In vitro detection of canine anti-human antibodies following intratumoral injection of the hu14.18-IL2 immunocytokine in spontaneous canine melanoma

Short title: Analysis of canine anti-human antibodies

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Abstract

Background: Canine and human malignant melanoma are naturally occurring cancers with many similarities, making the dog an important parallel patient population to study both diseases. However, development of canine anti-human antibodies (CAHA) needs to be considered when evaluating humanized biotherapeutics in dogs.

Objectives: Characterize CAHA in sera from dogs with spontaneous melanoma receiving radiotherapy and intratumoral immunocytokine (IT-IC) with humanized 14.18-IL2.

Methods: Serum samples were obtained pre-treatment and at several post-treatment times from 12 dogs with locally advanced or metastatic melanoma treated with radiotherapy to the primary site and regional lymph nodes (when clinically involved) followed by IT-IC of humanized 14.18-IL2. Two CAHA assays were developed. A sandwich enzyme-linked immunosorbent assay (ELISA) was developed to detect antibodies against the humanized IgG component of hu14.18-IL2. A flow cytometry assay was developed to determine the ability of CAHA to inhibit binding of a mouse anti-GD2 monoclonal antibody to its target.

Results: Post-treatment sera from 7 of 12 dogs developed CAHA levels over pre-treatment that were identified by ELISA as significant increases at Day 30 and/or Day 60. Day 10, Day 30, and Day 60 post-treatment sera from 10 of 12 dogs significantly inhibited the binding of anti-GD2 monoclonal antibody to its target compared to pre-treatment. Significant binding inhibition was also detected in 2 of 12 dogs after local RT but before IT-IC (Day 1). Normal canine sera did not mediate binding inhibition.

Conclusions: This study advances CAHA detection strategies and reports the kinetics of CAHA following IT-IC in dogs with spontaneous melanoma.

Introduction

Companion (pet) dogs with spontaneous tumors provide unique and clinically relevant insights into human tumor biology, therapy, and drug development [1, 2]. Dogs develop spontaneous tumors, similar to humans [3]. This contrasts with many laboratory mouse models where tumors are experimentally induced [4]. Moreover, the molecular and genetic characteristics of many canine cancers resemble those of human cancers [5]. While no model perfectly recapitulates human cancer, the companion dog melanoma patient population holds a distinctive position in oncology research and has received attention from the scientific community as being somewhat parallel to human melanoma patients, particularly for evaluation of immunotherapy studies [6-10]. In that regard, development of antidrug antibodies (ADAs) against immunotherapeutic monoclonal antibodies (mAbs) is well documented in human patients, and the presence of ADAs can impact the safety and efficacy of immunotherapy [11-15]. Moreover, the immune response is highly species-specific and relatively few immunotherapeutic mAbs are caninized, i.e., canine speciated [16]. Therefore, it will be critical to monitor ADAs in the setting of canine immunotherapy, particularly when the immunotherapeutic is a mAb of a species ‘foreign’ to the dog (e.g., a humanized mAb).

The humanized mAb Hu14.18-IL2 and the chimeric ch14.18-IL2, both GD2-targeting mAbs linked to IL2 and known as immunocytokines (IC), have been studied *in vitro* and in preclinical mouse models with GD2-expressing tumors [17-20]. Clinical trials evaluating intravenous (IV) delivery of hu14.18-IL2 IC in patients with melanoma or neuroblastoma have demonstrated immune activation and had manageable safety

87 profiles [21-25]. Although hu14.18-IL2 is a humanized mAb, some patients still
88 developed antibodies to the therapeutic IC [22, 26]. In these patients, antibodies were
89 detected to both the idiotype, i.e., the antigen binding end, and the Fc-IL2 end of the IC
90 [22, 26]. A substantial fraction of patients develop a detectible anti-IC antibody
91 response, and some patients develop a strong anti-IC response and functional, in vivo
92 "neutralization" of detectible IC. Namely, following detection of a strong anti-IC
93 response, blood obtained immediately after an IC infusion does not contain the
94 anticipated level of detectible IC, based on the IC level in the blood obtained from
95 samples collected prior to development of the anti-IC response [22, 26]. A neutralizing
96 anti-human antibody (HAHA) or anti-chimeric antibody (HACA) has been reported for
97 some patients treated with humanized or chimeric hu14.18 mAb [27, 28].

98 The immunogenicity of the humanized hu14.18-IL2 is potentially a greater
99 concern when administering this molecule to a "foreign" species, e.g., to a mouse or
100 dog, as ADAs could interfere with the pharmacokinetics of this therapy, potentially
101 reducing its efficacy [29]. When huKS1/4-IL2, a humanized antibody directed against
102 the human epithelial cell adhesion molecule, was administered intravenously to mice
103 with adenocarcinoma, the mice mounted significant ADAs in the form of mouse anti-
104 human antibodies (MAHAs) resulting in rapid clearance of additional doses of the
105 therapeutic [18]. Delivering hu14.18-IL2 intratumorally in mouse models also induced a
106 MAHA response, similar to what was seen following IV-IC therapy [30]. In addition,
107 ADAs in dogs have been observed in other studies, including one report following
108 subcutaneous administration of a therapeutic IC [31-33]. However, the development of

109 canine anti-human antibodies (CAHAs) following intratumoral immunotherapy with an IC
110 has not been reported.

111 Here we present CAHA assay development and CAHA monitoring of companion
112 dogs with spontaneous melanoma enrolled in a clinical trial of local RT plus intratumoral
113 hu14.18-IL2. An in-house ELISA was developed and used to monitor CAHA against the
114 IgG portion of the IC. Previous studies have demonstrated the ability of ELISA to detect
115 ADAs against antibody-based biopharmaceuticals [26, 34-37]. Our ELISA is
116 customizable, cost-effective, and uses common laboratory reagents and equipment. We
117 also examined if CAHA interferes with the ability of the GD2-binding mAb part of the IC
118 to recognize GD2 in a cell-based flow cytometry assay. Details about safety, clinical
119 activity, and tumor biopsy analyses from this trial, as well as another trial, are separately
120 reported [38, 39].
121

122 Material and Methods

123 Trial eligibility and enrollment

124 Client-owned companion (pet) dogs weighing ≥5 kg with a readily palpable and
125 accessible oral malignant melanoma (histologically confirmed) of at least 2.0 cm
126 (longest diameter) were included in the study. Both treatment naïve dogs and dogs with
127 surgically recurrent tumors were allowed, provided the recurrence met the minimum
128 size criteria. All dogs were required to have a pretreatment constitutional clinical sign
129 status of 0 or 1 (normal or mild lethargy over baseline; diminished activity from pre-
130 disease level but able to function as an acceptable pet) according to VCOG-CTCAE v2

131 criteria at study entry [40]. Dogs determined to have any significant co-morbid illness
132 were excluded.
133

134 Study design

135 Client-owned companion dogs with locally advanced or metastatic melanoma
136 were randomized (6 dogs per Arm) to receive either a single 8 Gy fraction (Arm A) or
137 three 8 Gy fractions delivered over 1 week (Arm B) to the primary site and regional
138 lymph nodes (when clinically involved) with the single or last fraction 5 days prior to IT-
139 IC at 12 mg/m^2 on 3 consecutive days. Prior to treatment, a baseline physical
140 examination with tumor measurements was performed, blood was collected for clinical
141 assessments, and blood was collected for banking of serum, plasma, and peripheral
142 blood mononuclear cells (PBMC) for subsequent *in vitro* analysis. External beam
143 radiation therapy (RT) was delivered by image-guided intensity modulated radiation
144 using helical tomotherapy (TomoTherapy HiArt Treatment System, Accuray Inc.,
145 Sunnyvale, CA, USA). The hu14.18-IL2 was provided as lyophilized vials (4 mg/vial;
146 each ml prior to lyophilization contained 4 mg/ml hu14.18-IL2, 2% sucrose, 80mM L-
147 arginine, 10mM citric acid, 0.2% polysorbate 20, pH 5.5) and was reconstituted with
148 either 0.5 ml or 0.25 ml 0.9% (w/v) NaCl for injection at a concentration of either 8
149 mg/ml or 16 mg/ml. The total volume was administered using multiple needle re-
150 directions into the target lesion within and surrounding the lesion. Injections were
151 performed on 3 consecutive days. PBMC were obtained at pre-treatment and various
152 times post-treatment. Etomidate (1-2 mg/kg, IV) and/or Propofol (4-5 mg/kg IV) were
153 used to induce anesthesia in dogs receiving RT. Isoflurane (1.2-1.3%, inhalation) and/or

