

1 **Title**
2 Tasmanian devil CD28 and CTLA4 capture CD80 and CD86 from adjacent cells

3
4 **Running title**
5 Intercellular protein transfer of immune checkpoints in Tasmanian devils

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7 **Article Type**
8 Short Communications

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33 **Highlights**

- 34 • Key immune checkpoint receptor-ligand interactions are conserved in marsupials.
- 35 • Live cell-based assays show Tasmanian devil CD28 and CTLA4 can capture CD80
36 and CD86 *in trans* from adjacent cells.
- 37 • Mutation of the conserved CTLA4_{MYPPPY} ligand binding motif to CTLA4_{MYPPPA}
38 reduces binding to CD80 and intercellular protein transfer.
- 39 • Removal of conserved CTLA4_{YVKM} protein recycling binding motif in CTLA4 results
40 in bidirectional intercellular protein transfer between CTLA4 and CD80.
- 41 • Highly successful human immune checkpoint immunotherapies have the potential to
42 be translated for veterinary and conservation medicine.

43

44 **Abstract**

45 Immune checkpoint immunotherapy is a pillar of human oncology treatment with potential for
46 non-human species. The first checkpoint immunotherapy approved for human cancers targeted
47 the CTLA4 protein. CTLA4 can inhibit T cell activation by capturing and internalizing CD80
48 and CD86 from antigen presenting cells, a process called trans-endocytosis. Similarly, CD28
49 can capture CD80 and CD86 via trogocytosis and retain the captured ligands on the surface of
50 the CD28-expressing cells. The wild Tasmanian devil (*Sarcophilus harrisii*) population has
51 declined by 77% due to transmissible cancers that evade immune defenses despite genetic
52 mismatches between the host and tumours. We used a live cell-based assay to demonstrate that
53 devil CTLA4 and CD28 can capture CD80 and CD86. Mutation of evolutionarily conserved
54 motifs in CTLA4 altered functional interactions with CD80 and CD86 in accordance with
55 patterns observed in other species. These results suggest that checkpoint immunotherapies can
56 be translated to evolutionarily divergent species.

57

58 **Keywords**

59 immune checkpoint, wild immunology, transmissible cancer, trans-endocytosis, trogocytosis,
60 intercellular protein transfer

61 **Abbreviations**

62 intercellular protein transfer (IPT), devil facial tumour (DFT), interferon gamma (IFN γ), major
63 histocompatibility complex (MHC), Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4),
64 blue fluorescent protein (BFP)

65

66 **1. Introduction**

67 The initial USA Food and Drug Administration approval for an immune checkpoint
68 inhibitor was in 2011 when a monoclonal antibody targeting CTLA4 was approved for
69 metastatic melanoma (U.S. Food and Drug Administration, 2011). Since then, targeting
70 immune checkpoints has proved effective for many types of cancer, and a single monoclonal
71 antibody targeting the PD1 pathway is currently approved for 15 types of cancer (U.S. Food
72 and Drug Administration, 2020). Despite the massive potential for veterinary medicine and
73 One Health projects, functional comparative studies of immune checkpoints studies are limited,
74 and have focused primarily on domestic animals (Achleitner et al., 2011; Bernard et al., 2007;
75 Folkl et al., 2010; Hansen et al., 2009; Hartley et al., 2016; Ikebuchi et al., 2014, 2013, 2011;
76 Maekawa et al., 2014, 2017, 2016; Nemoto et al., 2018; Rebech et al., 2020; Tagawa et al.,
77 2016).

78 The Tasmanian devil (*Sarcophilus harrisii*) immune system has received attention in
79 recent years due to two transmissible cancers that have decimated the wild Tasmanian devil
80 population (Pearse and Swift, 2006; Pye et al., 2016). The original transmissible devil facial
81 tumor (DFT1) was first observed in 1996 and has since spread across most of the island state,
82 leading to an average 77% population decline in areas where the DFT1 is present (Lazenby et

83 al., 2018). In 2014, a second independent transmissible devil facial tumor (DFT2) was
84 discovered in southern Tasmania (Pye et al., 2016). DFT1 and DFT2 are lethal and the available
85 evidence suggests that both tumors have arisen from Schwann cells (Murchison et al., 2010;
86 Patchett et al., 2020). A primary means of immune evasion by DFT1 cells is downregulation
87 of MHC-I. However, MHC-I can be upregulated in response to IFN γ (Siddle et al., 2013). The
88 DFT2 cells examined to date generally express MHC-I (Caldwell et al., 2018). This suggests
89 that these transmissible cancers employ additional immune evasion mechanisms to induce
90 tolerance to tumor allografts and damage-associated molecular patterns.

91 We have shown that the immune checkpoint protein PDL1 binds to PD1 on DFT cells
92 and developed devil-specific monoclonal antibodies that can block this receptor-ligand
93 interaction (Flies et al., 2020a, 2016). Furthermore, exposure to IFN γ upregulates PDL1 on
94 DFT cells, matching the pattern observed in many human and mouse cancers. Comparative
95 sequence analysis of other key checkpoint proteins in devils showed that key regions in mouse
96 and human CD28 and CTLA4 proteins are conserved in devils (Flies et al., 2017). Additionally,
97 binding of CTLA4 to its ligands CD80 and CD86 has been confirmed using soluble
98 recombinant proteins (Flies et al., 2020a). However, functional analyses of cell surface protein
99 interactions and protein trafficking remain unexplored.

100 In humans and mice, CTLA4 can capture CD80 and CD86 from the surface of adjacent
101 cells via trans-endocytosis, which results in internalization and degradation of CD80 and CD86
102 in the capturing cell. CTLA4 is continuously recycled from the cell surface to endosomal
103 compartments, which allows a single CTLA4 protein to capture multiple ligands (Qureshi et
104 al., 2011; Robles et al., 2012). CTLA4-mediated depletion of CD80 and CD86 from antigen
105 presenting cells results in reduced T cell activation due to impaired CD28 costimulation
106 (Qureshi et al., 2011).

107 CD28 is known to capture CD80 and CD86 from the surface of adjacent cells, but the
108 captured ligands remain bound to CD28 on the surface of the capturing cell (Briggs, 2014;
109 Hwang et al., 2000; Qureshi et al., 2011). This process of trogocytosis has also been well-
110 documented for capture of MHC-I and MHC-II by T cells, NK cells, and basophils, and can
111 result in immune suppression due to depletion of MHC from target cells (Akkaya et al., 2019;
112 Chung et al., 2014; Daubeuf et al., 2006; Gu et al., 2012; Joly and Hudrisier, 2003; Lemaoult
113 et al., 2015; Nakayama et al., 2011).

