI) such as anti-PD(L)1 antibodies [2].
51 However, the efficacy of ICI in treating brain malignancies such as GBM has been proven insufficient to
52 improve patient overall survival [3–5]. Durations of response were nevertheless greater in patients treated
53 with the anti-PD1 antibody nivolumab compared to those treated with the standard-of-care in the
54 Checkmate 143 trial (NCT02017717) [4]. Clinical trials notably informed on the need for an effective
55 minimally-invasive method to select patients who will benefit from ICI and monitor the immune response
56 at the central nervous system (CNS) level.

57 PD-L1 expression by neoplastic tissues or its microenvironment is the most predictive biomarker of
58 anti-PD(L)1 therapy response in most tumors [6,7]. It has not been investigated in GBM as PD-L1
59 expression is usually evaluated by immunochemistry on tumor biopsies. Moreover, PD-L1 expression is
60 known to be spatially heterogeneous and to change over time with treatments [8]. Repeated biopsies over
61 the therapy course are neither always feasible nor acceptable, particularly for CNS localizations. In this
62 regard, non-invasive detection of biomarkers by immunoPET has emerged as a powerful tool to monitor
63 response to ICI therapy. ImmunoPET imaging using radiolabeled antibodies targeting PD-(L)1 allows
64 quantification and the *in vivo* assessment of the inter- and intratumoral heterogeneity of the biomarker
65 expression [9]. However, immunoPET imaging within the CNS remains challenging due to the inability of
66 antibodies to cross the blood-brain barrier (BBB). Niemeijer *et al.* and Nienhuis *and al.* obtained uneven
67 accumulation between lesions of the anti-PD1 antibody ⁸⁹Zr-nivolumab or ¹⁸F-adnectins targeting PD-L1 in
68 brain metastases of patients [9,10]. This uptake in some brain metastases but not all within a patient
69 probably reflects the tumor-induced loss in the integrity of the BBB rather than the local expression of
70 targeted biomarkers. It is therefore essential to propose strategies to overcome the BBB and improve the
71 brain kinetics of radiolabeled antibodies to enable quantitative estimation of immune biomarkers such as
72 PD-L1 in infiltrative brain tumors like GBM using immunoPET [11].

73 The engineering of antibodies is currently investigated to improve their brain delivery [12]. This
74 includes modulation of neonatal Fc receptor (FcRn) mediated transcytosis. Reducing the affinity of an
75 antibody for the FcRn has notably been proposed to optimize the peripheral and brain kinetics of
76 radiolabeled antibodies for immunoPET [13]. According to the tissue considered, FcRn is responsible for
77 the recycling and transcytosis of the Fc-containing proteins [14]. FcRn's physiological function is to bind
78 endogenous immunoglobulin G (IgG) and albumin at acidic pH to protect them from lysosomal
79 degradation, maintaining their serum homeostasis [15]. The loss of affinity for the FcRn obtained by
80 substituting two key amino acids of the Fc-domain of an IgG (H310A and H435Q) decreases the plasma
81 half-life of antibodies [16,17]. Besides the advantages for PET imaging, the affinity loss for the FcRn could
82 impact IgG brain distribution. FcRn is highly expressed by the CNS endothelium [18]. Although there has
83 yet to be a consensus regarding the role of FcRn in controlling IgG transport across brain endothelial cells.
84 Some studies on the brain distribution of IgG in FcRn knock-out mice led to the conclusion of an absence
85 of a FcRn mediated transcytosis at the BBB [19,20]. However, those studies are subject to discussion due
86 to potential compensatory mechanisms associated with FcRn depletion. Moreover, some evidence supports
87 a FcRn-mediated efflux of IgG from the brain to the circulation. After intracranial injection, brain clearance
88 of IgG with improved FcRn affinity was faster compared with IgG with reduced FcRn affinity [21]. This
89 property was used effectively to promote IgG-mediated amyloid plaque removal in a mouse model of
90 Alzheimer's disease [22]. To our knowledge, the particular role of FcRn at the blood-tumoral barrier (BTB)
91 has never been investigated.

92 Transcranial application of low-intensity focused ultrasound (FUS) with the injection of microbubbles
93 (MB) was shown to enable the brain delivery of drugs by reversibly disrupting the BBB. Local mechanic
94 and shear stress induced by the oscillations of MB (alternation of expansion and compression of the
95 gaseous core of the MB) loosen the tight junctions and increase the pinocytic activity of endothelial cells
96 [23,24]. FUS are investigated in preclinical models and clinical trials to treat numerous CNS diseases,
97 notably GBM [25–27]. FUS-aided immunoPET was useful in demonstrating the increased brain delivery of

98 radiolabeled antibodies from a therapeutic perspective [28,29]. Meng et al. reported enhanced delivery of
99 trastuzumab across the BBB with magnetic resonance-guided FUS in patients with HER2-positive breast
100 cancer and brain metastases. In this study, the HER2-positive status of the brain metastases was determined
101 on surgical specimens previously collected at different temporality before treatments [30]. The
102 NCT05879120 clinical trial aims to estimate the median overall survival of patients with recurrent GBM
103 with FUS BBB opening and neo-adjuvant pembrolizumab [31]. The inclusion criteria of patients in this
104 study do not include the determination of the brain tumors' PD(L)1 status. FUS-aided immunoPET could
105 provide a companion theranostic approach to assess the likelihood of response of brain tumors to
106 immunotherapy.

107 Here, we aimed to investigate the potential of FUS-aided immunoPET to provide quantitative imaging
108 of PD-L1 expression by glioblastoma tumors within the brain. The human recombinant IgG1, C4, targeting
109 human and murine PD-L1 and its engineered low FcRn affinity (H310A/H435Q) mutant (C4^{Fc-MUT}) were
110 used to perform FUS-aided immunoPET in a syngeneic glioblastoma murine model (**Scheme 1**). Kinetic
111 modeling was performed to interpret brain PET data, estimate PD-L1 expression, and elucidate the
112 importance of FcRn function on the transport of the radiolabeled antibody across the BBB/BTB.

113 114 **METHODS**

115 **Production of the C4 ligands.** C4 is a human recombinant IgG1 cross-reacting with human and murine
116 PD-L1 [32]. The C4 ligands production has been performed as already described in Bouleau *et al.*[17].
117 Briefly, they were obtained by transient transfection of HEK293 Freestyle™ cells (Thermo-Fisher) with
118 AbVec2.0-IGHG1 and AbVec1.1-IGLC plasmids corresponding to the IgG C4 heavy and light chains,
119 respectively. After transient cell transfection, the supernatant was collected and purified using Lambda
120 FabSelect columns (GE Healthcare).

121
122 **FcRn binding assay.** IgG/human FcRn and IgG/murine FcRn affinity were measured using the Lumit™
123 FcRn Binding Immunoassay kit (Promega) following the protocol described by Nath *et al.*[34]. To measure
124 IgG/mFcRn interaction, hFcRn was substituted by recombinant mFcRn with terminus biotin
125 (ACROBiosystems). The recombinant mFcRn was introduced at a concentration of 0.25 mg/mL.
126 Experiments were run in triplicate. Normalized luminescence data were generated by assigning 100% to the
127 maximum bioluminescent signal obtained in the absence of IgG.