154 Sevoflurane (2.4%, inhalation) were used to maintain anesthesia and the gas
155 percentages were adjusted depending on the canine patient's clinical response to
156 anesthesia under guidance of the clinician. The duration of anesthesia was
157 approximately 30 minutes. Butorphanol (0.2-0.4 mg/kg IM or IV) and/or midazolam (0.2
158 mg/kg IV) were used to sedate the dogs during IT-IC administration, the duration of
159 which was approximately 45 minutes. Pulse, respiratory and heart rates were monitored
160 throughout anesthesia and at least every 15 minutes until the dog was ambulatory.
161 Lidocaine was administered at the site of the IT-IC administration for pain (0.5-0.75 ml
162 2% Lidocaine, intradermal). Following IT-IC administration, pain was alleviated with
163 analgesics for 3 days or longer if the clinician or owner perceived that the dog was
164 experiencing continued discomfort. Analgesics included either Carprofen (2.2 mg/kg,
165 orally, 2x daily), Deracoxib (3-4 mg/kg, orally, 1x daily), Meloxicam (0.1-0.2 mg/kg,
166 orally, 1x daily), and/or Tramadol (1-5 mg/kg, orally, every 8 hours). Physical
167 examination and canine patient history were assessed at each clinic visit. Dogs were
168 monitored by the dog's owner and clinician for animal health and behavior, as well as
169 the development of adverse events (AE). AEs were graded and attributed according to
170 VCOG-CTCAE v2 [40]. The attending clinician prescribed appropriate treatment and/or
171 supportive care if an AE was observed**.** All efforts were made to minimize suffering. The
172 study endpoint was the time the dog developed progressive disease as determined by
173 physical examination and/or thoracic radiographs. Owners could remove a dog from the
174 study for any reason, or the attending clinician could remove a dog from the study if it
175 was determined there were severe AEs not manageable with supportive care.
176 Euthanasia was not a study endpoint nor was it part of this study and was only

177 performed at the request of a dog's owner. If requested, the American Veterinary
178 Medical Association guidelines were followed [41]. The treatment schema and blood
179 collection times are shown in Fig 1.
180
181 **Fig 1. Treatment Schema.** All dogs receive radiation therapy (RT) to the local site
182 either in 1x 8 Gy (Arm A) or 3x 8 Gy fractions over 1 week (Arm B). RT was delivered on
183 Day -4 (Arm A) or on Days -8, -6 -4 (Arm B). Intratumoral (IT) injection of IC is
184 administered (IT-IC) on three consecutive days starting five days after completion of RT.
185 All days were allowed ± 1-2 days leeway for flexible scheduling around holidays, subject
186 vacations, and clinic scheduling.
187

188 ## IACUC approvals

189 The authors confirm that the appropriate ethical review committee approval has
190 been received. All procedures and treatments performed on client-owned companion
191 dogs were approved by the Institutional Animal Care and Use Committees of the
192 University of Wisconsin-Madison School of Veterinary Medicine (Approval V006037)
193 and by the Animal Care Committee at the William S. Middleton Memorial Veterans
194 Hospital. Written informed consent was obtained from all companion dog caregivers
195 prior to entry into this trial. Protocol treatments were administered at the University of
196 Wisconsin Veterinary Care (UWVC) hospital.
197

198 ## Canine Anti-Human ELISA

199 A standard protocol was adapted to develop an ELISA to detect canine
200 antibodies against human IgG [42]. Nunc MaxiSorp™ Flat-Bottom 96-well plates
201 (Thermo Scientific Inc., USA) were coated with ChromPure Human IgG (Jackson
202 ImmunoResearch Laboratories, Inc., USA) [10μg/mL, 50μL/well in ELISA Coating Buffer
203 (Bio-Rad Laboratories, Inc., USA)], which served as the "capture" agent, and incubated
204 overnight at 4°C. The coating solution was removed, and the wells washed three times
205 (3x) with distilled water (DI). The plate was blocked with Neptune™ Block
206 (ImmunoChemistry Technologies, USA) (200μL/well) for 1 hour at 25°C followed by
207 washing as above. Canine serum was diluted 1:20 in PBS and added to wells in
208 quadruplicate at 50μL/well and incubated for 1 hour at 25°C. A murine mAb against
209 human IgG (ab436, Abcam, USA) was used as a positive control, as we are not aware
210 of any commercially available canine anti-human IgG to serve as a positive control. The
211 mAb was diluted 1:1,000 in PBS and added to wells in quadruplicate at 50μL/well and
212 incubated for 1 hour at 25°C. PBS was added to wells as a negative control. Wells were
213 washed as above. Rabbit anti-mouse IgG (H+L) antibody, affinity purified against human
214 serum proteins conjugated to horseradish peroxidase (HRP) (# 315-035-045, Jackson
215 ImmunoResearch Laboratories Inc., USA) was the secondary (i.e.: "detection") antibody,
216 was diluted 1:40,000 in PBS, added to wells at 75μL/well, and incubated for 30 minutes
217 (min.) at 25°C. Wells were washed as above. The plate was developed with1-Step™
218 Ultra TMB-ELISA solution (Thermo Scientific Inc., USA) (75μL/well) and incubated for
219 15 min. at 25°C. The reaction was stopped by addition of TMB stop solution (Sera Care,
220 USA) (75μL/well), and the absorbance at 450nm read on a SpectraMax M3 Micro-Plate
221 reader.

222

223 ## Cell culture

224 The GD2+ M21 human melanoma cell line was cultured in RPMI-1640
225 supplemented with 10% fetal bovine serum (GeminiBio, BenchMark™), 25 mM HEPES,
226 2 mM L-glutamine, 1 mM sodium pyruvate, and 1X non-essential amino acids
227 (Mediatech). M21 was obtained as a gift from Dr. Ralph Reisfeld (Scripps Research
228 Institute, La Jolla, CA, USA).

229

230 ## Detection of GD2 binding inhibition by CAHA using flow
231 ## cytometry

232 Mouse anti-GD2 mAb 14G2a-PE (BioLegend, #357303) (100 μl) was added to
233 2.5 μl canine sera from this study (Baseline, Day 1, Day 10, Day 30, Day 60) or to
234 serum from healthy dogs (2.5 μl or 20 μl) (Antibodies Inc, #54-490-0005) and adjusted
235 to a total volume of 120 μl using 1% FBS/PBS flow cytometry wash buffer (WB). A
236 sample without canine sera was used as a staining control. The light-protected mixture
237 was incubated for 30 min. at 4°C. The serum-14G2a mixture was added to GD2+ M21
238 cells aliquoted to flow cytometry tubes and incubated for 30 min.at 4°C, followed by 2x
239 washes with WB. Cells were fixed with Cytofix Fixation Buffer (BD, #554655) for 15 min.
240 at 25°C, washed 1x with WB, resuspended in WB, and held at 4°C until analysis. Data
241 were acquired on a BD LSR Fortessa, single cells were gated, and the Median
242 Fluorescence Intensity (MFI) was determined with FlowJo™ Software (BD Life

Sciences). Binding inhibition was calculated as follows where X equals Day 1, Day 10, Day 30, or Day 60:

$$\%\ \text{Binding Inhibition} = 100 \times \frac{\text{Baseline MFI} - \text{Day X MFI}}{\text{Baseline MFI}}$$

Statistical Analysis and Considerations

Animal specific kinetic levels of CAHA following IT-IC were summarized in terms of means and standard errors. Profile plots were generated to examine changes over time. Changes from baseline to Day 10, 30, and 60 were evaluated using a generalized linear model. Model assumptions were validated by examining residual and normal probabilities plots. Dunnett's test was used to control the type I error (<0.05) when conducting multiple comparisons for changes from baseline to Day 10, 30, and 60. All reported P values are two-sided and P<0.05 was used to define statistical significance. Statistical analyses were conducted using SAS software (SAS Institute, Cary NC), version 9.4. Plots were generated using R software version 4.2.1 with graphical packages [43-45].

