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Evidence for induction of humoral and cytotoxic immune responses against devil facial tumor disease cells in Tasmanian devils (*Sarcophilus harrisii*) immunized with killed cell preparations

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ABSTRACT

Tasmanian devils (*Sarcophilus harrisii*) risk extinction from a contagious cancer, devil facial tumour disease (DFTD) in which the infectious agent is the tumor cell itself. Because devils are unable to produce an immune response against the tumor cells no devil has survived 'infection'. To promote an immune response we immunized healthy devils with killed DFTD tumor cells in the presence of adjuvants. Immune responses, including cytotoxicity and antibody production, were detected in five of the six devils. The incorporation of adjuvants that act via toll like receptors may provide additional signals to break 'immunological ignorance'. One of these devils was protected against a challenge with viable DFTD cells. This was a short-term protection as re-challenge one year later resulted in tumor growth. These results suggest that Tasmanian devils can generate immune responses against DFTD cells. With further optimization of immune stimulation it should be possible to protect Tasmanian devils against DFTD with an injectable vaccine.

1. Introduction

Devil Facial Tumour Disease (DFTD) is a clonal [1] and fatal transmissible cancer that arose in a single female Tasmanian devil (*Sarcophilus harrisii*), prior to 1996 [2,3]. It has been spread by biting [2] and killed more than 80% of devils in the island state of Tasmania. Extinction is possible [4].

DFTD is a cancer of Schwann cells [5]. Initially it was believed that a lack of genetic diversity accounted for why the tumor cells were not immunologically rejected [6]. More recently it has been identified that DFTD cells do not express surface class I Major Histocompatibility Complex molecules [7] and are therefore ignored by the host immune system. DFTD progresses with no sign of leukocyte infiltration [8,9]. There is no, or limited, evidence for any devils recovering from DFTD [10,11]. This suggests the tumor cells are not rejected as a typical allograft, even though devils have robust humoral and cell mediated immune responses [9,12–14]. Skin allografts can be rejected, exhibiting $CD8^+$ T cell infiltration and xenogeneic tumor cells can be killed via antibody dependent cytotoxicity, a property of NK cells [14,15]. Preliminary experiments with two devils immunized with DFTD cells failed to detect an immune response in either devil [15]. Since then we have shown that in mice DFTD cells are immunogenic and that sonicated, freeze/thawed and irradiated cells can induce immune responses [16]. As a prelude toward the development of a vaccine, the objective of this study was to further investigate if an immune response could be generated against DFTD cells.

Tasmanian devils are an endangered species and access to animals for research purposes was limited. This restricted sample size and the capacity to incorporate a diverse range of vaccine formulations. We hypothesized that there is sufficient genetic difference between DFTD cells and host devils for an immune response to be induced. This could be promoted in the presence of adjuvants. We used Montanide and CpG. Montanide, is a proprietary water in oil adjuvant (Seppic S.A., France) that has been shown to enhance both cellular and humoral immunity in humans [17–20]. CpG included the oligonucleotides CpG1668 and CpG1585, as they increase the

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effectiveness of standard adjuvants [21–26]. They act via Toll-like Receptor 9 (TLR9) and have been used successfully with tumor antigens in Montanide ISA51 [21,27].

The principle aim of this study was to provide evidence that, in the presence of adjuvants, cultured DFTD cell preparations that were inactivated by either γ-irradiation, sonication, or repeated freeze thawing could induce humoral or cytotoxic responses in Tasmanian devils.

2. Methods

2.1. DFTD cell lines

The DFTD cell lines ½Pea, 2112 (both strain 2) and C5065 (strain 3) [1,28] were maintained in complete RPMI medium, which was RPMI 1640 medium (Life Technologies, Grand Island, USA) supplemented with 10% vol/vol heat inactivated fetal calf serum (Bovogen Biological, Victoria, Australia), 5 mM L-glutamine (Sigma–Aldrich, St. Louis, USA) and 100 IU of gentamicin sulfate (Pfizer, Western Australia, Australia) at 35 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Tasmanian devils

The care of Tasmanian devils was in accordance with Department of Primary Industries, Parks, Water and Environment of Tasmania guidelines. Their endangered status limited access. All devils were adults and their ages ranged from two to four years at commencement. There was a mixture of males and females. The University of Tasmania Animal Ethics Committee approved all procedures under permit numbers A9215 and A11436. Blood collection, immunization and live cell challenges were performed under anesthesia induced with 5% Isofluorane® (Abbot Australasia Pty Ltd., Botany, Australia) reducing to 3% via a mask.

2.3. Preparation of DFTD cells for immunization

Vaccines consisted of 10^8 inactivated DFTD tumor cells. The devils TD1 and TD2 received an equal mixture of the strain 2 cell lines ½ Pea and 2112. All other devils (including TD2 on re-immunization) received the strain 3 cell line C5065. As this cell line labeled with Cr^{51}, it was suitable for cytotoxicity assays. They were either freeze/thawed (six to eight quick cycles of freezing in liquid nitrogen and thawing in a 37 °C water bath), irradiated (20 Gray of γ radiation using a Varian Clinac 23-EX linear accelerator (Varian Medical Systems Inc., California, USA), or sonicated (disruption with four ultra-sonic cycles of 60 s at input 6, Microson XL-2000 ultrasonic cell disruptor, VWR International, Radnor, USA) with 60 s intervals on ice. For all preparations, lack of cell viability was confirmed using trypan blue dye and cell culture. Cell preparations in 1000 μL of sterile PBS were vigorously mixed with 300 μL of Montanide ISA71VG adjuvant (Seppic S.A., France) and, with the exception of frozen/thawed cells, 200 μL (200 μg) of CpG was also included. The mixture was vigorously mixed and kept on ice until required. The initial immunizations of TD1 and TD2 were at closer time intervals than the other devils. After these first two devils it was deemed more appropriate (ethically and biologically) to space the immunizations further apart.

2.4. Immunizations, blood collection and live DFTD cell challenge

Immunizations were subcutaneous injections with a 21 gauge needle into the rump (schedule summarized in Table 1). Previously we used the shoulder [15] but the rump is a more convenient site to monitor for signs of inflammation and the unlikely possibility of tumor growth. Due to limited availability of devils, individual animals were assessed rather than groups. The different vaccine compositions were chosen to maximize the number of combinations. Before and 14 days after each immunization approximately 12 ml of blood was collected from the jugular vein. Up to 4 ml of blood was placed into clot activating tubes (Greiner Bio-one, Frickenhausen, Germany) for serum analyses and the remainder into lithium heparin anticoagulant tubes (BD Biosciences, NJ, USA) for cell analyses.