128
129 **Radiolabeling.** The anti-PD-L1 C4 ligands were radiolabeled according to a previously published protocol
130 [17]. First, the p-isothiocyanatobenzylferrioxamine (p-NCS-Bz-DFO, Macrocylics) was conjugated to
131 the C4, and then the radiolabeling with ⁸⁹Zr-oxalate (PerkinElmer) was performed. After the purification of
132 the DFO-anti-PD-L1 ligands with a PD-10 column (GE Healthcare), DFO-anti-PD-L1 ligands were
133 incubated with [⁸⁹Zr]Zr-oxalate for 1 h at 37°C. The ⁸⁹Zr-labeled DFO-anti-PD-L1 ligand conjugates were
134 then purified with a PD-10 column and buffer exchanged in HEPES solution (Gibco) with a Vivaspin
135 centrifugal concentrator (Sartorius). Radiochemical purity was assessed by instant thin-layer
136 chromatography (iTLC) and high-performance liquid chromatography (HPLC) analyses (Figure S1).

137
138 **Cell culture.** Murine glioma's cell line GL261 transfected to produce Green Fluorescent Protein (GL261-
139 GFP) were obtained from the Institute of Neurophysiopathology, Aix-Marseille University. Cells were
140 cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10 % heat-inactivated fetal
141 bovine serum and 1% penicillin-streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 95%
142 air and 5% carbon dioxide.

143
144 **Animals.** Animal experiments were performed on six weeks old female C57BL/6 NRj mice (Janvier Labs).
145 Animal experiments were performed according to the European Directive 2010/63/EU and its transposition
146 into French law (Decree No. 2013-118). The research project was conducted at the CEA-SHFJ imaging
147 platform (authorization D91-471-105) and was approved by a local ethics committee (CETEA-CEA DSV
148 IdF). Mice were housed in standard conditions (microisolator polycarbonate cages, aspen wood as bedding
149 material, 5 mice in each cage, room temperature 22 °C, humidity 40%) under a regular 12-h dark/light
150 cycle. Food and water were available ad libitum.

152 **GL261 orthotopic model.** 24 mice were orthotopically implanted with the syngeneic cell line GL261-GFP,
153 $5 \cdot 10^4$ cells in 1 μ L PBS into the striatum. With bregma as origin, implantations coordinates were X= 0mm,
154 Y=+2mm, Z=-3mm. Mice were anesthetized with isoflurane (3% for induction and 2% for maintenance) in
155 100% O₂. 0.05 mg.kg⁻¹ of buprenorphine was subcutaneously administered at the end of the intervention to
156 prolong analgesia.

157
158 **MRI.** 14 days after GL261 implementation, anatomical T2-weighted and T1-weighted contrast-enhanced
159 MRI were acquired with a 7T/90mm bore hole MRI scanner (Pharmascan scanner, Bruker). A Gadolinium-
160 based contrast agent (Dotarem[®], 1nm diameter, 100 μ L by animal) was intravenously injected via a
161 catheter. T1-weighted images were then acquired (MSME sequence, TE/TR = 8/340 ms, matrix = 256 \times
162 256 \times 64, resolution = 0.15 \times 0.15 \times 0.60 mm³, 10 averages, acquisitions time = 6 min). T2-weighted
163 images were acquired through a RARE sequence (TE/TR = 5/1800 ms, RARE factor = 16, matrix = 256 \times
164 256 \times 64, resolution = 0.12 \times 0.12 \times 0.12 mm³).

165
166 **Blood-brain barrier disruption.** A focused transducer (active diameter 25 mm, focal depth 20 mm, axial
167 resolution 5 mm, lateral resolution 1 mm, Imasonic) centered at 1.5 MHz was used to disrupt the BBB. The
168 transducer was connected to a single-channel programmable generator (Image Guided Therapy) and
169 mounted on a motorized XYZ-axis stage. and positioned above the mouse head maintained under
170 anesthesia with isoflurane (3% for induction and 1.5% for maintenance) in a 50:50 mixture of air-O₂. The
171 device was coupled to the mouse skull using a latex balloon (filled with deionized and degassed water) and
172 centrifugated coupling gel. The distance between the transducer and the skull was adjusted by the
173 displacement of the motorized axis (Z) and the filling of the balloon in order to get the center of the of the
174 brain, at the focal distance (*i.e.*, 20 mm). SonoVue[®] microbubbles (Bracco) were intravenously
175 administrated in the tail vein via a bolus (50 μ L) before the beginning of the FUS or sham sessions. The
176 FUS sequence was similar to the one described in Felix *et al.* and already validated for efficient and safe
177 BBB disruption on healthy mice [28,35]. **Reversibility of the BBB opening within 24h after FUS was**
178 **confirmed in mice using [¹⁸F]2-fluoro-2-deoxy-sorbitol, a PET marker of BBB integrity (Figure S2).**
179 Briefly, the FUS sequence is composed of quasi-continuous ultrasonic waves transmitted with duty cycle of
180 69% at a peak negative pressure of 420 kPa (considering a transmission through mouse's skull of 80% at
181 1.5 MHz). A raster scan (XY-axis) of 6 mm x 6 mm was synchronized to the generator output in order to
182 induce a whole brain BBB opening (Scheme S1). The sequence of 5.1s was repeated 25 times for a total
183 exposure of 126.75 s.

184
185 **microPET/CT imaging.** On day 15 post GL261-GFP implementation, a 60-min dynamic PET scan was
186 performed concurrently with radioligand injections of ⁸⁹Zr-DFO-C4 (3.5 \pm 0.3 MBq, 4.97 MBq/nmol, n=8)
187 or ⁸⁹Zr-DFO-C4^{Fc-MUT} (3.5 \pm 0.5 MBq, 9.89 MBq/nmol, n=16) under camera after the FUS protocol. The
188 radioligand injection was performed quickly after the end (1.7 \pm 0.2 min) of the FUS BBB opening
189 protocol. 20-min static PET scans were subsequently acquired at selected times post-injection (5 h, 22 h, 46
190 h, 70 h, and 7 days). A sham workflow (without emission of ultrasound waves) was applied on 6 tumor-
191 bearing animals before the injection of ⁸⁹Zr-DFO-C4^{Fc-MUT} and imaged accordingly.

192 PET emission scans were performed using an Inveon microPET scanner and an Inveon
193 microPET/CT scanner (Siemens). After each PET scan, a transmission scan or a CT scan were performed
194 for photon attenuation correction. PET images were reconstructed with the Inveon Acquisition Workspace
195 software (2.1) using a three-dimensional ordinary Poisson ordered-subset expectation maximization
196 followed by a maximum a posteriori algorithm (OP-OSEM3D-MAP). Normalization, as well as corrections
197 for dead-time, scatter, decay and attenuation, were applied to all PET data.

198 Dynamic PET acquisitions were reconstructed in 24 frames averaging signal on the period from
199 0.5 to 5 min resulting in a sequence of images of 3x30, 5x60, 5x120, 3x180, 3x240, 4x300, and 1x150 s.

200 Image analysis was performed with the PMOD software (v3.9). A volume of interest (VOI) was
201 defined in the left cardiac ventricle to obtain blood radioligand concentration. MRI and brain PET
202 acquisitions were all repositioned with the T2w MRI as reference. VOI were defined in selected brain
203 areas, defining the T1w contrast-enhanced volume, the PET contrast-enhanced volume, and the
204 contralateral hemisphere. Concentrations in VOI are expressed as percentage of injected dose (%ID/cc =
205 activity (Bq/cc) / injected dose (Bq)) and time activity curves (TACs) were extracted (Table S1-4).

