Title: Mitogen activated Tasmanian devil blood mononuclear cells kill Devil Facial Tumour Disease cells

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Abstract:

Devil Facial Tumour Disease (DFTD) is a transmissible cancer that has brought the host species, the Tasmanian devil, to the brink of extinction. The cancer cells avoid allogeneic immune recognition by down-regulating cell surface MHC I expression. This should prevent $CD8^+$ T cell, but not NK cell, cytotoxicity. The reason why NK cells, normally reactive to MHC-negative cells, are not activated to kill DFTD cells has not been determined. The immune response of wild devils to DFTD, if it occurs, is uncharacterised. To investigate this, we tested twelve wild devils with DFTD, and found suggestive evidence of low levels of antibodies against DFTD cells in one devil. Eight of these devils were also analysed for cytotoxicity, however, none showed evidence for cytotoxicity against cultured DFTD cells. To establish whether mimicking activation of anti-tumour responses could induce cytotoxic activity against DFTD, Tasmanian devil peripheral blood mononuclear cells (PBMC) were treated with either the mitogen Concanavalin A, the toll-like receptor (TLR) agonist Poly I:C, or recombinant Tasmanian devil IL-2. All induced the PBMC cells to kill cultured DFTD cells, suggesting that activation does not occur after encounter with DFTD cells *in vivo,* but can be induced. The identification of agents that activate cytotoxicity against DFTD target cells is critical for developing strategies to protect against DFTD. Such agents could function as adjuvants to induce functional immune responses capable of targeting DFTD cells and tumours *in vivo*.

INTRODUCTION

The Tasmanian devil, a carnivorous marsupial endemic to the island of Tasmania in Australia, has recently come under threat of extinction from an emerging disease, Devil Facial Tumour Disease (DFTD). Cytogenetics has established that DFTD is a clonal neoplasm [1], and evidence drawn from cytology, molecular genetics and functional studies confirmed it is a transmissible cancer derived from a Schwann cell [1-3]. The disease is transmitted as an allograft when tumour cells enter wounds acquired during biting, which commonly occurs between Tasmanian devils during feeding and mating [1, 4].

Since it was first identified in 1996, DFTD has spread across most of the devil's geographical range, causing severe population declines [5]. Remarkably, a second transmissible cancer was identified in 2014 [6]. This second transmissible cancer is similar the first, but the precise tissue of origin has yet to be defined. Consequently DFTD refers to the transmissible facial cancers, which includes DFT1 (first identified in 1996) and DFT2 (first identified in 2014). Although conservation programs have established captive populations to ensure the survival of the species, they are confined to small areas of the Tasmanian mainland or offshore islands. In order to preserve the natural ecosystem and ensure the Tasmanian devil continues to function as an important scavenger and the major predator in the environment, it is imperative that a strategy to induce protective immune responses against DFTD is developed. This would not only help control DFTD levels in the wild but would ensure the safe release of insurance populations, which would otherwise be threatened by any persisting reservoir of DFTD in remaining wild devils.

Although marsupial immune systems have many similar characteristics to those of placental mammals, the most striking differences are related to the long lasting primary immune response of marsupials. IgG levels have been reported to last from nine weeks in the tammar wallaby (*Macropus eugenii*) and 15 weeks in the brushtail possum (*Trichosurus vulpecula*) [7] to as long as 37 weeks in the Brazilian opossum (*Monodelphis domestica*) [8]. This prolonged primary immune response has made it difficult to characterise a truly secondary response in marsupials. Conversely, cellular immune responses in marsupials appear to be similar to those of the eutherians, and studies with the tammar wallaby showed that these responses have hallmarks of T cell activation observed in placental mammals [9]. The Tasmanian devil appears to have a functional immune system [10], but this does not protect from allogeneic DFTD cell transmission.

As the name suggests, DFTD is characterised by the appearance of large tumours on the face, in and around the mouth of the animal [11]. DFTD is consistently fatal, with death occurring due to

starvation, infection or metastases [11, 12]. Although no resistant wild animals have been formally identified [13], there is some evidence for protection, with two healthy wild devils showing low levels of antibodies recognising DFTD cells [14]. While it is possible that these antibodies were specific for DFT1 cells, it remains to be determined whether they were DFTD specific or merely cross-reactive. In preliminary experiments, two devils immunized four times with irradiated DFTD cells failed to mount a detectable immune response [15]. Since then, we have shown that a modified protocol of repeated immunisations with sonicated, freeze/thawed and irradiated cells can induce humoral immune responses [16].

