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1 **Intra-articular delivery of full-length antibodies through the use of an *in situ* forming**
2 **depot**

3
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20

21 **1. ABSTRACT**

22 Monoclonal antibodies (mAbs) are large size molecules that have demonstrated high
23 therapeutic potential for the treatment of cancer or autoimmune diseases. Despite some
24 excellent results, their intravenous administration results in high plasma concentration. This
25 triggers off-target effects and sometimes poor targeted tissue distribution. To circumvent
26 this issue, we investigated a local controlled-delivery approach using an *in situ* forming
27 depot technology. Two clinically relevant mAbs, rituximab (RTX) and daratumumab
28 (DARA), were formulated using an injectable technology based on biodegradable PEG-
29 PLA copolymers. The stability and controlled release features of the formulations were
30 investigated. HPLC and mass spectrometry revealed the preservation of the protein
31 structure. *In vitro* binding of formulated antibodies to their target antigens and to their
32 cellular FcγRIIIa natural killer cell receptor was fully maintained. Furthermore,
33 encapsulated RTX was as efficient as classical intravenous RTX treatment to inhibit the *in*
34 *vivo* tumor growth of malignant human B cells in immunodeficient NSG mice. Finally, the
35 intra-articular administration of the formulated mAbs yielded a sustained local release
36 associated with a lower plasma concentration compared to the intra-articular delivery of
37 non-encapsulated mAbs. Our results demonstrate that the utilization of this polymeric
38 technology is a reliable alternative for the local delivery of fully functional clinically
39 relevant mAbs.

40

41 **2. KEYWORDS**

42 Full length monoclonal antibodies; Intra-articular; Local delivery; biodegradable copolymers;
43 rituximab; daratumumab.

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48 3. INTRODUCTION

49 A considerable number of monoclonal antibodies (mAbs) have been approved in clinics and
50 are a fast growing group among new therapeutics as they offer exquisite specificity[1]. This
51 immunotherapy has revolutionized the way to treat patients in many therapeutic areas, including
52 oncology and auto-immune diseases. Due to their intrinsic biochemical nature, antibodies are
53 not used orally and are mostly administrated intravenously or subcutaneously [2]. Both routes
54 of administration can lead to side effects related to their initial elevated concentration. The large
55 size of antibodies, together with neonatal Fc receptor (FcRn)-mediated recycling, confer them
56 a long half-life generally comprised between two to three weeks. This makes them amenable to
57 convenient dosing regimen. Hence, and in contrast to therapeutic peptides or small proteins,
58 mAbs are not usually considered for drug delivery systems [3]. However, the large mAb size
59 can be problematic to reach certain tissues or compartments, which are poorly vascularized or
60 present a barrier for protein infiltration[4]. This results in a lower treatment efficacy or a higher
61 toxicity due to the necessity to increase the administered dose[5]. Developing strategies to
62 achieve local delivery would improve mAbs efficacy and could reduce the treatment costs as a
63 consequence of the administered dose reduction. Sophisticated technologies relying either on
64 protein engineering or on drug carrier modifications would provide a specific recognition of
65 the targeted tissue or cell type while significantly increasing the manufacturing cost or affecting
66 the production yield [6–10]. A more straightforward approach is the direct injection of the drug
67 in the targeted tissue or compartment. It has been for instance successfully used in ocular
68 applications with intravitreal injections of anti-VEGF antibodies or antibody fragments such as
69 ranibizumab, bevacizumab or aflibercept for the treatment of macular diseases[11]. However,
70 even in a close compartment like the eye, the intravitreal injection of anti-VEGF agents
71 presented systemic effects with the suppression of plasma-free VEGF for several days, even at
72 a dose more than 100-fold lower than the one used in oncology via a systemic delivery [12]. In
73 the oncology field, intratumor injection of checkpoint inhibitors, *e.g.* anti-CTLA4, has been
74 explored to reduce their toxicity profile [13]. Still, a direct injection of the therapeutic protein

75 in a targeted compartment does not necessarily translate in a prolonged, high local exposure of
76 the molecule. For instance, an intra-articular injection of a protein-containing saline solution
77 does not result in long-term residence of the protein, which is quickly retrieved from the
78 systemic circulation due to the efficient lymphatic drainage of the joint[14]. Conversely,
79 targeting the joint using a systemic approach is complicated as the synovial fluid is an
80 ultrafiltrate of the plasma. Indeed, after intravenous injection, a small fraction of proteins can
81 be detected in the synovial fluid. Moreover, the larger the protein, the lower the synovial to
82 serum concentration ratio will be[15]. As a consequence, the expected synovial fluid
83 concentration of a mAb is 3 to 10 times lower than the serum one, a feature that could explain
84 the lack of efficacy of certain molecules evaluated for the treatment of osteoarthritis. There is
85 therefore a need to design long-acting injectables for local, intra-articular, mAb delivery that
86 would sustain high local concentrations and improve treatment efficacy for a condition
87 (osteoarthritis) with current unmet medical needs.

88 The aim of the present work is to design a sustained release formulation of a full-length model
89 mAb for intra-articular delivery, using an *in situ* forming depot (ISFD) long-acting technology
90 registered under the name of BEPO[®]. BEPO[®] technology relies on the use of copolymers based
91 on polyethylene glycol (PEG) or methoxy-PEG (mPEG) and poly D,L lactic acid (PDLLA).
92 Specifically, on the combination of one triblock (PDLLA-PEG-PDLLA) and one diblock
93 (mPEG-PDLLA) copolymer. These copolymers are designed for not being soluble in water and
94 need to get solubilized in an organic solvent to prepare an injectable formulation. Upon
95 injection into an aqueous environment, the formulation forms a solid polymeric depot due to
96 the diffusion of the organic solvent and the subsequent precipitation of the copolymers. If a
97 therapeutic molecule is incorporated into the formulation, it will be trapped within the
98 precipitated polymeric matrix and will be delivered in a controlled fashion thereafter[16]. The
99 release kinetics of the formulated drug can be modulated by varying different parameters such
100 as polymer composition or polymer content in the initial formulation. This technology has the
101 potential to provide sustained release formulations for the local or systemic delivery of small

102 molecules with release kinetics varying from weeks to months. It was also successfully used to
103 formulate a 55 kDa bispecific antibody in a half-life extension strategy and the functionality of
104 the formulated protein was fully established[17]. However, the efficacy of this technology to
105 formulate full-size mAbs is unknown. This is clinically relevant because the vast majority of
106 Abs used nowadays in therapeutics present molecular weights higher than 140 kDa.
107 Anti-CD20 Rituximab (RTX) and anti-CD38 Daratumumab (DARA), two clinically validated
108 and well characterized mAbs, were chosen in the present study to evaluate the capacity of
109 BEPO[®] technology to successfully deliver them in the intra-articular region. Formulated mAbs
110 were extensively characterized for their physico-chemical properties and biological activity
111 while *in vivo* pharmacokinetic studies were conducted in mice after intra-articular (IA) injection
112 of ISFD formulations to evaluate the delivery of the mAb in the synovial fluid.

113

114 **4. MATERIALS AND METHODS**

115 **4.1. Materials**

116 RTX and DARA proteins were obtained from the commercial products MabThera[®] and
117 Darzalex[®], respectively. Protein sequences for the murine version of RTX were taken from the
118 following reference [18] and the mouse RTX (mRTX) protein was produced by Evitria AG,
119 Switzerland. The rat IgG2a isotype control was purchased from BioXCell (Lebanon, NH,
120 USA). Triblock (TB) and diblock (DB) copolymers were produced by CM Biomaterials
121 (Tucker, GA, USA). If not otherwise stated, all chemicals were obtained from Sigma-Aldrich
122 and used without further purification.

123 BEPO[®] polymeric vehicles (*i.e.* a solution of copolymers in tripropionin) used in this study are
124 summarized in Table 1. Triblocks (TB) and diblocks (DB) are presented with the molecular
125 weights in kDa of (m)PEG and PDLA in the copolymers. The total polymer content is
126 expressed as a mass percentage. For instance, the vehicle V1 from Table 1 was obtained by
127 solubilizing a triblock with 3 kDa PEG and 9.8 kDa PDLA (presented as 3-9.8 in Table 1),
128 and a diblock with 1 kDa PEG and 6.5 kDa PDLA (presented as 1-6.5 in Table 1) in

129 tripropionin. The weight fraction of copolymers in the final vehicle was 20 % (w/w) with a 1:1
130 TB:DB weight ratio.

131 **Table 1** : *Composition of vehicles used to make BEPO[®]:protein formulations*

Vehicle	TB:DB couple	Triblock	Diblock	Polymer content (% w/w)	TB:DB weight ratio
V1	TB1:DB1	3-9.8	1-6.5	20	1:1
V2	TB1:DB1	3-9.8	1-6.5	20	3:1
V3	TB1:DB2	3-9.8	2-27.8	20	1:1

132
133 In the manuscript, Vx refers to a polymer vehicle (copolymers solubilized in tripropionin) while
134 Fx refers to a drug loaded formulation where a spray dried cake has been dispersed into the
135 corresponding vehicle.

136 **4.2. Spray Drying of antibody solutions**

137 RTX or DARA commercial solutions were buffer exchanged through the use of desalting PD-
138 10 columns (Merck). In the final solution, protein concentration was comprised between 7 and
139 10 mg/mL. Then, antibody solutions were spray dried using a Mini B290 apparatus (Büchi,
140 Switzerland). Inlet temperature and liquid feed rate were set at 85°C and 1.5 mL/min,
141 respectively. Aspirator was set at 100 % and air flow at 55 on the device. The outlet temperature
142 recorded during the process was comprised between 55°C and 58°C. Generally, the process
143 yield was comprised between 70% and 90%. Powder was collected and dried overnight in a
144 vacuum oven at 30°C. Particle size analysis was performed on the dry powder using a
145 MorphologyG3 instrument (Malvern). The dry powder was also submitted to SEM imaging.
146 Thermal analysis was conducted using the Tycho NT.6 instrument (NanoTemper GmbH,
147 Munich, Germany). The instrument relies on the protein intrinsic fluorescence and records the
148 ratio of emitted fluorescence at 350 nm over 330 nm over a temperature ramp between 35°C
149 and 95°C. Temperatures at which a transition occurs are called inflection temperatures (T_i).
150 Both the native RTX and sdRTX proteins were analyzed at a concentration of 0.5 mg/mL in

151 samples in PBS. The protein content in the spray dried (sd) cake was determined by solubilizing
152 the powder in PBS and quantifying the protein by SEC-HPLC. Trehalose content was also
153 determined using an enzymatic assay based on trehalase activity (Libios, France).

154

155 **4.3. Preparation of protein ISFD formulations and in vitro release (IVR) studies**

156 Vehicles were made by dissolving the copolymers (TB and DB) in tripropionin overnight. Then,
157 the sd cakes containing the antibodies were weighed and dispersed into the polymer vehicles to
158 reach the targeted final protein loading. Good powder dispersion and formulation homogeneity,
159 assessed visually, were achieved after 30 min under magnetic stirring. Drug assay was
160 performed as follows. Briefly, around 50 mg of formulation (in triplicate) were weighed
161 precisely in a 1.5 mL polypropylene tube. After centrifugation (10 min, 13000 rpm), the
162 supernatant (polymer vehicle) was removed, the pellet was washed three times with 500 μ L of
163 ethyl acetate and finally dried for 1 hour in vacuum oven. Dry pellet was solubilized in 1 mL
164 of PBS and the protein concentration was determined by SEC-HPLC. Experiments were carried
165 out in triplicate. Drug assay samples will be noted using a “da” prefix in the following text.

166 *In vitro* release (IVR) tests of BEPO[®]:protein formulations were initially performed in SEC-
167 Tween buffer (50 mM sodium phosphate buffer pH 6.8, 100 mM NaCl and 0.005% Tween80).
168 The presence of Tween80 ensured minimal protein adsorption at low concentrations. However,
169 to ensure full compatibility of the release medium with cell-based assays, regular PBS was later
170 substituted to SEC-Tween as release medium. Around 100 mg of each formulation were
171 injected into 18 mL of buffer in glass vials that were capped and placed at 37°C under
172 continuous orbital shaking. At given time points, the release medium (samples noted with the
173 “iv” prefix) was collected and replaced by preheated buffer. The released protein concentration
174 in the medium was determined by SEC-HPLC and a cumulative release profile was built for
175 each formulation considering the initial protein cargo. IVR studies were performed in triplicate.
176 During IVR studies and after extensive drying of the polymer depot in vacuum oven at 30°C,

177 the protein was also extracted from the polymer depot (samples note with the “dep” prefix)
178 following the same procedure as for the drug assay and analyzed. For stability studies,
179 formulations were stored at 4°C in 2 mL crimped vials capped with PTFE coated stoppers.

180

181 **4.4. Liquid chromatography methods**

182 4.4.1. SEC-HPLC method

183 Size-exclusion chromatography was carried out using a BEH SEC 200 column, 7.8x300 mm,
184 3.5 µm particle size (Waters, Milford, MA, USA). The eluent was a 50 mM sodium phosphate
185 buffer at pH 6.8 with 250 mM NaCl and 0.02% of sodium azide. 40 µL of sample were injected
186 and elution was carried out at room temperature at a 1 mL/min flow rate. Protein detection was
187 achieved with a fluorescence detector (W2475) using the intrinsic protein fluorescence with
188 excitation and emission wavelengths at respectively 284 nm and 335 nm. RTX eluted at a
189 relative retention time (RRT) of 8.25 min. The chromatogram presented also a high molecular
190 weight aggregated fraction (HMW) at 7.52 min (RRT of 0.91) and a low molecular weight
191 (LMW) specie eluting at 10.06 min (RRT of 1.22) and corresponding most probably to a Fab
192 fragment. The SEC-HPLC method was used to both monitor the HMW or LMW species and
193 quantitate accurately the protein.

194 4.4.2. RP-HPLC method

195 RTX samples in PBS were submitted to IdeS (Genovis AB, Sweden) digestion following the
196 manufacturer recommendations and were further reduced using TCEP at 20 mM final
197 concentration. Samples were then analyzed by RP HPLC using the BioResolve RP mAb
198 polyphenyl column from Waters (450 Å, 2.7 µm, 4.6x150 mm). Injection volume was 10 µL
199 and the column temperature was set at 60°C. The eluents were H₂O with 0.1% TFA and
200 acetonitrile with 0.1% TFA. Protein subunits elution was achieved at 1 mL/min using a gradient
201 from 30 % to 40 % acetonitrile in 20 min. The three protein subunits eluted in the Fc/2, LC and
202 Fd sequence order with the following retention times: 6.5, 8.1 and 13.6 min, respectively. Total

203 impurity level could be reported and for a particular impurity, its level was reported as the
204 percentage of total area relative to the related RTX moiety.

205 4.4.3. SCX-HPLC method

206 As the isoelectric point of RTX is greater than 8, cation-exchange is very well suited to try to
207 separate the charge variants of the protein. Hence, SCX-HPLC was carried out at pH 6.2 using
208 a BioResolve SCX mAb, 3 μ m, 4.6x100 mm column from Waters. The eluent was 20 mM MES
209 at pH 6.2. The protein was eluted with a NaCl gradient (from 75 mM to 150 mM NaCl in
210 12 min) at a flow rate of 0.5 mL/min.

211 4.5. Mass spectrometry analyses

212 For intact-mass analysis, deglycosylated samples (PNGase) were diluted in ultrapure water and
213 10 μ g of protein were injected in Matrix Assisted Laser Desorption Ionisation device (MALDI-
214 TOF-TOF) 4800 Applied Biosystems device.

215 Full tryptic peptide analysis of native RTX and formulated samples (daRTX, ivRTX and
216 depRTX) was carried out with the following procedure. Samples were acidified with 0.1% TFA
217 (trifluoro acetic acid) up to 80 μ L. Protein content of the sample was extracted with RP-W
218 cartridges on AssayMap BRAVO automate (Agilent, Lexington, USA). Samples were further
219 reduced with dithiothreitol (DTT) during one hour at 56°C. Free cysteines were alkylated with
220 iodoacetamide (IAA) by adding 7.5 μ L of 125 mM IAA and incubating 30 minutes in the dark,
221 at 56°C. Finally, proteins were digested with trypsin (0.5 μ g) overnight at 37°C. Digestion was
222 stopped by adding 5 μ L of formic acid and peptides were cleaned and desalted with C18 Tips
223 before being dried on Speedvac. After resuspension in 60 μ L of 2% acetonitrile/0.1% formic
224 acid/97.9% water, 7 μ L of supernatant were injected on nanoElute (Bruker Daltonics,
225 Massachusetts, USA). NanoFlow LC was coupled to Q-TOF MS instrument (Impact II, Bruker
226 Daltonics, Massachusetts, USA) through captive spray ion source (1200V, dry gas: 3 L/min at
227 150°C) operating with nanobooster (0.2 Bar of Nitrogen boiling in acetonitrile). In the LC part,
228 samples were desalted and pre-concentrated on-line on a PepMap u-precolumn (300 μ m x 5
229 mm, C18 PepMap 100, 5 μ m, 100 Å, ThermoFisher, Waltham, MA, USA). To perform the

230 separation, peptides were transferred to analytical column (75 μm x 500 mm; Acclaim Pepmap
231 RSLC, C18, 2 μm , 100 \AA , ThermoFisher, Waltham, MA, USA).
232 A gradient consisting of 5-26 % B for 192 min and 90% B for 10 min (A = 0.1% formic acid,
233 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at 400 nL/min, 50°C, was used
234 to elute peptides from the reverse-phase column.
235 For peptide identification, data dependent acquisition (DDA) was performed with a lock-mass
236 as internal calibrator (m/z 1222, Hexakis “ ^1H , ^1H , ^4H -hexafluorobutyloxy” phosphazine,
237 Agilent Technologies, Santa Clara, USA). Using Instant Expertise software (Bruker Daltonics,
238 Massachusetts, USA), the most intense ions per cycle of 3 seconds were selected and then active
239 exclusion was used (after 1 spectrum for 2 minutes unless the precursor ion exhibited intensity
240 higher of three times than the previous scan). All MS/MS spectra were searched against
241 homemade Rituximab database by using the Mascot v 2.6.0 algorithm (Matrix Science,
242 <http://www.matrixscience.com/>) with the following settings: (1) enzyme: trypsin, (2) variable
243 modifications: oxidation (M) and deamidated (N,Q), (3) fixed modifications: carbamidomethyl
244 (C), (4) missed cleavages: 2, (5) instrument type CID: ESI-QUAD-TOF, (6) peptide tolerance:
245 10.0 ppm, (7) MS/MS tolerance: 0.05 Da, (8) peptide charge: 1+, 2+ and 3+, (9) mass:
246 monoisotopic, (10) C13: 1, (11) minimum peptide length: 5, (12) peptide decoy: ON, (13) adjust
247 FDR [%]: 1, (14) percolator: on, (15) ions score cut-off: 12, (16) ions score threshold for
248 significant peptide IDs: 12.