206

207 **Immunofluorescence.** After the last imaging session, mice were sacrificed, and their brains were collected,
208 immersed in isopentane, and frozen in liquid nitrogen. A set of fixed frozen brain sections (10 μm) were
209 incubated with a rat anti-mouse PD-L1 primary antibody (1:500, clone 10F.9G2, Biolegend). The slides
210 were then incubated with an AF546-conjugated donkey anti-rat secondary antibody (1:1000, Jackson
211 Laboratories). Adjacent brain sections were incubated with an AF546-conjugated goat anti-human
212 secondary antibody (1:1000, Jackson Laboratories) to stain the injected C4. Adjacent slides were used for
213 hematoxylin/eosin (H&E) staining. Slides were fixed in neutral buffer formalin 10%, then stained with
214 Harris hematoxylin (Sigma) and Eosin Y (Sigma) according to previously reported protocol [36].
215 Immunofluorescent and H&E stained sections were scanned with a 20x objective using an AxiObserver Z1
216 microscope (Zeiss).

217

218 **Blood pharmacokinetic.** Plasmatic concentrations were calculated from image-derived blood activity
219 concentration drawn in the left cardiac ventricle. Considering that antibodies are restricted to the serum, a
220 blood-to-plasma concentration ratio of 0.55 was used. A bicompartmental model with a first-order
221 elimination function was individually fitted using Phoenix WinNonlin (v.8.3.1, Certara®). Parameters and
222 equations of the model are detailed in supplemental materials. Parameters of the plasma kinetic were intra-
223 individually fixed to apply the same input function to all brain VOI.

224

225 **Brain kinetics.** Brain kinetic parameters were obtained by fitting regional TACs to either a modified 1-
226 tissue compartment model (m1TCM) or a modified 2-tissue compartment model (m2TCM) in Phoenix
227 WinNonlin (v.8.3.1, Certara®). These two models were modified to match the transient character of FUS-
228 induced BBB disruption. Parameters and equations of the model are detailed in SI App. Goodness-to-fit of
229 models was determined by generating the Akaike Information Criterion, plots of residuals over time, and
230 plots of individual prediction versus observed concentration (Figure S3-6). The influence of C4 format on
231 kinetic parameters estimations was assessed with a two-factor ANOVA followed by pairwise comparison
232 of mean with Bonferroni's p-value adjustment in R v.4.0.2. Significance was set to 95%.

233

234 RESULTS

235

236 **Characterization of the native and the mutant C4.** First, we sought to verify that introducing the H310A
237 and H435Q mutations in the Fc domain of the C4 anti-PD-L1 antibody ($\text{C4}^{\text{Fc-MUT}}$) led to significant
238 differences in affinities for the FcRn. Affinities for the FcRn of both IgG were measured by a competition
239 assay. Incubation with the native C4 led to a concentration-dependent decrease in bioluminescent signal
240 signing the interaction with human ($\text{IC}_{50}= 10.2 \mu\text{g/mL}$) and murine FcRn ($\text{IC}_{50}= 5.2 \mu\text{g/mL}$). No signal
241 inhibition was observed with the $\text{C4}^{\text{Fc-MUT}}$, which confirmed the abolition of interaction with the FcRn
242 induced by the mutation (Figure 1A-B).

243

244 **Lifting Fc/FcRn interaction for brain PET imaging.** We evaluated the potency of anti-PD-L1 IgGs with
245 low affinity for the FcRn receptor for brain PET imaging by comparing the kinetics of ^{89}Zr -DFO-C4 and
246 ^{89}Zr -DFO- $\text{C4}^{\text{Fc-MUT}}$ in an orthotopic syngeneic mouse model of GBM. Post-contrast T1-weighted images
247 depicted comparable tumor growth between the two mice groups (Figure S7).

248 As expected, the plasma clearance of ^{89}Zr -DFO- $\text{C4}^{\text{Fc-MUT}}$ was faster compared with ^{89}Zr -DFO-C4
249 (Figure 1E). A bicompartmental model with a first-order elimination function was individually fitted to
250 plasma kinetics. Parameters' estimations of the two C4 formats plasmatic kinetic are reported in Table 1.
251 ^{89}Zr -DFO- $\text{C4}^{\text{Fc-MUT}}$ is cleared from the central compartment promptly compared with ^{89}Zr -DFO-C4,
252 therefore associated with a shorter mean terminal half-life of 89.7 h versus 176.4 h for ^{89}Zr -DFO-C4 (p -
253 value = 0.009). ^{89}Zr -DFO- $\text{C4}^{\text{Fc-MUT}}$ peripheral distribution volume is twice higher than ^{89}Zr -DFO-C4, and
254 its transfer rate from peripheral to the central compartment (k_{21}) is significantly lower than IgG C4's one
255 ($0.020\pm 0.009 \text{ h}^{-1}$ versus $0.055\pm 0.032 \text{ h}^{-1}$). Those differences in kinetics are consistent with higher retention
256 of the low FcRn affinity antibody in the liver and the spleen (Figure S8).

257 FUS enabled the entry into the brain of both radiolabeled antibodies. At 1h post-injection, ^{89}Zr -
258 DFO-C4 and ^{89}Zr -DFO- $\text{C4}^{\text{Fc-MUT}}$ mean concentrations in the brain reached $3.64\pm 0.73 \text{ \%ID/cc}$ and
259 $3.75\pm 0.41 \text{ \%ID/cc}$, respectively. Reflecting its blood kinetic, the ^{89}Zr -DFO- $\text{C4}^{\text{Fc-MUT}}$ concentrations in the

260 contralateral hemisphere decreased at a higher rate than the concentrations of ^{89}Zr -DFO-C4 (Figure 1F).
261 The tumor distribution of the two C4 formats differed heavily over time. After reaching the maximal
262 concentration of 5.1 ± 1.5 %ID/cc, ^{89}Zr -DFO-C4^{Fc-MUT} concentration in the GBM tumor decreased at a
263 slower pace than in the contralateral hemisphere. It resulted in a significant difference of tissue to plasma
264 ratio between the tumor and the contralateral hemisphere from 5h p.i. (Figure 1F). Tumoral uptake of ^{89}Zr -
265 DFO-C4, on the contrary, increased from 48h post-injection to reach a maximal uptake 7 days post-
266 injection. Over time, those differences in brain distribution led to the highest PET contrast in the tumor
267 observed at 22h post-injection for ^{89}Zr -DFO-C4^{Fc-MUT} versus 7 days post-injection for the ^{89}Zr -DFO-C4.