Due to ethical considerations, it was only possible to challenge two devils. Both were initially challenged with strain 2 DFTD cells. TD1 did not produce an immune response and was not boosted prior to the challenge. Effectively this devil served as a 'control' for tumor growth. TD2 produced an immune response and was boosted prior to challenge. This devil was the 'test' for protection against tumor growth. 25,000 viable tumor cells in 500 μL of PBS were injected in two sites; subcutaneous tissue of the right cheek and oral mucosa of the left lower jaw. This dose was selected as balance between the number of cells transmitted during natural transmission and a dose that would not be large enough to overwhelm the immune response. These sites were selected to reflect the most common sites that DFTD develops in wild devils. The devils were examined monthly under anesthesia for evidence of tumors. A second challenge took place in one of these devils. Again with 25,000 viable tumor cells, but this time with strain 3 DFTD cells and on the left and right subcutaneous tissue of the cheeks. The oral mucosa was not used as it was difficult to determine when tumors appeared, it caused inconvenience to the devil and could not be surgically removed.

Table 1 summarizes the immunization preparation, adjuvants, immunization times and challenges of devils.

Table 1
Summary of DFTD immunizations, challenges and tumor development.

Devil	Year 1	Year 2	Year 3	Year 4
TD1	••• • ↓ ✚			
TD2	••• • • ↓	◊ ✚	•	• • •
TD3			• • •	
TD4			• • •	• • •
TD5			• • •	
TD6			• • •	

• Immunisation ↓ Challenge strain 2 ◊ Challenge strain 3 ✚ Tumour development
TD1 and TD2 were immunized with strain 2
TD3, TD4, TD5 and TD6 were immunized with strain 3
TD2 was re-immunized with strain 3

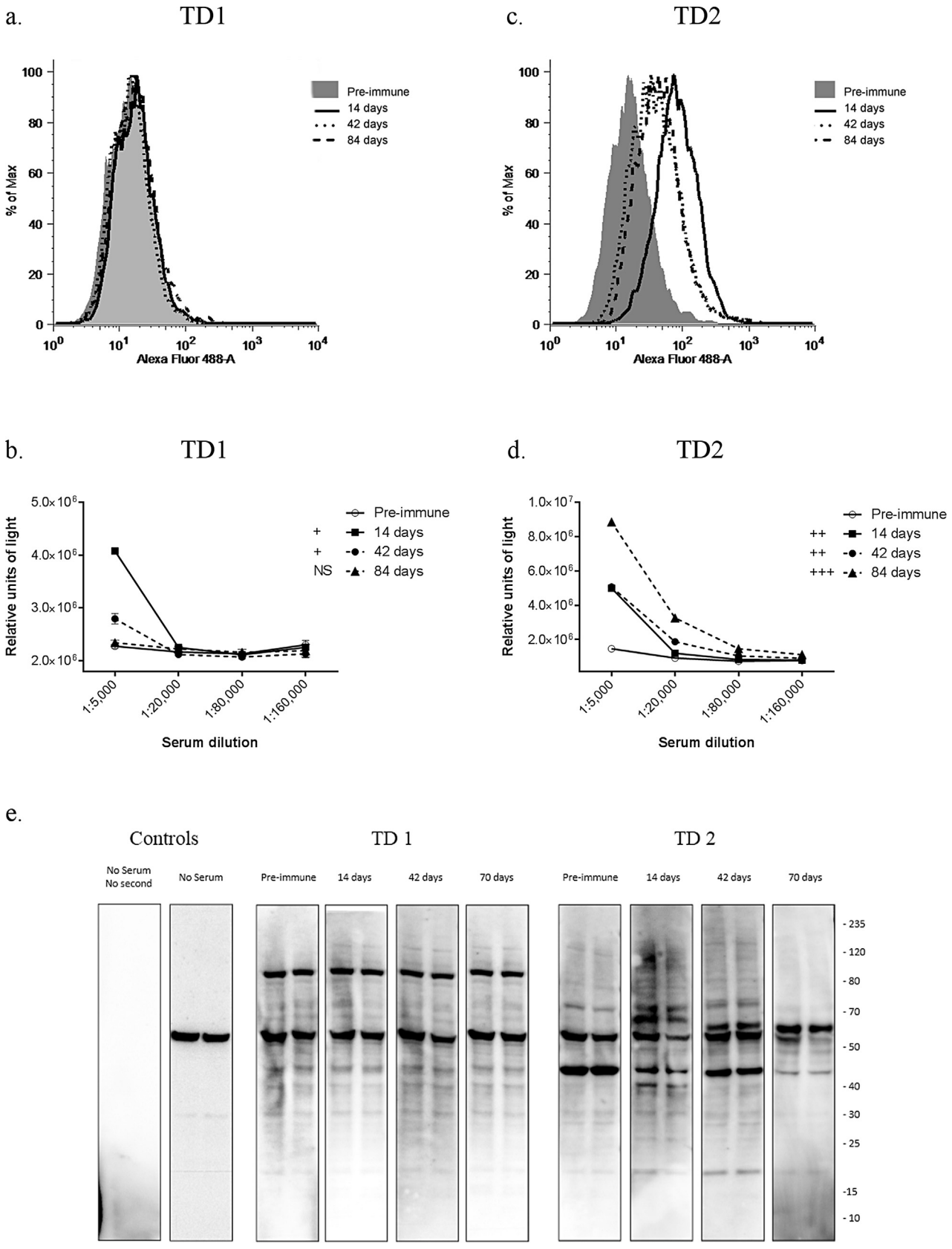

Fig. 1. Antibody responses and DFTD tumor development of devils that received frozen/thawed cells plus Montanide ISA71MVG. (a) and (c) Flow cytometry. Evidence for antibodies in TD2: (b) and (d) ELISA. Evidence for low levels of antibodies in TD1, but higher levels in TD2. (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution up to twice as high as pre-immune, (++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution 2–4 times higher than pre-immune, (+++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution more than 4 times higher than pre immune: (e) Western blot. Evidence for DFTD specific antibodies in TD2.

2.5. Cytotoxicity assays

A radioactive chromium (^{51}Cr) release assay was used [15]. Devil mononuclear cells were obtained by gradient centrifugation over Histopaque 1077 (Sigma–Aldrich, St. Louis, USA) and resuspended in complete RPMI medium. DFTD cells were labeled with ^{51}Cr for 2 h and added to triplicate wells of a round bottom 96 well plate containing devil mononuclear cells. After incubation at 37 °C with 5% CO_2 in air for 18 h, culture supernatants were analyzed for γ radioactivity using a Genesys γ radiation counter (Laboratory Technologies Inc., IL, USA). Mean percent cytotoxicity values at each ratio were calculated thus:

Percent cytotoxicity = ((sample CPM − mean negative control CPM)/(mean maximum control CPM − mean negative control CPM)) × 100 (%).