114 We developed a panel of immune checkpoint proteins fused to fluorescent reporter
115 proteins and used coculture assays to monitor protein transfer using flow cytometry to
116 determine if intercellular protein transfer patterns evolutionarily conserved in Tasmanian
117 devils. This approach revealed that CTLA4 and CD28 can capture costimulatory ligands from
118 neighboring cells, and that key protein motifs for ligand binding and protein trafficking were
119 conserved in devils. The conserved functional interactions of these proteins across the 160
120 million years since the divergence of eutherian and metatherian mammals (Luo et al., 2011)
121 suggests strong stabilising selection and that there is potential to replicate successful human
122 immunotherapies in devils and other species.

123 **2. Materials and methods**

124 ***2.1 Design of expression vectors***

125 Complete methodological details with step-by-step instructions for (a) *in silico*
126 characterization of protein structure, (b) design and (c) assembly of expression vectors, and (d)
127 transfection of mammalian cells has been previously published (Flies et al., 2020a, Flies et al.,
128 under review at Bio-protocol).

129 Open reading frames for the devil genes of interest (CD28, CTLA4, CD80, and CD86)
130 were retrieved from Genbank and Ensembl databases (Table S1). *In silico* analysis of the
131 protein coding sequences for genes-of-interest in this study were performed in a previous study

132 (Flies et al., 2017). The expression vector design included the full-length open reading frames
133 for gene-of-interest fused to a tobacco etch virus cleavage tag, a linker peptide, a 6x-Histidine
134 purification tag, and monomeric fluorescent reporter protein. The direct fusion of the protein-
135 of-interest to a fluorescent reporter protein allow for protein tracking in live cells (Flies et al.,
136 2020a). The vectors contain an all-in-one transfection system that includes antibiotic resistance
137 and genes-of-interest within a Sleeping Beauty transposon cassette (Kowarz et al., 2015), and
138 the Sleeping Beauty transposase outside of the transposon cassette (Mátés et al., 2009).
139 CTLA4, CD28, CD80, and CD86 were fused to mCitrine (Addgene # 135923), mOrange
140 (Addgene # 135928), mTagBFP (Addgene # 135924), and mNeptune2 (Addgene # 135927),
141 respectively.

142 ***2.2 Restriction digest of expression vectors***

143 2 µg of base expression vectors were digested using NotI-HF (NEB # R3189S) and
144 SmaI (NEB # R0141S) overnight at room temperature. Antarctic phosphatase (NEB #
145 M0289S) was then added to the vector digests and incubated for 60 minutes at 37°C, then for
146 a further 2 minutes at 80°C to inactivate the enzymes. The reactions were then run at 100V in
147 a 1% agarose gel and the digest vectors were excised and purified using a NucleoSpin® Gel
148 and PCR Clean-up Kit (Macherey-Nagel, USA) according to instructions set by the
149 manufacturer. DNA concentrations were measured and analysed using a NanoDrop™ 1000
150 Spectrophotometer (NanoDrop Technologies, USA).

151 ***2.3 Overlap extension PCR to create vector inserts***

152 Overlap extension PCR was used to extend open reading frames and remove the stop
153 codon from the genes of interest to allow for Gibson assembly of plasmids pCW1, pCW2,
154 pCW3, and pCW5 (Gibson et al., 2009). Primers and reaction conditions are available in Table
155 S2. PCR amplicons were isolated using gel electrophoresis run at 100 V for 30 minutes in a
156 1% agarose gel. DNA was purified and quantified as described above. CTLA4 mutants with a

157 truncated C-terminus (pCW9; CTLA4_{truncated}; amino acids 211-223 deleted) or the YVKM
158 protein trafficking motif deleted (pCW8; CTLA4_{del}; amino acids 201-223 deleted) were
159 developed as previously described by Qureshi et al. (2012) with minor modifications. pCW8
160 and pCW9 were made by amplifying with a reverse primer that amplified the DNA upstream
161 of the deleted region and extension that overlapped the expression vector (Table S2). The
162 CTLA4 expression vector (pCW10) with an altered binding motif was produced by substituting
163 an alanine for the for the second tyrosine in the MYPPPY ligand binding motif (Y139A) to
164 create the CTLA4_{MYPPPA} mutant (Morton et al., 1996). The mutation was coded into new
165 forward and reverse primers (Table S2), and were used to amplify and extend one fragment
166 upstream of the mutation, and one fragment downstream of the mutation. The two fragments
167 were purified, combined in a PCR reaction, and re-amplified using the forward from the 5'
168 fragment and the reverse primer from the 3' fragment to form one large fragment. This fragment
169 was purified and assembled using NEBuilder as described above.

170 ***2.4 Assembly and transformation of expression vectors***

171 The DNA for the gene-of-interest with overlap extensions were ligated into the
172 digested vectors using NEBuilder[®] HiFi DNA Assembly Cloning Kit (NEB # E5520S) by
173 incubating at 50 °C for one hour according to the manufacturer's instructions. A negative
174 control was established by transforming DH5 α with the linearised vector. A positive control
175 on the other hand was established by transforming DH5 α bacteria with NEBuilder[®] Positive
176 Control (New England Biolabs, USA). After transformation, the bacteria were grown overnight
177 at 37°C on Luria Broth Agar plates added with 100 μ g/mL of ampicillin.

178 Colony PCR was used to identify colonies with the predicted insert size. 10 μ L of
179 OneTaq Quick-Load 2X Master Mix (NEB # M0486L) was used for all colony PCR. 7 μ L of
180 deionized-distilled water was added to the mix, and then 1 μ L of forward (pSB_EF1a.FOR:
181 GCCTCAGACAGTGGTTCAAAG) and reverse (pSB_BGH.REV:

182 AGGCACAGTCGAGGCTGAT) primers at 10 μ M concentration were diluted into the mix. 4
183 to 6 bacterial colonies were picked using sterile pipette tips and transferred to separate tubes
184 containing 10 μ L of PBS for assessing. 1 μ L of bacteria in PBS was then transferred to the PCR
185 tube to achieve a final volume of 20 μ L and primer concentration of 0.5 μ M. The mix was then
186 denatured at 94 $^{\circ}$ C for 5 minutes, and then subjected to 30 cycles of denaturation at 94 $^{\circ}$ C for
187 15 seconds, annealing at 58 $^{\circ}$ C for 15 seconds, and extension at 68 $^{\circ}$ C for 105 seconds, before
188 a final extension step at 72 $^{\circ}$ C for 3 minutes. 10 μ L of the reaction was then added directly to
189 a 1% agarose gel and run at 100 V for 25-35 minutes. DNA bands were imaged under UV light
190 using Gel DocTM XR+ Gel Documentation System (Bio-Rad, USA).