268
269 **FUS-aided immunoPET imaging of glioblastoma.** To validate the benefits of applying FUS to target PD-
270 L1 with ^{89}Zr -DFO-C4^{Fc-MUT}, immunoPET imaging was performed in a GBM model with (FUS group) and
271 without (sham group) FUS-induced BBB permeabilization before the injection. A similar imaging protocol
272 to the one described earlier was applied and we verified that tumor growth was similar between the sham
273 and the FUS group on post-contrast T1-weighted MRI (Figure S7). In addition to the brain, FUS protocol
274 had a significant impact on the spleen uptake of the ^{89}Zr -DFO-C4^{Fc-MUT} (Figure S8). As expected, BBB
275 permeabilization by FUS significantly enhanced the brain uptake of ^{89}Zr -DFO-C4^{Fc-MUT} (Figure 2A). One-
276 hour post-injection, brain uptake of ^{89}Zr -DFO-C4^{Fc-MUT} reached 3.75 ± 0.41 %ID/cc with FUS versus
277 1.92 ± 0.45 %ID/cc without FUS. The difference between the two groups remained significant up to 168h
278 post-injection, even in the contralateral hemisphere (Figure 2B). Immunostaining with a secondary
279 antibody targeting the ^{89}Zr -DFO-C4^{Fc-MUT} confirmed the specificity of the signal observed (Figure 2C). The
280 injected antibody was only detectable in the tumor when FUS were applied, even though PD-L1 was
281 expressed. No evidence of brain damage was observed into the brain after FUS protocol on
282 hematoxylin/eosin staining (Figure S9).

283
284 **Kinetic modeling of ^{89}Zr -labeled C4 brain PET data.** To further decipher the role of FcRn at the BBB
285 and characterize the impact of FUS on the entry of antibodies in the brain, compartmental modelling was
286 performed (Figure 3A). As no specific binding is expected in the contralateral hemisphere, a 1-Tissue
287 compartment model was chosen. For the tumoral volume, a 2-Tissue compartment model was fitted (Figure
288 3B). Mean predicted and observed data of ^{89}Zr -DFO-C4 and ^{89}Zr -DFO-C4^{Fc-MUT} versus time in each brain
289 volume are displayed in figures 3C and 3D, respectively.

290 Our structural model introduced two supplemental parameters, t_{FUS} which describes the time for
291 the BBB to recover integrity after FUS, and K_{FUS} , which describes the perfusion-dependent transfer rate of
292 IgG across the permeabilized BBB (Figure 3B). K_{FUS} and t_{FUS} estimates did not differ between the two ^{89}Zr -
293 DFO-C4 formats or across brain regions (Table 2). FUS enabled the entry of the two antibodies at a mean
294 rate of 0.163 ± 0.071 mL/h/g of tissue for 12.1 ± 4.6 minutes after the end of the FUS protocol.

295 The impaired interaction of ^{89}Zr -DFO-C4^{Fc-MUT} with FcRn significantly decreased the efflux rate
296 constant (k_2) from the healthy brain tissue to plasma (0.015 ± 0.027 h⁻¹) compared with ^{89}Zr -DFO-C4
297 (0.300 ± 0.218 h⁻¹, p-value = 0.002). It also impacted the influx rate constant (K_1) post-FUS, with a 10-fold
298 decrease in the rate constant for transfer from plasma to tissue (Table 2). However, in the tumoral volumes,
299 there was no significant difference between both ^{89}Zr -DFO-C4 formats' K_1 and k_2 estimates.

300 301 DISCUSSION

302 Focused ultrasound is an emerging technology that could change the paradigms of glioblastoma
303 treatment. If associated with immunotherapy, there is a need for a companion methodology to select
304 patients that will respond to such therapy. Improving the kinetic of antibodies in brain tumors is essential
305 for therapy and imaging purposes. The difficulty in addressing personalized therapy for GBM prompted us
306 to propose a theranostic approach based on quantifying PD-L1 expression in brain lesions.

307 Antibody design with low FcRn affinity has been described as a promising strategy to overcome
308 some pharmacokinetic limitations of immunoPET imaging. Bouleau *et al.* demonstrated that using an
309 H310A/H435Q mutant-IgG offers more advantages for immunoPET imaging than using smaller objects,
310 which mainly aims to decrease the plasma half-life. They obtained significantly higher tumoral uptake of
311 the ^{89}Zr -C4^{Fc-MUT} compared with the corresponding ^{89}Zr -Fab while benefiting from a fairly fast blood
312 clearance resulting in high contrast PET images at only 24h post-injection [17]. Although human IgG1 has
313 a stronger affinity for murine FcRn than for human FcRn, which could impact the prediction of a human

314 antibody pharmacokinetic from mice models, the H310A/H435Q mutation has a translational value as it
315 abolishes IgG1 interaction with both the human and the murine FcRn [14,16]. Moreover, the importance of
316 FcRn functions on the transcytosis and recycling of IgGs is preserved across species [37].

317 The overall brain exposure of $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ was lower than that of $^{89}\text{Zr-DFO-C4}$. This
318 decrease in brain disposition was similarly observed by Chang *et al.*, who compared the brain kinetics of
319 the i.v administered human IgG1 trastuzumab and its (I253A/H310A/H435A) mutant, which is unable to
320 bind FcRn [38,39]. The retention of the mutated C4 due to the binding to PD-L1, enabled by FUS, did not
321 compensate for the reduced brain disposition compared with the $^{89}\text{Zr-DFO-C4}$. The dose-normalized
322 simulation of both C4 formats kinetic in the tumor considering a single intravenous injection and FUS-
323 induced BBB disruption shows that tumor IgG exposure overtime was higher for the $^{89}\text{Zr-DFO-C4}$ (AUC_{0-}
324 $_{168\text{h}, \text{C4}}=433 \mu\text{g}\cdot\text{h/mL}$) compared to the mutated IgG ($\text{AUC}_{0-168\text{h}, \text{C4}(\text{Fc-MUT})}=247 \mu\text{g}\cdot\text{h/mL}$) (Figure 4).
325 Therefore, using IgG with a modified Fc to reduce their affinity for FcRn does not appear advantageous for
326 therapeutic purposes. These results are the consequence of the significantly reduced plasma half-life of
327 $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ due to the abolition of FcRn-mediated transcytosis and recycling. In fact, the high brain
328 exposure of $^{89}\text{Zr-DFO-C4}$ might result from the BBB disruption associated with GBM at an advanced stage
329 at 21 days post-implementation, in addition to the long plasma half-life of IgGs [40,41]. $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$
330 foremost benefits from improved kinetic properties for PET imaging of brain tumors expressing PD-L1 as
331 it generates an optimal contrast between the brain tumor and the contralateral hemisphere at 22h p.i. for the
332 $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ against 168h for $^{89}\text{Zr-DFO-C4}$. Kinetic modeling was used to decipher the non-specific
333 and the specific binding contribution to the observed PET signal. It enlightens the potency of $^{89}\text{Zr-DFO-}$
334 $\text{C4}^{\text{Fc-MUT}}$ to measure target engagement with the specific binding being predominant as soon as 12h post-
335 injection, versus 48h post-injection for the full IgG, $^{89}\text{Zr-DFO-C4}$ (Figure 4). Moreover, the low disposition
336 of $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ in the brain without FUS-induced BBB disruption, associated with its fast plasma
337 clearance, implies that the PET image predominantly reflects PD-L1 expression at the time of injection.
338 The same assumption cannot be made for the $^{89}\text{Zr-DFO-C4}$ which uptake may depend on the growth and
339 evolution of the tumor between the time of injection and optimal time for PET acquisition.

340 FUS were necessary to image GBM with the $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$. Although BBB disruption was
341 induced on both brain hemispheres, the binding of $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ was mostly observed in the GBM
342 tumor volume, thus confirming the specificity of the PET signal for PD-L1 expression. The maximum
343 concentration in the GBM tumor was attained at 5h post-injection versus 1h post-injection in the
344 contralateral hemisphere. This delay reflects the time of association of $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ liberated in the
345 interstitial fluid of the brain parenchyma to its target, PD-L1. Safety of the FUS protocol used was
346 previously validated by contrast-enhanced T1w and T2w MRI in addition to histological analysis
347 [28,42,43]. Concordantly, no structural impact of FUS was observed on the brain of mice on H&E staining.