2.6. Antibody measurement by flow cytometry

Washed DFTD tumor cells (100 μL at 10^6/mL diluted in washing buffer – 1% BSA in PBS) were placed in wells of a round bottom 96 well plate on ice. Serum samples were diluted 1:100 (final dilution 1:200) with washing buffer, mixed with C5065 tumor cells (strain 3) and incubated on ice for 30 min. Cells were washed twice with washing buffer, and incubated with 50 μL of 2 μg/mL of a monoclonal mouse anti-devil IgG [29] for 30 min, washed and incubated with 50 μL of 0.2 μg/mL of alexa fluor 488-conjugated goat anti-mouse IgG antibody for 30 min (Molecular Probes, Leiden, The Netherlands). After washing, cells were resuspended in 200 μL of washing buffer containing 3 μM of propidium iodide (Sigma–Aldrich, St. Louis, USA). Viable cells were analyzed on a BD Canto II flow cytometer (Becton Dickinson, New Jersey, USA). The controls (DFTD cells labeled with the secondary and tertiary antibodies, but no devil serum) did not show background fluorescence.

2.7. Antibody measurement by ELISA

White opaque 96-well plates (ThermoFisher Scientific, Waltham, USA) were coated overnight at 4 °C with 10 μg/mL of DFTD protein extracted from cultured DFTD tumor cells using RIPA buffer (Thermo Scientific, Rockford, IL) and diluted in PBS. Wells were washed three times with 0.05% of Tween 20 (Sigma-Aldrich, St. Louis, USA) in PBS (PBS-T) and non-specific protein binding was blocked with protein blocker (Dako, Glostrup, Denmark). After three washes with PBS-T, 100 μL of serum samples were serial diluted in PBS in triplicate wells, incubated overnight at 4 °C, washed three times with PBS-T and 100 μL of 2 μg/mL of unconjugated mouse anti-devil IgG [29] added and incubated for 1 h at room temperature. After three washes with PBS-T, 100 μL of 0.05 ng/mL of HRP goat anti-mouse antibody (Dako, Glostrup, Denmark) was placed in each well, incubated for 1 h and washed three times with PBS-T. 100 μL per well of chemiluminescent peroxidase substrate for ELISA (Sigma–Aldrich, St. Louis, USA) was added for 1 min and relative light units (RLUs) measured at 425 nm (SpectraMax M2, Molecular Devices, Sunnyvale, USA). Controls consisting of wells with no protein, no serum or no mouse anti-devil IgG did not bind non-specifically. All experiments with the tested samples were performed at least twice. Results are expressed as units of light at all dilutions.

Statistics to compare pre-immune with immune samples of individual devils were performed with two-way ANOVA (GraphPadPrism).

2.8. Western blot

Total cell protein from a DFTD cell line was extracted with 1 ml of RIPA buffer (Thermo Scientific, Rockford, IL) containing 10 μl of HALT™ protease inhibitor cocktail (Thermo Scientific, Rockford, USA) for approximately 40 mg of cell pellet. The sample was sonicated, centrifuged at 16,000 × *g* for 20 min at 4 °C and the supernatant transferred to a new tube for protein quantification (EZQ™ Protein Quantitation Kit, Life Technologies, Eugene, USA). 20 μg of protein sample was added to Bolt™ LDS Sample Buffer (4×) (Life Technologies, Carlsbad, USA) and Bolt™ Sample Reducing Agent (10×) with the remaining volume made up by Milli-Q water to 10 μl. The samples were vortexed, centrifuged and heated at 70 °C for 10 min. Samples and a molecular weight marker were loaded on Bolt® 4–12% Bis-Tris Plus mini-gels. The gels were run at 165 V for 50 min using Bolt™ MES SDS running buffer and the Bolt® mini gel tank.

Electroblotting was performed using the iBlot® dry blotting system using 20 V for 7.5 min (Life Technologies, Carlsbad, USA). Membranes were incubated overnight at 4 °C with an animal protein-free blocker buffer (Vector Laboratories, Burlingame, USA) in TBS containing 0.05% tween (TBS-T). Membranes were incubated for 2 h with pre-immune and immune serum from two immunized devils (TD1 and TD2) at a dilution of 1:125 in blocking buffer. Negative controls were no devil serum and no serum plus no secondary antibody. After four washes with TBS-T, 2 μg/mL of unconjugated mouse anti-devil IgG was applied for 1 h, followed by four washes and incubation with 0.05 ng/mL of HRP goat anti-mouse antibody (Dako, Glostrup, Denmark). Chemiluminescence detection was performed with Immobilon™ Western HRP substrate (Millipore, Billerica, USA) for 5 min and imaged using the Image Station 4000MM Pro system (Carestream Health, Woodbridge, USA).

3. Results

3.1. Immunization with frozen/thawed DFTD cells in Montanide adjuvant

3.1.1. Antibody responses

TD1 had negligible anti-DFTD antibody responses by flow cytometry, but there was a small increase in antibodies by ELISA 14 and 42 days after the first immunization, but this did not persist (Fig. 1a and b). TD2 showed evidence for DFTD specific antibodies by flow cytometry and ELISA (Fig. 1c and d).

3.1.2. Western blot

To confirm that the antibody responses from TD2 were DFTD specific, western blots were performed (Fig. 1e). The immune serum of TD2 showed reactivity, especially 14 days after the first immunization. This coincided with the highest flow cytometric response. In contrast no obvious bands were produced with the immune serum of TD1.

3.1.3. Cytotoxic responses

A DFTD cell line that labeled sufficiently with Cr^{51} was not available at the time TD1 and TD2 were first tested.

3.1.4. Challenge with live tumor cells

Both devils were challenged with live DFTD tumor cells. TD1 developed tumors at both sites, 84 days after the challenge. The tumor stained positively with periaxin, a marker for DFTD cells. 126 days after tumor development the cheek tumor was surgically removed due to its large size. For ethical reasons TD1 was euthanized 56 days after removal. There was evidence for metastases only in the right submandibular lymph node. TD2 did not develop palpable tumors on either site for 190 days. To determine whether the protection was long-term, a second challenge was carried out, at Day 476. Small sized tumors (less than 1 cm in diameter) were detected in both challenged sites 154 days after the second

Table 2
Summary of immune responses generated for all Tasmanian devils.

Devil	Immunization	Analysis	Antibody response		Cytotoxicity
			Flow cytometry	ELISA	Chromium release assay
TD1	Frozen/thawed + Montanide				
	Day 0				
	Day 7				
	Day 14	Day 14	–	+	ND
		Day 42	–	+	ND
	Day 70	Day 84	–	NS	ND
	Challenge Day 287	Tumor developed 84 days later			
TD2 1st test	Thawed/frozen cells + Montanide				
	Day 0				
	Day 7				
	Day 14	Day 14	+++	++	ND
		Day 42	++	++	ND
	Day 70	Day 84	++	+++	ND
	Day 217				
	First challenge Day 287	No tumor development			
	Second challenge Day 476	Tumor developed 154 days later			
TD2 2nd test	Sonicated cells + Montanide + CpG1668				
	Day 0 (day 1127)	Day 14	+	+++	+++
	Day 28	Day 42	+	+++	+++
	Day 56	Day 70	–	+++	ND
TD3	Irradiated cells + Montanide + CpG1668				
	Day 0	Day 14	–	NS	++
	Day 28	Day 42	–	+	NS
	Day 56	Day 70	ND	ND	ND
TD4 1st test	Irradiated cells + Montanide + CpG1668				
	Day 0	Day 14	–	++	+++
	Day 28	Day 42	–	+	+++
	Day 56	Day 70	+++	++	+++
TD4 2nd test	Sonicated cells + Montanide + CpG1668				
	Day 0 (day 162)	Day 14	+	+++	+++
	Day 28	Day 42	+	+++	++
	Day 56	Day 70	–	+++	ND
TD5	Irradiated cells + Montanide + CpG1585				
	Day 0	Day 14	–	NS	+
	Day 28	Day 42	–	NS	+
	Day 56	Day 70	+	++	NS
TD 6	Irradiated cells + Montanide + CpG1585				
	Day 0	Day 14	–	NS	++
	Day 28	Day 42	–	NS	+
	Day 56	Day 70	–	NS	NS