191 ***2.5 Purification and sequencing of expression vectors***

192 Colonies that yielded amplicons that matched the predicted amplicon size were cultured
193 overnight for plasmid amplification. 9 μ l of bacteria in PBS was added to 5 mL of Luria Broth
194 with 100 μ g/ml of ampicillin. The bacterial cultures were incubated at 37 $^{\circ}$ C and 200 rpm
195 overnight. The plasmids were isolated from bacterial culture using PureLinkTM Quick Plasmid
196 Miniprep Kit (Invitrogen, USA) or NucleoSpin[®] Plasmid EasyPure Miniprep Kit (Macherey-
197 Nagel, USA) according to the manufacturers' instructions. Concentrations of purified plasmids
198 were quantified using a NanoDropTM 1000 Spectrophotometer (NanoDrop Technologies,
199 USA). The plasmids were stored at -20 $^{\circ}$ C. The plasmids were sequenced using a BigDyeTM
200 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The BigDyeTM
201 Terminator was removed using Agencourt[®] CleanSEQ[®] (Beckman Coulter, USA) according
202 to the protocols set by the manufacturer before loading samples to the Applied Biosystems[®]
203 3500xL Genetic Analyzer (Applied Biosystems, USA) for sequencing using a fluorescence-
204 based capillary electrophoresis analytical technique.

205 ***2.6 Transfection of Chinese Hamster Ovary (CHO) cells***

206 CHO-K1 (ATCC CCL-61) cells were cultured in cRF10 (complete RPMI media
207 supplemented with 10% fetal bovine serum) in 75 cm² culture flasks (Corning, USA) at 37°C
208 in a humidified atmosphere with 5% CO₂. CHO cells were transfected using a modified
209 polyethylenimine-based (PEI) method. 300,000 cells/well were seeded into 6-well plates and
210 were grown overnight at 37°C with 5% CO₂. 2 µg of plasmid DNA and 6 µg of PEI were
211 diluted separately to 100 µL of PBS in separate microfuge tubes. The solution from the DNA
212 tube was then added to PEI tubes and mixed by gentle pipetting. Tubes with a total volume of
213 200 µl of reagents were incubated for 15 to 20 minutes at room temperature. While the
214 DNA:PEI solution was incubating the media on the target CHO cells in 6-well plates was
215 replaced with fresh cRF10. The transfection was accomplished by adding the entire contents
216 of DNA:PEI solution (200 µL) dropwise to the cells. Cells were then incubated with
217 transfection reagents overnight and examined for fluorescence on the Leica-DM IRB
218 fluorescence microscope (Leica, Germany). After fluorescence was detected indicating that
219 transfection was successful, the media was removed and replaced with cRF10 containing 1
220 mg/mL of hygromycin B solution from *Streptomyces hygroscopicus* (Invitrogen, USA). Media
221 was refreshed as needed until drug selection was complete and all cells were fluorescent. The
222 cells were then cultured in cRF10 with a maintenance dose of 0.2 mg/mL of hygromycin B.

223 ***2.7 Intercellular protein transfer coculture assay***

224 The coculture assay to assess intercellular protein transfer was modified from Briggs
225 (2014). The lysosomal inhibitor chloroquine was used to block degradation of captured proteins
226 that were endocytosed (Qureshi et al., 2011). Chloroquine was diluted to 100 µM in cRF5
227 (complete RPMI with 5% fetal bovine serum) and 100 µL were added into appropriate wells
228 in a 96-well round bottom plate (Costar, USA). The plate was incubated at 37°C with 5% CO₂
229 until cell lines were ready to be cocultured. Stably-transfected CHO cells were harvested,
230 washed, and resuspended to 5 x 10⁵ cells/mL in cRF5. 50 µL (25,000 cells) of cell line A (e.g.

231 CD28) and cell line B (e.g. CD80) were aliquoted into appropriate wells to achieve a 1:1 ratio
232 and a total of 50,000 cells/well. The final volume was 200 μ L/well with a final chloroquine
233 concentration of 50 μ M. The plates were then incubated at 37°C with 5% CO₂ for either 3 hours
234 or 20 hours.

235 **2.8 Flow cytometry**

236 The coculture assay plates were centrifuged at 500g for 3 minutes at 4°C. The 96-well
237 plate was then inverted, and the media was discarded by briskly dumping the media out. 100
238 μ L of PBS was added into appropriate wells to wash cells and the plate was spun again at 500g
239 for 3 minutes at 4°C. After removing media, 100 μ L of Trypsin-EDTA was added into each
240 well and incubated at 37°C with 5% CO₂ for 5 to 10 minutes or until cells were dislodged from
241 the bottom of the well. 150 μ L of cRF10 was then added to each well and the cells were
242 resuspended by pipetting. The plate was then centrifuged at 500g for 3 minutes at 4°C and
243 media was discarded. 200 μ L of flow cytometry buffer was added into each well. Following
244 this, the plate was spun again at 500g for 3 minutes at 4°C. Media was discarded and cells were
245 resuspended in 250 μ L of flow cytometry fixation buffer (Flies et al., 2020a). The coculture
246 plate was placed on a rocking platform and incubated at room temperature and protected from
247 light for at least 20 minutes. The incubated plate was subsequently spun at 500g for 3 minutes
248 at 4°C and the media was discarded (Flies et al., 2020a). Cells in each well were resuspended
249 in 250 μ L of flow cytometry buffer, wrapped in foil, and stored at 4°C for at least 2 hours before
250 flow cytometric analysis.

251 **2.9 Data Processing and Gating Strategy for Flow Cytometry Analysis**

252 Flow cytometry data was analysed using the FCS Express 6 Flow Cytometry Software
253 version 6 (De Novo Software). CHO cells were primarily gated by forward scatter height (FSC-
254 H) and side scatter height (SSC-H) to exclude cell debris, and then sub-gated for singlets using
255 FSC-A (area) and FSC-H. CHO cells that did not express a fluorescent protein or expressed a

256 single fluorescent protein were used as controls for setting voltages and compensation. A
257 minimum of 10,000 cells was acquired from each experimental sample, and all cell
258 combinations were done with n=3 technical replicates.

259 Intercellular protein transfer was identified according to the schematic in **Fig. 1A**. The
260 upper left quadrant (UL) and lower right quadrant (LR) indicate cells that have only a single
261 color of fluorescent protein (**Fig. 1A**). The upper right quadrant (UR) indicates cells that are
262 positive for two colors, indicating the intercellular transfer of a fluorescent protein occurred
263 (Briggs, 2014). Additionally, a shift in both cell lines to the UR quadrant indicates a
264 bidirectional protein exchange, whereas a shift from one of the cell lines to the UR quadrant
265 indicates a one-way protein transfer (**Fig. 1A**).