348 Kinetic modeling of the impact of FUS on the brain kinetics of a radiolabeled compound was
349 previously described using a classic 1-tissue compartment model [44]. This model implies that the entry
350 rate of the radiotracer is constant throughout the observation period. In this study, we considered a
351 discontinuous entry function to match the transient character of FUS-induced BBB disruption. To this end,
352 we introduced two additional parameters related to the molecule uptake, K_{FUS} and t_{FUS} , that corresponds
353 respectively to the clearance and to the time of BBB/BTB sealing after the transient disruption with FUS.
354 The similarity of these two parameters in all brain regions where FUS were applied reinforces the
355 hypothesis that they only are related to the dynamics of BBB permeation/recovery rather than the intrinsic
356 kinetic properties of tested antibodies. Considering all brain regions and for both C4 formats, the mean
357 K_{FUS} is $0.163\pm 0.071 \text{ mL/h/g}$ of tissue. The same order of magnitude with the uptake clearance calculated
358 for $^{89}\text{Zr-Cetuximab}$ passage has been observed by Tran *et al.* ($\text{CL}_{\text{up Cetuximab}}= 0.78\pm 0.36 \text{ mL/h/g}$ of tissue)
359 [28]. The mean t_{FUS} was determined to be $10.4\pm 4.6 \text{ min}$, which is consistent with the theoretical closure
360 half-life of the BBB from Marty *et al.* [45]. Their estimation, based on semi-empirical observation with
361 MRI revealed a half-life of 17.8 min after FUS-enhanced delivery of particles with a hydrodynamic
362 diameter similar to a diameter of an antibody ($\approx 10 \text{ nm}$). Additionally, in our case, implementing a
363 discontinuous permeability function improved the model's fit with the observed concentrations in the tumor
364 volume delineated by contrast-enhanced T1w-MRI. In this particular volume, the BBB is already disrupted
365 by the GBM presence, allowing diffusion of gadoteric acid in the tumor. This suggests that FUS transiently
366 induced a stronger disruption of the BTB than the one induced by this particular tumor model. Enhanced
367 BTB permeability induced by FUS remains to be further investigated in other preclinical models. Of note,

368 this was not observed by Brighi *et al.*, who performed FUS-aided immunoPET imaging of a patient-derived
369 glioma model in mice [46].

370 The robustness of the estimation of K_{FUS} and t_{FUS} allowed the characterization of our two C4
371 formats brain kinetic to assess the influence of FcRn affinity quantitatively. In the contralateral hemisphere
372 devoid of tumoral tissue, the ^{89}Zr -DFO-C4^{Fc-MUT} is associated with a lower efflux rate between the brain
373 and plasma. The influx rate constant was also lower when affinity with FcRn is lost. These results suggest
374 that antibody-FcRn interaction controls the mAbs transcytosis across the BBB in both directions. This
375 conclusion is consistent with the slower clearance from the brain of IgG with reduced affinity observed
376 when the IgG is intracranially injected [21,47]. In regions associated with GBM tumoral tissues, transfer
377 rate constants of ^{89}Zr -DFO-C4^{Fc-MUT} were higher than those in the contralateral hemisphere and were
378 similar to that observed with the unmodified ^{89}Zr -DFO-C4. The absence of exchange constants difference
379 between the PET contrast-enhanced and the T1w MRI contrast enhanced volume indicates that mAbs
380 tumoral distribution does not relate to antibody-FcRn interaction or BBB integrity before FUS application
381 but mainly on the antigen presence.

382 Abolishing Fc-FcRn interaction confers improved kinetic properties to ^{89}Zr -DFO-C4^{Fc-MUT} for
383 immunoPET imaging with a better contrast obtained sooner in brain tumoral tissues. FUS-aided BBB/BTB
384 disruption enables quantitative imaging of PD-L1 expression by glioblastoma tumors within the brain and
385 allowed us to study the effect of Fc-FcRn interaction on the brain distribution of antibodies.

386 This study demonstrates the potency of FUS-aided BBB disruption with the smart design of
387 radiolabeled antibodies to enable quantitative immunoPET imaging of PD-L1 within the brain..

388

389 **Abbreviations:** BBB: blood-brain-barrier; BTB: blood-tumoral barrier; CNS: central nervous system;
390 FcRn: neonatal Fc receptor; FUS: focused ultrasound; GBM: glioblastoma; HPLC: high-performance liquid
391 chromatography; ICI: immune checkpoint inhibitor; iTLC: immunoglobuline G; MB: microbubble; MRI:
392 magnetic resonance imaging; PD1: programmed cell death protein 1; PD-L1: programmed death ligand 1;
393 PET: positron emission tomography.

394

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401 D.K., E.S., B.J., H.N., C.T. performed research; C.C., A.N., A.D., S.D., D.K., E.S., B.J., B.L., H.N., C.T.
402 contributed new reagents/analytic tools, C.C., N.T., S.D., D.K., E.S., B.J., B.M., H.N., C.T., analyzed data
403 and, C.C., A.N., N.T., B.M., H.N., C.T. wrote the paper;

404 **Competing interest statement:** The authors declare no competing interest.

405

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529

531 Tables

532 Table 1. Plasma pharmacokinetic parameters of the ^{89}Zr -DFO-C4 and the ^{89}Zr -DFO-C4^{Fc-MUT}.
533

Parameter	^{89}Zr -DFO-C4		^{89}Zr -DFO-C4 ^{Fc-MUT}		p-value
	Mean	Sd	Mean	Sd	
V _c (mL)	1.5	0.1	1.7	0.3	0.45
k ₁₀ (h ⁻¹)	0.014	0.004	0.076	0.011	<u>7.4E-06</u>
k ₁₂ (h ⁻¹)	0.119	0.058	0.094	0.047	0.48
k ₂₁ (h ⁻¹)	0.055	0.032	0.02	0.009	<u>0.03</u>
V _p (mL)	3.4	0.5	7.8	2.7	<u>0.01</u>
Initial t _{1/2} (h)	4.3	1.5	4.2	1.3	0.84
Terminal t _{1/2} (h)	176.4	50	89.7	30.9	<u>0.009</u>
AUC _{0-∞} (μg.h.mL ⁻¹)	5954	1411	569	108	<u>1.1E-05</u>

534

535 Mean parameters estimates and associated standard errors obtained by individually fitting a
536 bicompartmental kinetic to plasmatic concentration of ^{89}Zr -DFO-C4 (n=6) and ^{89}Zr -DFO-C4^{Fc-MUT} (n=8). p-
537 value shown are results from Student comparison of mean. V_c: volume of the central compartment; k₁₀:
538 elimination constant from the central compartment; k₁₂: transfer constant from the central to the peripheral
539 compartment; k₂₁: transfer constant from peripheral to central compartment; V_p: volume of the peripheral
540 compartment; Initial t_{1/2}: initial half-life; Terminal t_{1/2}: terminal half-life.
541

542 **Table 2. Mean estimates and standard deviation of parameters of the different models applied to**
 543 **brain VOI data to characterize $^{89}\text{Zr-DFO-C4}$ and $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ kinetic.**