Flow cytometry assay: (–) Median fluorescence intensity (MFI) not higher than pre-immune, (+) MFI up to twice as high as pre-immune, (++) MFI 2 to 4 times higher than pre-immune, (+++) MFI above 4 times higher than pre-immune. *ELISA*: (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution up to twice as high as pre-immune, (++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution 2 to 4 times higher than pre-immune, (+++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution more than 4 times higher than pre-immune. *Chromium release assay*: (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and between 11 and 20% cytotoxicity, (++) Significantly different to pre-immune and between 21 and 30% cytotoxicity, (+++) Significantly different to pre-immune and greater than 30% cytotoxicity. ND: not done.

challenge (Table 2). The tumors were confirmed to be DFTD as described above and wide margin excision surgery was performed to remove the tumors. As there was no recurrence after one year, this was regarded as a surgical 'cure', and the devil was included in further experiments, 490 days after the surgery.

3.2. Devils immunized with irradiated DFTD cells in Montanide adjuvant with CpG 1668 oligonucleotides

3.2.1. Antibody responses

TD3 showed evidence for low levels of anti-DFTD antibodies at day 42, but this was only evident with the ELISA (Fig. 2a and c). TD4 had anti-DFTD antibody responses, which were evident with ELISA and flow cytometry. The responses were highest at day 70, which was 14 days after the third immunization (Fig. 2d and e).

3.2.2. Cytotoxic responses

The pre-immune responses from TD3 and TD4 were usually below 10% cytotoxicity; hence 10% was considered the cut-off. TD3 showed evidence for cytotoxicity 14 days after the first immunization, but this had subsided by day 42 (Fig. 2c). TD4 had consistent responses, with cytotoxicity above 30% after each of the immunizations. The highest was 43% after the third and final immunization (Fig. 2f).

3.3. Devils immunized with irradiated DFTD cells in Montanide adjuvant with CpG 1585 oligonucleotides

3.3.1. Antibody responses

TD5 had undetectable anti-DFTD antibodies after the first two immunizations (days 14 and 42), but antibody was detected after the third immunization (day 70) (Fig. 3a and b). TD6 had no detectable anti-DFTD antibody responses (Fig. 2d and e).

3.3.2. Cytotoxic responses

Although both devils had the highest responses 14 days after the first immunization, neither devil provided convincing evidence for cytotoxicity (Fig. 3c and f).