249 **4.6. Surface Plasmon Resonance (SPR) experiments**

250 The surface plasmon resonance (SPR) experiments were performed on a BIACORE T200 at
251 25 °C with PBS containing 0.05% of Tween20 (Cytiva) as running buffer. For Fc γ R
252 measurements, the capturing anti-his-tag antibody (R&D) was immobilized on three flow cells
253 Fc2,3,4 of a CM5 chip (Cytiva) at 8,000–9,000 RU using a standard amine coupling procedure.
254 Fc1 was taken as a control submitted to the same procedure without any protein. After the
255 capture of Fc γ R (100-200 RU), samples containing formulated RTX were injected at
256 100 $\mu\text{L}/\text{min}$ at five increasing concentrations in a single run. After a dissociation time of 600 s

257 with running buffer, the chip surface was regenerated using a 30 s injection of 10 mM glycine-
258 HCl pH 1.7. Bulk refractive index differences were corrected by subtracting the response
259 obtained on the reference flow cell. Binding kinetic titrations were analyzed by BiaEvaluation
260 software (T200Evaluation 3.2) using a two-state binding model. For some of these interactions,
261 featuring too fast dissociation to allow the determination of kinetic constants, the affinity
262 constant K_D was determined by steady state analysis. For binding analysis to FcRn, hFcRn was
263 covalently immobilized on CM5 by amine coupling (200-250 RU) and samples presenting a
264 protein concentration of 150 nM were injected at a flow rate of 30 μ L/min in 50 mM PBS buffer
265 (pH 6.0) and 0.05% Tween20. After a dissociation step of 400 s with running buffer, the sensor
266 surfaces were regenerated using two pulses of PBS buffer (pH 7.4). To compare the binding
267 kinetics of the different samples on immobilized FcRn, responses (in RU) at three time points
268 on the curves, 180, 200, and 580 s, designated as binding, stability 1, and stability 2,
269 respectively, were measured. Data were read directly on the curve to be independent of fitting.
270 The stability of the coated surface was checked by injection of RTX (50 nM) as a positive
271 control at the beginning and end of the series of experiments.

272 **4.7. Cell lines and cell culture**

273 Raji and Daudi (B cell lines derived from Burkitt lymphomas), Molm-13 (derived from acute
274 monocytic leukemia (AML-M5a)), PLH (Epstein-Barr Virus (EBV) transformed
275 lymphoblastoid cell line) and K-562 (derived from chronic myelogenous leukemia) were
276 obtained from ATCC. The primary BCL-P2 cells (derived from a B-cell lymphoma patient)
277 have already been described elsewhere[19,20]. All cells were cultured in RPMI Glutamax™
278 medium supplemented with FBS (10%), at 37°C in a humidified 5% CO₂ atmosphere incubator
279 in a BSL-2 facility without any antibiotics. All cell lines were tested for potential mycoplasma
280 contamination using a commercial kit (Lonza). Prior to any medium change, cells were counted
281 using trypan blue exclusion or a Muse cell counter device (Millipore). Expanded human NK
282 cells (eNKs) were produced from umbilical cord blood (UCB) as previously described [21].
283 Briefly, after PBMCs ficoll extraction, CD3+ cells were depleted with an EasySep™ kit (Stem

284 Cells). CD56 positive cells were determined by a CD3-/CD56+ cells staining on the Muse
285 device. According to CD56 number, PLH irradiated cells (4:1) were added to NK cells culture
286 with RPMI-FBS (10%) supplemented with IL-2 and IL-15 (100 UI/mL and 5 ng/mL,
287 respectively) After 14-21 days, eNK were ready to use [21].

288 **4.8. Antibody Dependent Cellular Cytotoxicity assay (ADCC) on B cell lines**

289 Sensitive target cells (Raji, BCL-P2, PLH, Daudi) were stained with CellTrace™ Violet cyto
290 trace violet (CTV; 4 µM) in PBS for 20 min at 37°C. Resistant target cells (Molm-13) were
291 stained with CytoTracker™ Green (CTG; 4 µM) and used as internal control. Cells were then
292 centrifuged (5 min, 500 g, RT) and resuspended in 14 mL of RPMI medium (without FBS)
293 during 30 min at 37°C. Then, sensitive and resistant cells and effector (eNK) cells were placed
294 together in a 96-well plate at 0.5 million cells in 200 µL per well. After centrifugation (5 min,
295 450 g), cells were resuspended in RPMI-10% FBS containing native or formulated RTX
296 overnight at 37 °C with eNKs at the expected ratios. Afterwards, cells were washed and stained
297 in 100 µL during 20 min at 4°C with fluorescent antibodies and chemicals. CD56-PE staining
298 and target cell tracker staining were used to better distinguish effector and target cell
299 populations. Viobility (FL9) for Daudi and 7AAD (FL4) staining for Raji were used to assess
300 cell death. Cells were washed and analysed by FACs in a Gallios 3L (Beckman) using Kaluza
301 software [22,23]. eNK natural cytotoxicity was measured in absence of antibody. After doublet
302 exclusion, death staining signal was observed over CD56 negative population.

303 **4.9. NFAT reporter activation assay**

304 Effector and target cells from the ADCC Reporter Bioassay (Promega) were thawed the day of
305 the experiment and resuspended in RPMI 1640 medium with 4% low IgG FBS. Effector cells
306 were added to each well (75,000 cells per well) of a V-bottom 96-well plate. Target cells were
307 then added at 6:1 (E:T) ratio. 25 µL of RTX samples were added for a final volume of 75 µL
308 and samples incubated during 8 hours at 37°C (5% CO₂). Afterwards, the plate was placed 15
309 minutes at RT prior the addition of the Nano-Glo®Dual-luciferase® reagent. Then, a sequential

310 quantification of luminescence at t=10-30 min was performed using a bioluminescence reader.
311 Samples were analyzed in triplicate.

312 **4.10. CD20 binding assay**

313 Raji cells were plated in 96-well round bottom plates at 1×10^6 cells/mL in 200 μ L. The different
314 RTX samples were added for 30 min at 4°C and cells were washed three times in PBS 2% FBS.
315 RTX was targeted using an AlexaFluor647 conjugated anti-human IgG (H+L) antibody
316 (Jackson ImmunoResearch) at an 800-fold dilution for 30 min at 4°C. Cells were washed three
317 times in PBS and resuspended in 250 μ L of PBS with 2 % FBS before FACS analysis. 10 000
318 events were analysed by FACs in a Gallios 3L (Beckman) using Kaluza software.

319

320 **4.11. RTX and formulated RTX antitumor activity**

321 BCL-P2 cells (5×10^6 in 50 μ L PBS) were injected subcutaneously (flank area) in 6-10 week-
322 old NOD scid gamma (NSG) mice. Four days later, mice were randomized in the different
323 treatment groups and at day 6, RTX was administered intravenously as a saline solution or
324 subcutaneously for the RTX ISFD formulation at a dose of 10 mg/kg. At 5, 13 and 20 days, 5
325 $\times 10^6$ eNK were injected. Mice weight and tumor size were measured at 5, 7, 13, 19, 20, 21, 26,
326 28, 33, 35, 38, 42, 48, and 53 days after RTX administration.

327 **4.12. RTX, mRTX, DARA and ADA detection by ELISA**

328 In-house ELISA assay was developed using a pair of anti-RTX antibodies (HCA186 for the
329 capture, MCA2260P for the detection) (BioRad). As both antibodies recognize RTX paratopes,
330 mRTX could be quantified as well using the same assay. Briefly, 96 half-well plates were
331 coated overnight at 4°C with 50 μ L of HCA186 at 1 μ g/mL in PBS. After washing in PBST
332 (PBS + 0.05% Tween20) and blocking with PBST supplemented with 5% BSA during 90 min
333 at room temperature (RT), standards and samples were adequately diluted in PBST and 50 μ L
334 were incubated during 1 hour at RT. Detection was achieved using the HRP conjugated
335 antibody MCA2260P at 1 μ g/mL in blocking buffer (5% BSA in PBST) and incubated with
336 3,3',5,5'-tétraméthylbenzidine (TMB) peroxidase substrate. The optical density was measured

337 at 450 nm after HCl 1N addition. Each sample was analyzed in duplicate. The RTX standard
338 curve (0.6 to 450 ng/mL) was fit using a 4-parameter logistic regression. As the limit of
339 detection in the ELISA assay was around 2 ng/mL, associated with a maximal sample dilution
340 of 5-fold, the RTX LOQ (Limit of Quantification) was around 10 ng/mL.

341 DARA quantification in mice serum or RPMI after knee collection was made using an ELISA
342 kit for Human IgG (ThermoFisher), following the manufacturer recommendations.

343 Anti-RTX antibodies were detected through a simple ELISA assay using RTX as a probe
344 (overnight coating at 1.5 µg/mL in PBS), incubation of the samples after adequate dilution (50-
345 fold and 20-fold dilutions for the serum and synovial samples, respectively) during 1 hour at
346 room temperature and detection of the anti-RTX mice antibodies with a goat anti-mouse IgG
347 HRP (Abcam).

348 **4.13. Biocompatibility and pharmacokinetics studies after IA delivery**

349 In vivo studies were performed using 10-week old C57BL/6j male mice. Experiments were
350 performed in accordance with the European Community guidelines (86/609/EEC) and the
351 French National Committee (87/848) for the care and use of laboratory animals and were
352 approved by the regional ethics committee for animal experimentation in Languedoc Roussillon
353 (Reference APAFIS#21840-2019081912221252, n°036; approval number: CEEA-LR-12163).

354 Test items (physiological serum, saline protein solutions and ISFD formulations or vehicles)
355 were injected IA (2.5 µL) into the right knee of animals to achieve a RTX dose of 25 µg. Blood
356 sampling was performed at 1, 7, 14, 21 and 28 days. Knee joints were recovered at day 1, 14,
357 21 and 28 and incubated for 1 hour at 37°C in 1 mL of RPMI medium after rupture of the
358 synovial capsule to obtain the synovial fluid. RTX concentration in synovial fluid was adjusted
359 considering arbitrarily a knee synovial fluid volume of 5 µL. Recovered knees were fixed 7
360 days in 3.7% formaldehyde at ambient temperature. Knees were then rinsed and decalcified in
361 5% formic acid solution for 7 days. After paraffin inclusion, three frontal sections of 7 µm in
362 thickness and spaced by 100 µm were recovered from each tibia and stained with
363 hematoxylin/eosin or safranin O/fast green staining. Synovitis was graded on

364 hyperplasia/enlargement of synovial lining layer and the degree of inflammatory infiltration as
365 follows: no synovitis (0), slight (1), moderate (2) and strong synovitis (3).

366 Osteophyte formation was attributed a score from 0 (absence of osteophytes) to 3 (osteophyte
367 with the highest volume) depending on the osteophyte volume at the edge of lateral and median
368 tibial plateaus. The final score is the mean of the scores attributed for the 3 sections obtained
369 from each sample.

370 **4.14. Statistical analysis**

371 All statistics were performed using Graph Pad Prism version 9.1.0 All tests were performed on
372 at least three experiments within technical triplicates. All graphs present means, and standard
373 deviation obtained by row stats or column stats tools. Multiple comparisons of repeated assays
374 were done over each group within each experiment (mean, n and SD) using Two-way ANOVA
375 to compare the different conditions. All XY data were analysed by spearman correlation.

376

377 **5. RESULTS**

378 The proteins were formulated in solid state. For this purpose, copolymers were first solubilized
379 in tripropionin, a small chain triglyceride that presents a low solubility into water, to obtain a
380 so-called polymer vehicle. Then, a protein solid cake was dispersed into the polymer vehicle to
381 achieve the targeted protein loading (Suppl. Fig. S1). Preliminary experiments had shown that
382 a spray dried (sd) protein cake was more suitable than a lyophilized sample to achieve high
383 drug loading, and easy to resuspend homogenous formulation (data not shown). The first step
384 was hence to produce a sd mAb cake.

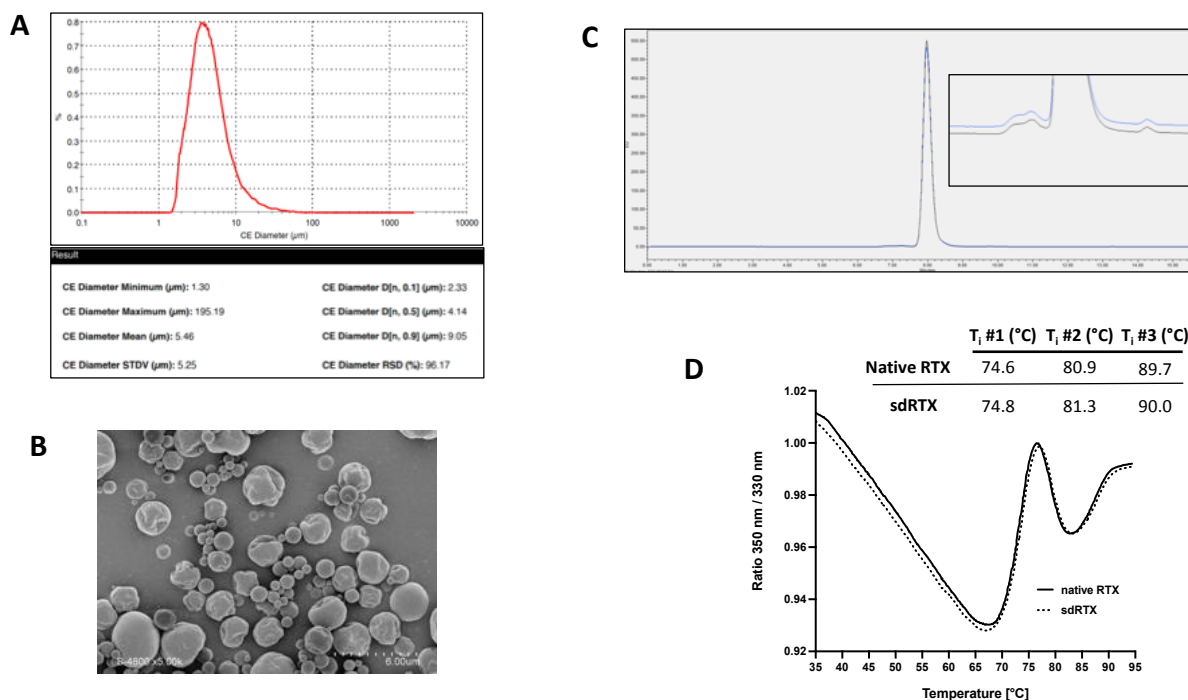
385 **5.1. mAb ISFD formulations**

386 MabThera[®] solution was spray dried without any prior manipulation. The particle size
387 distribution analysis of the obtained powder revealed a mean particle size of 5.5 μm and a
388 narrow particle size distribution (D_{90} of 9.1 μm) (Fig. 1A). SEM imaging of the spray dried
389 cake confirmed the presence of a homogenous population with most particles presenting a dry
390 grape shape (Fig. 1B). The RTX commercial product should theoretically lead to a powder

391 presenting 37 % (w/w) protein content along with salt (NaCl) and buffering agent (sodium
392 citrate). After solubilization of the spray dried cake into water, no visible particles could be
393 observed, and the protein was quantified either by SEC-HPLC or by using a micro-BCA assay.
394 Both methods retrieved the expected protein content value, which confirmed the fact that the
395 drying process did not induce a preferential loss of the protein. However, after a centrifugation
396 step (10 min, 16000 g), a 2 % protein loss was observed with the BCA assay and not by SEC-
397 HPLC, which could be an indication of the presence of some subvisible particles in the protein
398 solution. The SEC-HPLC analysis of the spray dried RTX (sdRTX) showed also that the drying
399 process induced a slight increase from 0.6 % to 1.2 % of the HMW fraction (Fig. 1C). Finally,
400 a thermal analysis was conducted on the native RTX and the sdRTX protein solubilized in PBS,
401 displaying superimposable profiles with very similar transition temperatures (Fig. 1D) and
402 showing that the first inflection temperature was over 70°C while the observed outlet
403 temperature during the spray drying process was below 60°C. Finally, mass spectrometry
404 analysis was performed on the full-length proteins (native RTX and sdRTX) after
405 deglycosylation showing the same molecular weight for both proteins corresponding to lysine-
406 clipped heavy chains and the presence of pyro-glutamate as a N-terminal residue in both the
407 light and heavy chains (Suppl. Fig. S2).

408

409



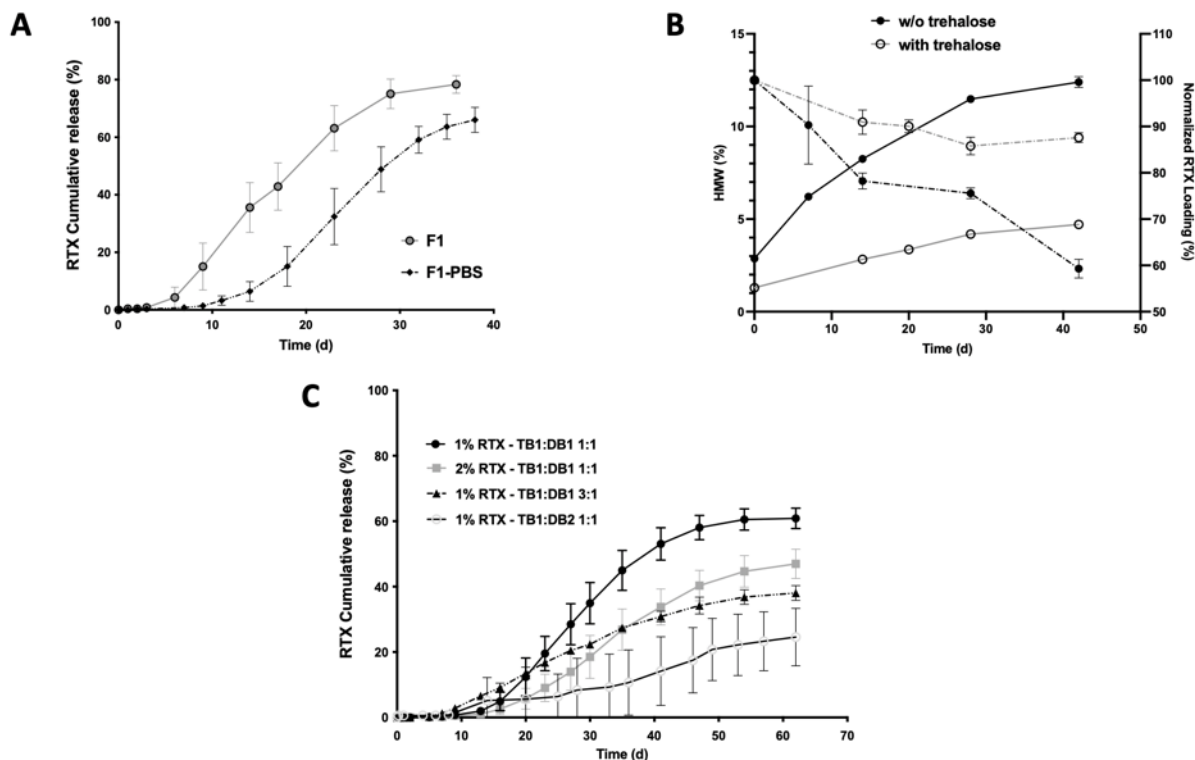
410
 411
 412 **Fig. 1: Characterization of spray dried RTX.** (A) Particle size distribution analysis of the spray
 413 dried protein. (B) SEC-HPLC chromatograms of native RTX (black trace) and sdRTX (blue
 414 trace). The inset is a zoom on the elution peak baseline for a better visualization of HMW
 415 species. (C) SEM imaging of sdRTX powder. (D) Thermal analysis of native RTX (black trace)
 416 and sdRTX (dashed line). The ratio between the emitted fluorescence at 350 nm and 330 nm is
 417 represented in function of the temperature. Inflection temperatures (T_i) are indicated for both
 418 proteins.