DFT1 cells do not express surface MHC I due to an epigenetic down regulation of antigen processing molecules, an adaptation that could allow them to evade the immune response. [17] Expression can be increased following exposure to interferon gamma (IFN-γ) [17], but it is unknown if this translates to increased immunogenicity *in vivo.* While MHC I is the obligatory ligand for $CD8^+$ cell cytotoxicity, its absence could make DFT1 cells targets for NK cell-mediated cytotoxicity. Previous studies with Tasmanian devils immunised with K562 tumour cells suggest a role of NK cells in anti-tumour immunity [15]. NK cells can be non-specifically activated to display anti-tumour activity through a variety of mechanisms, including mitogen stimulation and activation with cytokines [18-20]. These methods for activation have not yet been tested on Tasmanian devil cells. We have shown that Tasmanian devil peripheral blood mononuclear cells (PBMC) proliferate in response to stimulation by mitogens such as concanavalin (Con) A, phytohaemagglutinin A (PHA) and pokeweed mitogen (PWM) [21]. Consequently a functional analysis of such non-specifically activated cells appeared warranted.

Another molecule that has recently been assessed in many studies for its stimulatory and adjuvant activity is the toll-like receptor (TLR) agonist polyinosinic:polycytidylic acid (Poly I:C), which can induce cytotoxicity by NK cells [22]. Treatment of mononuclear cells with Poly I:C activates NK cells and induces the production of IFNγ, which augments other facets of an anti-tumour response [23]. Other agents that can non-specifically activate NK cells in other species include cytokines such as interleukin (IL)-2 [19], IL-15 [24] and IFNγ [20] and mitogens such as Con A [18]. Many of these non-antigen specific agents, particularly cytokines, have been used in immunotherapy trials in animal models or for human therapy (reviewed in [25]). Consequently, stimulation with cytokines or mitogenic non-specific activators may provide methods to activate anti-tumour immune responses against DFTD in Tasmanian devils.

In this study we assessed the effects of Poly I:C, Con A and IL-2 treatment on the cytotoxic capacity of isolated Tasmanian devil PBMC against DFT1 cells, with a view to developing methods for use in immunotherapy and immunisation for the disease.

RESULTS*:*

Tasmanian devils with DFTD do not show evidence of cytotoxicity or antibody responses against DFT1 tumour cells:

Tasmanian devils with DFTD were analysed for antibody and cytotoxicity responses to DFT1 tumour cells. Indirect immunofluorescence, analysed by flow cytometry, was used to measure antibody responses in serum, and a Cr^{51} release assay was used to measure cytotoxicity by PBMC of Tasmanian devils with DFTD tumours. Serum and PBMC from a DFT1-immunised and naive captive devils were used as positive and negative controls, respectively. Figure 1 (and supplementary table for median fluorescence intensity values) shows results from representative Tasmanian devils with DFTD. Twelve devils were analysed for antibody responses. Based on median fluorescence intensity of at least 1.5 times average median fluorescence intensity of the captive devils, there was a weak antibody responses in one devil (Dd13). The Cr^{51} release assay did not provide any evidence for cytotoxicity against DFT1 cells in the nine devils analysed. Such absent cell-mediated and weak/absent humoral immune responses to DFT1 cells are consistent findings for devils with DFTD.

Tasmanian devil PBMC stimulated by the mitogen Con A kill DFT1 tumour cells in vitro

Activation of lymphocytes by the mitogen Con A induces proliferation and cytotoxicity. To determine whether Con A stimulation of Tasmanian devil PBMC could induce cytotoxicity against DFT1 cells, Con A was incorporated during an 18 h Cr^{51} cytotoxicity assay. This did not cause statistically significant levels of cytotoxicity in the two devils tested, compared the control, in which the Con A was not included during the 18 h cytotoxicity assay. However, if Con A stimulation was performed for 48 hs prior to an 18 h Cr^{51} cytotoxicity assay, statistically significant levels of cytotoxicity were observed with cells from all four devils tested, compared to PBMC cultured for 48 h in the absence of Con A (Fig. 2). Thus Con A activated Tasmanian devil PBMC can kill DFT1 cells.