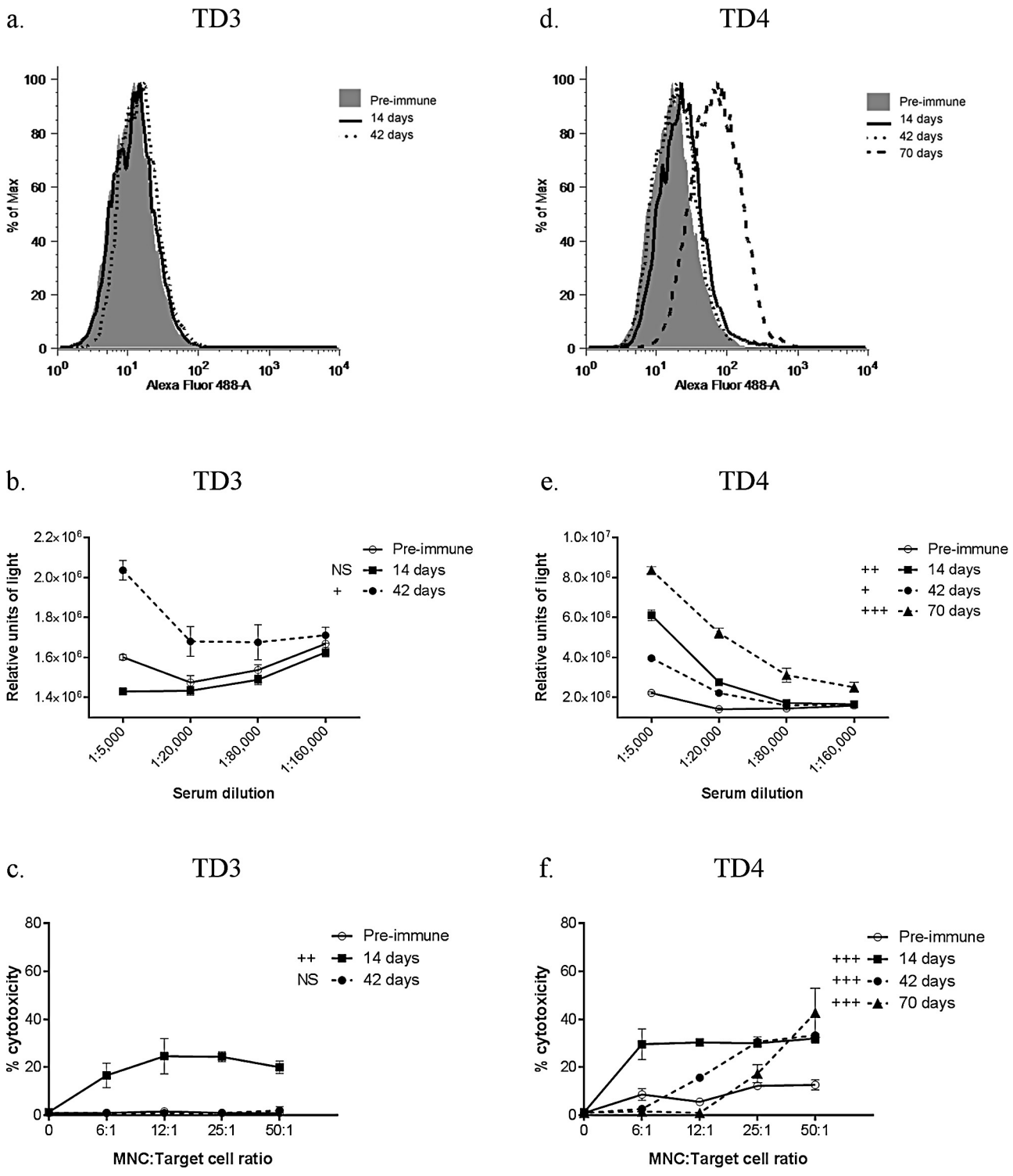

Fig. 2. Antibody and cytotoxic responses of devils that received irradiated cells, Montanide ISA71MVG and CpG1668. (a) and (d) Flow cytometry assay. Evidence for antibodies in TD4 (but not TD3), but only after 70 days: (b) and (e) ELISA. Evidence for low levels of antibodies in TD3, but only after 42 days and evidence for antibodies in TD4 from 14 days: (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution up to twice as high as pre-immune, (++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution 2 to 4 times higher than pre-immune, (+++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution more than 4 times higher than pre immune: (c) and (f) Chromium release assay. Evidence for cytotoxicity in TD3 after 14 days, but not after 42 days and in TD4 after all timepoints. (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and between 11 and 20% cytotoxicity, (++) Significantly different to pre-immune and between 21 and 30% cytotoxicity, (+++) Significantly different to pre-immune and greater than 30% cytotoxicity.

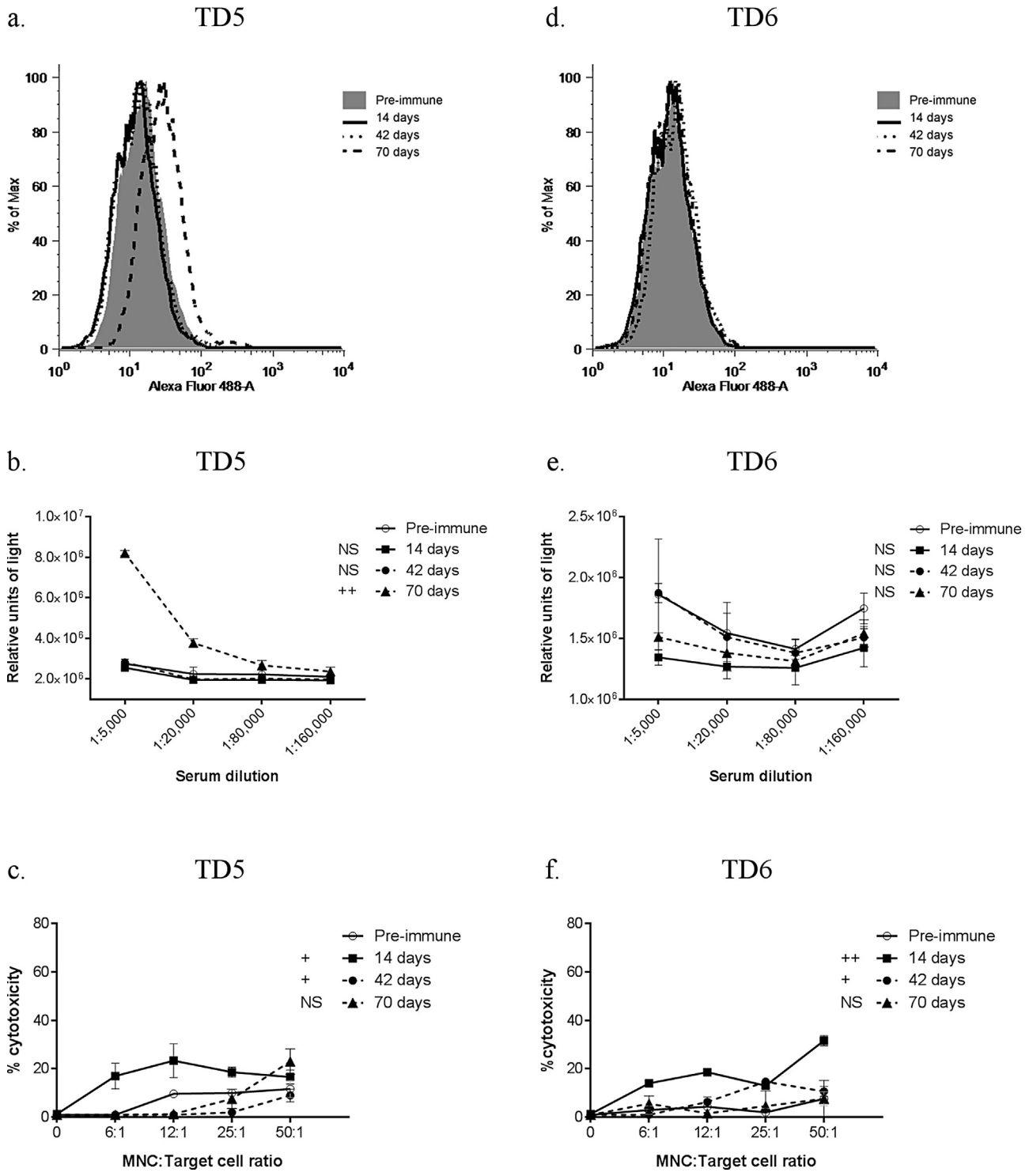

Fig. 3. Antibody and cytotoxic responses of devils that received irradiated cells, Montanide ISA71MVG and CpG1585. (a) and (d) Flow cytometry assay. Evidence for antibodies in TD5 (but not TD6) but only after 70 days: (b) and (e) ELISA. Evidence for low levels of antibodies in TD5, but only after 70 days and no evidence for antibodies in TD6. (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution up to twice as high as pre-immune, (++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution 2 to 4 times higher than pre-immune, (+++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution more than 4 times higher than pre immune. (c) and (f) Chromium release assay. Evidence for cytotoxicity in TD5 and TD6 after 14 and 42 days, but not after 70 days. (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and between 11 and 20% cytotoxicity, (++) Significantly different to pre-immune and between 21 and 30% cytotoxicity, (+++) Significantly different to pre-immune and greater than 30% cytotoxicity.

3.4. Devils immunized with sonicated DFTD cells in Montanide adjuvant with CpG 1668 oligonucleotides

These two devils had been immunized previously (896 days previously for TD2 and 92 days previously for TD4). At the time of 're-testing' the anti-DFTD antibody and cytotoxicity levels were very low and considered equivalent to pre-immune serum.