266 **2.10 Analysis of CTLA4 binding and protein trafficking motifs across species**

267 CTLA4 protein sequences were obtained from the National Center for Biotechnology
268 Information (NCBI) (<https://www.ncbi.nlm.nih.gov/gene/1493/ortholog/>). Descriptive
269 information for CTLA4 for each class (*Amphibia*, *Reptilia*, *Aves*, *Mammalia*) were exported
270 separately from NCBI as tabular data (e.g. comma separated values spreadsheet). The files
271 were manually updated to include a new column containing the class (e.g. *Aves*). The
272 descriptive spreadsheets were then combined to create a spreadsheet with descriptive
273 information for all species. The RefSeq protein sequences were exported from NCBI as a
274 FASTA file. FASTA sequences were imported into CLC Main Workbench (CLC, 2020), and
275 the "Show Table" tab was used to determine the length of each CTLA4 protein sequence. This
276 table was copied and saved as a .CSV file for subsequent merging with other spreadsheets. The
277 "Motif Search" function in CLC was then used to identify and annotate all sequences containing
278 a greater than 90% match to the CTLA4_{MYPPPY} and CTLA4_{YVKM} motifs. A new .CSV file was
279 created for the CTLA4_{MYPPPY} and the CTLA4_{YVKM} matches. The four .CSV files were then
280 merged in R version 3.6.1 using the "merge" function (R Core Team, 2019). The script is

281 available with the supplementary material (r_CTLA4_merge_master.R) and the merged data
282 are available in Table S3.

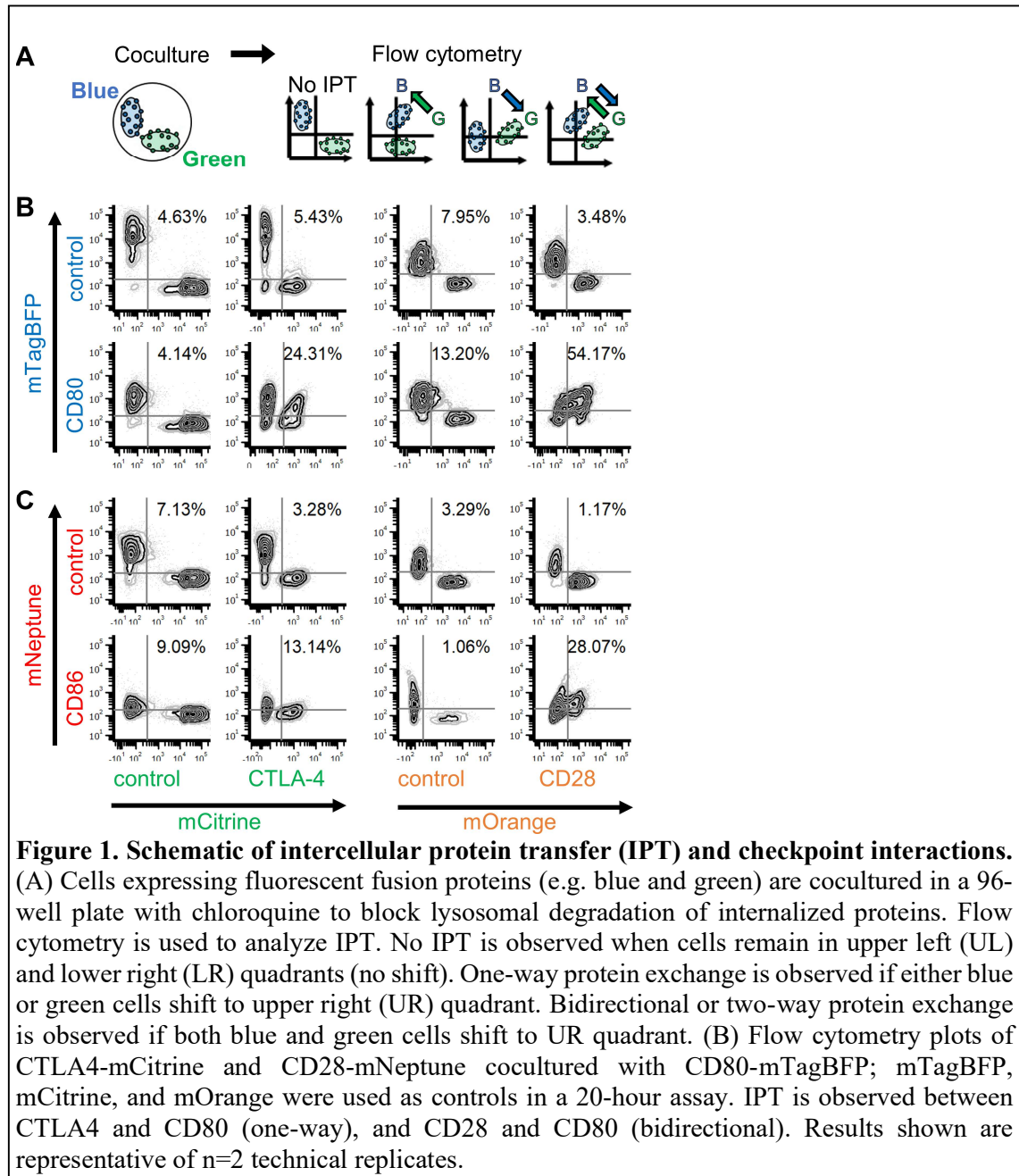
283 **3. Results and Discussion**

284 CHO cells expressing either unfused mCitrine or mTagBFP controls resulted in the
285 cells remaining primarily positive for only a single color after coculture (**Fig. 1B**). Likewise,
286 cells remained mostly single color positive after coculture of CTLA4-mCitrine with mTagBFP
287 control or mCitrine control with CD80-mTagBFP (**Fig. 1B**). However, coculture of CTLA4-
288 mCitrine with CD80-mTagBFP for 20 hours resulted in 24% of the cells in the upper right
289 quadrant. CTLA4 cells shifted from the lower right quadrant to the upper right quadrant
290 indicating positive receptor-ligand binding and protein transfer from CD80-mTagBFP to
291 CTLA4-mCitrine (**Fig. 1B**). The coculture between cells transfected with CD28-mOrange and
292 CD80-mTagBFP suggests that bidirectional protein transfer occurred as both single color
293 populations shifted, with 54% of the cells were in the upper right quadrant after coculture.
294 CTLA4 also captured CD86, whereas transfer of CD28 and CD86 again appeared to be
295 bidirectional (**Fig. 1C**). The interaction of CTLA4 and CD28 with CD80 appears stronger than
296 with CD86, but this could be an artefact of the suboptimal excitation of the mNeptune
297 fluorescent protein; mNeptune has peak excitation at 600 nm, but our available flow cytometer
298 had a 633 nm laser.