Results

Companion dog characteristics

Twelve dogs met eligibility criteria for the trial and were enrolled and randomized into one of the two treatment groups from May 2020 to December 2021 [38]. Companion dog breed, tumor characteristics, and randomization results are summarized in Table 1.

264 All 12 dogs completed the treatment phase of the protocol, and none experienced

265 significant or unexpected adverse events [38].

266

Table 1. Characteristics and status of dogs receiving 12 mg/m^2 IC and randomized to RT Arm A or RT Arm B

Patient ID	Breed	Sex	Age (yr)	Wt (kg)	Tumor Site & Stage at Diagnosis	RT Arm[a]	Sera assayed from all timepoints?
ITIC-04	Labrador Retriever	SF	10	38.9	Left Maxilla/Pulmonary nodule (T2N0M1)	A	Yes
ITIC-05	Burnese Mountain	NM	7	43.4	Right hindlimb subcutaneous (T2N2M0)	B	Yes
ITIC-06	Min. Poodle mix	NM	10	6.6	Right Mandible (T2N1M0)	A	No[b]
ITIC-07	Aust. Cattle mix	NM	13	31.2	Left Maxilla (T2N0M0)	B	Yes
ITIC-08	Beagle mix	NM	11	15.8	Soft palate (T2N1M0)	B	Yes
ITIC-09	Great Dane	SF	10	51.7	Left Maxilla (T2N1M1)	A	Yes
ITIC-10	German Shepherd	NM	9	26.3	Right Maxilla (T2N0M0)	A	Yes
ITIC-11	Mix Breed	NM	11	26.5	Right Mandible (T2N0M1)	B	Yes
ITIC-12	Pom mix	SF	11	9.0	Left Maxilla (T3N1M0)	A	No[b]
ITIC-13	Mix Breed	NM	10	7.0	Cutaneous (TxN1M1)	A	Yes
ITIC-14	Collie mix	SF	11	25.0	Left Maxilla (T2N1M0)	B	Yes
ITIC-15	Mix Breed	NM	9	36.1	Lingual (T2N0M0)	B	Yes

IC, immunocytokine; RT, radiation therapy; SF, spayed female; NM, neutered male
[a]Arm A, a single 8 Gy fraction to the primary tumor followed 5 days later with hu14.18-IL2 IC (12 mg/m^2 for 3 consecutive days); Arm B, three 8 Gy fractions delivered on a

Monday, Wednesday and Friday schedule followed 5 days later with hu14.18-IL2 IC (12 mg/m^2 for 3 consecutive days).
[b]Serum not available from Day 60.

267

268 ## Detection of CAHAs by ELISA

269 We developed an in-house sandwich ELISA to detect antibodies in canine sera
270 specific to human IgG — the antibody portion of hu14.18 is humanized IgG. For this
271 assay to work, a secondary antibody specific for, or cross-reactive to, canine
272 immunoglobulin is required. Additionally, to avoid the need for multiple secondary
273 reagents, reactivity to mouse immunoglobulin, the species the positive control is raised
274 in, was preferred. We first tested our potential secondary reagents by optimizing their
275 ability to generate a strong signal when detecting mouse anti-human IgG mAb as the
276 positive control (in place of the canine serum), as the mouse anti-human IgG was
277 serving as the positive control reagent, namely the analyte. Our initial “checkerboard”
278 trials tested various concentrations of human IgG coating antigen, mouse anti-human
279 IgG mAb as the analyte, and rabbit anti-dog HRP secondary antibody. As expected, the
280 measured absorbance was dependent on the concentration of the rabbit anti-dog HRP
281 secondary, i.e., absorbance decreased as concentration of the secondary decreased. In
282 contrast, the absorbance was not dependent on the concentration of either the coating
283 human IgG or the mouse anti-human IgG mAb (data not shown). To address these
284 issues, we first troubleshot reagents and/or steps, e.g., testing different blocking buffers,
285 including 5% normal rabbit sera, as well as non-mammalian protein buffers, increasing
286 the blocking time, and testing different wash buffers. These changes did not resolve the
287 high background issue, which was also observed in negative control wells, i.e., in wells
288 incubated with PBS instead of the mouse anti-human IgG mAb.

289 These data suggested that the rabbit anti-dog HRP secondary may be binding to
290 the human IgG coating on the plate. Immunoglobulins from different species share
291 similar protein structures [42]. Therefore, antibodies against one species are likely to
292 cross-react with a number of other species — a property which, as discussed below,
293 was leveraged to optimize the assay. We adsorbed the rabbit anti-dog secondary
294 antibody with excess human IgG; however, recognition of human IgG by this adsorbed
295 reagent was only slightly minimized (data not shown). We are not aware of a
296 commercially available anti-dog secondary antibody adsorbed to human serum proteins.
297 Using the cross-reactivity of different species' immunoglobulins, we next tested a
298 commercially available rabbit anti-mouse IgG secondary antibody adsorbed to ensure
299 minimal cross-reactivity with human serum proteins. The human adsorbed rabbit anti-
300 mouse IgG did not bind to plate-bound human IgG while the cross-reactivity to the
301 positive control mouse IgG was maintained. This one reagent then permitted the use of
302 a single secondary antibody to effectively detect both CAHA and mouse-anti-human
303 antibody (MAHA).
304 Post-treatment sera from seven of twelve dogs tested positive for anti-human IgG
305 antibodies determined via our in-house CAHA ELISA (Fig 2). Pre-treatment sera was
306 used as baseline and as an internal control for each dog's post-treatment samples. Two
307 of six and five of six dogs in Arm A and Arm B, respectively, demonstrated significant
308 increases in anti-human IgG compared to the pre-treatment absorbance (Fig 2). The
309 post-treatment anti-human IgG was significantly increased compared to pre-treatment
310 anti-human IgG at Day 30 for five dogs and at Day 60 for five dogs. For Arm A, both
311 samples reaching significance were from Day 30 (K906 and K909). The sera from

312 K909 no longer had a significant increase in CAHA at Day 60 and no sera was available
313 for Day 60 CAHA analysis from K906. For Arm B, anti-human IgG antibody levels in
314 three dogs were elevated similarly at Day 30 and Day 60 (K905, K911, K915), one dog
315 had an elevated level at Day 30 that declined by Day 60 (K914), and one had an
316 elevated level that was only detected at Day 60 (K908). No dogs had detectable anti-
317 human IgG antibodies following RT but before IT-IC (Day 1) or at Day 10 post-
318 treatment. There were no substantial differences in anti-human IgG levels between
319 negative control wells with PBS only and pre-treatment samples for the 12 dogs
320 enrolled in this study.
321
322 **Fig 2. CAHA is induced in some dogs receiving IT-IC injections.** Mean CAHA levels,
323 measured as O.D. values by ELISA, from sera assayed in quadruplicate collected at
324 Baseline (before radiation), Day 1 (after radiation, but prior to IT-IC), and Days 10, 30
325 and 60 (after IT-IC) with standard deviation error bars are shown. Day 60 timepoint was
326 not available for two dogs, K906 and K912, in Arm A. Increases in absorbance from
327 Baseline to Day 1, Day 10, Day 30, or Day 60 for each dog were analyzed and
328 significance indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
329

330 Sera from dogs treated with hu14.18-IL2 inhibit in vitro
331 binding to GD2

332 We modified a flow cytometry assay to determine the potential for CAHA in
333 canine sera to inhibit the binding of IC to GD2 on cells [30]. In this assay, sera are first
334 incubated with 14G2a-PE, a mouse mAb that recognizes GD2 at a dilution of ~1:48.