Cytokines, including Interleukin 2, induce cytotoxic responses in Tasmanian devil PBMC

Stimulation of lymphocytes with Con A is known to induce cytokine production. Consequently the production of cytokines during the 48 h Con A culture could contribute to cytotoxic responses against DFT1 cells. Interleukin-2 (IL-2) is a candidate cytokine likely to be present in the supernatants and to cause the effects observed. Conditioned medium (25% vol/vol) from the 48 h Con A cultures, or 1% (vol/vol), recombinant Tasmanian devil IL-2 purified from Sf21 insect cell supernatants was added to cytotoxicity assays to determine if cytokine addition could induced cytotoxic responses in devil PBMC. 25% vol/vol 'Conditioned medium' did not induce cytotoxicity if included during a 4 h cytotoxicity assay in the two devils tested (Fig 3a), but induced statistically significant cytotoxicity responses during an 18 h assay compared to the cytotoxicity assays that did not include the 'Conditioned Medium' in all four devils tested (Fig. 3b). IL-2 alone induced statistically significant, cytotoxic responses at 1% vol/vol IL-2 in both devils tested (corresponding to 5 μg/mL; Fig. 3c).

The Toll-like receptor 3 (TLR3) agonist Poly I:C induces cytotoxic responses in Tasmanian devil PBMCs

The TLR3 agonist Poly I:C can activate cytotoxicity in NK cells. The TLR3 gene has been identified in Tasmanian devils and shown to be functional [26]. We therefore used Poly I:C to evaluate the function of Tasmanian devil TLR3 to induce cytotoxicity in PBMCs, presumably by NK cells. Poly I:C stimulated cells from both devils tested exhibited statistically significant cytotoxic responses when included in the 18 h cytotoxicity assays (Fig. 4).

DISCUSSION

Devil Facial Tumour Disease (DFTD) is one of only three examples of a naturally occurring transmissible cancer [1, 27, 28]. The transmissible nature suggests that the tumour cells are capable of evading the host immune response. For DFTD, histology has provided evidence for a lack of anti-tumour activity against DFTD, with low levels of lymphocyte infiltration in only seven percent of samples [29]. There is no convincing evidence for disease resistance among wild devils [13]. The disease rapidly progresses after infection, with death occurring within only a few months of tumour appearance [30]. This had led to the assumption that immune responses against DFTD tumours are absent. However, this assumption has not been confirmed using functional studies of lymphocyte cytotoxicity and antibody production against DFTD tumour cells [15]. The results presented herein provide the functional evidence that wild Tasmanian devils with DFTD fail to mount a specific anti-

tumour immune response against the tumour cells. There was no evidence for cytotoxicity in any of the devils tested. A more sensitive method was the analysis of antibody levels. Only one devil showed evidence for the presence of anti-DFTD antibodies, but this was, at most, a very weak response. Despite prolonged exposure to the disease, involving large numbers of DFTD cancer cells, their immune system failed to identify and eliminate the tumour cells. This finding is consistent with the immunohistochemical evidence of low lymphocyte numbers within DFTD tumours [29].

With Tasmanian devil population numbers diminished by up to 95% in areas where DFTD has been observed for many years [31], intervention may soon become necessary to protect declining numbers in the wild. However, the use of standard cancer therapies, such as surgery, chemotherapy or radiation is impractical on a population scale. Consequently, the most appropriate option for intervention may be to induce a beneficial host immune response against DFTD tumours using immunotherapy or vaccination.

This study sought to identify techniques that could activate the immune cells of Tasmanian devils to target and kill DFT1 cells. Given that DFT1 cells do not normally express MHC I, non-specific stimulation of NK cells could be a potential strategy to induce anti-tumour activity in infected devils. Candidate stimuli for non-specific activation of anti-tumour responses in NK cells included cytokines, TLR agonists and mitogens.

Con A, a non-specific activator of anti-tumour immune responses in T and NK cells, has previously been shown to activate other responses, such as proliferation, in Tasmanian devil cells [21]. *In vitro* assays demonstrated that activation of devil PBMCs by Con A consistently caused the killing of DFT1 cells. Activity was low when cells were stimulated for only 18 h within the assays but increased greatly when PBMC were pre-treated for 48 h before cytotoxicity was assessed. The longer incubation may have induced proliferation, hence the relative number of effector cells had increased. Activation of PBMC with Con A provides evidence that DFT1 cells can be targeted and killed by immune cells.