299 To assess the role of key functional motifs in CTLA4 we developed three CTLA4
300 mutants (**Fig. 2A**). Here, we used 3-hour and 20-hour coculture assays to document IPT over
301 time, and also to minimise potential side effects of extended cell culture with chloroquine. The
302 results show a one-way IPT from CD80-mTagBFP to CTLA4-mCitrine in 3-hour (17%) and
303 20-hour (46%) assays (**Fig. 2B**). The devil mutant truncated CTLA4_{truncated}-mCitrine (**Fig. 2B**)
304 also showed one-way protein exchange in both 3-hour (16%) and 20-hour (44%) assays with
305 CD80 (**Fig. 2B**). The YVKM motif in the cytoplasmic domain of CTLA4 is important for

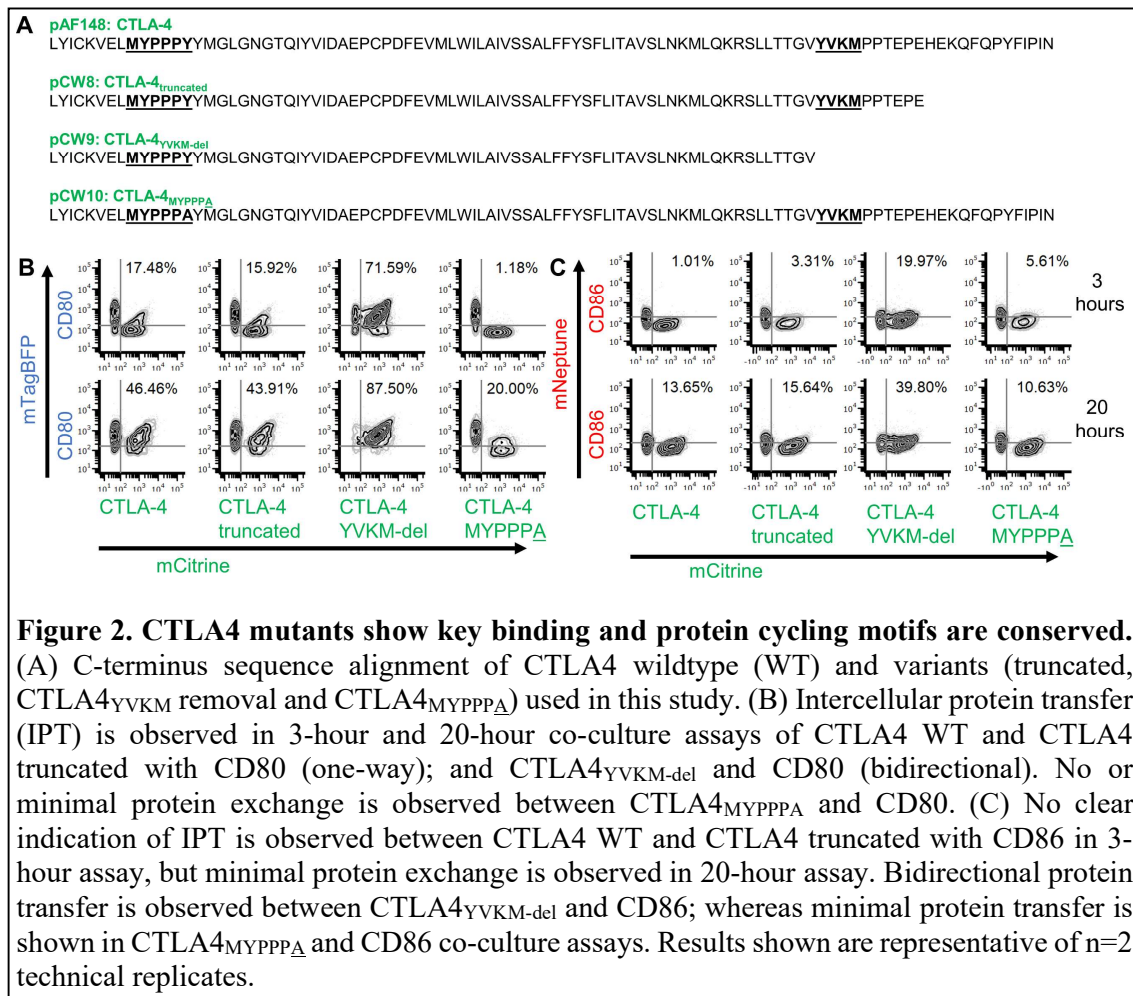
306 protein recycling, so we hypothesized that CTLA4-CD80 binding would remain intact in a
307 CTLA4 mutant with the YVKM motif removed, but that CTLA4 would have reduced capacity
308 to trans-endocytose CD80 and CD86. As expected, deletion of the YVKM dramatically
309 resulted in a pattern more closely resembling the interaction of CD28-CD80 than the CD80
310 with the other CTLA4 variants (**Fig. 2B**). The CTLA4 and CD86 interaction was also changed
311 in the CTLA4_{YVKM-del} mutant, with the CTLA4 and CD86 populations merging towards a single
312 population after coculture.

313 In all species studied to date, the MYPPPY motif is required for ligand binding, so we
314 hypothesized that IPT would be reduced in cocultures using the CTLA4_{MYPPPA} mutant
315 compared to other CTLA4 variants (**Fig. 2A**). Coculture of CD80 with mutant CTLA4_{MYPPPA}
316 showed minimal or no protein transfer (3-hours: 1%; 20-hours: 20%) (**Fig. 2B**), suggesting that
317 the MYPPPY motif is critical for CTLA4-CD80 binding interactions in Tasmanian devils. The
318 coculture of devil CTLA4 WT and CTLA4 mutants with CD86 ligand did not reveal a clear
319 indication of intercellular protein exchange in the 3-hour assay even though the flow cytometry
320 results showed a slight shift of CTLA4 (WT and mutants) to the UR quadrant (**Fig. 2C**).
321 However, 20-hour coculture assays revealed a one-way protein exchange from CD86-
322 mNeptune to CTLA4 WT-mCitrine (14%) and CTLA4 truncated-mCitrine (16%) (Fig. 2C).
323 Additionally, cells expressing mutant CTLA4_{YVKM-del} cocultured with CD86 showed
324 bidirectional protein transfer between both cell lines in both 3-hour (20%) and 20-hour (40%)
325 assays (Fig. 2C). Lastly, CD86-mNeptune cocultured with mutant CTLA4_{MYPPPA} exhibited
326 minimal protein exchange in 20-hour assay (11%) (**Fig. 2C**).