335 The mixture is subsequently added to GD2+ M21 cells, and the level of PE fluorescence
336 is determined. 14G2a is an IgG2a-class switch variant of the mouse IgG3 mAb clone
337 14.18, and thus has the same idiotype as that of the humanized immunocytokine
338 hu14.18-IL2 [46]. Consequently, some of the CAHA in dog sera that recognizes 14G2a
339 may in effect recognize the idiotype shared by 14G2a and hu14.18-IL2, while some of
340 the CAHA might also recognize other regions of the IC. Thus, in this assay, the PE
341 fluorescence of the 14G2a-PE mAb bound to the M21 cells will be decreased if any of
342 the CAHA sin sera from treated subjects recognize the idiotype of 14G2a and effectively
343 block its binding to GD2 on the M21 cells. In contrast, substantial PE fluorescence will
344 be detected if the serum antibodies do not block the binding of 14G2a to GD2.
345 In preliminary experiments, we assayed healthy canine sera for components in
346 dog serum that might interfere with the flow cytometry assay. Serum is a complex
347 biologic that contains carbohydrates, proteins, and lipids that can interfere with the
348 ability of antibodies to bind their targets. Healthy serum was spiked into PBS and
349 incubated with the 14G2a anti-GD2 mAb before adding the mixture to M21 cells, as
350 described above. Compared to M21 stained in the absence of healthy sera, the addition
351 of sera substantially inhibited 14G2a binding to GD2 in a concentration dependent
352 manner. Substantial inhibition (~50.2%) was observed at 1:6 ratio of normal sera to PBS
353 whereas at a 1:48 ratio inhibition was largely undetected (> 0.1%) (S1 Fig). As the
354 observed interference of healthy control serum on 14.G2a-PE binding to M21 was
355 ameliorated at the 1:48 ratio, we used this same ratio to test sera from the clinical trial.
356 We included heathy donor sera diluted 1:6 and 1:48 with PBS as positive and negative,

357 respectively, controls in each assay. Each dog's pre-treatment sample was used as its
358 baseline.

359 Varying levels of binding inhibition were observed in 10 of 12 dogs (all but K913
360 and K914) when canine sera were mixed with 14G2a mAb before the mixture was used
361 to stain M21 cells (Table 2, Fig 3, and S2 Fig for select data). The binding was
362 significantly inhibited at Days 10, 30 and 60 compared to pre-treatment in all 10 of these
363 same dogs (save for subjects K906 and K912, that did not have day 60 serum sample
364 collected). GD2 binding was also significantly inhibited by Day 1 sera from 2 of these
365 same 10 dogs, one dog in each Arm, (K910 and K915), but for these 2 dogs, the
366 calculated binding inhibition on Day 1 was not as high as at any of the other days tested
367 for those same 2 dogs. In the remaining 10 dogs, that did not have significant binding
368 inhibition on Day 1, the binding of 14G2a to GD2 was increased at Day 1 for 8 of them
369 (all but K905 and K908), i.e., the % inhibition calculated using the formula in the
370 methods section was negative, corresponding to a slight increase in binding at Day 1
371 compared to the pre-treatment serum. However, the increase in binding seen on Day 1
372 (i.e., the negative binding inhibition value) was significant in only one of these eight
373 dogs (K909) (median -8.7%, range -0.3 to -21.2%) (Fig 3 and Table 2). These relatively
374 small positive or negative binding inhibition values detected on Day 1 (prior to any IT-IC
375 treatment, but after RT), which are significant only for 3 dogs (two positive and one
376 negative), were not anticipated and are discussed below; for all 3 of these dogs, all 3
377 subsequent time points for each dog show higher and statistically significant binding
378 inhibition. In contrast to the 10 dogs that did show significant binding inhibition, sera

379 from the other 2 dogs (K913 and K914), did not inhibit GD2 binding at any timepoint (Fig

380 3 and Table 2). Select flow cytometry data are shown in S2 Fig.

381

Table 2. Binding Inhibition (%) of anti-GD2 mAb to GD2 target in the presence of canine serum

Patient ID	RT Arm	Day 1	Day 10	Day 30	Day 60
ITIC-04	A	-7.6	88.3	98.2	57.2
ITIC-05	B	-1.5	92.8	96.6	98.8
ITIC-06	A	-3.5	74.2	97.3	n/a
ITIC-07	B	-5.6	12.5	91.3	40.6
ITIC-08	B	-0.3	26.4	96.4	89.9
ITIC-09	A	-21.2	93.3	96.1	97.0
ITIC-10	A	17.3	36.1	91.4	53.2
ITIC-11	B	-9.7	23.1	47.1	34.0
ITIC-12	A	-10.4	37.0	91.0	n/a
ITIC-13	A	-11.0	-23.2	-0.6	-6.9
ITIC-14	B	-10.7	13.1	10.2	14.6
ITIC-15	B	12.5	20.9	85.9	75.8

mAb, monoclonal antibody; RT, radiation therapy
Fluorescently labelled anti-GD2 antibody was incubated with canine serum and then used to stain GD2+ M21 tumor cells. % Binding Inhibition of post-treatment samples compared to baseline samples was determined by flow cytometry as described in Materials and Methods.

382

383 **Fig 3. In vitro inhibition of binding of anti-GD2 antibody to GD2 target.** The %

384 binding inhibition at various timepoints compared to Baseline. Data shown are

385 calculated from the mean of triplicates as described in Materials and Methods. Sera was

386 collected at Baseline (before radiation), Day 1 (after radiation, but just prior to IT-IC),

387 and Days 10, 30 and 60 (after IT-IC). Timepoints for each dog are presented and

388 connected by a line. Day 60 timepoint was not available for two dogs, K906 and K912,

389 in Arm A. Significance indicated is based on mean of triplicates at Baseline compared to
390 post-treatment timepoints, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
391

392 Comparison of CAHA Detection by ELISA and binding
393 inhibition by *in vitro* flow cytometry assay

394 Significant CAHA at various timepoints was detected by ELISA in 7 of 12 dogs: i)
395 K906 and K909 from Arm A at Day 30; ii) K908 from Arm B at Day 60; iii) K914 from Arm
396 B at Day 30; and iv) K905, K911, and K9 15 from Arm B at Day 30 and Day 60 (Fig 2).
397 Except for the Day 30 sera from K914, the above sera also significantly inhibited 14G2a
398 from binding to GD2 in the flow cytometry assay (Fig 3). To evaluate the relationship
399 between the ELISA and binding inhibition, the values for each serum sample for each
400 subject are plotted in Fig 4, with the ELISA O.D. value plotted on the Y axis, and the %
401 binding inhibition value plotted on the X-axis. There were no significant correlations
402 between the two assays within or for both arms combined detected. Significant binding
403 inhibition was detected earlier than significant CAHA: i) at Day 1 in 2 dogs; and ii) at
404 Day 10 in 10 dogs. Overall, inhibition was detected in 10 of 12 dogs: i) 5 of 6 Arm A
405 dogs and 5 of 6 Arm B dogs at Day 10; ii) 5 of 6 Arm A dogs and 5 of 6 Arm B dogs at
406 Day 30; iii) 3 of 4 Arm A dogs and 5 of 6 Arm B dogs at Day 60. Sera from the two dogs
407 that did not inhibit binding in the flow cytometry assay (K913 and K914) were also
408 CAHA negative by ELISA except for the Day 30 sera from K914 (Figs 2 and 3). Healthy
409 canine sera at the same dilution as sera from the clinical trial dogs were negative for
410 CAHA and did not inhibit the binding of 14G2a to M21 (Fig B in S1 Fig).