<i>Brain region</i>	Parameter	$^{89}\text{Zr-DFO-C4}$		$^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$		p-value
		Mean Estimate	Sd	Mean Estimate	Sd	
<i>Contralateral</i>						
	K_{FUS} (mL/h/g of tissue)	0.167	0.156	0.094	0.037	ns
	t_{FUS} (h)	0.165	0.099	0.215	0.065	ns
	K_1 (mL/h/g of tissue)	0.030	0.019	0.003	0.005	<u>0.005</u>
	k_2 (h^{-1})	0.300	0.218	0.015	0.027	<u>0.002</u>
	vB	0.064	0.006	0.077	0.014	ns
<i>TIw MRI CE</i>						
	K_{FUS} (mL/h/g of tissue)	0.179	0.046	0.200	0.087	ns
	t_{FUS} (h)	0.142	0.077	0.179	0.102	ns
	K_1 (mL/h/g of tissue)	0.053	0.021	0.062	0.028	ns
	k_2 (h^{-1})	0.566	0.188	0.601	0.308	ns
	vB	0.052	0.013	0.053	0.009	ns
	k_3 (h^{-1})	0.027	0.017	0.111	0.063	<u>0.031</u>
	k_4 (h^{-1})	0.005	0.006	0.020	0.011	<u>0.039</u>
<i>PET CE (wo TIw MRI CE)</i>						
	K_{FUS} (mL/h/g of tissue)	0.155	0.049	0.188	0.077	ns
	t_{FUS} (h)	0.150	0.037	0.161	0.070	ns
	K_1 (mL/h/g of tissue)	0.069	0.033	0.051	0.026	ns
	k_2 (h^{-1})	0.742	0.393	0.491	0.298	ns
	vB	0.058	0.010	0.063	0.013	ns
	k_3 (h^{-1})	0.017	0.013	0.077	0.047	<u>0.036</u>
	k_4 (h^{-1})	0.000	0.000	0.015	0.007	<u>0.003</u>

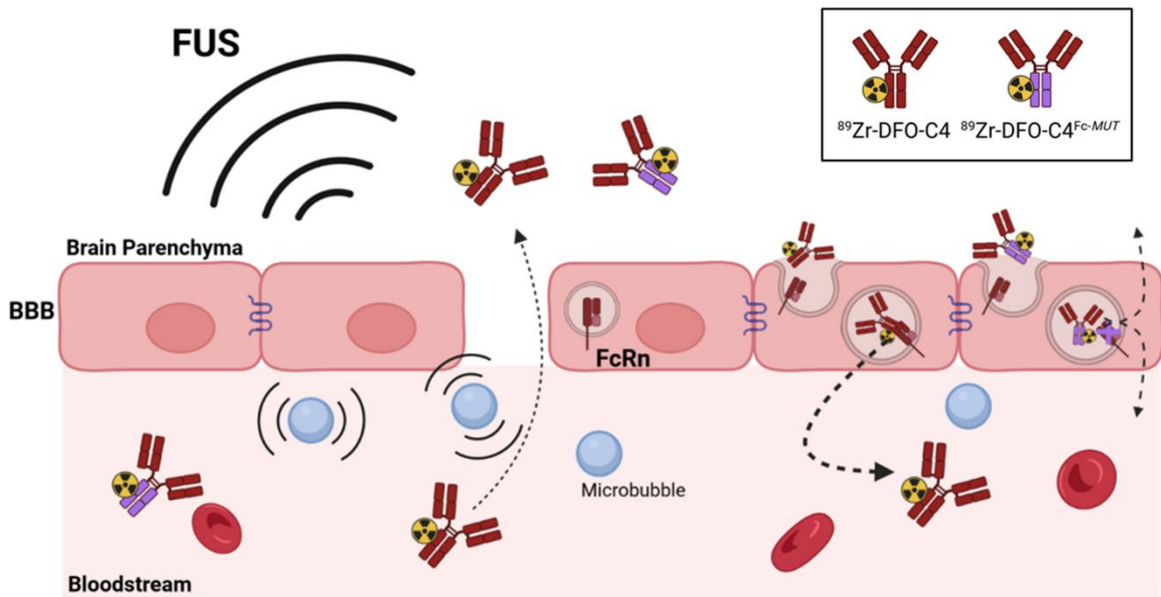
544 K_{FUS} and K_1 : perfusion dependent entry constant from plasma to free/non-specifically bound compartment.

545 t_{FUS} : time of significant closure of the BBB. k_2 : transfer constant from free/non-specifically bound

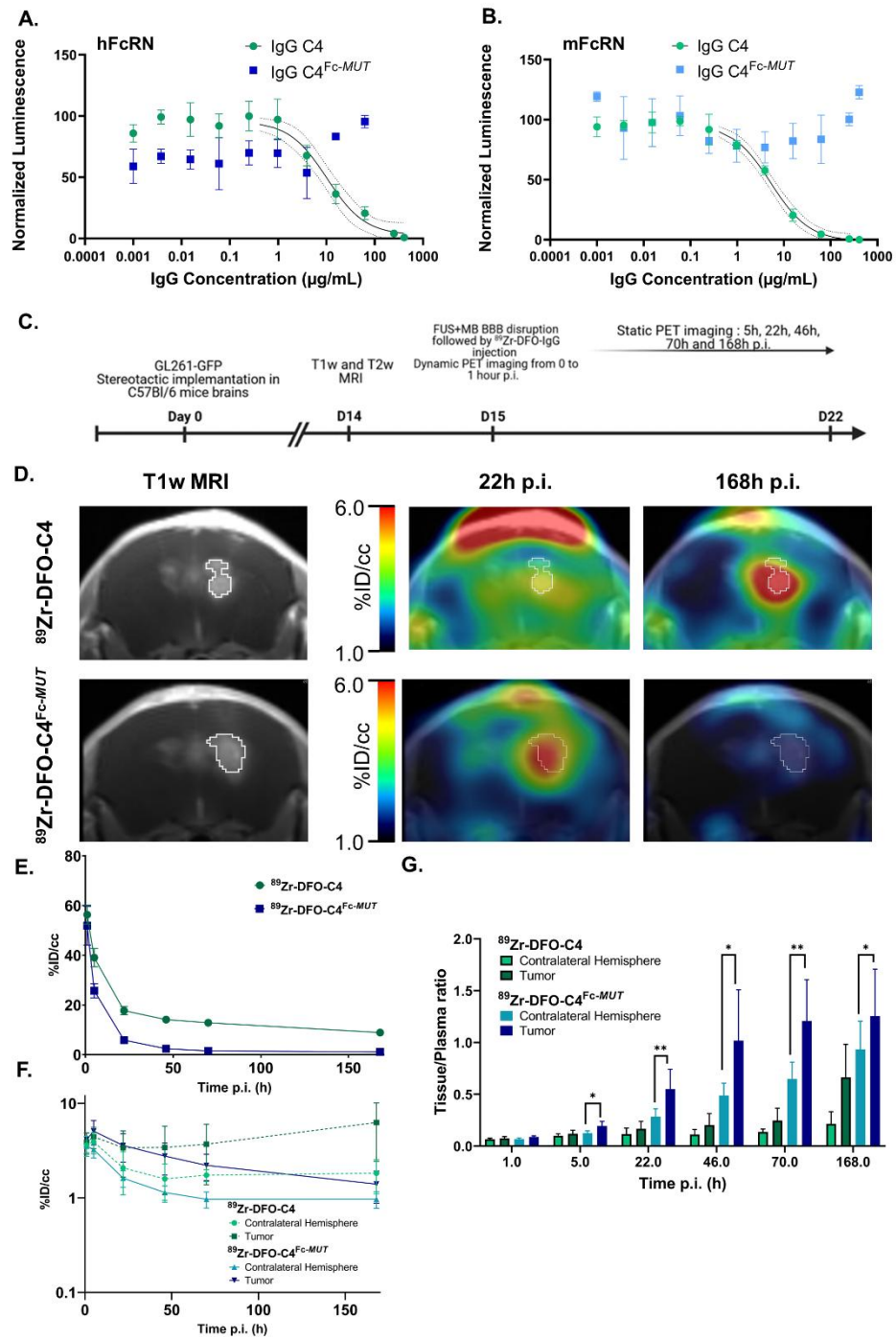
546 compartment to plasma. k_3 and k_4 : transfer constant between free/non-specifically bound compartment and