327

328



329

330 CTLA4 orthologues for 233 species are listed in the GenBank data base (accessed 8-

331 May-2020; Table S3) (Benson et al., 2012). The CTLA4^{MYPPPY} and CTLA4^{YVKM} motifs are

332 conserved in 137 and 138, respectively, of the 140 mammalian species. The CTLA4^{MYPPPY}

333 motif is conserved in all 76 bird species in GenBank with CTLA4 orthologues, and the

334 CTLA4^{MYPPPY} motif is conserved in 75 bird species. Only 3 reptiles have the CTLA4^{MYPPPY}

335 motif and 4 have the CTLA4^{YVKM} motif. All three amphibians have the CTLA4^{MYPPPY} motif,

336 but none have the CTLA4^{YVKM} motif. The coculture assays described here is amenable for

337 functional testing of cell surface interactions in any of the above species, without the need to

338 purify recombinant proteins. However, primary cells for each species or amphibian, reptilian,

339 avian cell lines should be used instead of mammalian cell lines.

340 In summary, our results and analyses suggest that strong stabilizing selection has
341 operated to maintain the function of these key immune checkpoint genes. This opens the door
342 to immunotherapies that target these proteins for most mammalian species, and potentially for
343 most bird species and some reptiles and amphibians. Although we have focused on marsupials
344 in this manuscript, we believe that these methods can potentially be applied to the study of
345 receptor-ligand interactions in invertebrates. We have published step-by-step protocols to
346 facilitate comparative immunology studies in other non-model species (Flies et al., 2020b).

347 **Supplementary materials**

348 **Table S1. Summary of genes and plasmids**

349 **Table S2. Primer sequences for plasmid assembly**

350 **Table S3. Primer sequences for Sanger sequencing**

351

352 **Acknowledgments**

353 We Amanda Patchett, Camila Espejo, Chrissie Ong, Rob Gasperini, and Ruth Pye for
354 assistance in the laboratory and general advice on this project.

355

356 **Funding**

357 ARC DECRA grant # DE180100484, ARC Linkage grant # LP0989727, ARC Discovery
358 grant # DP130100715, University of Tasmania Foundation Dr Eric Guiler Tasmanian Devil
359 Research Grant through funds raised by the Save the Tasmanian Devil Appeal (2013, 2015,
360 2017, 2018), and Entrepreneurs' Programme - Research Connections grant with Nexvet
361 Australia Pty. Ltd. # RC50680.

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364

365 **Author contributions**

366 ASF and CW designed the study; ASF, CW, JMD, PRL, PRM, and TLP developed the
367 technology; CW performed the experiments; ASF and CW created the figures; ASF, CW,
368 TPL, ABL, and GMW analyzed the data; CW, ASF, and GMW wrote the manuscript and all
369 authors edited the manuscript.

370

371 **Availability of data and materials**

372 Data is available upon request, and will be made freely available in the University of
373 Tasmania Research Data Portal (<https://rdp.utas.edu.au>) following peer-reviewed publication.

374

375 **Conflict of interest**

376 The authors have no conflict of interest to report.

377

378 **References**

- 379 Achleitner, A., Clark, M.E., Bienzle, D., 2011. T-regulatory cells infected with feline
380 immunodeficiency virus up-regulate programmed death-1 (PD-1). *Vet. Immunol.*
381 *Immunopathol.* 143, 307–313. <https://doi.org/10.1016/j.vetimm.2011.06.009>
- 382 Akkaya, B., Oya, Y., Akkaya, M., Al Souz, J., Holstein, A.H., Kamenyeva, O., Kabat, J.,
383 Matsumura, R., Dorward, D.W., Glass, D.D., Shevach, E.M., 2019. Regulatory T cells
384 mediate specific suppression by depleting peptide–MHC class II from dendritic cells.
385 *Nat. Immunol.* 20, 218–231. <https://doi.org/10.1038/s41590-018-0280-2>
- 386 Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J.,
387 Sayers, E.W., 2012. GenBank. *Nucleic Acids Res.* 41, D36–D42.
388 <https://doi.org/10.1093/nar/gks1195>
- 389 Bernard, D., Hansen, J.D., Du Pasquier, L., Lefranc, M.P., Benmansour, A., Boudinot, P.,

- 390 2007. Costimulatory receptors in jawed vertebrates: Conserved CD28, odd CTLA4 and
391 multiple BTLAs. *Dev. Comp. Immunol.* 31, 255–271.
392 <https://doi.org/10.1016/j.dci.2006.06.003>
- 393 Briggs, Z.L., 2014. CD28 costimulation in T cells: requirements, outcomes and regulation.
394 PhD Thesis.
- 395 Caldwell, A., Coleby, R., Tovar, C., Stammnitz, M.R., Mi Kwon, Y., Owen, R.S., Tringides,
396 M., Murchison, E.P., Skjødt, K., Thomas, G.J., Kaufman, J., Elliott, T., Woods, G.M.,
397 Siddle, H.V.T., 2018. The newly-arisen devil facial tumour disease 2 (DFT2) reveals a
398 mechanism for the emergence of a contagious cancer. *Elife* 7.
399 <https://doi.org/10.7554/eLife.35314>
- 400 Chung, B., Tor, John, Beilhack, G., Andersen, E., Brian, Jeffrey, Peter, 2014. Antigen-
401 Specific Inhibition of High-Avidity T Cell Target Lysis by Low-Avidity T Cells via
402 Trogocytosis 8, 871–882. <https://doi.org/10.1016/j.celrep.2014.06.052>
- 403 CLC, 2020. CLC Main Workbench.
- 404 Daubeuf, S., Puaux, A.-L., Joly, E., Hudrisier, D., 2006. A simple trogocytosis-based method
405 to detect, quantify, characterize and purify antigen-specific live lymphocytes by flow
406 cytometry, via their capture of membrane fragments from antigen-presenting cells. *Nat.*
407 *Protoc.* 1, 2536.
- 408 Flies, A.S., Blackburn, N.B., Lyons, A.B., Hayball, J.D., Woods, G.M., 2017. Comparative
409 analysis of immune checkpoint molecules and their potential role in the transmissible
410 Tasmanian devil facial tumor disease. *Front. Immunol.* 8, 513.
411 <https://doi.org/10.3389/fimmu.2017.00513>
- 412 Flies, A.S., Darby, J.M., Lennard, P.R., Murphy, P.R., Ong, C.E.B., Pinfold, T.L., De Luca,
413 A., Lyons, A.B., Woods, G.M., Patchett, A.L., 2020a. A novel system to map protein
414 interactions reveals evolutionarily conserved immune evasion pathways on transmissible