411

412 **Fig 4. The relationship of CAHA and binding inhibition by *in vitro* flow cytometry**
413 **assay.** The % binding inhibition related to CAHA at various timepoints is shown.
414 Timepoints for each dog are presented. Timepoints with significant CAHA responses are
415 shown with significance indicated, these timepoints also had significant binding
416 inhibition except for K914 Day 30. Sera from 10 of 12 dogs significantly inhibited binding
417 of anti-GD2 antibody to GD2 target at Day 10, 30 and 60 with the exceptions of K913
418 (shown as open symbols) and K914 (shown as black symbols). Day 1 sera from two
419 dogs (K910 and K915) inhibited binding. A single serum sample, K914 Day 30, showed
420 a significant CAHA response but did not inhibit binding (shown as the black circle).

421

422 # Discussion

423 We developed two distinct assays to monitor and assess the CAHA response in
424 dogs receiving treatment of a readily palpable oral malignant melanoma with local RT
425 plus IT injection of a humanized IC. An ELISA was designed to detect CAHAs
426 specifically targeting human IgG – which is analogous to the Fc portion of hu14.18-IL2
427 [46]. We also utilized a flow cytometry assay to investigate the inhibition of anti-GD2
428 mAb binding to GD2 by sera from dogs receiving RT and IT-IC. This approach
429 employed ELISA as a tool to identify the presence of ADA in serum, followed by a
430 subsequent flow cytometry analysis to provide additional characterization of the ADA
431 response.

432 Although there are commercially available assays to detect various anti-species

433 antibodies, e.g., mouse anti-human (MAHA) and human anti-mouse (HAMA), an off-the-
434 shelf assay is not available to detect CAHA. The absence of a commercially available
435 ELISA specifically designed to detect CAHAs is an unmet need within the comparative
436 immuno-oncology field. This gap becomes increasingly relevant as the canine model
437 gains recognition for its value in studying human disease. The primary objective of
438 developing our in-house CAHA ELISA was to create a monitoring tool that would be
439 easy to use in veterinary and translational research laboratories. Additionally, we sought
440 to develop a cost-effective and flexible assay. The binding specificity of the CAHA could
441 also be explored in more depth by modifying the whole human IgG coating reagent that
442 was used in this report (e.g., whole IgG, Fc fragment, Fab or $F(ab')_2$).

443 A challenge in developing an ELISA to detect canine antibody that binds human
444 IgG was the lack of commercially available anti-canine IgG HRP conjugates that were
445 cross absorbed against human IgG. This cross-absorption step is crucial for reducing
446 the assay background and ensuring specificity. To address this limitation, we used a
447 rabbit anti-mouse IgG HRP conjugate that had been cross absorbed against human
448 IgG. Our testing during assay optimization demonstrated sufficient cross-reactivity
449 between mouse and canine-derived IgG, enabling us to detect CAHA to human IgG
450 (data not shown). In addition, we included a commercially available mouse IgG specific
451 for human IgG as a control analyte sample for detection by the rabbit anti-mouse HRP
452 in each assay. The selection of antibodies and the incorporation of cross-absorption
453 steps contributed to the assay's specificity and adaptability for potential use in both
454 veterinary and translational research lab settings.

455 Our CAHA ELISA is primarily designed to detect free ADAs in serum, by
456 detecting their binding to plate-bound human IgG. In contrast, the in vitro binding
457 inhibition flow cytometry assay allows the ADAs to interact with the soluble mouse
458 14.G2a mAb (which shares the idiotype of the hu14.18-IL2 IC) in solution, and
459 evaluates detection of GD2 binding in a cellular context, enabling the detection of ADAs
460 that inhibit or interfere with the binding of a therapeutic drug to its specific target in its
461 natural form on the cell surface, e.g., GD2 on tumor cells. This distinction is critical
462 because antigens immobilized to plastic, as in an ELISA, may not accurately mimic the
463 natural conformations of antigens on cell surfaces [47], potentially leading to an
464 underestimation of certain ADAs by this ELISA that flow cytometry can detect.

465 It is of interest that two dogs (K910 and K915) inhibited the binding of the anti-
466 GD2 mAb to GD2 on target cells at Day 1. The Day 1 sample was collected the day of,
467 but prior to, the first injection of hu14.18-IL2. Therefore, these responses following RT
468 may reflect pre-existing canine anti-human or anti-mouse antibodies, cross species-
469 reactive antibodies, or assay interference [48-50]. The first step in the flow cytometry
470 binding inhibition assay is the incubation of canine sera with the mouse anti-GD2 mAb
471 14G2a prior to incubating the mixture with the GD2+ M21 cells. Therefore, a possible
472 explanation for the Day 1 binding inhibition is pre-existing canine anti-mouse antibodies
473 as previously reported [49], but these would have needed to appear, or be boosted,
474 following baseline, as the positive binding inhibition is detected in comparison to the
475 baseline value for that same dog. We did not prospectively survey clients regarding their
476 pet's known allergies or exposures to other mammalian species, e.g., mouse or rabbit,
477 which could potentially induce a humoral response. mAbs of both mouse and rabbit

478 origin are used in our in-house assays and could result in assay background or
479 interference. However, neither of these two dogs were CAHA+ by ELISA at Day 1. Anti-
480 human IgG was not detected by ELISA until Day 30 in any dog, nor from healthy canine
481 sera, suggesting exposure to hu14.18-IL2 as the origin of the antibodies. However, we
482 have not formally tested for pre-existing anti-mouse or anti-rabbit antibodies in these
483 dogs. These seemingly discordant data could reflect the different assay sensitivities and
484 different assay modalities. Moreover, serum from healthy humans and healthy dogs can
485 both contain detectable levels of autoantibodies including rheumatoid factors and anti-
486 idiotypic antibodies which may cause assay interference [48, 50, 51].

487 Additionally, activation of B cells, generation of anti-tumor antibodies and
488 establishment of B cell memory following a treatment regimen similar to that used in this
489 trial have been reported in a mouse melanoma model [52]. As IgM typically arises early
490 in an immune response, the CAHA response detected at early timepoints (e.g., Day 1,
491 Day 10) in this study may be of IgM isotype which our CAHA ELISA may also detect. in
492 contrast, the flow cytometry assay does not detect a particular antibody isotype and
493 thus may detect ADA despite lack of CAHA detected by ELISA in the same serum. The
494 detection of binding inhibition at early time points may also result from the isotype
495 agnostic nature of the flow cytometry assay. The increase in binding inhibition observed
496 in two dogs (K910 and K915) from baseline to Day 1 was unexpected as the dogs had
497 not been exposed to hu14.18-IL2 at the time of serum collection on either day.
498 Moreover, the timeframe from baseline to Day 1 in this study was between 5-9 days,
499 representing rapid kinetics for a *de novo* humoral response. However, we have not
500 determined the fine specificity of the antibody responses in this study. All dogs treated

501 in this study received radiation, thus there is potential for radiation-induced antigen
502 shedding by tumor to trigger a humoral response [53]. Tumor-specific and self-specific
503 antibodies have been found in sera of cancer patients in several studies [54, 55]. Thus,
504 the binding inhibition at Day 1 in these two dogs could be due to endogenous cross-
505 reactive antibodies or those recognizing cryptic epitopes or neoantigens [56].