Cytokines are produced as a result of Con A stimulation [32]. Consequently cytokines produced by the activated cells could be responsible for the induction of anti-tumour responses. Candidate cytokines for this activity included IL-2 and IFNγ. Cytotoxicity assays performed using recombinant Tasmanian devil IL-2 produced cytotoxic responses. As these responses were relatively weak, higher concentrations, or longer incubation periods, might be required to reach maximum activation and cytotoxicity.

As DFT1 cells do not usually express MHC I [17] it more is likely that NK cells, rather than $CD8^+$ T lymphocytes, mediated the cytotoxicity following PBMC activation. This is supported by the activation by poly I:C, which is more effective at activating NK cells than T cells. We have previously identified cells resembling NK cells in the peripheral blood of Tasmanian devils using immunocytochemistry [15]. In order to determine more thoroughly if NK cells are mediating the killing in these assays, direct observation of their contact with the tumour cells will be required.

The reason DFT1 cells are not targeted by NK cells from diseased devils remains unknown as the absence of MHC I expression should make DFT1 cells susceptible to cytotoxicity from NK cells. DFT1 cells may have developed immune evasion strategies to resist the activity of cytotoxic ($CD8^+$ T lymphocyte and NK) cells *in vivo*, but DFT1 cells are vulnerable to activated cytotoxic cells *in vitro*. This is consistent with our murine model of DFTD, which clearly shows that DFT1 cells are susceptible to *in vitro* cytotoxicity [33].

The inability of Tasmanian devils to resist DFTD appears to be primarily due to a failure of recognition and subsequent activation of immune cells. Stimulation of cytotoxic cells (presumably NK cells) by cytokines, either directly or indirectly via Con A stimulation, provides the activation needed to target and kill DFT1 cells. It is most likely that NK cells are activated and responsible for the killing as they will target cells that don't express MHC. IL2 contributes to this activation, but it is likely that other cytokines (e.g. IL15) are required for optimal stimulation. Potentially these activated NK cells could produce cytokines such as IFN-γ, that would increase MHC expression on DFT1 cells *in vivo* [17], potentially providing a target for $CD8^+$ T cells. The evidence that PBMC of Tasmanian devils can kill DFT1 cells has implications for an immunotherapy or targeted vaccine for use against DFTD. The combined evidence that immunised Tasmanian devils can produce antibodies against DFTD [16], that PBMC from K562 immunised devils can kill K562 cells *in vitro* [8], and the demonstration here that activated cells can kill DFT1 cells *in vitro*, suggest that devils have the capacity to respond to, and to target, DFT1 cells. In the future, it is of enormous interest to establish whether these observations can be replicated *in vivo* as a successful vaccination or immunotherapy strategy.

In summary, the development of a vaccination strategy or immunotherapy is potentially the best option to conserve wild Tasmanian devil populations. Captive breeding and isolation, whether on private land, peninsulas or Tasmanian islands, will not ensure the conservation of Tasmanian devil populations across the entire species range. As population numbers have already dramatically declined, and the impact has already been observed on the Tasmanian ecosystem, the need for a

targeted vaccine or immunotherapy is paramount. Evaluation needs to be *in vivo*, to reflect the natural situation. This manuscript provides a basis for further research that may prove crucial in the conservation of the Tasmanian devil.

METHODS:

Cell culture

The DFTD cell line used in the following experiments was C5065, a DFT1 cell line that was established from a strain 3 [34] primary DFTD tumour biopsy sample taken under the approval of the Animal Ethics Committee of Tasmania's Park and Wildlife Services (permit numbers 33/2004–5 and 32/2005–6). It was provided by A-M. Pearse and K. Swift, Tasmanian Department of Primary Industries, Parks, Wildlife and Environment (DPIPWE). The C5065 DFTD cells were cultured in 75 cm^2 tissue culture flasks (Corning, New York, USA) in RPMI-10FCS, which consisted of RPMI1640 culture medium (GIBCO, New York, USA) supplemented with 10% vol/vol heat inactivated foetal calf serum (Bovogen Biological, Victoria, Australia), 5 mM L-glutamine (Sigma Aldrich, Ayrshire, UK) and 200 IU/L of gentamicin sulfate (Pfizer, Western Australia, Australia) at 35 °C in a humidified atmosphere of 5% CO_2/95% air. Cell number and viability counts were performed using Trypan blue exclusion on an improved Neubauer haemocytometer.