- 415 cancers. *Sci. Adv.* 6, eaba5031. <https://doi.org/10.1126/sciadv.aba5031>
- 416 Flies, A.S., Darby, J.M., Murphy, P.R., Pinfold, T.L., Patchett, A.L., Lennard, P.R., 2020b.
- 417 Fluorescent Adaptable Simple Theranostic (FAST) Proteins. *Bio-protocol*.
- 418 Flies, A.S., Lyons, A.B., Corcoran, L.M., Papenfuss, A.T., Murphy, J.M., Knowles, G.W.,
- 419 Woods, G.M., Hayball, J.D., 2016. PD-L1 Is Not Constitutively Expressed on
- 420 Tasmanian Devil Facial Tumor Cells but Is Strongly Upregulated in Response to IFN- γ
- 421 and Can Be Expressed in the Tumor Microenvironment. *Front. Immunol.* 7, 581.
- 422 <https://doi.org/10.3389/fimmu.2016.00581>
- 423 Folkl, A., Wen, X., Kuczynski, E., Clark, M.E., Bienzle, D., 2010. Feline programmed death
- 424 and its ligand: Characterization and changes with feline immunodeficiency virus
- 425 infection. *Vet. Immunol. Immunopathol.* 134, 107–114.
- 426 <https://doi.org/10.1016/j.vetimm.2009.10.019>
- 427 Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C. a, Smith, H.O., Iii,
- 428 C.A.H., America, N., 2009. Enzymatic assembly of DNA molecules up to several
- 429 hundred kilobases. *Nat. Methods* 6, 343–5. <https://doi.org/10.1038/nmeth.1318>
- 430 Gu, P., Fang Gao, J., D'Souza, C. a, Kowalczyk, A., Chou, K.-Y., Zhang, L., 2012.
- 431 Trogocytosis of CD80 and CD86 by induced regulatory T cells. *Cell. Mol. Immunol.* 9,
- 432 136–146. <https://doi.org/10.1038/cmi.2011.62>
- 433 Hansen, J.D., Pasquier, L. Du, Lefranc, M.P., Lopez, V., Benmansour, A., Boudinot, P.,
- 434 2009. The B7 family of immunoregulatory receptors: A comparative and evolutionary
- 435 perspective. *Mol. Immunol.* 46, 457–472. <https://doi.org/10.1016/j.molimm.2008.10.007>
- 436 Hartley, G., Faulhaber, E., Caldwell, A., Coy, J., Kurihara, J., Guth, A., Regan, D., Dow, S.,
- 437 2016. Immune regulation of canine tumour and macrophage PD-L1 expression. *Vet.*
- 438 *Comp. Oncol.* n/a-n/a. <https://doi.org/10.1111/vco.12197>
- 439 Hwang, I., Huang, J.F., Kishimoto, H., Brunmark, A., Peterson, P.A., Jackson, M.R., Surh,

440 C.D., Zeling, C., Sprent, J., 2000. T cells can use either T cell receptor or CD28
441 receptors to absorb and internalize cell surface molecules derived from antigen-
442 presenting cells. *J. Exp. Med.* 191, 1137–1148. <https://doi.org/10.1084/jem.191.7.1137>

443 Ikebuchi, R., Konnai, S., Okagawa, T., Yokoyama, K., Nakajima, C., Suzuki, Y., Murata, S.,
444 Ohashi, K., 2014. Influence of PD-L1 cross-linking on cell death in PD-L1-expressing
445 cell lines and bovine lymphocytes. *Immunology* 142, 551–561.
446 <https://doi.org/10.1111/imm.12243>

447 Ikebuchi, R., Konnai, S., Okagawa, T., Yokoyama, K., Nakajima, C., Suzuki, Y., Murata, S.,
448 Ohashi, K., 2013. Blockade of bovine PD-1 increases T cell function and inhibits bovine
449 leukemia virus expression in B cells in vitro. *Vet. Res.* 44, 1–14.
450 <https://doi.org/10.1186/1297-9716-44-59>

451 Ikebuchi, R., Konnai, S., Shirai, T., Sunden, Y., Murata, S., Onuma, M., Ohashi, K., 2011.
452 Increase of cells expressing PD-L1 in bovine leukemia virus infection and enhancement
453 of anti-viral immune responses in vitro via PD-L1 blockade. *Vet. Res.* 42, 103–117.
454 <https://doi.org/10.1186/1297-9716-42-103>

455 Joly, E., Hudrisier, D., 2003. What is trogocytosis and what is its purpose? *Nat. Immunol.* 4,
456 815. <https://doi.org/10.1038/ni0903-815>

457 Kowarz, E., Löscher, D., Marschalek, R., 2015. Optimized Sleeping Beauty transposons
458 rapidly generate stable transgenic cell lines. *Biotechnol. J.* 10, 647–653.
459 <https://doi.org/10.1002/biot.201400821>

460 Lazenby, B.T., Tobler, M.W., Brown, W.E., Hawkins, C.E., Hocking, G.J., Hume, F.,
461 Huxtable, S., Iles, P., Jones, M.E., Lawrence, C., Thalmann, S., Wise, P., Williams, H.,
462 Fox, S., Pemberton, D., 2018. Density trends and demographic signals uncover the long-
463 term impact of transmissible cancer in Tasmanian devils. *J. Appl. Ecol.* 55, 1368–1379.
464 <https://doi.org/10.1111/1365-2664.13088>

- 465 Lemaoult, J., Caumartin, J., Daouya, M., Switala, M., Rebmann, V., Arnulf, B., Carosella,
466 E.D., 2015. Trogocytic intercellular membrane exchanges among hematological tumors.
467 J. Hematol. Oncol. 8, 25–27. <https://doi.org/10.1186/s13045-015-0114-8>
- 468 Luo, Z.X., Yuan, C.X., Meng, Q.J., Ji, Q., 2011. A Jurassic eutherian mammal and
469 divergence of marsupials and placentals. Nature 476, 442–445.
470 <https://doi.org/10.1038/nature10291>
- 471 Maekawa, N., Konnai, S., Ikebuchi, R., Okagawa, T., Adachi, M., Takagi, S., Kagawa, Y.,
472 Nakajima, C., Suzuki, Y., Murata, S., Ohashi, K., 2014. Expression of PD-L1 on canine
473 tumor cells and enhancement of IFN-g production from tumor-infiltrating cells by PD-
474 L1 blockade. PLoS One 9, e98415. <https://doi.org/10.1371/journal.pone.0098415>
- 475 Maekawa, N., Konnai, S., Okagawa, T., Nishimori, A., Ikebuchi, R., Izumi, Y., Takagi, S.,
476 Kagawa, Y., Nakajima, C., Suzuki, Y., Kato, Y., Murata, S., Ohashi, K., 2016.
477 Immunohistochemical analysis of PD-L1 expression in canine malignant cancers and
478 PD-1 expression on lymphocytes in canine oral melanoma. PLoS One 11, e0157176.
479 <https://doi.org/10.1371/journal.pone.0157176>
- 480 Maekawa, N., Konnai, S., Takagi, S., Kagawa, Y., Okagawa, T., Nishimori, A., Ikebuchi, R.,
481 Izumi, Y., Deguchi, T., Nakajima, C., Kato, Y., Yamamoto, K., Uemura, H., Suzuki, Y.,
482 Murata, S., Ohashi, K., 2017. A canine chimeric monoclonal antibody targeting PD-L1
483 and its clinical efficacy in canine oral malignant melanoma or undifferentiated sarcoma.
484 Sci. Rep. 7, 8951. <https://doi.org/10.1038/s41598-017-09444-2>
- 485 Mátés, L., Chuah, M.K.L., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., Grzela,
486 D.P., Schmitt, A., Becker, K., Matrai, J., Ma, L., Samara-Kuko, E., Gysemans, C.,
487 Pryputniewicz, D., Miskey, C., Fletcher, B., VandenDriessche, T., Ivics, Z., Izsvák, Z.,
488 2009. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables
489 robust stable gene transfer in vertebrates. Nat. Genet. 41, 753–761.