419
 420 sdRTX was then dispersed into the V1 tripropionin-based polymeric vehicle presenting the
 421 TB1/DB1 polymer composition in a 1:1 ratio with a 20 % (w/w) total polymer content to yield
 422 the formulation named F1 (Table 1). The sd cake loading was 2.8 % (w/w) to achieve a 1.0 %
 423 (w/w) RTX loading in the final F1 formulation. The polymer concentration could have been
 424 increased to modify the sustained release properties of the formulation, but it was voluntarily
 425 kept low to make the formulation easily injectable through a 26 G needle for further *in vivo*
 426 evaluation. The *in vitro* dissolution profile of formulation F1 (TB1:DB1 polymer composition)
 427 was determined by making 100 mg depots (n=3) in the SEC-Tween release medium. We
 428 observed a S-shaped IVR profile consisting in an initial lag phase of 3 to 4 days followed by a

429 20-day steady release phase before reaching a plateau at around 70 % release of the injected
430 cargo (Fig. 2A). Interestingly, the substitution of PBS to SEC-Tween as a release buffer
431 accentuated the S-shaped release profile with a longer initial lag phase of around 10 days (Fig.
432 2A). For biocompatibility reasons, the PBS release medium was used for cell based and *in vivo*
433 studies (see below). Independently of the release medium, the F1 formulation displayed
434 promising *in vitro* sustained release properties and a good overall protein recovery as 10% to
435 15% of the initial protein cargo could be retrieved in the depots at the end of the release
436 experiment. Of note, the onset of protein release coincided with the release of lactic acid in the
437 release medium making the bridge between protein release and polymer degradation. However,
438 an early-stage stability study showed that the HMW fraction increased significantly upon
439 storage at 4°C for several weeks (Fig. 2B). In order to overcome this drawback, the composition
440 of the sd protein cake was adjusted by adding trehalose, a disaccharide well-known to stabilize
441 proteins during drying processes. Its presence substantially improved the protein stability
442 within the BEPO[®] formulation by reducing the formation of HMW species (Fig. 2B).

443 With trehalose present in the sdRTX cake, the influence on the IVR profile of different
444 parameters such as the diblock nature, the TB:DB ratio or the protein loading was investigated.
445 A more hydrophobic diblock (DB2) considerably slowed down the release kinetics but
446 conversely prevented a complete release of the protein cargo as the cumulative release profile
447 plateaued at a 30 % release of the initially encapsulated antibody (Fig. 2C). Only 15 to 20 % of
448 the drug cargo was retrieved at the end of the IVR study when protein was extracted and
449 quantified from the remaining depots, which could be the signature of some irreversible protein
450 binding to the polymer matrix that could be due to the hydrophobic nature of the diblock DB2
451 molecule. The change in the TB:DB ratio with a higher proportion of TB attenuated the S-
452 shaped release profile and resulted in an almost linear release kinetics with a release rate of
453 approximately 1 % of protein cargo per day, while unexpectedly the increase in protein loading
454 did not accelerate the release. For this last formulation, loaded with 2 % of protein, 40 % of the
455 initial protein cargo was retrieved in the depots at the end of the IVR study, leading to a similar

456 overall recovery as F1. Based on these results, for further investigations, we focused on the F1
457 formulation (1 % (w/w) RTX final loading in the V1 BEPO[®] vehicle).



458
459 **Fig. 2: In vitro dissolution profiles and stability of BEPO[®]:RTX formulations.** (A) In vitro
460 release profiles of F1 formulation (TB1:DB1 polymer composition). (B) Impact of the presence
461 of trehalose in the sd RTX cake on the stability of the F1 formulation stored at 4°C followed by
462 SEC-HPLC (HMW fraction in plain lines, normalized protein loading in dashed lines). (C)
463 Impact of variation of diblock, triblock type and ratio and drug loading in the formulation over
464 in vitro release profile.

465
466 Benefiting from the experience gathered while formulating RTX, we chose to formulate another
467 mAb, daratumumab (DARA), an anti-CD38 antibody commercialized under the brand name
468 Darzalex[®]. The commercial solution was buffer-exchanged for a trehalose containing solution
469 and spray dried using the same conditions as those used for RTX. The particle size distribution
470 of the sdDARA cake was similar to that of RTX and SEC-HPLC analysis demonstrated that
471 the spray dried protein had a similar profile as the commercial one, especially in terms of HMW
472 fraction (data not shown). sdDARA was then dispersed into the polymeric vehicle V1 to achieve

473 a 1 % (w/w) final protein loading. The resulting formulation presented an IVR profile similar
474 to the one loaded with RTX but with an earlier onset of protein release (Suppl. Fig. S3). This
475 supported our hypothesis that given our solid-state formulation approach, the identity of two
476 proteins of similar chemical nature would have a minimal impact on a formulation with a
477 selected polymer composition in terms of release characteristics.

478

479 **5.2. Physico-chemical characterizations of formulated RTX**

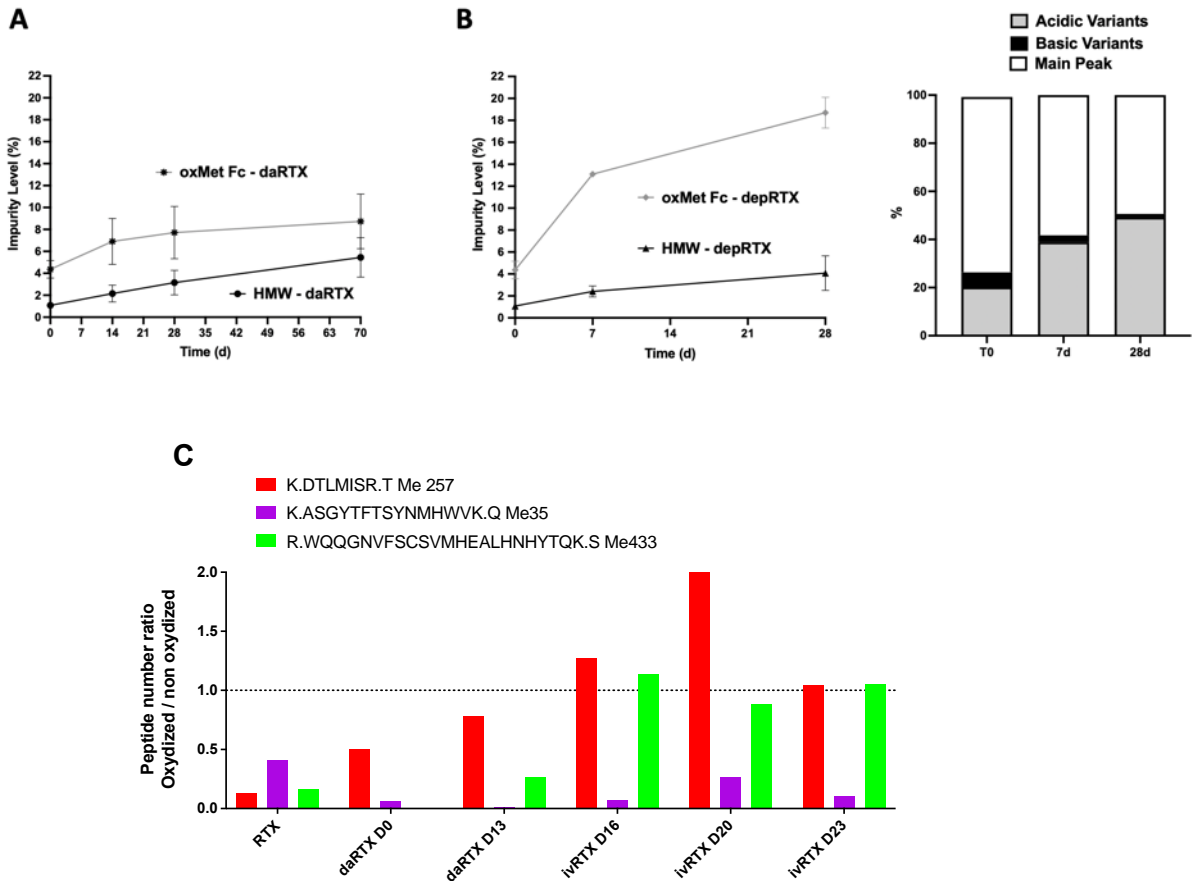
480 To monitor the potential generation of post-translational modifications (PTMs) during the
481 formulation process, different physico-chemical analyses were carried out on the formulated
482 protein. First, orthogonal LC methods were developed and RTX stability was assessed under
483 two angles. First, the stability of the protein in the formulation was monitored during the
484 subsequent storage of the formulation at 4°C during several weeks (daRTX samples, Suppl.
485 Fig. S4). Also, the status of the released protein was evaluated during the course of an IVR
486 study (ivRTX samples, Suppl. Fig. S4). In the latter, the concentration of the released protein
487 was often too low to allow for a valid analysis. In consequence, as a surrogate, to avoid any
488 protein concentration step that could be a bias, RTX was extracted from the solid polymer
489 depots incubated at 37°C (depRTX samples, Suppl. Fig. S4) as a reflect of the released protein
490 stability. RTX F1 formulation with trehalose was taken as the reference formulation for all these
491 analyses.

492 The SEC-HPLC analysis of three independent studies showed that the fraction of HMW species
493 increased steadily, almost linearly, upon storage at 4°C to reach around 5 % of HMW species
494 after 10 weeks. Of note, the HMW fraction of the commercial protein is 0.6 % (Fig. 3A). SCX-
495 HPLC analysis of the same samples showed a good stability of the charge variant distribution
496 with a stable proportion of both acidic and basic variants (data not shown). RP-HPLC analysis
497 after IdeS digestion highlighted the generation of a main impurity that was associated to the Fc
498 moiety and presented a relative retention time of 0.95 compared to the Fc elution peak. Further
499 mass spectrometry analysis showed that this impurity presented a mass adduct of +16 Da

500 corresponding most probably to the oxidation of a methionine residue. This impurity was noted
501 oxMet Fc and its content was reported as the fraction of the Fc moiety elution peak area.
502 Compared to the initial oxidized methionine content of 2.9 % for the native protein, methionine
503 oxidation level increased upon formulation (4.4 % for the daRTX T0 samples) and the trend
504 amplified during storage to reach almost 9 % after 10 weeks storage (Fig 3A). The stability
505 pattern was more contrasted regarding the released protein. If the protein extracted from the
506 depot (depRTX samples) presented a limited fraction of HMW species (4 % after four weeks
507 incubation at 37°C in the polymeric matrix), it displayed a pronounced oxidation of methionine
508 residues on the Fc moiety, up to 20% (Fig. 3B, left). The charge variant distribution changed
509 also noticeably in the depot. SCX-HPLC revealed that native RTX presented acidic and basic
510 variants that accounted respectively for 20 % and 6 % of the total peak area. The incubation
511 within the depot led to a progressive disappearance of the basic variants and a concomitant
512 increase of the proportion of acidic variants at the expense of the main peak relative area (Fig.
513 3B, right). To study more in-depth the stability profile of the formulated RTX, a full tryptic
514 peptide mapping was undertaken. RTX has a well-known peptidic signature, which includes
515 the following peptides: GLEWIGAIYPGNGDTSYNQK, ASGYTFTSYNMHWVK and
516 FSGSGSGTSYSLTISR. For all tested samples, we obtained a proper sequence coverage,
517 associated with a good confidence Mascot score and a reasonable quantity of detected peptides
518 (Suppl. Fig. S5). Afterwards, the modification of three peptides of the heavy chain, each of
519 them containing a methionine residue, was analyzed. The native RTX protein presented
520 minimal methionine oxidation, the most noticeable one being observed on the residue Met35.
521 Confirming previous observations made after IdeS digestion, LC-MS data showed that the
522 formulation process enhanced the oxidation of methionine Met257. Increasing levels of
523 oxMet257 were observed during formulation storage (daRTX samples), while highest levels
524 were observed for the released protein (ivRTX samples). These same samples showed also an
525 increased oxidation of the Met433 residue (Fig. 3C).

526

527



528 **Fig. 3: Stability of formulated RTX.** (A) Evolution of the HMW fraction and the Fc-related
 529 oxidation impurities (oxMet Fc) during a 10-week storage at 4°C of a BEPO®:RTX formulation.
 530 (B) Evaluation of the impurity pattern of depRTX samples for the protein extracted from the
 531 polymer depots after 7 days and 28 days of incubation at 37°C through the three LC methods.
 532 (C) For the same RTX samples, analysis by Q-tof Impact2 MS of the oxidation pattern of three
 533 heavy chain peptides carrying out one methionine residue potentially sensitive to oxidation.

534

535 The DTLMISR peptide carrying Met257 residue was under particular scrutiny, as several
 536 studies have shown that this conserved methionine residue located in the Fc moiety of human
 537 IgG1 is prone to oxidation, which could reduce the antibody binding affinity to the FcRn
 538 receptor and hence imparts the antibody pharmacokinetics properties [24–26]. In an effort to
 539 better evaluate the potential outcome of this PTM, the binding affinities of native and
 540 formulated RTX to CD16a (FcγRIIIa), including the potential V158F polymorphism, CD16b
 541 (FcγRIIIb) and FcRn receptors was measured *in vitro* by Surface Plasmon Resonance (SPR).

542 Table 2 shows that RTX extracted at different times from the formulation or polymer depots

543 showed similar binding affinities for those receptors as native protein, suggesting that the PTMs
 544 that had been evidenced on the Fc moiety had not reached a level that would alter RTX binding
 545 properties.

546 **Table 2: Affinity of formulated RTX samples for CD16a, CD16b and FcRn receptors**

<u>Proteins</u>	CD16a K _D pH 7.4 (M)	CD16a V158F K _D pH 7.4 (M)	CD16b K _D pH 7.4 (M)	FcRn K _D pH 6.0 (M)
Native RTX	2.74 x 10 ⁻⁷	9.52 x 10 ⁻⁷	15.40 x 10 ⁻⁷	2.73 x 10 ⁻⁸
daRTX T0	1.30 x 10 ⁻⁷	2.69 x 10 ⁻⁷	4.97 x 10 ⁻⁷	2.47 x 10 ⁻⁸
daRTX 5w 4°C	1.13 x 10 ⁻⁷	1.99 x 10 ⁻⁷	5.72 x 10 ⁻⁷	2.63 x 10 ⁻⁸
depRTX 7d	1.00 x 10 ⁻⁷	1.33 x 10 ⁻⁷	2.96 x 10 ⁻⁷	2.67 x 10 ⁻⁸
depRTX 13d	0.92 x 10 ⁻⁷	1.06 x 10 ⁻⁷	2.19 x 10 ⁻⁷	2.64 x 10 ⁻⁸

547

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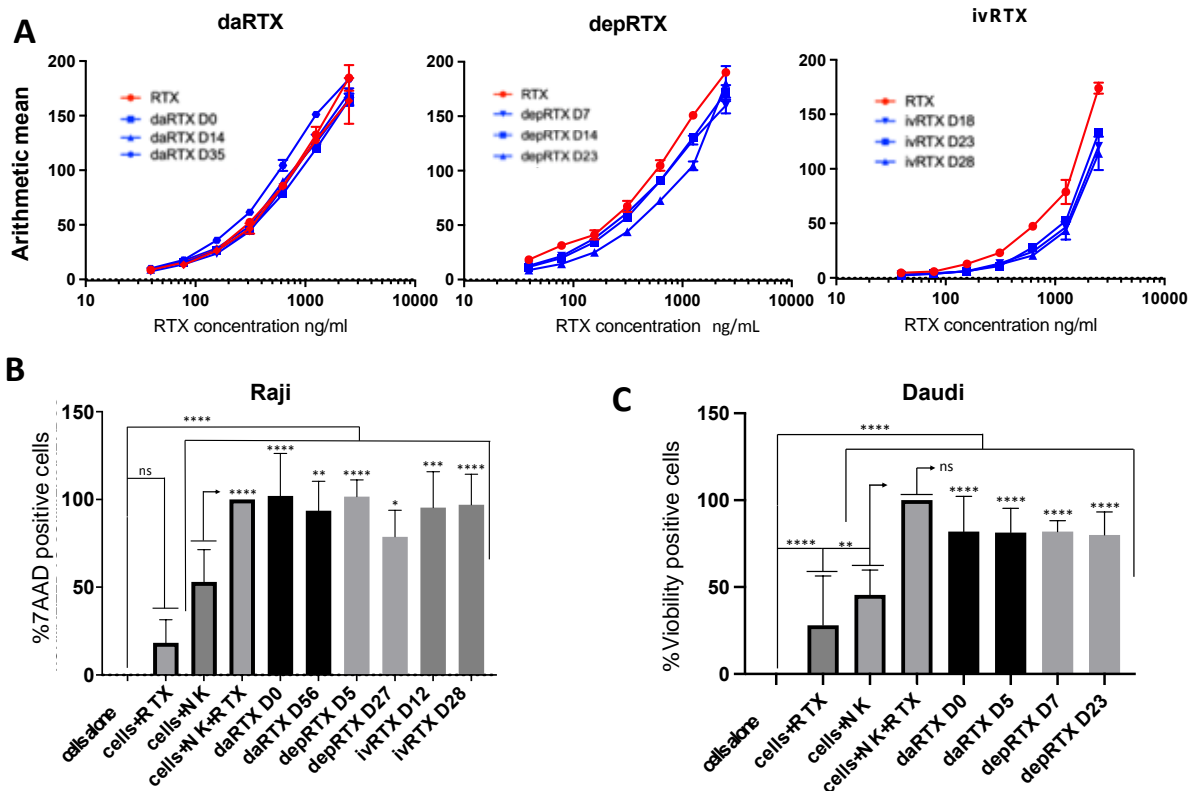
549 **5.3. Biological activity of formulated mAbs**

550 We then investigated the protein biological activity through different approaches. First, the
 551 capacity of sdRTX to generate antibody-dependent cell cytotoxicity (ADCC), a process that
 552 requires recognition of the Fc moiety by the CD16 receptor expressed by NK cells and the
 553 recognition of CD20 on the plasma membrane of target cells (*i.e.* Raji cells) by the Fab moiety,
 554 was analyzed. It was firstly observed that sdRTX bound Raji cells like RTX and induced ADCC
 555 with a similar efficiency than RTX (Suppl. Fig. S6). This showed that the spray drying process
 556 did not alter the protein functionality.