3.4.1. Antibody responses

TD2 and TD4 showed similar responses following 're-immunization'. Flow cytometry detected low levels of anti-DFTD

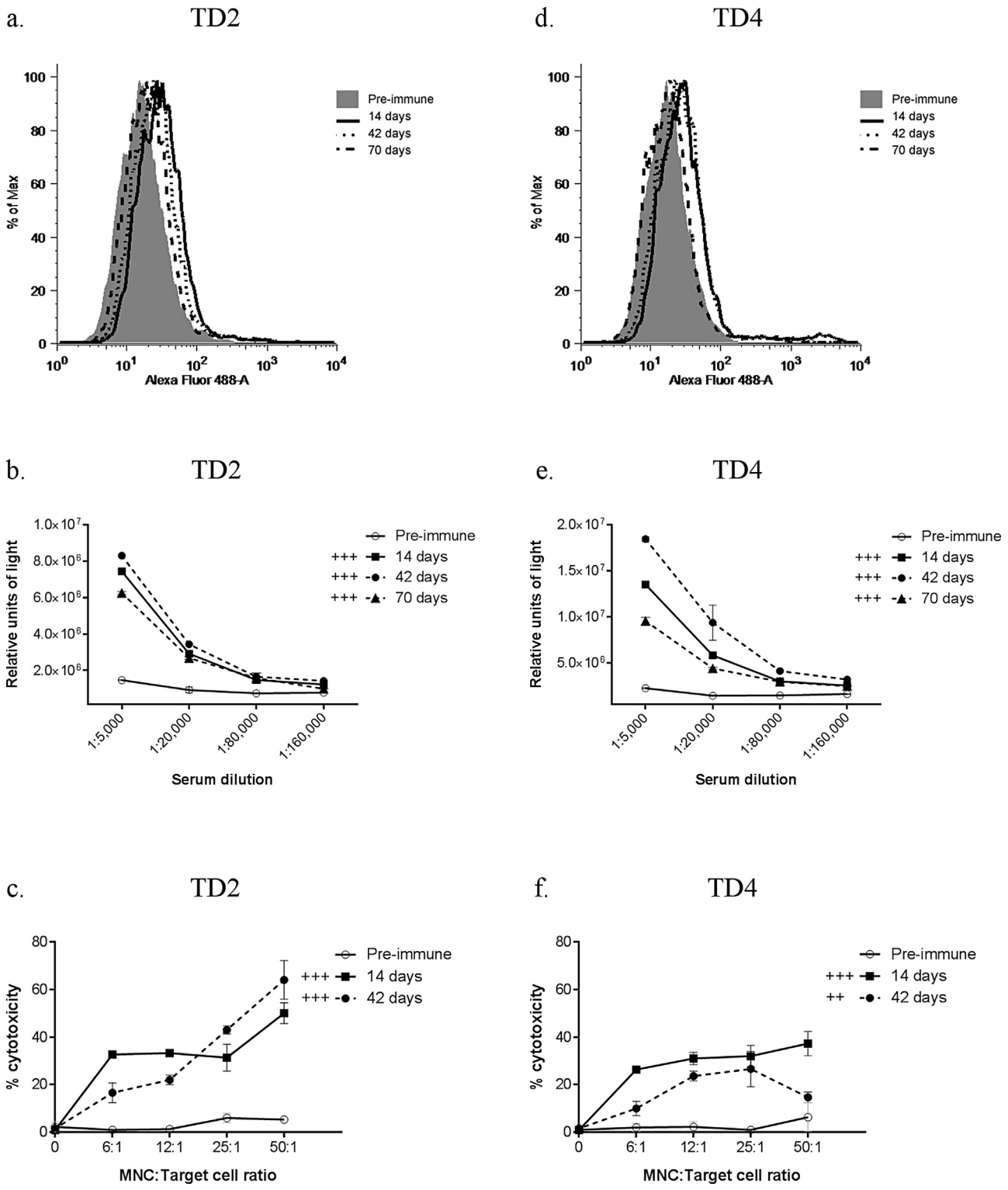

Fig. 4. Antibody and cytotoxic responses of devils that received sonicated cells, Montanide ISA71MVG and CpG1668. (a) and (d) Flow cytometry assay. Evidence for low levels of antibodies in TD2 and TD4 after 14 and 42 days but not after 70 days: (b) and (e) ELISA. Evidence for antibodies in TD2 and TD4 at all timepoints. (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution up to twice as high as pre-immune, (++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution 2 to 4 times higher than pre-immune, (+++) Significantly different to pre-immune and relative light unit intensity of 1:5000 dilution more than 4 times higher than pre immune. (c) and (f) Chromium release assay. Evidence for cytotoxicity in TD2 and TD4 after 14 and 42 days. (NS) Not significantly different to pre-immune, (+) Significantly different to pre-immune and between 11 and 20% cytotoxicity, (++) Significantly different to pre-immune and between 21 and 30% cytotoxicity, (+++) Significantly different to pre-immune and greater than 30% cytotoxicity.

antibodies for both devils (Fig. 4a and d). But the ELISA detected significantly high anti-DFTD antibodies levels at all time points (Fig. 4b and e).

3.4.2. Cytotoxic responses

TD2 and TD4 had increased cytotoxic responses after the first two 're-immunizations' (days 14 and 42) with TD2 producing the stronger response (Fig. 4c and f).

A summary of all responses is shown in Table 2.

4. Discussion

Devil facial tumour disease (DFTD) is a fatal and transmissible cancer. There is no evidence for an immune response against DFTD in wild Tasmanian devils [8,9,30]. This study evaluated the immune response against DFTD cells with devils immunized with inactivated cells in the presence of adjuvants. The endangered status of the Tasmanian devil limited access to animals for research purposes. Six devils were available of which two devils had to be used twice. To maximize the potential to identify responses, all six devils were vaccinated. It was not possible to include controls such as adjuvant alone and statistical comparison between devils was not possible. Despite this restriction there was evidence that Tasmanian devils can produce humoral and cell-mediated responses to DFTD cells.

ELISA was more sensitive than flow cytometry, most likely due to the ELISA detecting antibodies against total, rather than just cell surface, antigens. The Cr^{51} cytotoxicity assays provided evidence for cytotoxicity following immunizations in most of the devils tested. As DFTD cells do not express cell surface MHC-I [7] it is unlikely that cytotoxicity was mediated by $CD8^+$ T cells. There was no evidence for cytotoxicity in non-immunized devils. This suggests there was no spontaneous NK-cell activity against DFTD cells. We have previously provided evidence for devil NK cells that could mediate ADCC. It is unlikely that ADCC occurred in these cultures as they did not contain antibodies. As there was evidence for cytotoxicity one possibility is that the vaccination activated NK cells in vivo, which killed the DFTD cell in vitro. An alternative explanation is that interferon-γ (IFN-γ) was produced during the 18 h culture. This could have either activated NK cells or upregulated MHC-I on the DFTD cells making them targets for $CD8^+$ T cells.

To maximize immunogenicity cells were disrupted by freeze thawing [31] or by sonication. Of the two devils that were immunized with frozen/thawed cells, one produced an antibody response. Evaluation of cytotoxicity was not possible as there was not a DFTD cell line available at that time that could be used in a Cr^{51} release assay. The evidence of an antibody response was supported by western blots. Antibodies in the serum from the devil with the good immune response reacted with a range of DFTD antigens. Some of these are potentially tumor-associated antigens. These antigens may provide more targeted immunogens than whole tumor cells to induce a tumor specific immune response. This provided the important initial evidence that an immune response could be generated against DFTD proteins. Efforts to improve the response were performed by immunizing two devils with sonicated cells in the presence of CpG. Both devils produced good antibody and cytotoxic responses. Potentially, CpG in association with the disruption of DFTD tumor cells to expose multiple antigens may provide additional stimulatory signals. The effects of CpG on marsupials are unknown, but we have evidence for functional TLR9 expression on devil leukocytes (Patchett unpublished observations).

