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Tasmanian devil CD28 and CTLA4 capture CD80 and CD86 from adjacent cells

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Intercellular protein transfer of immune checkpoints in Tasmanian devils

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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.

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).

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

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

2. Materials and methods

2.1 Design of expression vectors

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

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

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

2.2 Restriction digest of expression vectors

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

2.3 Overlap extension PCR to create vector inserts

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

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

2.4 Assembly and transformation of expression vectors

The DNA for the gene-of-interest with overlap extensions were ligated into the digested vectors using NEBuilder[®] HiFi DNA Assembly Cloning Kit (NEB # E5520S) by incubating at 50 °C for one hour according to the manufacturer's instructions. A negative control was established by transforming DH5α with the linearised vector. A positive control on the other hand was established by transforming DH5α bacteria with NEBuilder[®] Positive Control (New England Biolabs, USA). After transformation, the bacteria were grown overnight at 37°C on Luria Broth Agar plates added with 100 µg/mL of ampicillin.

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

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

2.5 Purification and sequencing of expression vectors

Colonies that yielded amplicons that matched the predicted amplicon size were cultured overnight for plasmid amplification. 9 μ L of bacteria in PBS was added to 5 mL of Luria Broth with 100 μ g/ml of ampicillin. The bacterial cultures were incubated at 37°C and 200 rpm overnight. The plasmids were isolated from bacterial culture using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, USA) or NucleoSpin® Plasmid EasyPure Miniprep Kit (Macherey-Nagel, USA) according to the manufacturers' instructions. Concentrations of purified plasmids were quantified using a NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, USA). The plasmids were stored at -20°C. The plasmids were sequenced using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The BigDye™ Terminator was removed using Agencourt® CleanSEQ® (Beckman Coulter, USA) according to the protocols set by the manufacturer before loading samples to the Applied Biosystems® 3500xL Genetic Analyzer (Applied Biosystems, USA) for sequencing using a fluorescence-based capillary electrophoresis analytical technique.

2.6 Transfection of Chinese Hamster Ovary (CHO) cells

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

2.7 Intercellular protein transfer coculture assay

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

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

2.8 Flow cytometry

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

2.9 Data Processing and Gating Strategy for Flow Cytometry Analysis

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

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

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

2.10 Analysis of CTLA4 binding and protein trafficking motifs across species

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

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

3. Results and Discussion

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

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

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

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

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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.

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

Supplementary materials

Table S1. Summary of genes and plasmids

Table S2. Primer sequences for plasmid assembly

Table S3. Primer sequences for Sanger sequencing

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Author contributions

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

Availability of data and materials

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

Conflict of interest

The authors have no conflict of interest to report.

References

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

2007. Costimulatory receptors in jawed vertebrates: Conserved CD28, odd CTLA4 and multiple BTLAs. *Dev. Comp. Immunol.* 31, 255–271.
<https://doi.org/10.1016/j.dci.2006.06.003>

Briggs, Z.L., 2014. CD28 costimulation in T cells: requirements, outcomes and regulation. PhD Thesis.

Caldwell, A., Coleby, R., Tovar, C., Stammnitz, M.R., Mi Kwon, Y., Owen, R.S., Tringides, M., Murchison, E.P., Skjødtt, K., Thomas, G.J., Kaufman, J., Elliott, T., Woods, G.M., Siddle, H.V.T., 2018. The newly-arisen devil facial tumour disease 2 (DFT2) reveals a mechanism for the emergence of a contagious cancer. *Elife* 7.
<https://doi.org/10.7554/eLife.35314>

Chung, B., Tor, John, Beilhack, G., Andersen, E., Brian, Jeffrey, Peter, 2014. Antigen-Specific Inhibition of High-Avidity T Cell Target Lysis by Low-Avidity T Cells via Trogocytosis 8, 871–882. <https://doi.org/10.1016/j.celrep.2014.06.052>

CLC, 2020. CLC Main Workbench.

Daubeuf, S., Puaux, A.-L., Joly, E., Hudrisier, D., 2006. A simple trogocytosis-based method to detect, quantify, characterize and purify antigen-specific live lymphocytes by flow cytometry, via their capture of membrane fragments from antigen-presenting cells. *Nat. Protoc.* 1, 2536.

Flies, A.S., Blackburn, N.B., Lyons, A.B., Hayball, J.D., Woods, G.M., 2017. Comparative analysis of immune checkpoint molecules and their potential role in the transmissible Tasmanian devil facial tumor disease. *Front. Immunol.* 8, 513.
<https://doi.org/10.3389/fimmu.2017.00513>

Flies, A.S., Darby, J.M., Lennard, P.R., Murphy, P.R., Ong, C.E.B., Pinfold, T.L., De Luca, A., Lyons, A.B., Woods, G.M., Patchett, A.L., 2020a. A novel system to map protein 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,

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

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

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

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

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

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

Lazenby, B.T., Tobler, M.W., Brown, W.E., Hawkins, C.E., Hocking, G.J., Hume, F., Huxtable, S., Iles, P., Jones, M.E., Lawrence, C., Thalmann, S., Wise, P., Williams, H., Fox, S., Pemberton, D., 2018. Density trends and demographic signals uncover the long-term impact of transmissible cancer in Tasmanian devils. *J. Appl. Ecol.* 55, 1368–1379. <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. Trophocytic 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- γ 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, Giacioletto, 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., Skjødtt, K., Woods, G.M., Kaufman, J., Skjødtt, 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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