557 Next, we studied the CD20 binding activity of various formulated RTX samples by flow
 558 cytometry. The selected samples obtained from formulated, depot-extracted or in vitro released
 559 RTX displayed similar binding activity than the native RTX (Fig. 4A). Moreover, the same
 560 observations were essayed for CD16 binding using the Jurkat-NFAT reporter assay (Suppl. Fig.
 561 S7). Altogether, these results suggested that Fab and Fc moieties of RTX were both still
 562 functional during the formulation process.

563 To further investigate antibody-based effector functions, the capability of RTX to elicit ADCC
 564 against two CD20+ vcell lines, Raji and Daudi, was evaluated. RTX or NK cells alone induced

565 cell death, while their concomitant addition provoked a synergistic effect (Fig. 4B and C and
 566 Suppl. Fig. S8). Again, formulated RTX (daRTX, ivRTX and depRTX) samples induced
 567 similar ADCC than the native RTX (Fig. 4B and 4C). In summary, taken together, these results
 568 demonstrate that a BEPO[®] derived depot released a fully functional RTX protein. Additionally,
 569 the biological activity of RTX extracted from an ISFD formulation after several weeks of
 570 storage at 4°C was clearly established.

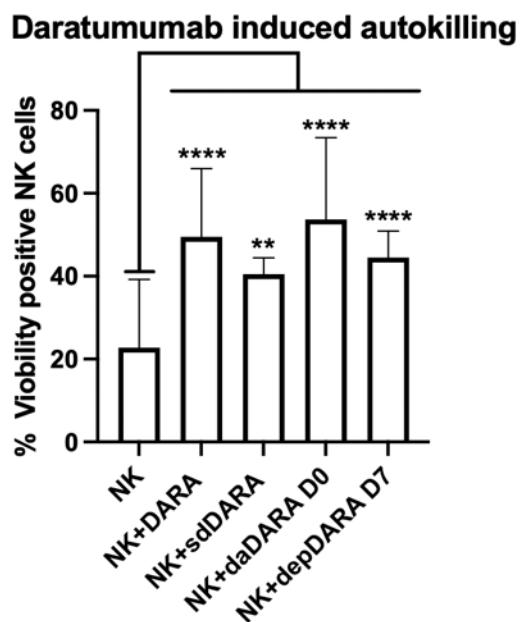


571

572 **Fig. 4: Functionality of formulated RTX.** All RTX samples were obtained from the same
 573 BEPO[®]:RTX formulation (F1 with trehalose) and were either extracted from the formulation
 574 after storage at 4°C (daRTX samples) or obtained during the release study through the
 575 evaluation of the released protein (ivRTX) or the extraction of the protein from the solid
 576 polymer depot (depRTX) at different time points. (A) Binding of RTX to CD20 on Raji cells, as
 577 expressed as the arithmetic mean fluorescence intensity (MFI). B-C) ADCC assay with different
 578 RTX samples (10 µg/ml), in presence of NK cells (E:T, 3:1) carried out overnight on Raji cells
 579 using 7AAD staining (B) or after 8 hours on Daudi cells using viabilityTM staining (C). Basal

580 cell death was normalized to 0% and commercial RTX+NK cells to 100%. Sample activity was
581 presented as % of native RTX activity. Graphs show mean \pm SD of a minimum of 2 experiments
582 performed in triplicate. Samples were compared by 2-ANOVA test, $n = 21$, * $p < 0.05$, ** $p <$
583 0.01 *** $p < 0.001$; **** $p < 0.0001$.

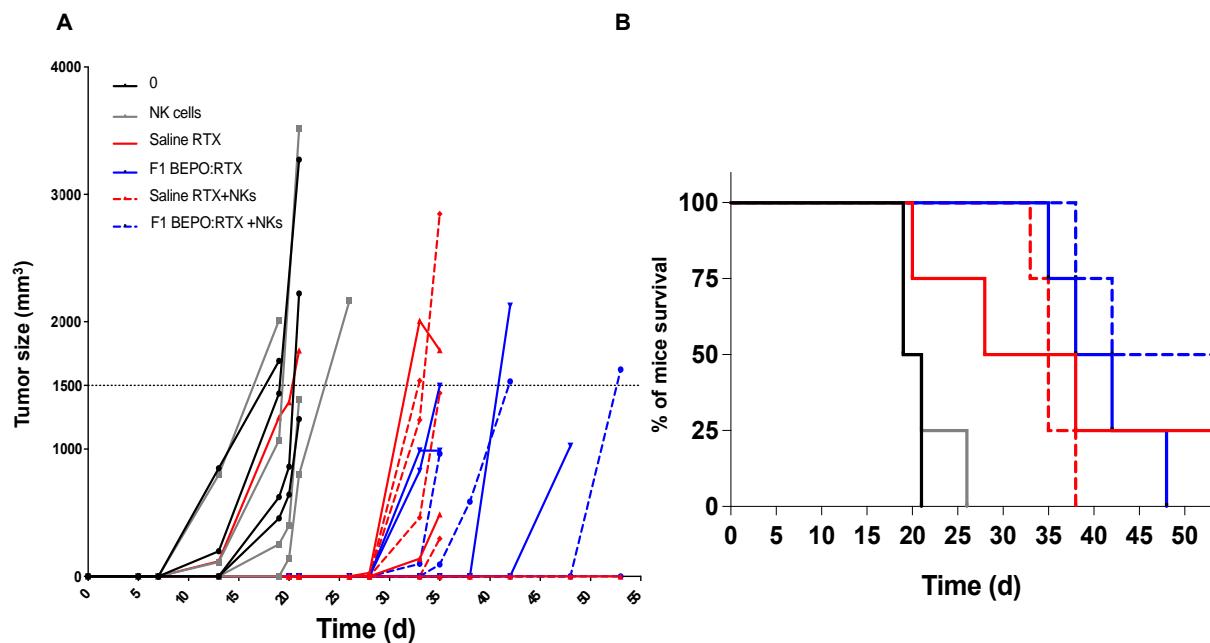
584
585 Functionality of formulated DARA was also evaluated using a pseudo-ADCC assay. As CD38
586 is present at the surface of NK cells, these cells served both as target and effector cells. In
587 consequence, the incubation of DARA with NK cells should lead to their rapid death if both
588 antibody moieties retain their functionality. Indeed, DARA significantly raised NK cell death
589 by an average of 26% and da, dep and sdDARA samples from formulated DARA exhibited
590 similar effects (Fig. 5).



591
592 **Fig. 5: Functionality of formulated DARA evaluated through NK cells autokilling.**
593 DARA samples were obtained and extracted from the BEPO[®]:DARA formulation presenting
594 the TB1:DB1 polymer composition. sdDARA was the spray dried protein solubilized in PBS,
595 daDARA D0 was the protein extracted from the formulation just after reconstitution while
596 depDARA D7 was obtained after the extraction of the protein from the solid polymer depot 7
597 days after depot formation.

598

599 The *in vivo* biological activity of the RTX F1 formulation was further assessed in a
 600 subcutaneous xenograft model of the human CD20+ lymphoma cell line BCL-P2 in NSG mice.
 601 A rapid tumor growth was observed and overall survival was shorter than 25 days (Fig. 6A).
 602 While non-significant improvements were made by regular NK cells injection, intravenous
 603 treatment with saline RTX largely decreased tumor growth and increased mice survival
 604 independently of NK cell engraftment. Subcutaneous injection of F1 formulation was as
 605 effective as saline RTX and, in fact, tumor initial growth was delayed and mice survived longer,
 606 demonstrating the full *in vivo* functionality of the formulated RTX (Fig. 6B).
 607



608 **Fig. 6: Evaluation of RTX functionality in a model of B cell lymphoma cell xenograft into**
 609 **NSG mice. 5 millions of BCL-P2 lymphoma B cells were grafted into NSG mice. (A) Evolution**
 610 **of the tumor size after tumor cell engraftment. (B) Survival curves representing the percentage**
 611 **of animals with tumor size under 1500 mm³ in function of days after tumor cell inoculation.**

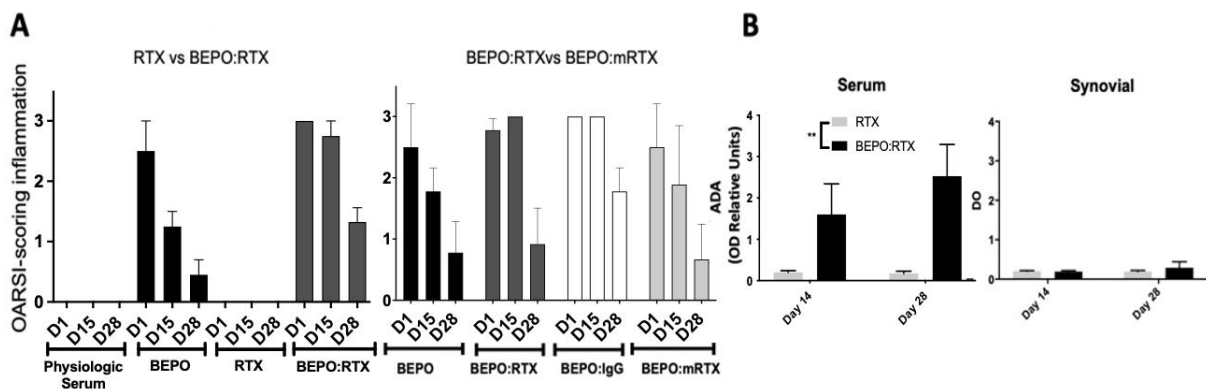
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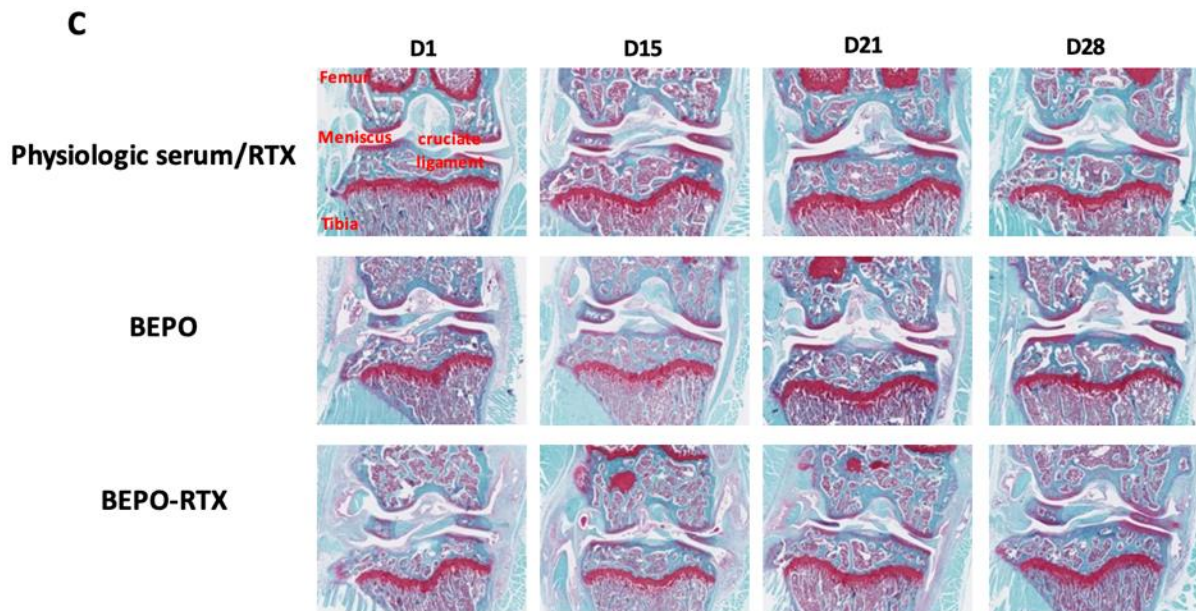
613 5.4. Intra-articular delivery

614 Because of the generation of a drug reservoir upon injection, *in situ* forming depot technologies
 615 are especially relevant to locally increase mAb concentration in, or close to, the target tissue.

616 In this study, mAb ISFD formulations were tested using the intra-articular (IA) route of

617 administration. First, biocompatibility was evaluated over 28 days after IA injection of the V1
618 polymeric vehicle with or without RTX. In contrast to the RTX saline solution, both vehicle
619 and RTX formulation induced an initial inflammatory reaction that partly resolved over time
620 (Fig. 7A, left). The pattern was typical of a foreign body reaction. The higher inflammatory
621 reaction noticed for the formulation could be due to the formulation of a non-murine protein as
622 RTX is a chimeric antibody that presents murine variable and human constant regions. Indeed,
623 anti-drug antibodies (ADAs), *i.e.* anti-RTX antibodies, were detected in the serum of treated
624 animals, which could be related with the exacerbated inflammation (Fig. 7B).
625 This hypothesis was supported by the fact that IA injection of a fully murine protein (mRTX)
626 formulated with BEPO[®] led to lower inflammation levels compared to those generated with the
627 chimeric antibody (Fig. 7A, right). Moreover, formulation of a rat IgG2A isotype generated a
628 similar inflammation than the humanized RTX (Fig. 7A, right). Still, inflammation was going
629 down at day 28. Of note, biocompatibility of the injected material was confirmed by the fact
630 that no cartilage or subchondral bone damage could be detected after staining with safranin O
631 Fast green (Fig. 7C).
632





633

634 **Fig. 7: Biocompatibility of BEPO[®] vehicles and formulations after IA delivery in mice.**

635 (A) 3 C57BL/6J mice/group/timepoint were injected with saline RTX or ISFD formulations of
 636 RTX, rat IgG2b or mRTX in the intra-articular space of their right knee to achieve a 25µg
 637 protein dose. The OARSI inflammation score ranged from 0 to 3 was analyzed at different times
 638 after treatment. (B) Anti-drug antibodies profile for RTX (grey) and BEPO[®]:RTX (black) in
 639 serum and synovial fluid at 14 and 28 days in mice from (A). (C) Representative knee sections
 640 from mice from (A) at day 1, 15, 21 and 28.

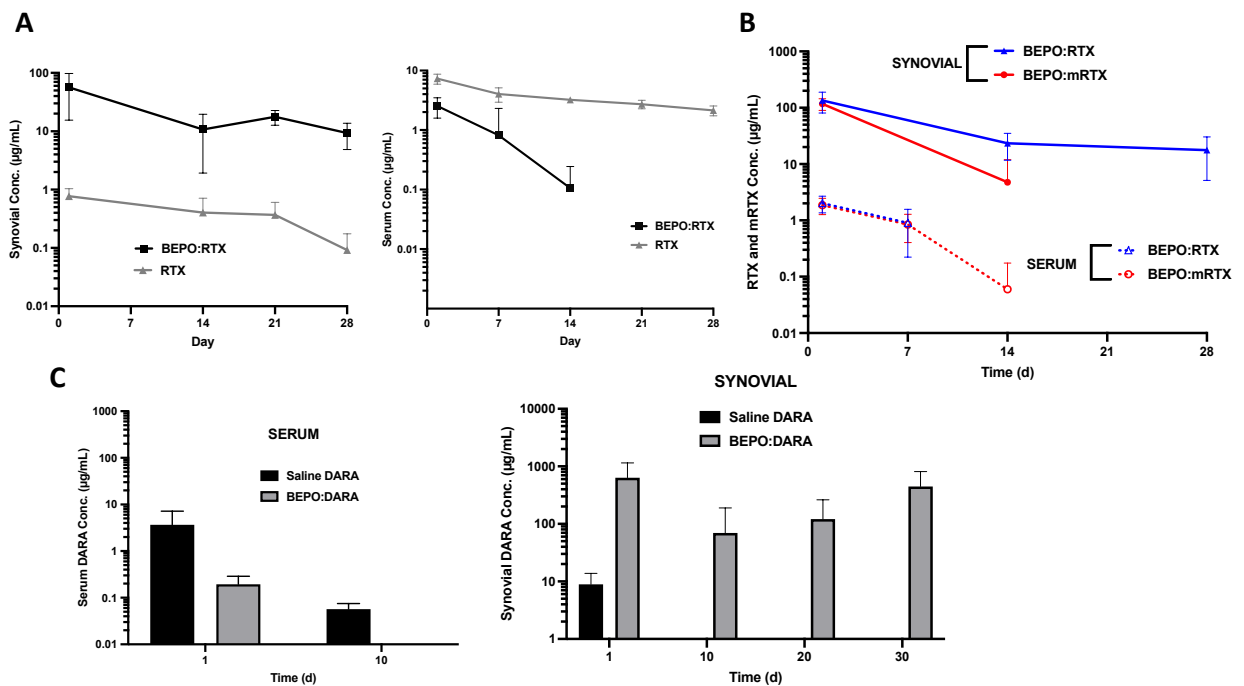
641

642 Next, we investigated the pharmacokinetics (PK) properties of the RTX F1 formulation. RTX
 643 was administered IA either as a saline solution or formulated with BEPO[®] (25 µg/2.5 µL). The
 644 resulting RTX concentrations in serum and in synovial fluid of the injected knee were measured
 645 over four weeks. In the saline group, the protein was mostly found in the systemic circulation
 646 as the C_{max} value normalized by the dose (7.3 (µg/mL)/(mg/kg)) was similar to the one that was
 647 obtained after subcutaneous administration of a RTX saline solution (Fig. 8A). RTX
 648 concentration in serum decreased following a 10-14 days half-life. As expected, the synovial
 649 fluid concentration was a fraction (around 10-fold lower) of that of serum. In contrast, IA
 650 injection of the F1 formulation allowed high synovial fluid concentrations sustained for four
 651 weeks with low systemic release. No RTX was retrieved in serum from 14 days post-inoculation

652 (Fig. 8A). The ratio between the synovial and serum concentrations at day 14 illustrated the
653 differences as it was 0.13 for the saline group and 101 for the BEPO[®] group.