Targeting the devil's immune response to cell surface antigens in order to improve efficacy may require whole cells, rather than cell fragments. Our previous study showed that mice immunized with live DFTD cells elicited a strong response [16]. The risk of tumor development in devils immunized with live cells was avoided by irradiating the DFTD cells. Our earlier experience with two devils immunized with irradiated DFTD cells did not provide evidence for antibody or cytotoxic responses [15]. This is potentially due to a lack of genetic diversity among Tasmanian devils and the failure of DFTD cells to express MHC antigens [6,7,32]. In the current experiments all four devils that received irradiated cells developed cytotoxic responses and three of them developed antibody responses. Inclusion of CpG distinguishes them from our previous study. This indicates that the inclusion of TLR9 ligands that activate innate immune cells [33] may provide the stimulus to break 'immunological ignorance'. The two devils that were used twice received sonicated cells and CpG after previously receiving irradiated or frozen/thawed cells. This delayed 'boost' may have increased the response, as marsupials tend to develop slower immune responses [34,35].

A potential vaccination strategy that incorporates all of the above may provide stronger and more consistent responses. DFTD cells could be disrupted to increase immunogenicity, CpG or other TLR agonists would promote the response via innate immune cells, multiple immunizations followed by a boost. Frozen/thawed, or sonicated, cells could initially direct the immune response toward cytoplasmic antigens and potentially expose additional stimulatory molecules such as heat shock proteins [36]. High-dose γ-irradiation is useful for inducing cell death for vaccination [37]. This would largely preserve the ultrastructure of the tumor cells to target immune responses against extracellular proteins.

Support for the induction of a protective immune response against DFTD was provided when one of the two devils challenged with live DFTD cells did not develop the tumor. Both devils received 25,000 strain 2 DFTD cells at each injection site. This provided a dose between the low number of cells that potentially would be transmitted through biting and large enough dose for tumor establishment. As TD1 developed tumors and TD2 develop a tumor on the second challenge, it was apparent that 25,000 cells were sufficient. Protection against tumor development is the most definitive test for effectiveness of anti-DFTD responses. The devil that did not develop DFTD at first challenge was the one that showed increased anti-DFTD antibody responses. The boost this devil received 70 days prior to the first challenge may have provided additional protection, compared to non-boosted TD1, suggesting that the protection was not long-term. This is supported by the second challenge for TD2, which occurred with strain 3, 37 weeks (259 days) after the last immunization. This resulted in tumor development at both injection sites 22 weeks (154 days) later. As the tumors developed simultaneously and were identified as strain 3, it is unlikely that the tumor that developed on the right side was latent or modified tumor growth from the first challenge, more than one year previously. Although the challenge was with strain 3 and the immunization with strain 2, the antibodies reacted equally well to both strains and would be equally protective.

A similar length of protection induced in wild devils may allow them to resist DFTD during, for example, the breeding season. This season extends for a period of 3–4 weeks [38] and aggressive displays of mating behavior during this time accounts for a high proportion of penetrating bite wounds capable of transmitting DFTD [39]. Use of a DFTD vaccine at this time would have the potential to increase the number of devils successfully breeding and fully weaning their young.

Ethically, it was not possible to challenge all devils as the first two challenged devils both developed tumors. It will remain unknown if the responses in any of the other devils would have translated to protection. Despite the evidence for short-term protection in one devil, a more robust immunization regime that produces consistent responses is required, prior to further challenges. Such approaches would include upregulating MHC-I

on the DFTD cells prior to immunization, modifying the adjuvant to activate toll like receptors, identification and incorporation of DFTD tumor-associated antigens.

The findings presented above reflect the challenges working with an endangered species. The small sample size makes it difficult to control for age and gender. The lack of specific Tasmanian devil reagents and assays routinely used in mouse and human immunology was also limiting. Despite these we provide credible evidence that under the right conditions an immune response to DFTD cells can be generated. This offers the first substantial advance toward a vaccine.

Authors' contribution

AK, GKB, GMW, and ABL: Conception and design of study.
AK, GKB, CT: Acquisition of data.
AK, GKB, CT, ABL, and GMW: Analysis and interpretation of data.
AK, GMW GKB, CT, and ABL: Drafting of the manuscript.
GMW, AK, GKB, CT, and ABL: Final approval.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