547 specifically bound compartment. vB: fraction of blood in the tissue.

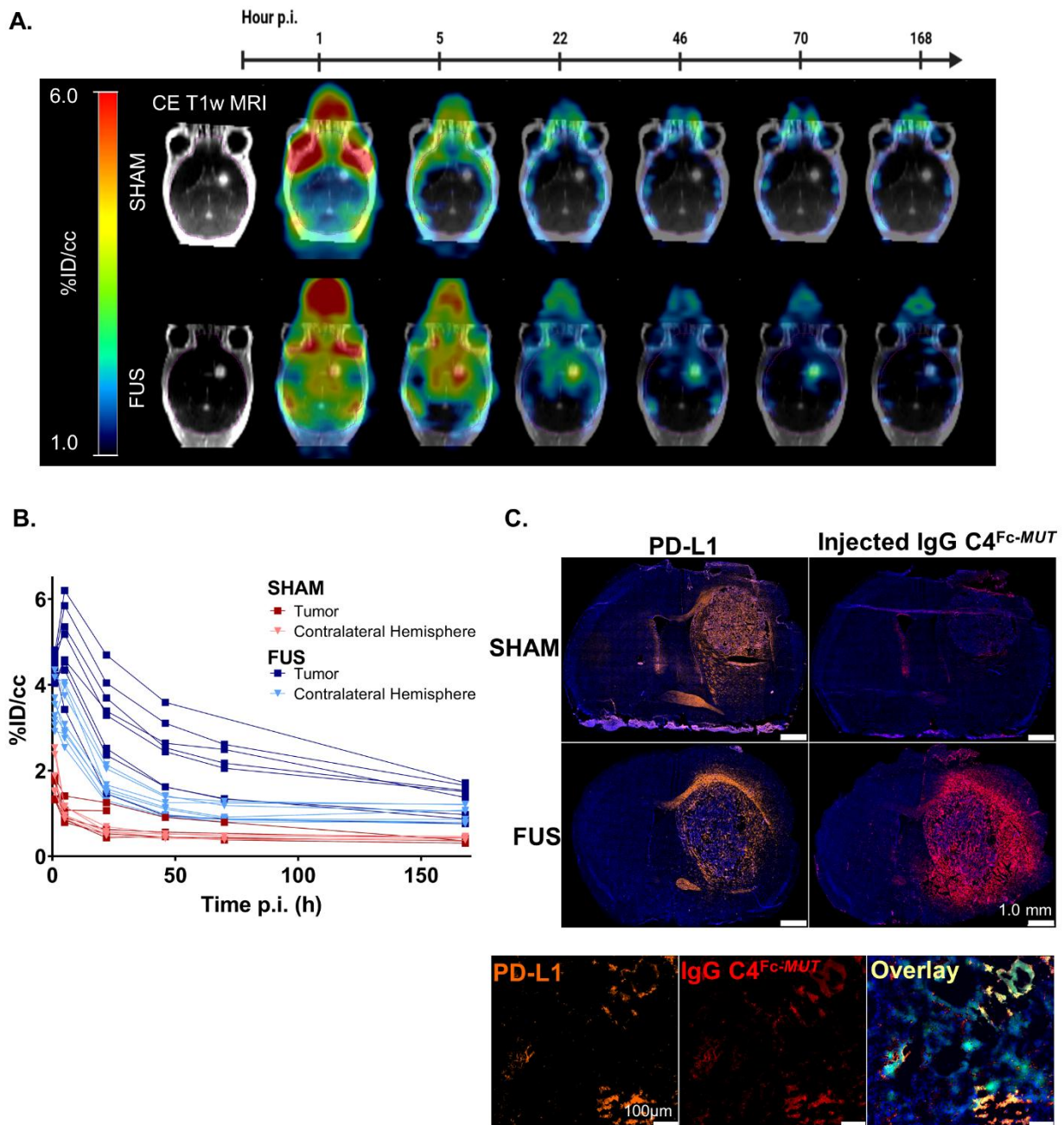
548



550
551 **Scheme 1.** Scheme describing the rational of focused ultrasound to deliver through the blood-brain barrier
552 a low FcRn affinity IgG in the brain parenchyma for immunoPET imaging. *The disruption of the tight*
553 *junctions of endothelial cells allows the paracellular diffusion of antibodies to the brain parenchyma. The*
554 *loss of affinity for the FcRn should modify the fate of the radiolabeled C4 antibody targeting PD-L1.*
555