654 As RTX triggered the generation of anti-RTX antibodies, we repeated the experiment using
655 murine RTX (mRTX). This latter formulation allowed sustained high synovial mRTX
656 concentrations for 14 days, which was less pronounced than for the RTX formulation (Fig 8B).
657 Still, the mRTX synovial to serum concentration ratio was greater than 1 at day 14. The faster
658 clearing of mRTX from synovial fluid could be due to its binding to Fc receptors present at the
659 surface of immune cells, which could accelerate synovial clearance.

660 To avoid generation of ADA and challenge our local delivery approach with a new mAb, we
661 administered IA the DARA protein into the right knee of immune suppressed NSG mice, which
662 lack B cells and hence antibody production. The clearance of human IgG1 is considerably
663 accelerated in these mice [27], a feature that we retrieved in our study as DARA was not
664 quantifiable anymore in the serum of the animals 10 days after IA injection of saline DARA
665 (Fig. 8C). DARA also had disappeared from synovial fluid at this time. We then dispersed
666 sdDARA into V1 polymeric vehicle at 1 % (w/w) and 25 µg of the resulting formulation was
667 IA injected. At day 10, DARA could not be detected in serum. However, it was still present in
668 synovial fluid 30 days after administration (Fig. 8C). In summary, these data demonstrate that
669 formulating mAbs with BEPO[®] allowed high local protein concentrations for several weeks.



671 **Fig. 8: Pharmacokinetics studies of BEPO:mAb formulations after intra-articular**
 672 **administration.** (A) 3 C57BL/6J mice/group/timepoint were injected with saline RTX or
 673 BEPO[®]:RTX formulation in the intra-articular space of their right knee to achieve a 25µg
 674 protein dose. RTX was quantified using a specific ELISA assay in both serum and knee synovial
 675 fluid at different times after treatment. (B) PK profiles of two BEPO[®] formulations
 676 incorporating either RTX or the fully murine mRTX protein. (C) 4 NSG mice/group were
 677 injected with saline DARA or BEPO[®]:DARA formulation in the intra-articular space of their
 678 right knee to achieve a 16 µg protein dose. DARA was quantified by ELISA in both serum and
 679 knee synovial fluid at different times after treatment.

680 6. DISCUSSION

681 The design of long-acting injectable protein formulations through the use of bioresorbable
 682 polymers has always represented a daunting task especially regarding the preservation of the
 683 protein integrity and functionality. In fact, there are currently no commercialized products of a
 684 therapeutic protein formulated with polyesters such as PLGA or PLA [28,29]. In order to
 685 minimize the generation of impurities due to interactions between protein and polymer, we
 686 opted for a strategy, in which the protein would stay in its solid state during the entire

687 formulation process. This approach had been used successfully to formulate a Fab fragment
688 with PLGA and, more recently, we managed to formulate a 55 kDa bispecific antibody with
689 PEG-PLA copolymers [17,30]. It requires the production of a protein powder, which in our case
690 was obtained via a spray drying process. Along lyophilization, spray drying has become a
691 method of choice to produce protein powders[31,32]. We confirmed that two clinically relevant
692 mAbs, namely RTX and DARA, were not altered by the spray drying process and retained their
693 functionality. Only a very slight increase of the HMW fraction was observed, an increase that
694 amplified during the formulation process. However, the generation of aggregates in the
695 formulation could be largely circumvented with the introduction of trehalose, a well-known
696 protein stabilizer during drying processes [33,34]. HMW fraction is a critical quality attribute
697 of therapeutic proteins as the presence of aggregates is often linked to immunogenicity issues.
698 A 5% limit is usually set as a specification for therapeutic mAb formulations in the
699 pharmaceutical industry and the presence of trehalose allowed to stay below this level even
700 after several weeks of storage at 4°C. Still, as most of antibody preparations present HMW
701 fractions below 2% [33,34], it should guide us to try to further improve the stability profile of
702 the formulated proteins.

703 Because local delivery requires usually the use of relatively low doses of the therapeutic
704 molecule, a low protein loading of 1 % (w/w) was chosen for the BEPO[®]:mAb formulation.
705 Also, polymer concentration was chosen to yield a formulation viscosity compatible with an
706 administration through a 26 G needle for further *in vivo* evaluations. Different IVR profiles
707 were generated, and we showed that different parameters in the formulation such as the polymer
708 composition could change the release kinetics. While the formulated protein showed a good
709 stability profile in the formulation stored at 4°C for several weeks, the protein released from
710 the polymer depots showed different PTMs revealed by a change in the charge variant
711 distribution as well as an increase of methionine oxidation on the Fc moiety. However, SPR
712 showed that these modifications did not alter the RTX binding affinities to several Fc receptors.
713 Moreover, *in vitro* cell-based assays showed that the functionality of formulated mAbs was

714 similar to those of the commercial products and full biological activity of formulated RTX was
715 demonstrated in a xenografted mice model. Thus, it can be concluded that our BEPO[®]:mAb
716 formulations released a protein with similar functional properties to the clinical ones.
717 Our second goal was to evaluate the pertinence of using such formulations for local delivery.
718 IA delivery is of particular interest as there are considerable unmet medical needs for the
719 treatment of rheumatic diseases and particularly of rheumatoid arthritis where 30% of patients
720 are resistant to biotherapies and osteoarthritis [35]. This delivery route associated with a drug
721 delivery system would undoubtedly improve the efficacy of treatments, as past and recent
722 clinical trial failures with systemic delivery of therapeutic proteins could be attributed to a lack
723 of synovial fluid exposure [36–38]. Here, we showed that the IA injection of BEPO[®]:mAb
724 formulations allowed to sustain high synovial fluid concentration of the antibody for several
725 weeks in the knee of mice in contrast to saline compositions. The formulation induced
726 inflammation locally, which could be related to the high quantity of foreign material injected
727 relative to the size of the mouse joint. In agreement with this hypothesis, the inflammatory
728 reaction disappeared overtime, probably in relation with the progressive polymer resorption.
729 Importantly, no cartilage or subchondral bone damage was detected. It would be interesting in
730 the future to better evaluate the inflammatory process in an animal species that presents a larger
731 synovial fluid volume. Another important observation was the higher inflammation levels that
732 were observed for BEPO[®]:mAbs formulations compared to the corresponding BEPO[®] vehicles
733 or protein saline solutions. This phenomenon could be related to the generation of ADAs
734 produced in BEPO[®]:RTX administered animals. The mouse immune system should recognize
735 RTX as a foreign antigen and reacts generating ADAs. B-cells can be activated to plasma cells
736 by antigens possessing repetitive epitopes which cross-link antigen-specific BCR, breaking
737 down B-cell tolerance [39,40]. The faculty of well-ordered structures to induce more potent
738 immune responses compared with monomeric proteins has been known for decades [41] and is
739 at the basis for new vaccine generation [42-45]. A breakdown in tolerance following the
740 formation of repetitive epitopes probably induces ADA [39]. The release process from the

741 polymeric depot, that is associated with the progressive solubilization of the embedded spray
742 dried protein cake should promote very high local RTX concentrations that could induce the
743 formation of subvisible particles or repetitive-like epitopes that can greatly enhance
744 immunogenicity. In addition, the presence of an inflammatory context due to the presence of a
745 foreign body, i.e. BEPO, could also play a role in the generation of ADA. Therefore, the
746 potential immunogenicity of a BEPO:RTX formulation will have to be studied more in-depth
747 in the future as high ADA titers could hamper the clinical development of such a product
748 especially for a chronic administration. However, it is interesting to note that very concentrated
749 epitopes, protein aggregation, repeat dosage and prolonged exposure may either break or lead
750 to tolerance [40,46], which suggests that the product final immunogenicity could be uncovered
751 only after its clinical use.

752 Finally, BEPO[®]:DARA formulation confirmed the results that we had generated with the RTX
753 protein in terms of preservation of protein integrity and functionality and the PK study in NSG
754 mice highlighted once again the pharmacokinetics promises held by such formulations. Taken
755 together, the results shown in the present paper demonstrate that intra-articular delivery of
756 BEPO[®]:mAb formulations could enable future treatments by increasing considerably the
757 synovial fluid:plasma concentration ratio, which could improve treatment efficacy while lower
758 potential side effects.

759

760 **7. CONCLUSION**

761 The BEPO[®] delivery platform offers a simple and effective tool to design long-acting injectable
762 therapeutic protein formulations, preserving their functionality and minimizing the generation
763 of PTMs. Moreover, after intra-articular administration, BEPO[®]:mAb formulations
764 demonstrated superior pharmacokinetics properties compared to IA saline bolus of the same
765 protein. This could improve the efficacy of future treatments in a therapeutic area where there
766 are still high unmet medical needs. It will be important in the future to confirm and expand our
767 data in larger animal species and exploit the BEPO[®] features for other modes of local delivery.

768

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776

777 **Author Contributions Statement**

778 A.F., W.L., S.G., D.N., P.LP. and M.V. designed experiments. A.F., K.T. and S.G. performed
779 experiments and analyzed results. A.F., M.V. and S.G. wrote the main manuscript text. All
780 authors reviewed and approved the manuscript.

781 **Ethical statement**

782 S.G. and A.LN. are MedinCell employees. S.G., W.L. and A.LN. are shareholders of
783 MedinCell. Other authors declare no competing financial interests.

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788 **Data Availability**

789 The authors confirm that the data supporting the findings of this study are available within the
790 article [and/or] its supplementary materials.

791

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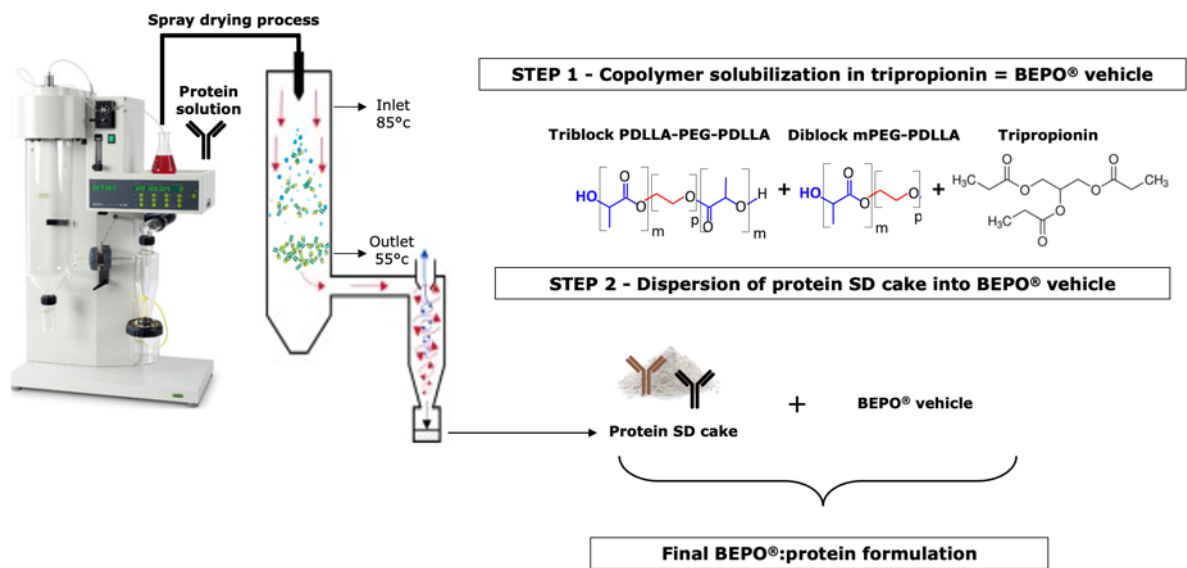
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Protein formulation process



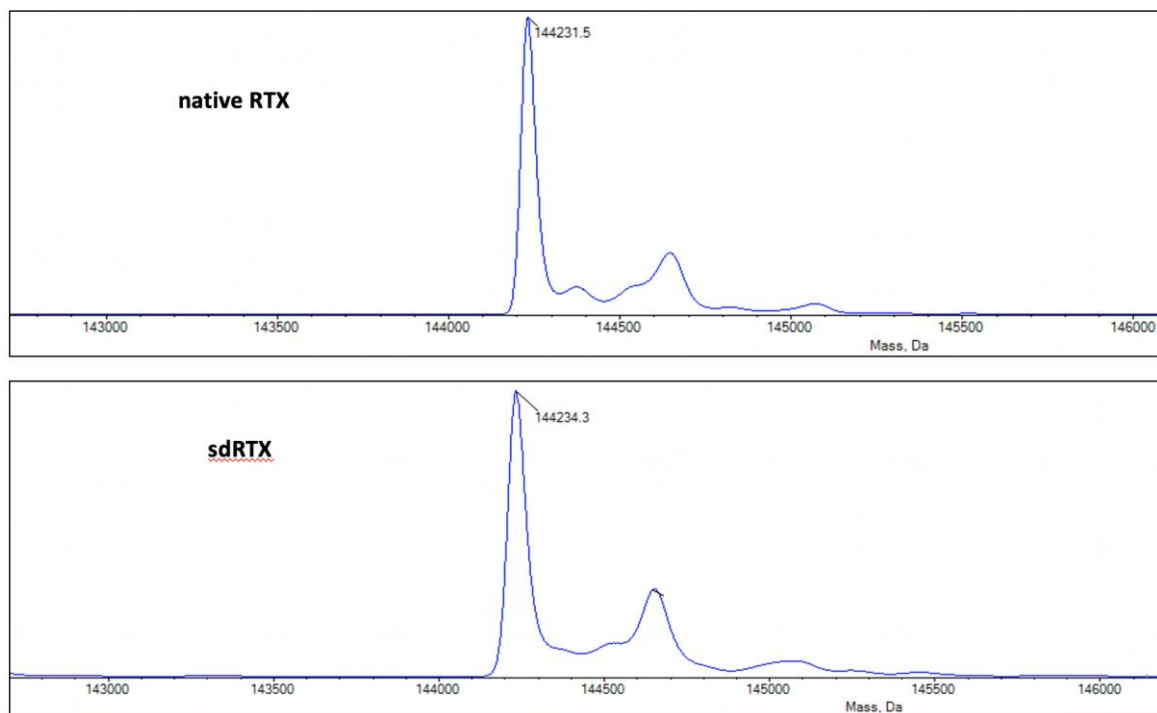
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957 *Supplementary figure S1: Illustration of the protein formulation strategy using BEPO®*

958 *copolymers.*

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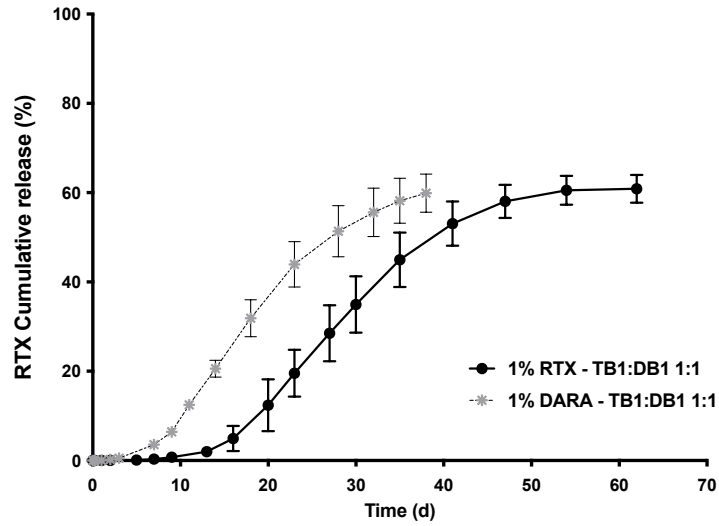


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962 *Supplementary figure S2: Intact Mass (LC-MS) analysis performed after deglycosylation of*
963 *the native RTX and sdRTX proteins.*

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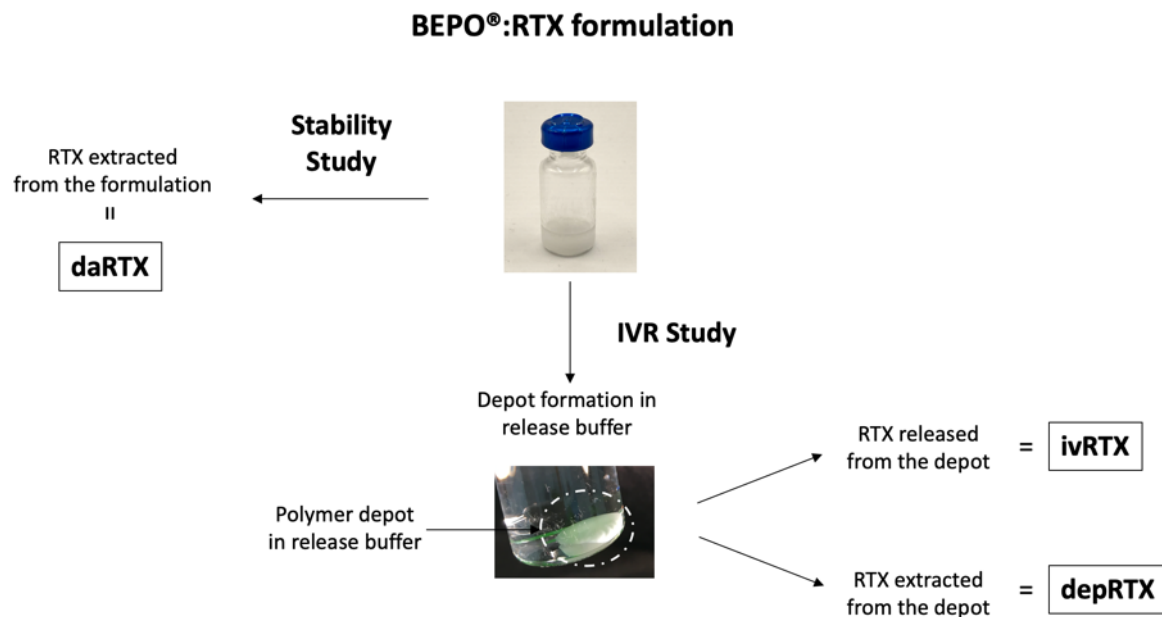
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967 *Supplementary figure S3: Comparison of the IVR profiles of spray dried RTX and DARA*

968 *proteins formulated with V1 BEPO[®] vehicle (TB1:DB1 polymer composition).*

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970



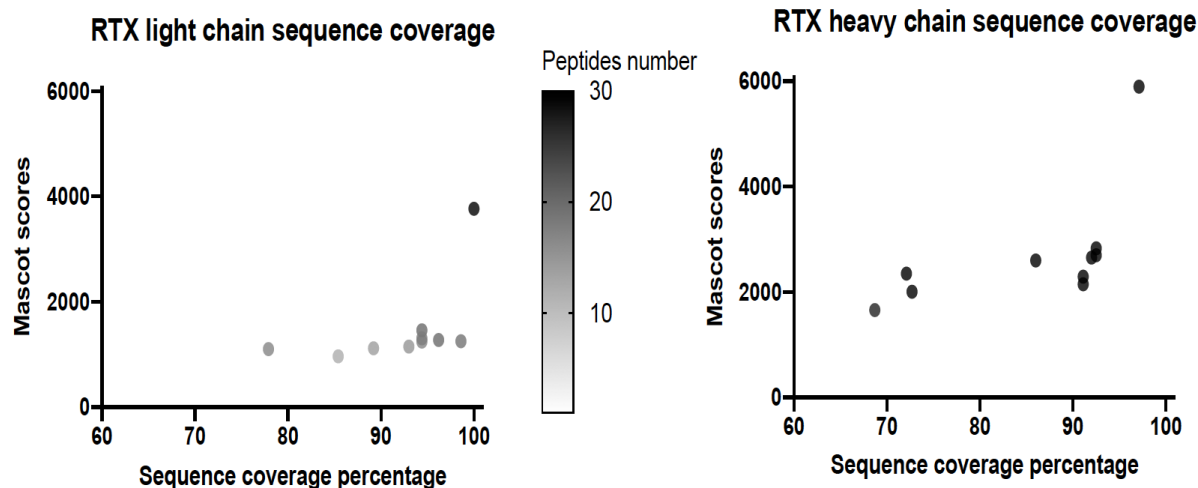
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973 **Supplementary figure S4:** Illustration to define the nomenclature that was used to name RTX

974 samples during the formulation process with BEPO[®] copolymers.