506 The IC has humanized IL2 linked to the mAb Fc region, thus the IL2 and/or the
507 linker between the Fc and the IL2 may be recognized as foreign in the dog triggering an
508 immune response. None of the dogs in this study experienced significant or unexpected
509 adverse events [38]. However, as dogs received only a single 3-day course of IT-IC, we
510 could not evaluate the impact of induced ADAs on subsequent pharmacokinetics of
511 hu14.18-IL2 or on the potential toxicity during subsequent courses. It will be important to
512 evaluate peak serum levels of hu14.18-IL2 as well as monitor anti-IL2 and anti-linker
513 responses to determine if CAHA+ sera affect antibody-mediated cellular cytotoxicity, as
514 seen in some humans receiving IC treatment [26].

515 Additional studies could further characterize the binding specificity in terms of
516 distinct IgG regions, e.g., Fc receptor, Fab hinge region [49]. Further, as in humans, IgG
517 is the most prevalent Ig in canine sera, however IgM and IgA are present at lower
518 concentrations [57]. The rabbit anti-mouse IgG HRP secondary reagent used in the
519 ELISA is reactive to both the heavy and light chain of mouse IgG. As such, this reagent
520 may also react with other immunoglobulin classes, e.g., IgM, IgA, since these all share
521 the same light chain. Therefore, subsequent testing could determine the isotype of the
522 CAHA responses, as well as species cross-reactivity – in particular to mouse or rabbit

523 IgG.

524 The timeline of the anti-human IgG CAHA ELISA response observed in our study
525 (7 of 12 dogs at Day 30) is similar to that observed in a previous study of dogs treated
526 with NHS-IL12, a humanized antibody against necrotic tumor regions linked to
527 humanized IL12. Dogs treated with NHS-IL12 developed ADA against both human IgG
528 (8 of 12 dogs) and against human IL12 (5 of 14 dogs) between 15 to 29 days after
529 treatment [31]. Factors that may explain differences in the ADA timeline between our
530 study and that of Paoloni et al., include the composition and target of the IC, IC dose
531 level, administration route, as well as the assays used to monitor the development of
532 ADA.

533 Conclusion

534 The CAHA detection assays in this report provide tools to monitor parallel canine
535 patient populations receiving treatment with human biopharmaceuticals. These assays
536 should be translatable to other treatment regimens delivering “foreign” protein
537 molecules *in vivo*, as well as to species other than the dog, provided appropriate
538 controls are available. The recent availability of immune checkpoint blockade in the form
539 of a caninized anti-PD1 therapeutic mAb (Gilvetmab, Merck) expands the treatment
540 options for dogs with melanoma. To this end, we are evaluating the addition of immune
541 checkpoint blockade, i.e., anti-PD1, to our novel treatment regimen of RT and IT-IC with
542 hu14.18-IL2 in dogs with melanoma. Local anti-tumor responses as well as systemic
543 immune responses, including the development of CAHA using the assays described

herein, will be investigated.

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794 # Supporting information

795 **S1 Fig. Binding inhibition assay controls.** PE-conjugated anti-GD2 antibody 14G2a
796 was mixed with either (A) 20 μl (open histogram with solid line) or (B) 2.5 μl (open
797 histogram with solid line) healthy canine donor sera before staining M21 cells. M21
798 stained with 14G2a-PE mixed with 20 μl or 2.5 μl PBS as a no-serum control is
799 represented as a gray histogram in both (A) and (B). Data are representative of
800 triplicates. Values represent % binding inhibition compared to no-serum control.
801

802 **S2 Fig. Serum from dogs treated with hu14.18-IL2 shows varied level of inhibition**
803 **of 14G2a binding to target GD2.** Prior to staining M21 cells, PE-conjugated anti-GD2
804 antibody 14G2a was mixed with baseline/pre-treatment sera or post-treatment sera
805 from different timepoints from (A) K914 or (B) K915. Baseline/pre-treatment data are
806 represented as gray histograms; post-treatment data are represented as open
807 histograms with solid lines. Data are representative of triplicates. Values represent %
808 binding inhibition compared to baseline/pre-treatment control.

Data availability statement

All relevant data are within the manuscript and its Supporting Information files.