- 490 <https://doi.org/10.1038/ng.343>
- 491 Morton, P. a, Fu, X.T., Stewart, J. a, Giacometto, K.S., White, S.L., Leysath, C.E., Evans, R.J.,
492 Shieh, J.J., Karr, R.W., 1996. Differential effects of CTLA-4 substitutions on the
493 binding of human CD80 (B7-1) and CD86 (B7-2). *J. Immunol.* 156, 1047–1054.
- 494 Murchison, E.P., Tovar, C., Hsu, A., Bender, H.S., Kheradpour, P., Rebbeck, C.A., Obendorf,
495 D., Conlan, C., Bahlo, M., Blizzard, C.A., Pyecroft, S., Kreiss, A., Kellis, M., Stark, A.,
496 Harkins, T.T., Marshall Graves, J.A., Woods, G.M., Hannon, G.J., Papenfuss, A.T.,
497 2010. The Tasmanian devil transcriptome reveals Schwann cell origins of a clonally
498 transmissible cancer. *Science.* 327, 84–87. <https://doi.org/10.1126/science.1180616>
- 499 Nakayama, M., Takeda, K., Kawano, M., Takai, T., Ishii, N., Ogasawara, K., 2011. Natural
500 killer (NK)-dendritic cell interactions generate MHC class II-dressed NK cells that
501 regulate CD4 + T cells. *Proc. Natl. Acad. Sci. U. S. A.* 108, 18360–18365.
502 <https://doi.org/10.1073/pnas.1110584108>
- 503 Nemoto, Y., Shosu, K., Okuda, M., Noguchi, S., Mizuno, T., 2018. Development and
504 characterization of monoclonal antibodies against canine PD-1 and PD-L1. *Vet.*
505 *Immunol. Immunopathol.* 198, 19–25. <https://doi.org/10.1016/j.vetimm.2018.02.007>
- 506 Patchett, A.L., Flies, A.S., Lyons, A.B., Woods, G.M., 2020. Curse of the devil: molecular
507 insights into the emergence of transmissible cancers in the Tasmanian devil (*Sarcophilus*
508 *harrisii*). *Cell. Mol. Life Sci.* <https://doi.org/10.1007/s00018-019-03435-4>
- 509 Pearse, A.-M., Swift, K., 2006. Allograft theory: transmission of devil facial-tumour disease.
510 *Nature* 439, 549. <https://doi.org/10.1038/439549a>
- 511 Pye, R.J., Pemberton, D., Tovar, C., Tubio, J.M.C., Dun, K.A., Fox, S., Darby, J., Hayes, D.,
512 Knowles, G.W., Kreiss, A., Siddle, H.V.T., Swift, K., Lyons, A.B., Murchison, E.P.,
513 Woods, G.M., 2016. A second transmissible cancer in Tasmanian devils. *Proc. Natl.*
514 *Acad. Sci. U. S. A.* 113, 374–379. <https://doi.org/10.1073/pnas.1519691113>

- 515 Qureshi, O.S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E.M., Baker, J.,
516 Jeffery, L.E., Kaur, S., Briggs, Z., Hou, T.Z., Futter, C.E., Anderson, G., Walker,
517 L.S.K., Sansom, D.M., 2011. Trans-endocytosis of CD80 and CD86: A molecular basis
518 for the cell-extrinsic function of CTLA-4. *Science*. 332, 600–603.
519 <https://doi.org/10.1126/science.1202947>
- 520 Rebech, G.T., Venturin, G.L., Siqueira Ito, L.T., Bragato, J.P., De Carvalho Fonseca, B.S.,
521 Melo, L.M., Costa, S.F., De Rezende Eugênio, F., Dos Santos, P.S.P., De Lima, V.M.F.,
522 2020. PD-1 regulates leishmanicidal activity and IL-17 in dogs with leishmaniasis. *Vet.*
523 *Immunol. Immunopathol.* 219, 109970. <https://doi.org/10.1016/j.vetimm.2019.109970>
- 524 Robles, A., Qureshi, S.E., Stephen, S.J., Wilson, S.R., Burden, C.J., Taylor, J.M., Robles,
525 J.A., Qureshi, S.E., Stephen, S.J., Wilson, S.R., Burden, C.J., Taylor, J.M., 2012.
526 Efficient experimental design and analysis strategies for the detection of differential
527 expression using RNA-Sequencing. *BMC Genomics* 13, 484.
528 <https://doi.org/10.1186/1471-2164-13-484>
- 529 Siddle, H. V., Kreiss, A., Tovar, C., Yuen, C.K., Cheng, Y., Belov, K., Swift, K., Pearse, A.-
530 M.A.-M., Hamede, R., Jones, M.E., Skjødt, K., Woods, G.M., Kaufman, J., Skjødt, K.,
531 Woods, G.M., Kaufman, J., 2013. Reversible epigenetic down-regulation of MHC
532 molecules by devil facial tumour disease illustrates immune escape by a contagious
533 cancer. *Proc. Natl. Acad. Sci.* 110, 5103–8. <https://doi.org/10.1073/pnas.1219920110>
- 534 Tagawa, M., Maekawa, N., Konnai, S., Takagi, S., 2016. Evaluation of costimulatory
535 molecules in peripheral blood lymphocytes of canine patients with histiocytic sarcoma.
536 *PLoS One* 11, e0150030. <https://doi.org/10.1371/journal.pone.0150030>
- 537 Team, R.C., 2019. R: a language and for statistical computing.
- 538 U.S. Food and Drug Administration, 2020. KEYTRUDA® (pembrolizumab) injection, for
539 intravenous use Initial U.S. Approval: 2014.

540 U.S. Food and Drug Administration, 2011. YERVOY (ipilimumab) injection, for intravenous

541 use Initial U.S. Approval: 2011. U.S. Food Drug Adm. 1–32.

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544