975



976

977 *Supplementary figure S5: Amino acid sequence coverage determined by Q-tof Impact2 MS*

978 *presented as function of percentage of sequence coverage (X axis), Mascot score (left axis)*

979 *and peptide detected numbers (right colored axis). Light chain (left) and heavy chain (right)*

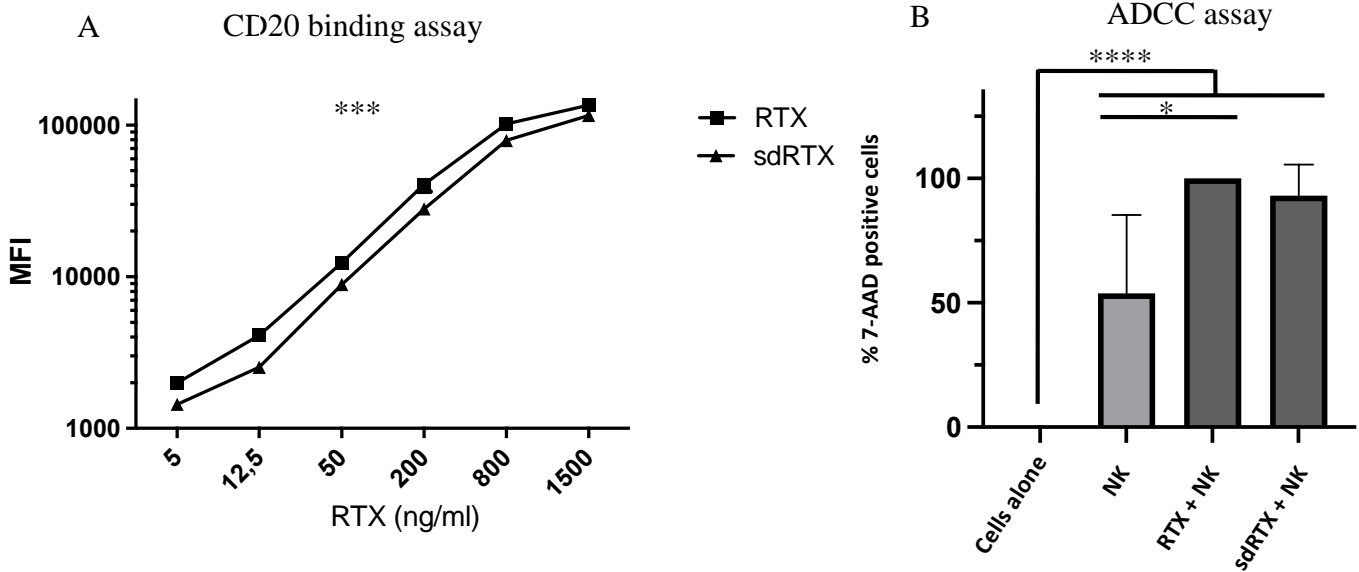
980 *are presented separately. Each dot represents a formulated RTX sample (daRTX and ivRTX)*

981 *taken at different time points. In both graphs, native RTX is the isolated dot in the top right*

982 *corner.*

983

984



985

986 **Supplementary figure S6: Binding and ADCC assays carried out with sdRTX.** (A) Binding to
 987 CD20 expressed by Raji cells. The depicted RTX samples were incubated for 30 min with Raji
 988 cells. RTX was revealed by an anti-human Fab-PE (1.875 µg/mL). The arithmetic mean
 989 fluorescence intensity (MFI) is represented. (B) ADCC on Raji cells. ADCC assay with RTX
 990 samples (10 µg/mL), in presence of NK cells (E:T, 3:1) was carried out overnight on Raji cells.
 991 Basal cell death was normalized to 0% and saline RTX+NK to 100%. Sample activity was
 992 presented as % of native RTX activity. Graph shows mean +/- SD of a minimum of two
 993 experiments performed in triplicate. Statistics in A are from spearman correlation over one
 994 experiment in triplicate ;*** $p < 0,0009-0,0002$; In B ADCC samples were compared by 2-
 995 ANOVA test, $n = 5$, * $p < 0,030$; **** $p < 0.0001$.

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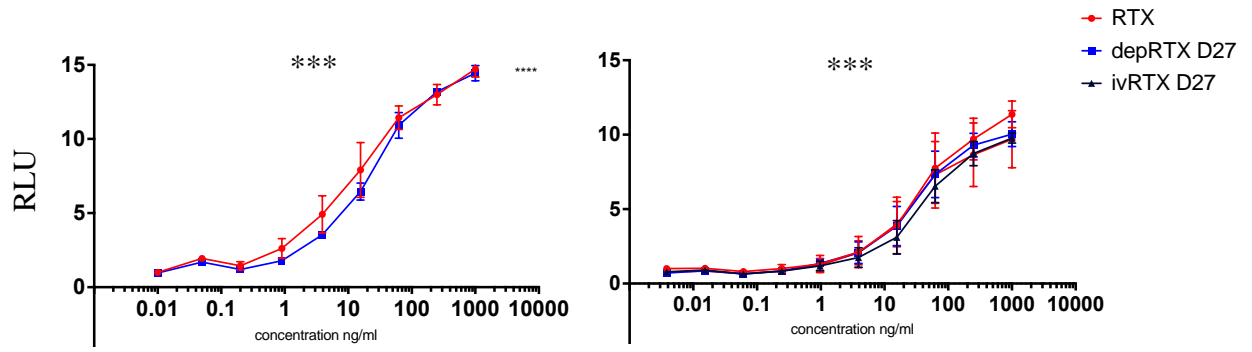
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Supplementary figure S7: NFAT Reporter Activation Assay. The capacity of formulated RTX

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samples to induce the NFAT signalling pathway in modified Jurkat cells was evaluated and

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compared to the one of native RTX. Signal is presented in Y-axis as relative luciferase units

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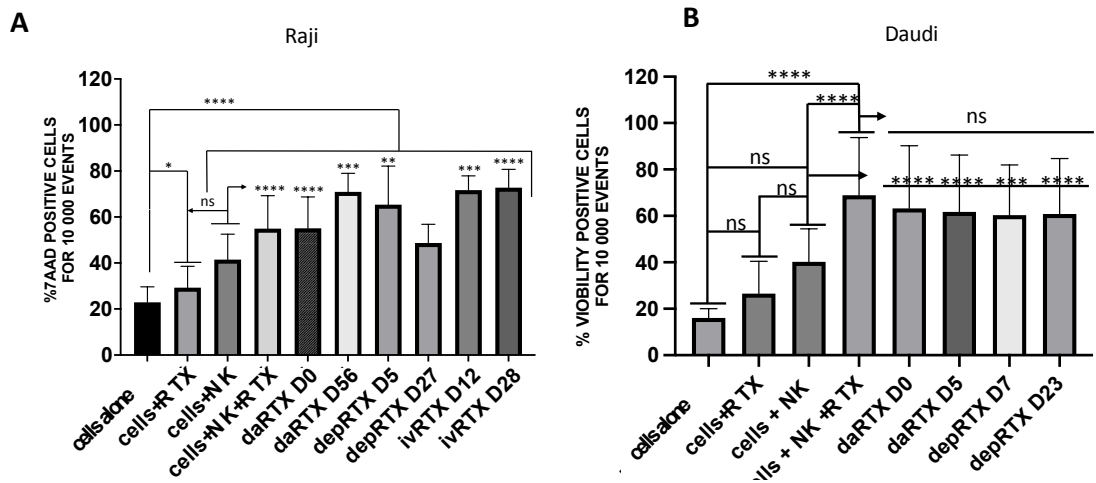
(RLU). Correlation was carried out by Spearman R test. Expressed as R square (0,903-1,000)

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and p-value ***; $p < (0,0008-0,0001)$.

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Supplementary figure S8: Functionality of formulated RTX. All RTX samples were obtained from the F1 BEPO®:RTX and were either extracted from the formulation after storage at 4°C (daRTX samples) or obtained during the release study through the evaluation of the released protein (ivRTX) or the extraction of the protein from the solid polymer depot(depRTX). ADCC assay with the different RTX samples (10 µg/mL), in presence of NK cells (E:T, 3:1) was carried out overnight on Raji cells (7AAD stained) (A) or after 8 hours on Daudi cells (viability stained) (B). Graphs show the mean +/- SD of a minimum of two experiments performed in triplicate. Samples were compared by 2-ANOVA test, n = 21, *p < 0.05, **p < 0.01, ***p=0.0001 and ****p<0.0001.

Vehicle	TB:DB couple	Triblock	Diblock	Polymer content (% w/w)	TB:DB weight ratio
V1	TB1:DB1	3-9.8	1-6.5	20	1:1
V2	TB1:DB1	3-9.8	1-6.5	20	3:1
V3	TB1:DB2	3-9.8	2-27.8	20	1:1

Table 1 : *Composition of vehicles used to make BEPO[®]:protein formulations*

Proteins	CD16a K_D pH 7.4 (M)	CD16a V158F K_D pH 7.4 (M)	CD16b K_D pH 7.4 (M)	FcRn K_D pH 6.0 (M)
Native RTX	2.74 x 10 ⁻⁷	9.52 x 10 ⁻⁷	15.40 x 10 ⁻⁷	2.73 x 10 ⁻⁸
daRTX T0	1.30 x 10 ⁻⁷	2.69 x 10 ⁻⁷	4.97 x 10 ⁻⁷	2.47 x 10 ⁻⁸
daRTX 5w 4°C	1.13 x 10 ⁻⁷	1.99 x 10 ⁻⁷	5.72 x 10 ⁻⁷	2.63 x 10 ⁻⁸
depRTX 7d	1.00 x 10 ⁻⁷	1.33 x 10 ⁻⁷	2.96 x 10 ⁻⁷	2.67 x 10 ⁻⁸
depRTX 13d	0.92 x 10 ⁻⁷	1.06 x 10 ⁻⁷	2.19 x 10 ⁻⁷	2.64 x 10 ⁻⁸

Table 2: Affinity of formulated RTX samples for CD16a, CD16b and FcRn receptors

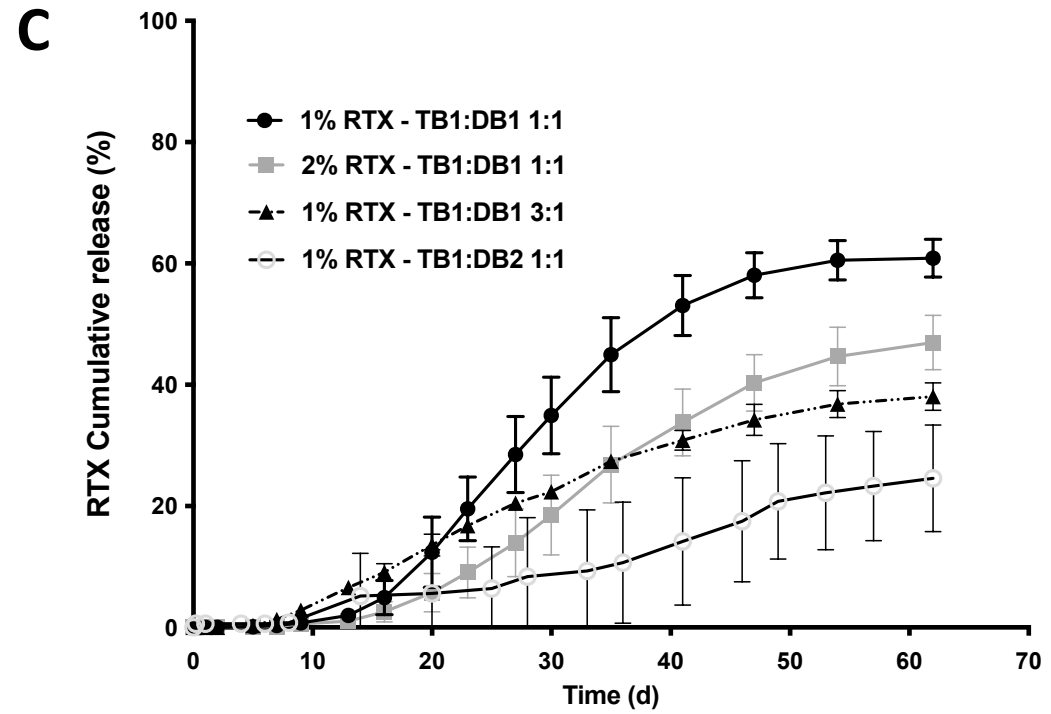
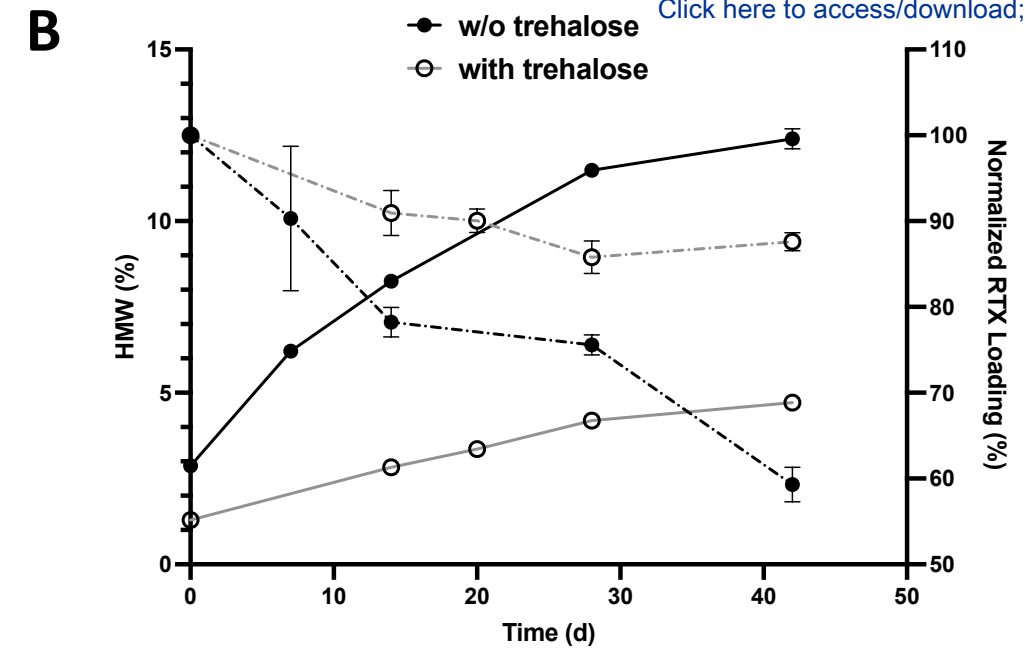
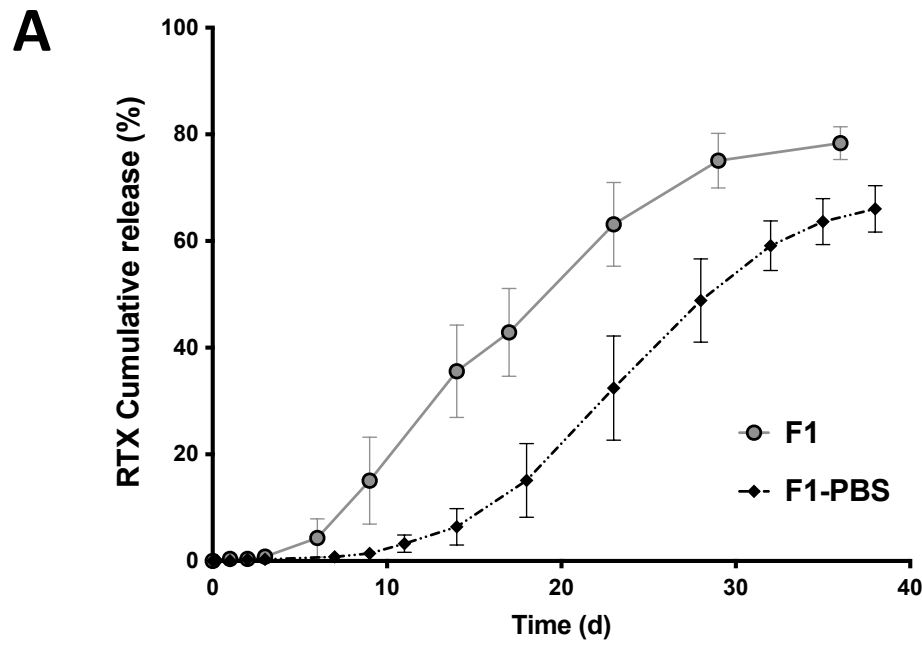
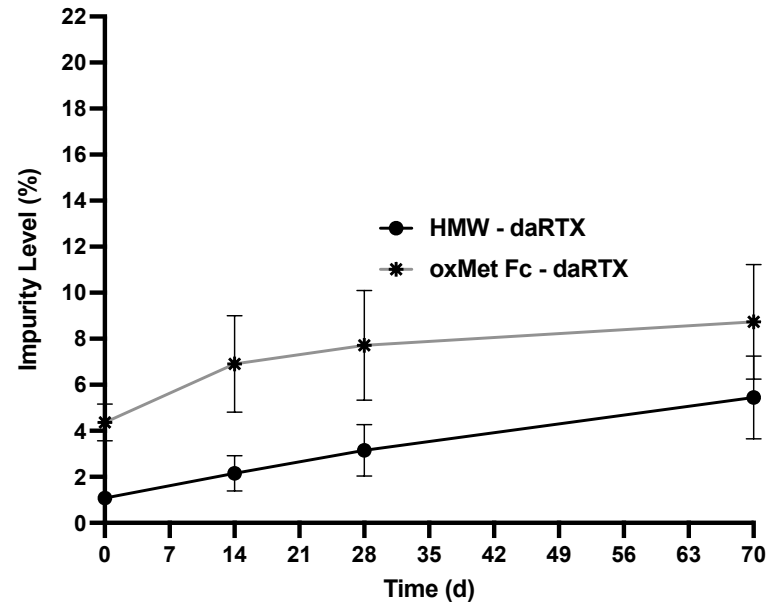
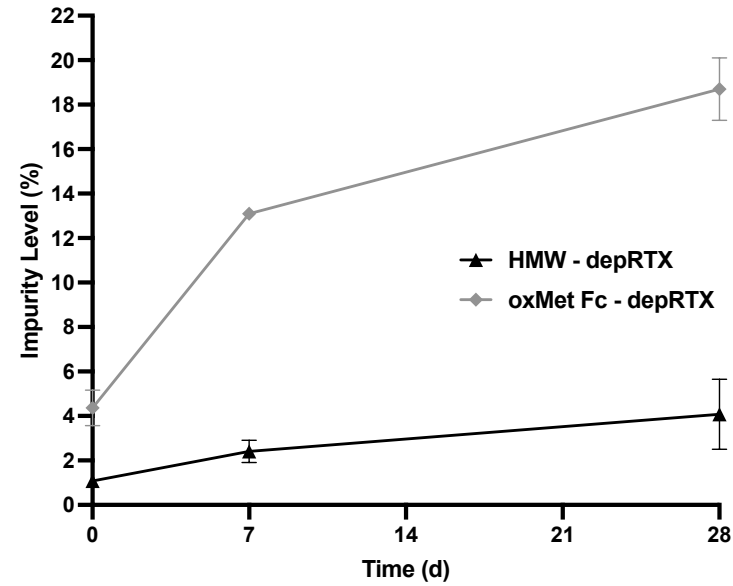