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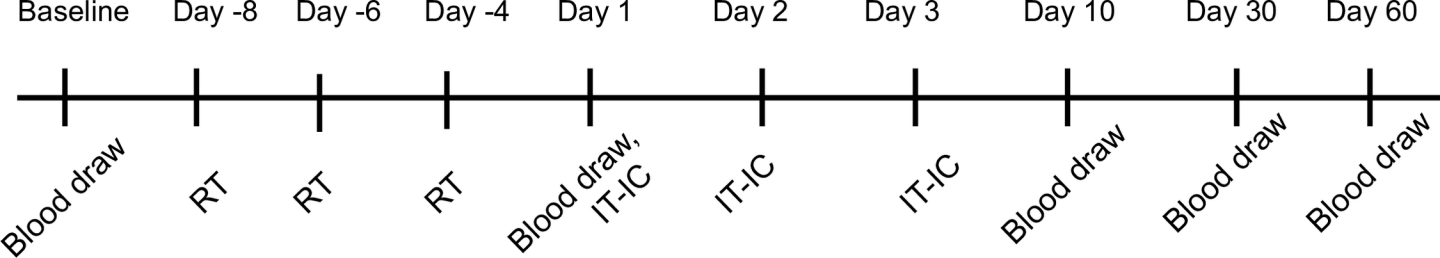
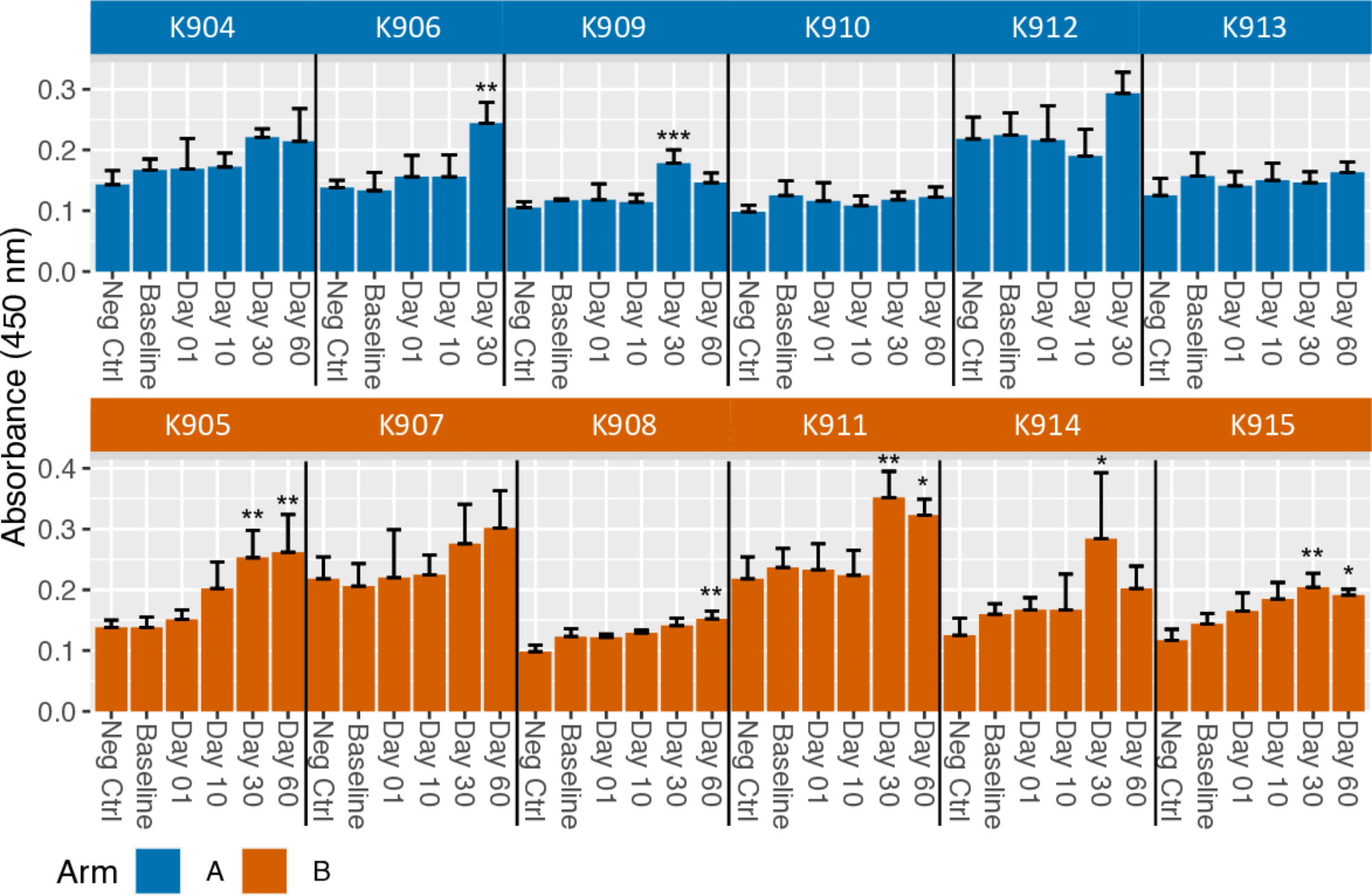
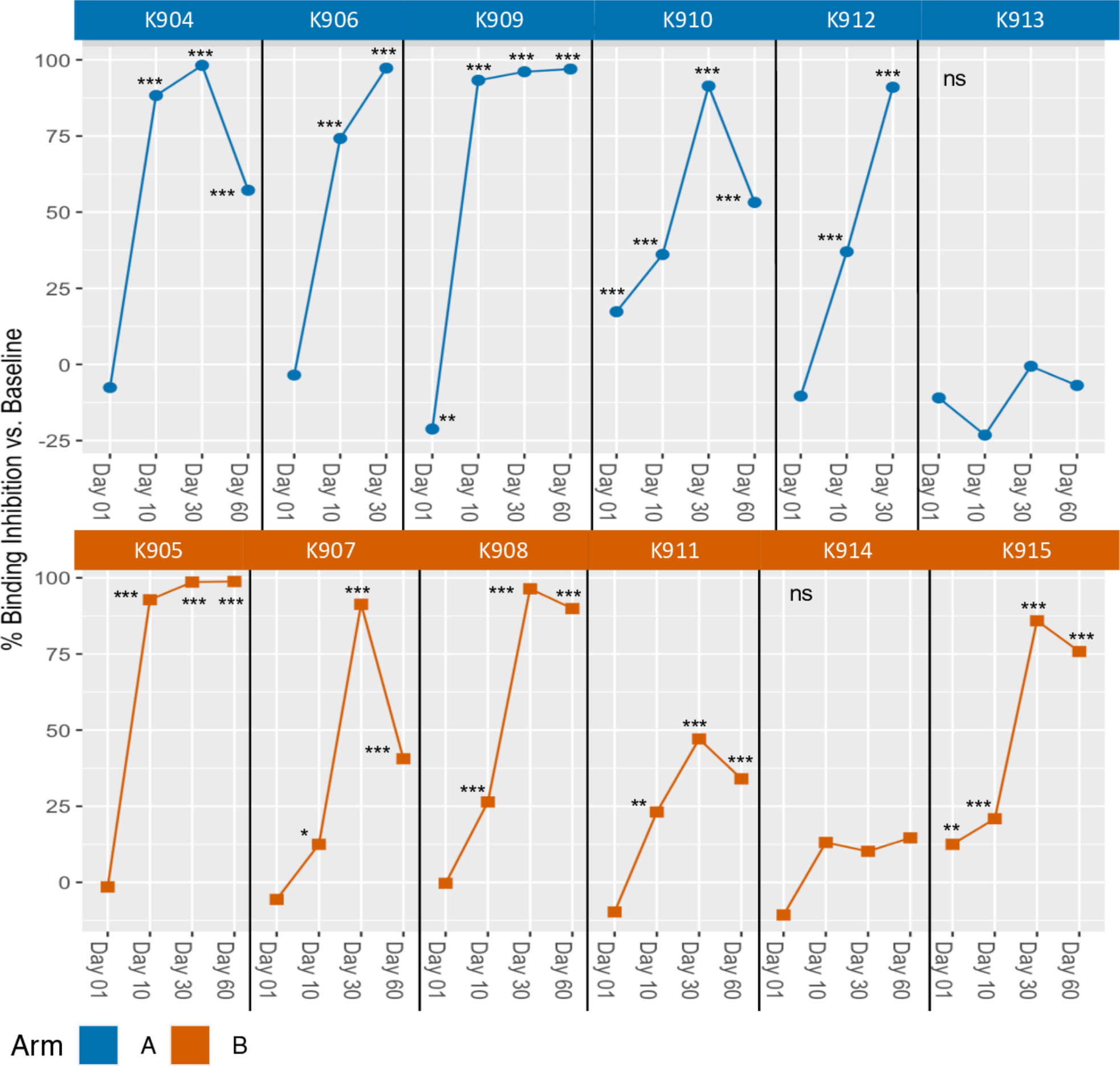
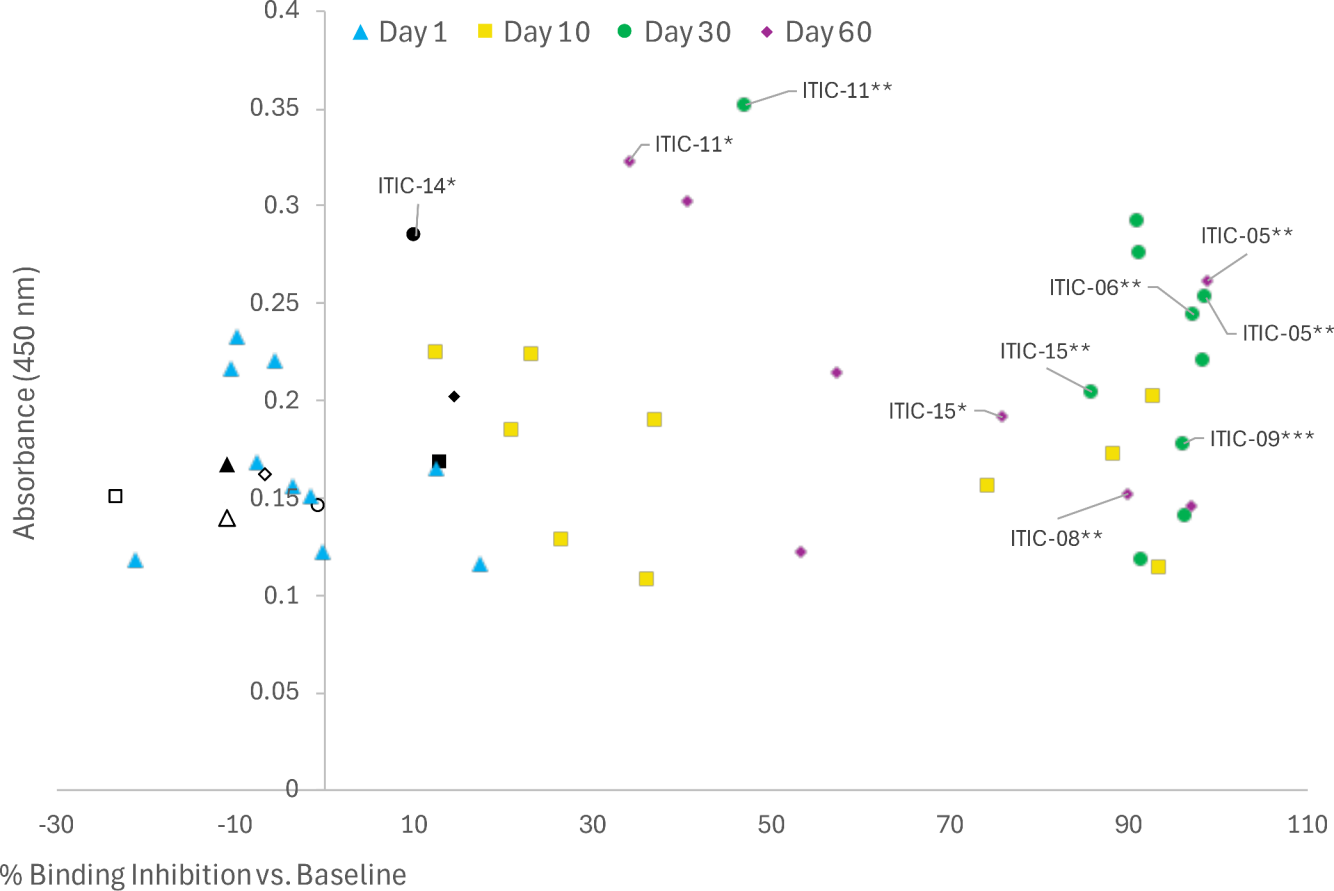
Competing interests