[1] Pearse AM, Swift K. Allograft theory: transmission of devil facial-tumour disease. Nature 2006;439:549.
[2] Hawkins CE, Baars C, Hesterman H, Hocking GJ, Jones ME, Lazenby B, et al. Emerging disease and population decline of an island endemic, the Tasmanian devil *Sarcophilus harrisii*. Biol Conserv 2006;131:307–24.
[3] Murchison EP, Schulz-Trieglaff OB, Ning Z, Alexandrov LB, Bauer MJ, Fu B, et al. Genome sequencing and analysis of the Tasmanian devil and its transmissible cancer. Cell 2012;148:780–91.
[4] McCallum H, Tompkins DM, Jones M, Lachish S, Marvanek S, Lazenby B, et al. Distribution and impacts of Tasmanian devil facial tumour disease. Ecohealth 2007;4:318–25.
[5] Murchison EP, Tovar C, Hsu A, Bender HS, Kheradpour P, Rebbeck CA, et al. The Tasmanian devil transcriptome reveals Schwann cell origins of a clonally transmissible cancer. Science 2010;327:84–7.
[6] Siddle HV, Kreiss A, Eldridge MD, Noonan E, Clarke CJ, Pyecroft S, et al. Transmission of a fatal clonal tumor by biting occurs due to depleted MHC diversity in a threatened carnivorous marsupial. Proc Natl Acad Sci U S A 2007;104:16221–6.
[7] Siddle HV, Kreiss A, Tovar C, Yuen CK, Cheng YY, Belov K, et al. Reversible epigenetic down-regulation of MHC molecules by devil facial tumour disease illustrates immune escape by a contagious cancer. Proc Natl Acad Sci U S A 2013;110:5103–8.
[8] Loh R, Bergfeld J, Hayes D, O'Hara A, Pyecroft S, Raidal S, et al. The pathology of devil facial tumor disease (DFTD) in Tasmanian devils (*Sarcophilus harrisii*). Vet Pathol 2006;43:890–5.
[9] Kreiss A, Fox N, Bergfeld J, Quinn SJ, Pyecroft S, Woods GM, et al. Assessment of cellular immune responses of healthy and diseased Tasmanian devils (*Sarcophilus harrisii*). Dev Comp Immunol 2008;32:544–53.
[10] Hamede R, Lachish S, Belov K, Woods G, Kreiss A. Reduced effect of Tasmanian devil facial tumor disease at the disease front. Conserv Biol 2011;26:124–34.
[11] Pyecroft SB, Pearse AM, Loh R, Swift K, Belov K, et al. Towards a case definition for devil facial tumour disease: what is it? Ecohealth 2007;4:346–51.
[12] Kreiss A, Obendorf DL, Hemsley S, Canfield PJ, Woods GM. A histological and immunohistochemical analysis of lymphoid tissues of the Tasmanian devil. Anat Rec (Hoboken) 2009;292:611–20.
[13] Kreiss A, Wells B, Woods GM. The humoral immune response of the Tasmanian devil (*Sarcophilus harrisii*) against horse red blood cells. Vet Immunol Immunopathol 2009;130:135–7.
[14] Kreiss A, Cheng Y, Kimble F, Wells B, Donovan S, Belov K, et al. Allorecognition in the Tasmanian devil (*Sarcophilus harrisii*), an endangered marsupial species with limited genetic diversity. PLoS ONE 2011;6:e22402.
[15] Brown GK, Kreiss A, Lyons AB, Woods GM. Natural killer cell mediated cytotoxic responses in the Tasmanian devil. PLoS ONE 2011;6:e24475.
[16] Pinfold TL, Brown GK, Bettiol SS, Woods GM. Mouse model of devil facial tumour disease establishes that an effective immune response can be generated against the cancer cells. Front Immunol 2014;5:251.
[17] Jang SI, Lillehoj HS, Lee SH, Lee KW, Lillehoj EP, Bertrand F, et al. Montanide ISA 71 VG adjuvant enhances antibody and cell-mediated immune responses to profilin subunit antigen vaccination and promotes protection against *Eimeria acervulina* and *Eimeria tenella*. Exp Parasitol 2011;127:178–83.
[18] Langermans JA, Hensmann M, van Gijlswiik M, Zhang D, Pan W, Giersing BK, et al. Preclinical evaluation of a chimeric malaria vaccine candidate in Montanide ISA 720: immunogenicity and safety in rhesus macaques. Hum Vaccin 2006;2:222–6.
[19] Lawrence GW, Saul A, Giddy AJ, Kemp R, Pye D. Phase I trial in humans of an oil-based adjuvant SEPPIC MONTANIDE ISA 720. Vaccine 1997;15:176–8.
[20] Quattrocchi V, Pappalardo JS, Langellotti C, Smitsaart E, Fondevila N, Zomorano P, et al. Early protection against foot-and-mouth disease virus in cattle using an inactivated vaccine formulated with Montanide ESSAI IMS D 12802 VG PR adjuvant. Vaccine 2014;32:2167–72.
[21] Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, et al. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8T cells through cross-priming. Proc Natl Acad Sci U S A 2007;104:8947–52.
[22] Eastcott JW, Holmberg CJ, Dewhirst FE, Esch TR, Smith DJ, Taubman MA, et al. Oligonucleotide containing CpG motifs enhances immune response to mucosally or systemically administered tetanus toxoid. Vaccine 2001;19:1636–42.
[23] Linghua Z, Xingshan T, Fengzhen Z. In vivo oral administration effects of various oligodeoxynucleotides containing synthetic immunostimulatory motifs in the immune response to pseudorabies attenuated virus vaccine in newborn piglets. Vaccine 2008;26:224–33.
[24] Mapletoft JW, Oumouna M, Townsend HG, Gomis S, Babiuk LA, van Drunen Littel-van den Hurk S, et al. Formulation with CpG oligodeoxynucleotides increases cellular immunity and protection induced by vaccination of calves with formalin-inactivated bovine respiratory syncytial virus. Virology 2006;353:316–23.
[25] Mendez S, Tabbara K, Belkaid Y, Bertholet S, Verthelyi D, Klinman D, et al. Coinjection with CpG-containing immunostimulatory oligodeoxynucleotides reduces the pathogenicity of a live vaccine against cutaneous Leishmaniasis but maintains its potency and durability. Infect Immun 2003;71:5121–9.
[26] Sun S, Zhang X, Tough DF, Sprent J. Type I interferon-mediated stimulation of T cells by CpG DNA. J Exp Med 1998;188:2335–42.
[27] Speiser DE, Lienard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, et al. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest 2005;115:739–46.
[28] Pearse AM, Swift K, Hodson P, Hua B, McCallum H, Pyecroft S, et al. Evolution in a transmissible cancer: a study of the chromosomal changes in devil facial tumor (DFT) as it spreads through the wild Tasmanian devil population. Cancer Genet 2012;205:101–12.
[29] Howson LJ, Morris KM, Kobayashi T, Tovar C, Kreiss A, Papenfuss AT, et al. Identification of dendritic cells, B cell and T cell subsets in Tasmanian devil lymphoid tissue; evidence for poor immune cell infiltration into devil facial tumors. Anat Rec (Hoboken) 2014;297:925–38.
[30] Woods GM, Kreiss A, Belov K, Siddle HV, Obendorf DL, Muller HK, et al. The immune response of the Tasmanian Devil (*Sarcophilus harrisii*) and devil facial tumour disease. Ecohealth 2007;4:338–45.
[31] Chiang CL, Benencia F, Coukos G. Whole tumor antigen vaccines. Semin Immunol 2010;22:132–43.
[32] Miller W, Hayes VM, Ratan A, Petersen DC, Wittekindt NE, Miller J, et al. Genetic diversity and population structure of the endangered marsupial *Sarcophilus harrisii* (Tasmanian devil). Proc Natl Acad Sci U S A 2011;108:12348–53.
[33] Higgins D, Marshall JD, Traquina P, Van Nest G, Livingston BD. Immunostimulatory DNA as a vaccine adjuvant. Exp Rev Vaccines 2007;6:747–59.
[34] Wilkinson R, Kotlarski I, Barton M. Koala lymphoid cells: analysis of antigen-specific responses. Vet Immunol Immunopathol 1992;33:237–47.
[35] Wilkinson R, Kotlarski I, Barton M. Further characterisation of the immune response of the koala. Vet Immunol Immunopathol 1994;40:325–39.
[36] Binder RJ. Functions of heat shock proteins in pathways of the innate and adaptive immune system. J Immunol 2014;193:5765–71.
[37] Santin AD, Rose GS, Hiserodt JC, Fruehauf J, Eck LM, Garcia RI, et al. Effects of cytokines combined with high-dose gamma irradiation on the expression of major histocompatibility complex molecules and intercellular adhesion molecule-1 in human ovarian cancers. Int J Cancer 1996;65:688–94.
[38] Guiler ER. *Sarcophilus harrisii* (Marsupialia: Dasyuridae) II. Reproduction, breeding, and growth of pouch young. Aust J Zool 1970;18:63–70.
[39] Hamede R, McCallum H, Jones ME. Seasonal, demographic and density-related patterns of contact between Tasmanian devils (*Sarcophilus harrisii*): implications for transmission of devil facial tumour disease. Austral Ecol 2008;33:614–22.