Fig. 2

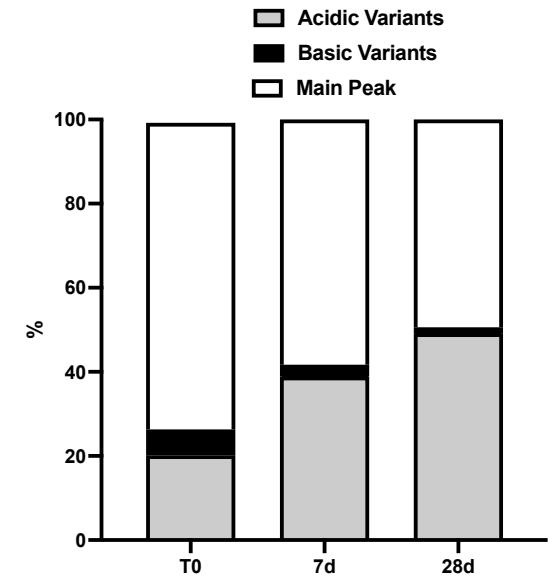
A



B



SCX Analysis of depRTX samples



C

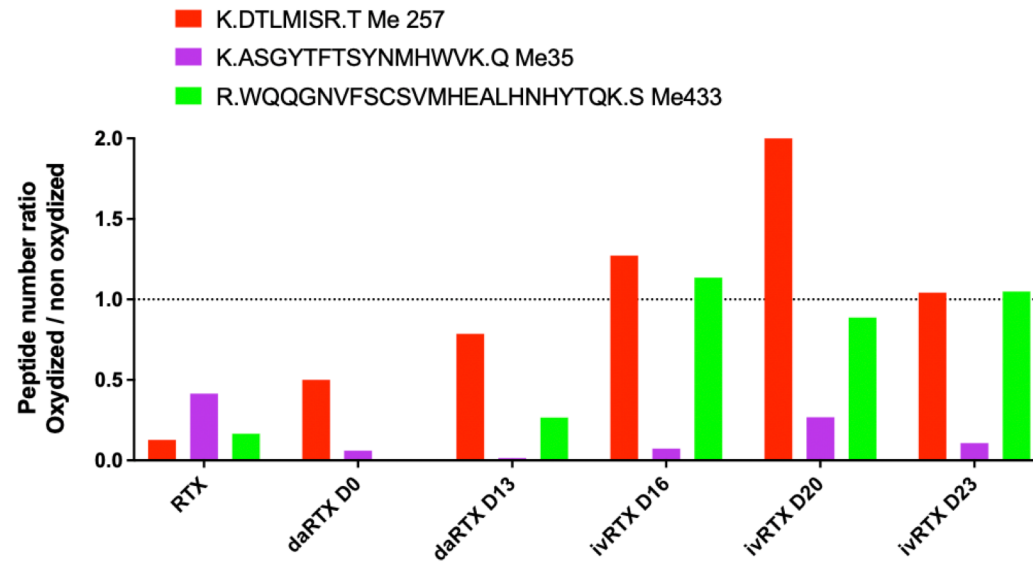


Fig. 3

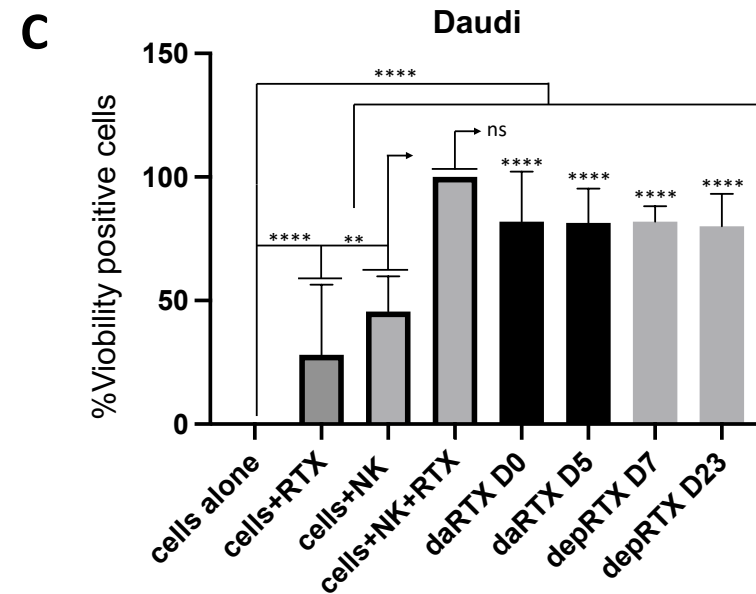
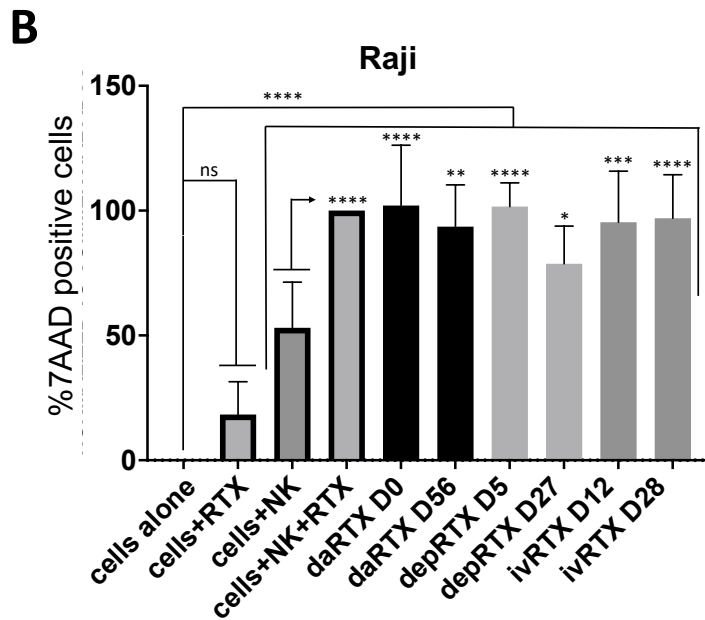
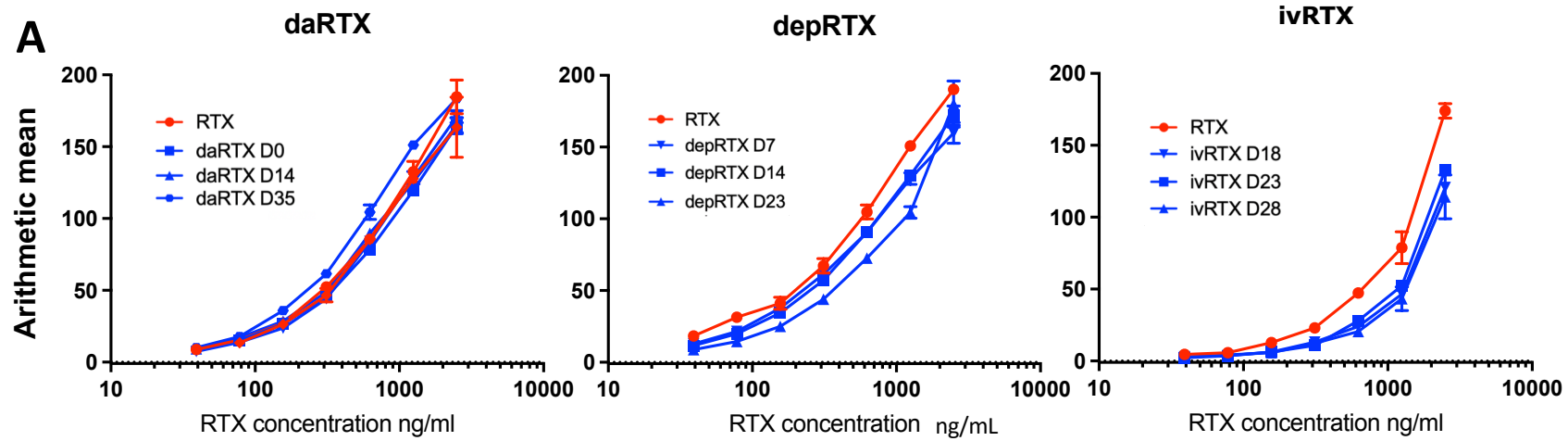


Fig. 4

Daratumumab induced autokilling

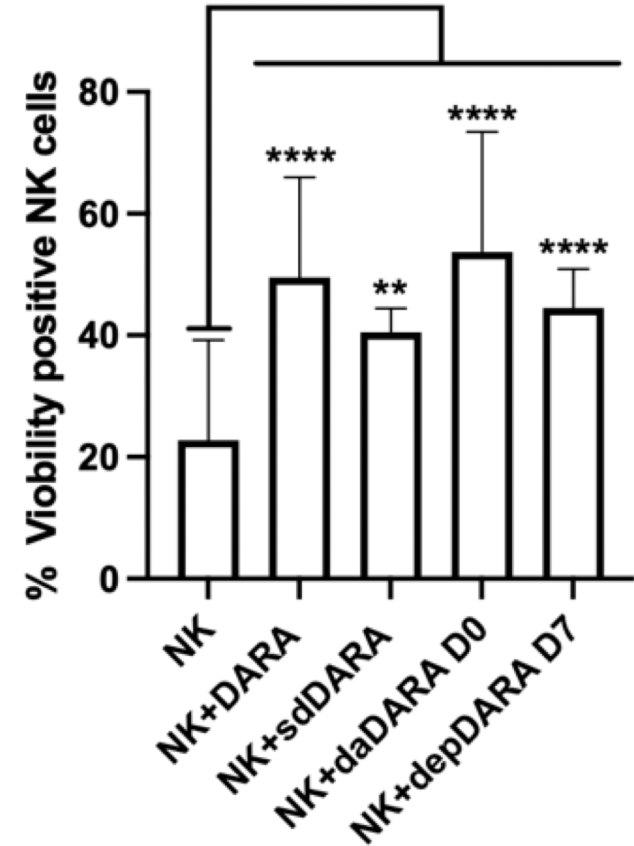


Fig. 5

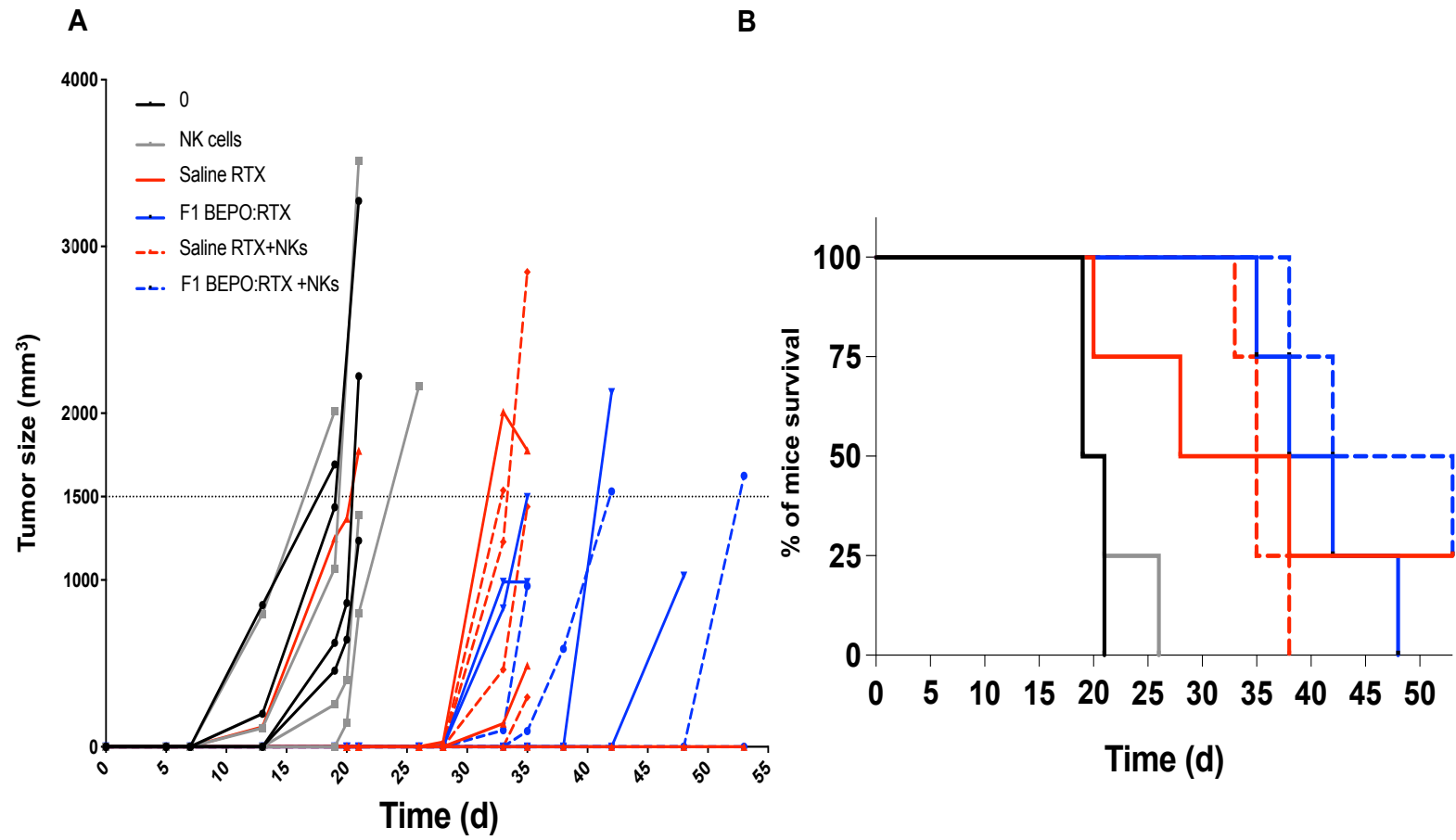


Fig. 6

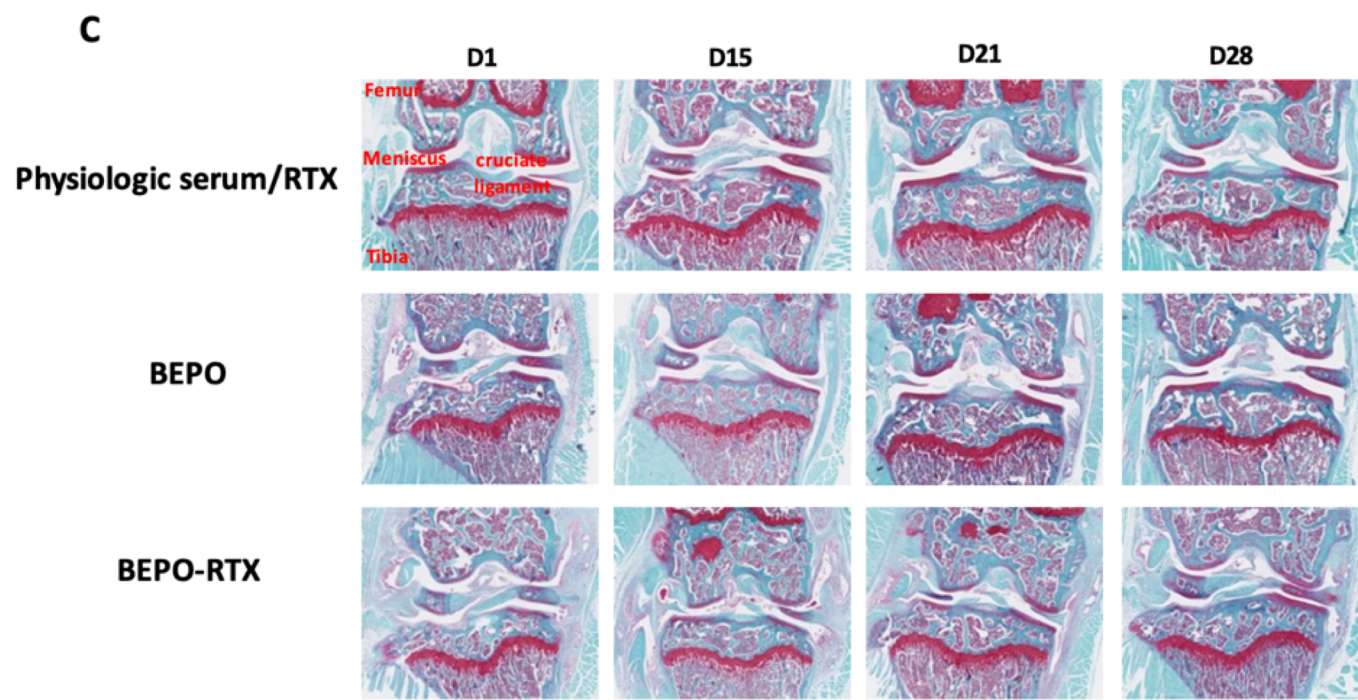
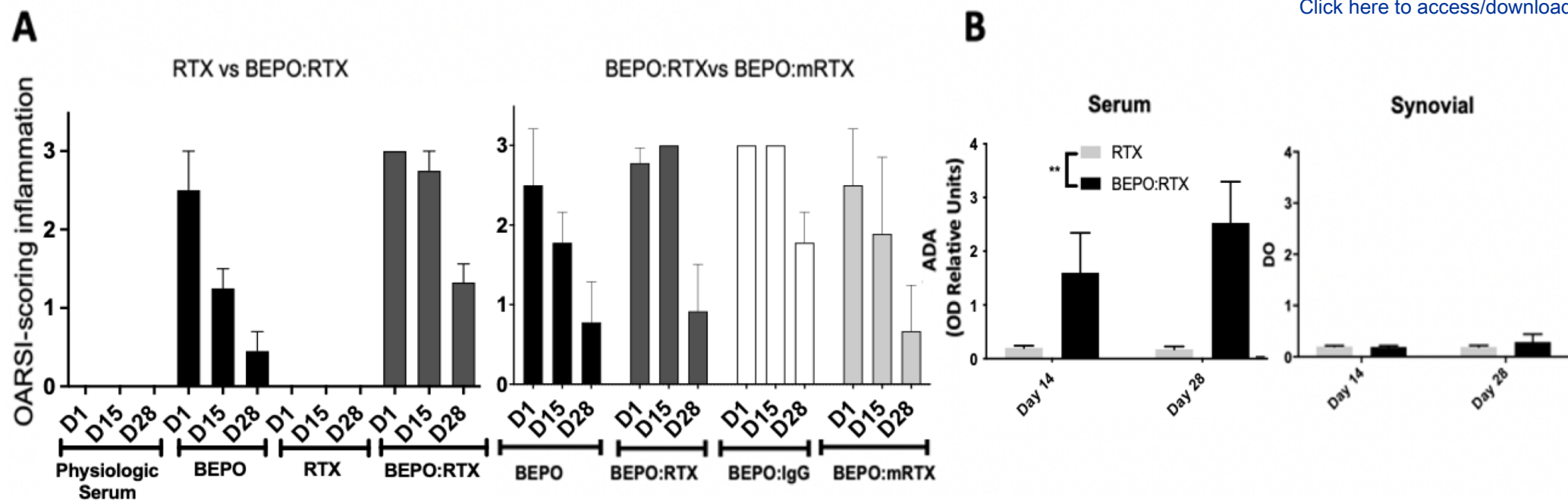