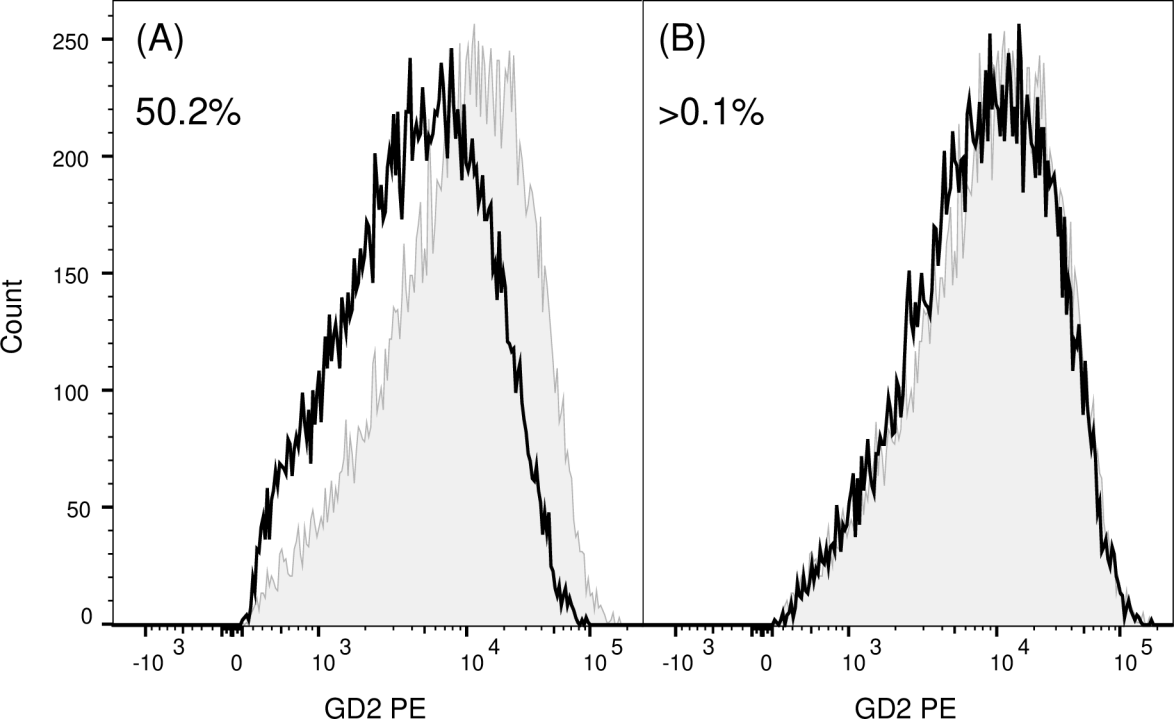
None

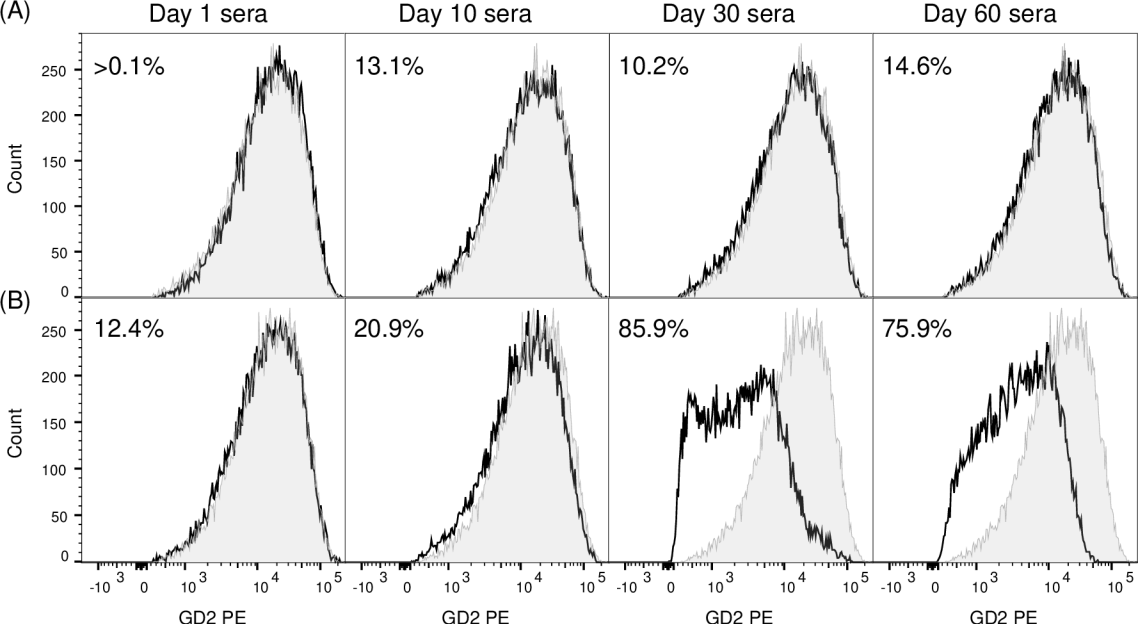

Baseline
Day -8
Day -6
Day -4
Day 1
Day 2
Day 3
Day 10
Day 30
Day 60
Blood draw
RT
RT
RT
Blood draw, IT-IC
IT-IC
IT-IC
Blood draw
Blood draw
Blood draw


K904
K906
K909
K910
K912
K913
K905
K907
K908
K911
K914
K915
Absorbance (450 nm)
0.0
0.1
0.2
0.3
0.4
Neg Ctrl
Baseline
Day 01
Day 10
Day 30
Day 60
**
***
*
Arm
A
B


K904
K906
K909
K910
K912
K913
K905
K907
K908
K911
K914
K915
% Binding Inhibition vs. Baseline
100
75
50
25
0
-25
Day 01
Day 10
Day 30
Day 60
ns
Arm
A
B


Day 1
Day 10
Day 30
Day 60
ITIC-11**
ITIC-11*
ITIC-14*
ITIC-05**
ITIC-06**
ITIC-05**
ITIC-15**
ITIC-15*
ITIC-09***
ITIC-08**
Absorbance (450 nm)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-30
-10
10
30
50
70
90
110
% Binding Inhibition vs. Baseline


(A)
50.2%
(B)
>0.1%
Count
250
200
150
100
50
0
-10 3
0
10 3
10 4
10 5
GD2 PE


(A)
Day 1 sera
Day 10 sera
Day 30 sera
Day 60 sera
>0.1%
13.1%
10.2%
14.6%
(B)
12.4%
20.9%
85.9%
75.9%
Count
250
200
150
100
50
0
-10 3
0
10 3
10 4
10 5
GD2 PE