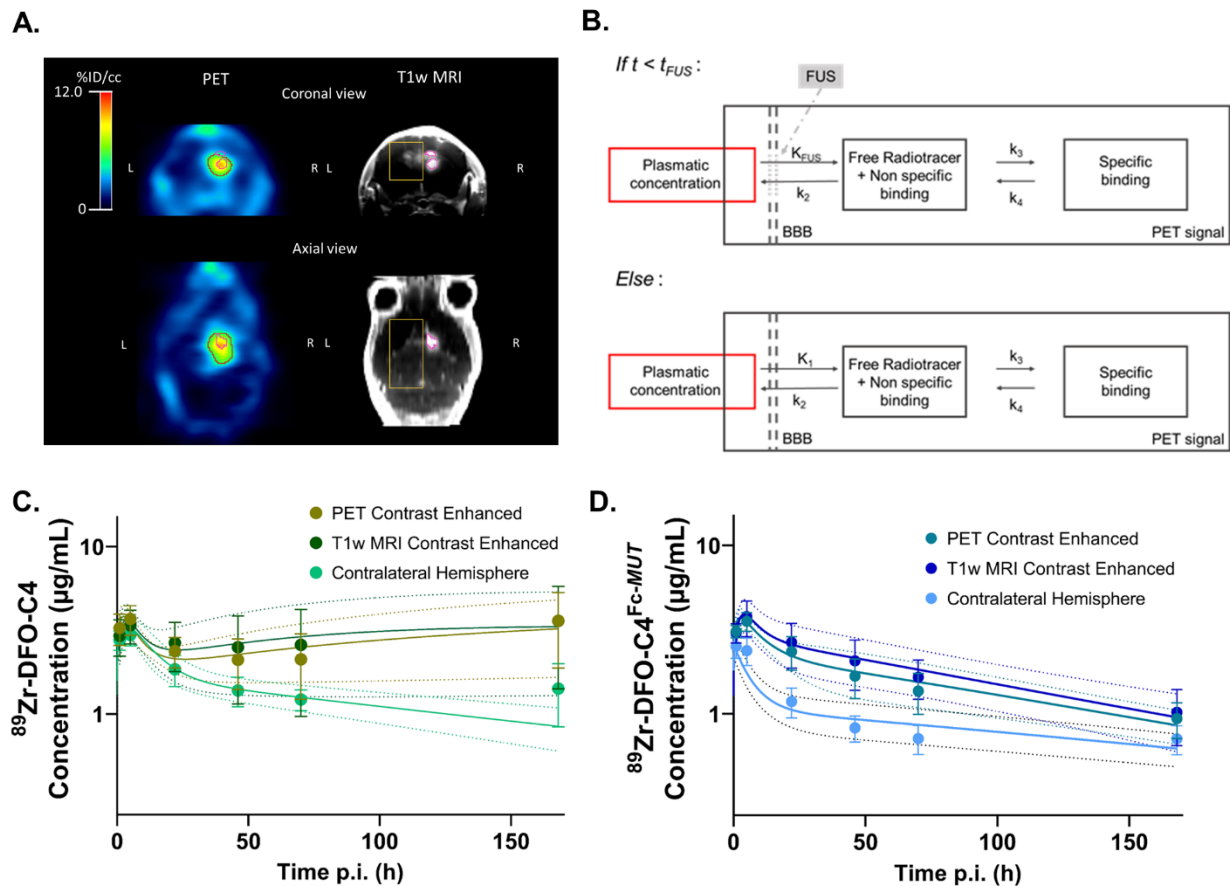


556 **Figure 1. *In vitro* and *in vivo* characterization of the C4 formats for brain PET PD-L1 imaging.** (A, B)
 557 Dose-dependent inhibition curves of both C4 formats with human and murine FcRn. Data represent the
 558 mean \pm SD of triplicate readings. (C) Timeline of the MRI and PET imaging protocol. (D) Representative
 559 brain PET- T1w MRI images of GL261-GFP bearing C57Bl/6 mice injected with ⁸⁹Zr-DFO-C4 (n=6) or
 560 ⁸⁹Zr-DFO-C4^{Fc-MUT} (n=8) at 22h and 168h p.i. Time activity curves in plasma (E) and in the brain (F) of the
 561 two C4 formats. TACs in the tumor and contralateral hemisphere are differentiated. (F) Tissue to plasma
 562 ratio of the two C4 radioligands in tumor and contralateral hemisphere.

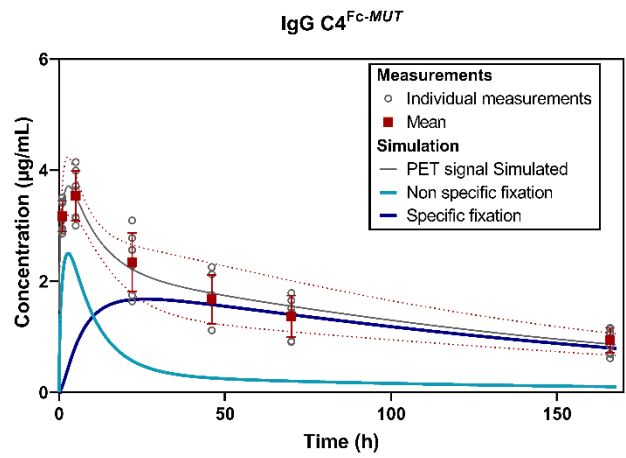
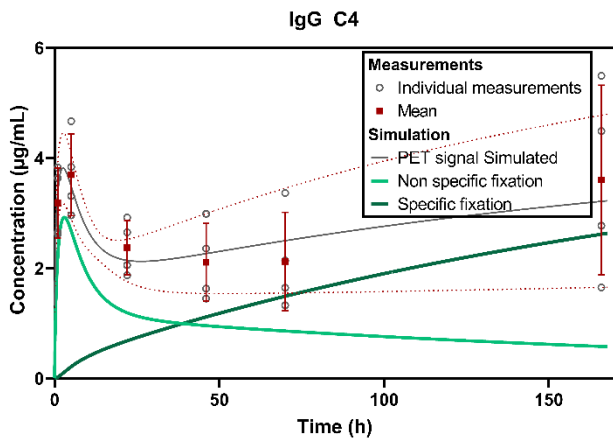


563
 564 **Figure 2. Brain kinetic of $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ after FUS-induced BBB permeabilization.** (A)
 565 Representative PET images overlaid on post-contrast T1-weighted MRI from the same mouse at different
 566 time points (1h, 5h, 22h, 46h, 70h and 168h after injection) for the sham (*top*) and the FUS (*bottom*)
 567 groups. (B) Time activity curves of the $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$ in the tumor enhanced on T1-weighted MRI
 568 and in the contralateral hemisphere in the sham (*red*, $n=5$) and the FUS (*blue*, $n=8$) groups. All data are
 569 represented as mean \pm SD. (C) Immunofluorescence staining of brain sections of $\text{C4}^{\text{Fc-MUT}}$ injected mice.
 570 Adjacent 10 μm cryo-sections were stained with either a rat anti-mouse-PD-L1 IgG and an AF546-goat-
 571 anti-rat IgG (orange) or AF546-goat-anti-human IgG (red) to detect the injected antibody.
 572 Immunofluorescence signal is overlaid on DAPI images (blue). Magnification of a cryo-section stained

573 with rat anti-mouse-PD-L1 IgG and an AF546-goat-anti-rat IgG (orange) or AF546-goat-anti-human IgG
574 (red) to detect the injected antibody. Immunofluorescence signal is overlaid on DAPI images (blue), GL261
575 cells express GFP (green). Supplementary immunofluorescence staining are shown in *SI Appendix* (Figure
576 S8).
577



578 **Figure 3. Brain kinetic modeling to elucidate FcRn's role at the BBB and the impact of FUS-induced**
 579 **BBB disruption on antibodies delivery.** (A) Representative example of VOI identifying the contralateral
 580 hemisphere (yellow), the tumor enhanced on post-contrast T1-weighted MRI (pink) and the tumor enhanced
 581 on PET images (red). (B) Structure of the 2-tissue compartment model applied on tumoral VOI. Observed
 582 (markers) and predicted (solid lines) brain concentration of $^{89}\text{Zr-DFO-C4}$ ($n=4$) (C) and $^{89}\text{Zr-DFO-C4}^{\text{Fc-MUT}}$
 583 ($n=7$) (D) according to the different VOI. All data are represented as mean \pm SD. K_{FUS} and K_1 : perfusion
 584 dependent entry constant from plasma to free/non-specifically bound compartment. t_{FUS} : time of significant
 585 closure of the BBB. k_2 : transfer constant from free/non-specifically bound compartment to plasma. k_3 and
 586 k_4 : transfer constant between free/non-specifically bound compartment and specifically bound
 587 compartment. *FUS +MB*: Focused ultrasound on microbubbles.
 588



589

590 **Figure 4. Simulated concentrations of the two C4 formats in GBM tumor with a preserved BBB**
 591 **integrity on T1w post-contrast MRI.** Concentrations were normalized to an injected dose of 70µg of
 592 antibody. The PET signal simulated is the resultant of the sum of the non-specific and specific fixation.
 593 Parameters used for the simulation were the mean of individual estimated parameters.