Fig. 7

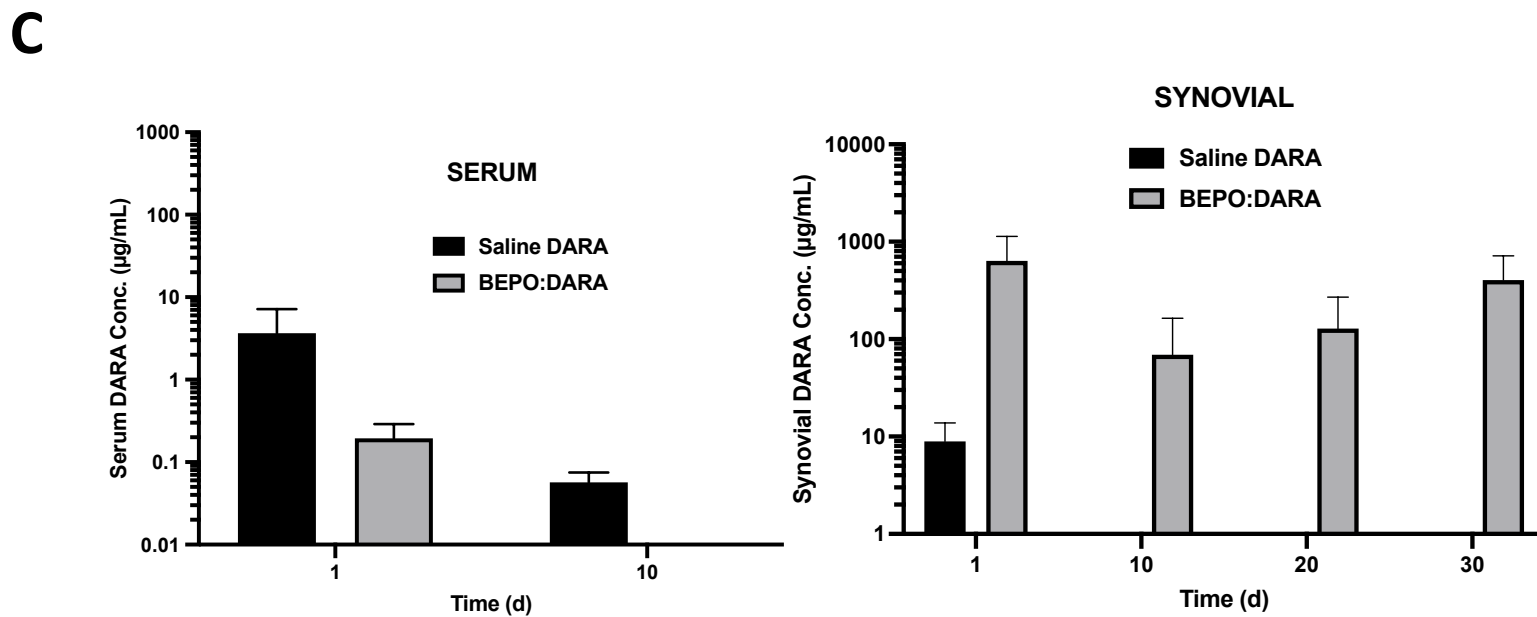
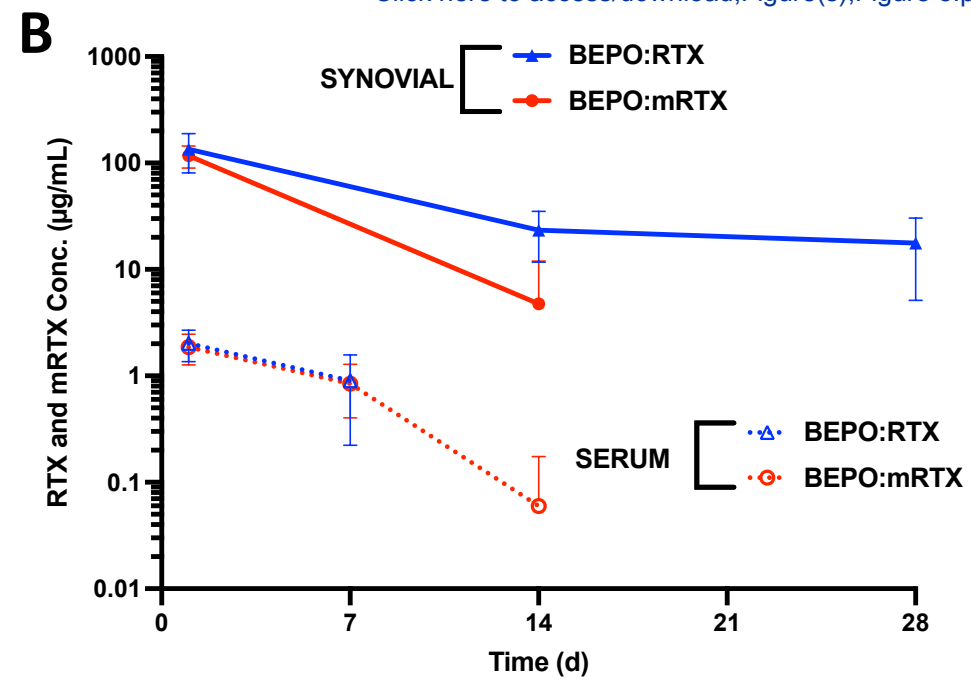
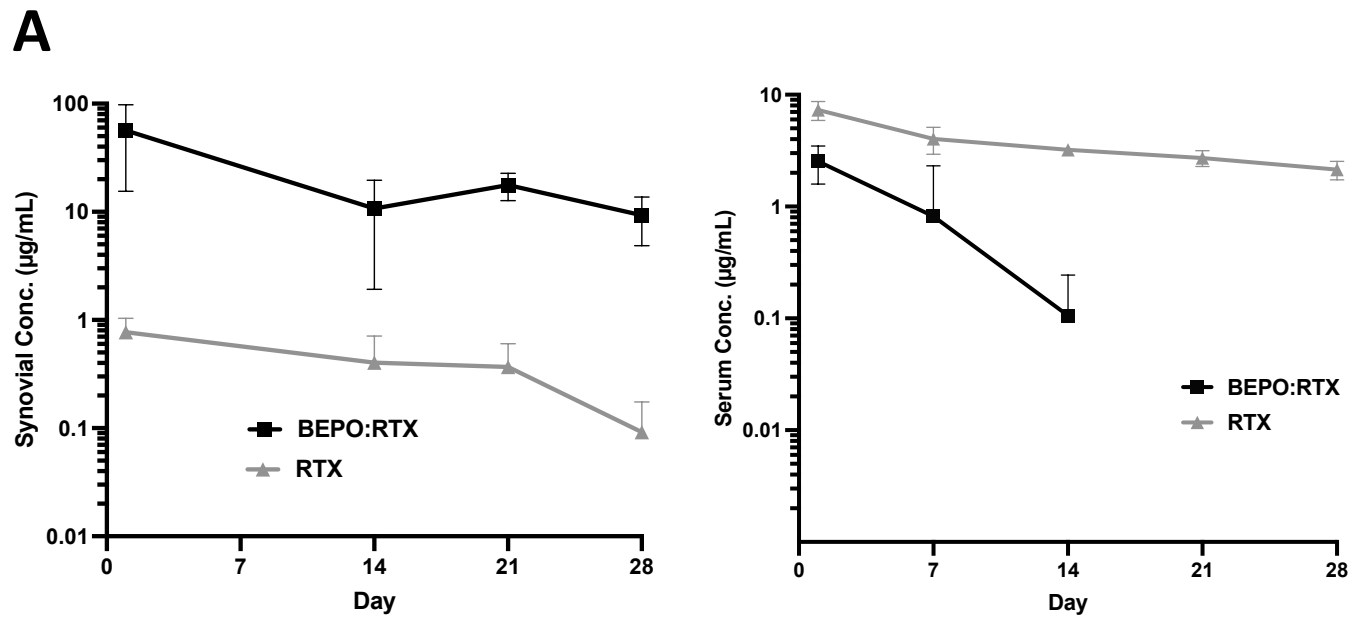


Fig. 8

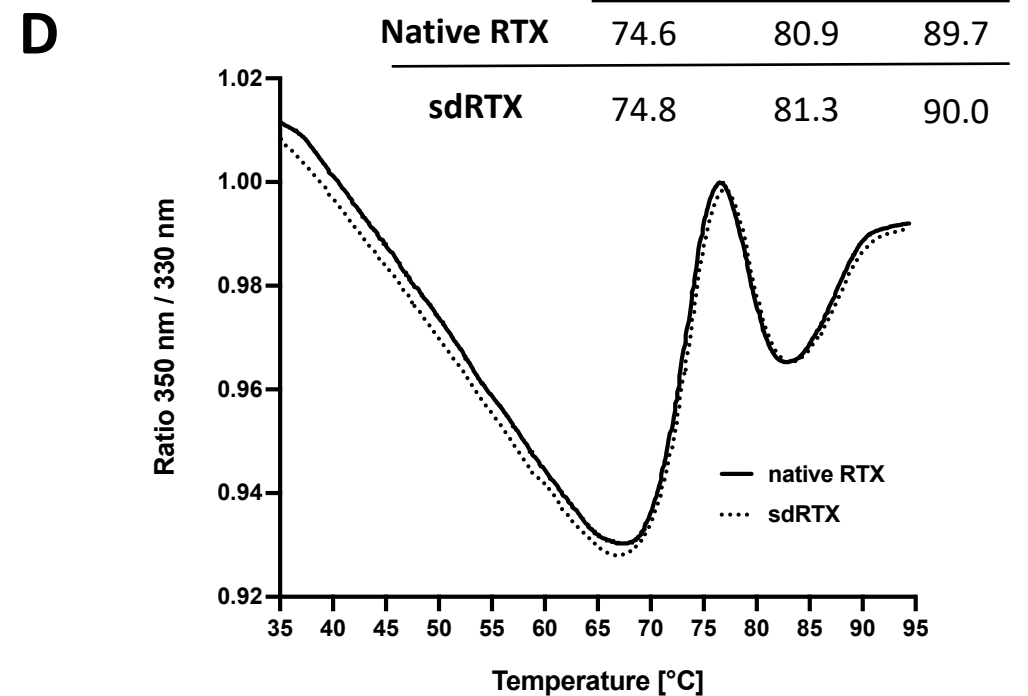
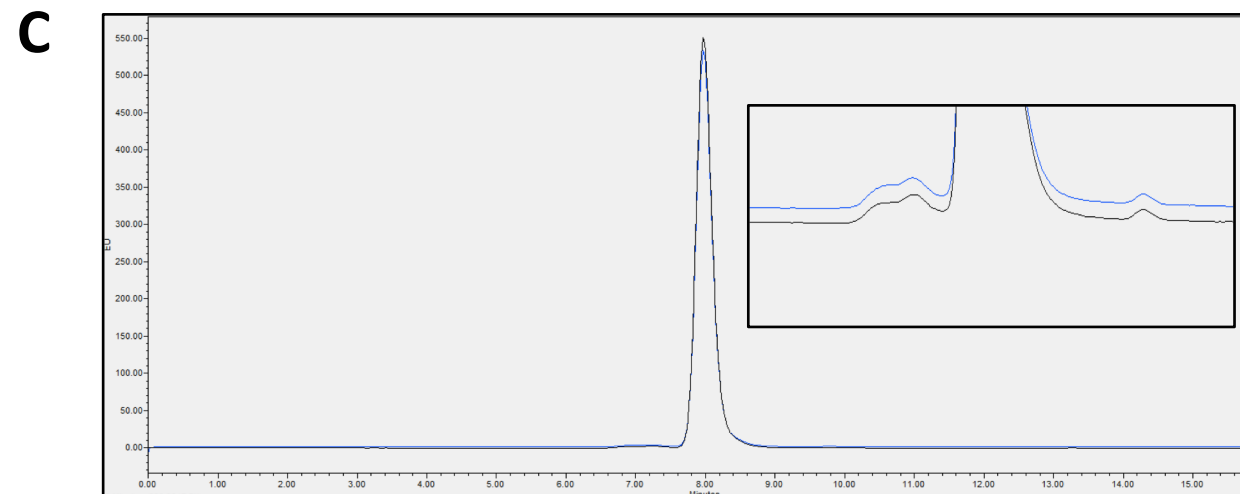
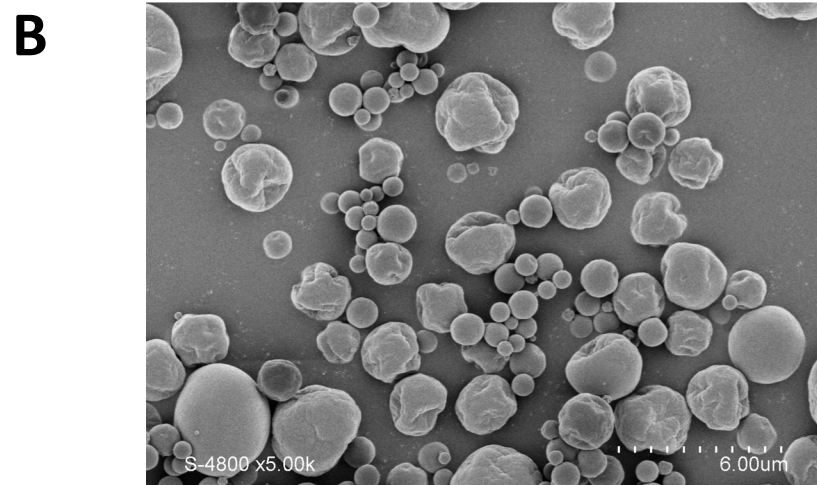
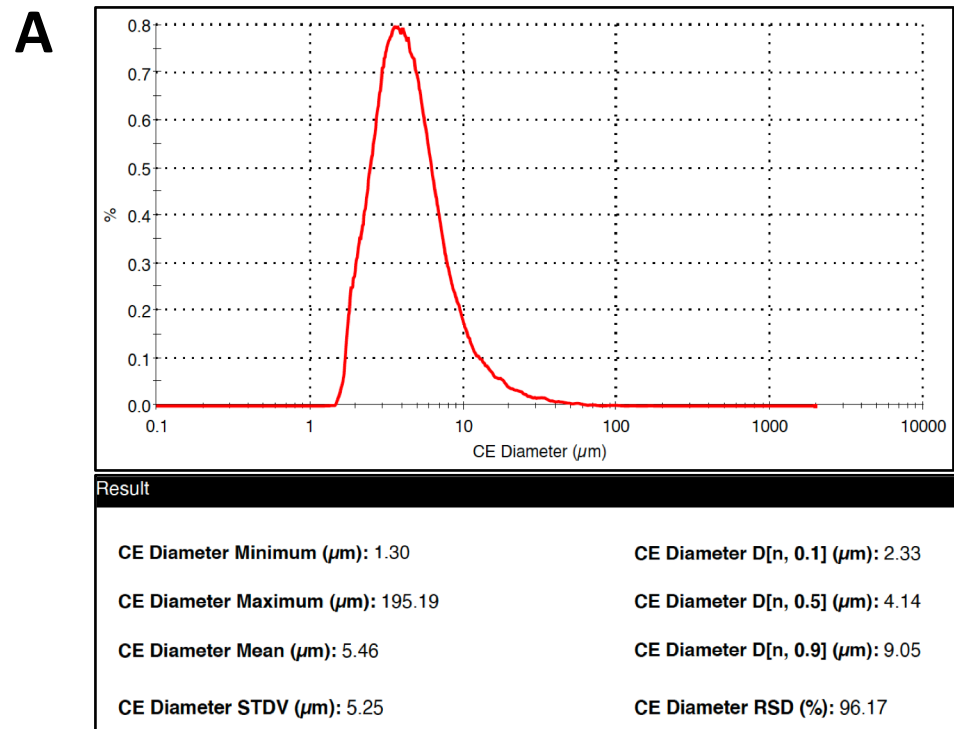


Fig. 1

Highlights

- Formulation of mAbs with an *in situ* forming depot technology using PEG-PLA copolymers.
- Formulated RTX showed full biological activity and minimal post-translational modifications.
- RTX formulation was as efficient as IV RTX treatment to inhibit *in vivo* tumor growth in NSG mice.
- mAbs formulations demonstrated superior PK properties after intra-articular delivery.

Author Contributions Statement

A.F., W.L., S.G., D.N., P.L.P. and M.V. designed experiments. A.F., K.T. and S.G. performed experiments and analyzed results. A.F., M.V. and S.G. wrote the main manuscript text. All authors reviewed and approved the manuscript.