Tasmanian devils

The experiments involving the use of Tasmanian devils were conducted under the approval of the University of Tasmania Animal Ethics Committee (permit number A0009215). The captive Tasmanian devils used in this study were provided by the Department of Primary Industries, Parks, Water and Environment, Tasmanian Government and housed under quarantine conditions. The immunised devil received three injections of irradiated cells and one injection of sonicated cells, modified from previous work [16]. In brief, the injections were performed every two weeks for eight weeks and blood collected two weeks after the final injection. They were fed, handled and anaesthetised according to the specifications given by Brown et al 2011 [35]. Wild Tasmanian devils affected by DFTD were randomly captured in pipe traps following a DFTD suppression trial in an isolated peninsula in the Southeast of Tasmania. Devils were anaesthetised using isofluorane gas anaesthesia and 10 ml of blood was collected from the heart immediately before euthanasia. The blood was injected into lithium heparin anticoagulant tubes (BD Biosciences, New Jersey, USA).

and stored at room temperature until arrival at the laboratory (<24 h). All subsequent sample processing and experiments were performed under sterile conditions.

Blood processing

PBMC were isolated from uncoagulated Tasmanian devil whole blood using density gradient centrifugation on Histopaque 1077 according to the manufacturer's protocol (Sigma Aldrich, St Louis, USA). The PBMC were washed with PBS, pelleted at 50 g then diluted for use in supplemented RPMI-10FCS culture medium.

Concanavalin (Con) A stimulation of PBMC for cytotoxicity and preparation of 'conditioned medium'

Isolated PBMC were diluted to 10^6 cells/ml in RPMI-10FCS culture medium containing 5 µg/ml Con A (C7275, Sigma-Aldrich, St. Louis, USA) and incubated for 48 h in 75 cm^2 culture flasks (353135, Corning, New York, USA) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The samples were pelleted in 15 ml conical centrifuge tubes (431651, Corning, New York, USA) at 250 g for 10 minutes, and the supernatant removed. The pellet was resuspended in RPMI-10FCS culture medium and the centrifugation step repeated. The residual Con A in the supernatant was removed by mixing with 15mg/ml of solid α-D Mannose for 5 minutes then the sample passed through a 25 mm 0.8/0.2 µm filter (4187, Pall Corporation, New York, USA) under sterile conditions. This comprised the 'conditioned medium'.

Production of recombinant devil IL2

A cDNA encoding full length devil IL2 (Ensembl reference ENSSHAG00000002065) bearing a 5' Kozak sequence (GAAACC) and a C-terminal fusion to a Gly_3His_6 tag was synthesized by DNA2.0 (California, USA) and subcloned into pFastBac1 as a BamHI-EcoRI fragment. The sequence was confirmed by Sanger sequencing (ANU Biomolecular Resource Facility, ACT) before the construct was transformed into DH10MultiBac cells and the bacmid prepared as described previously [36]. Sf21 cells were transfected according to established protocols [36], followed by two rounds of viral amplification to generate P3 virus. HighFive cells (0.5L) were cultured in 2.8L Fernbach flasks in ExpressFive medium (Life Technologies) shaking at 90 rpm, 27°C to a density of $3x10^6$ cells/mL before addition of P3 virus and continued incubation for 72 h. The volume of P3 virus per 10^6 cells required for optimal protein yield was determined empirically. Cells were pelleted by centrifugation and the supernatant was subjected to tangential flow concentration and three cycles of buffer exchange into mouse tonicity-phosphate buffered saline (MT-PBS; 149 mM NaCl, 16 mM

$Na_2HPO_42H_2O$; 4 mM $NaH_2PO_4.H_2O$) using a 10kD molecular weight cut off Pellicon 3 ultrafilter (P3C010C01). The concentrate was adjusted to 180mM NaCl, 10mM imidazole pH 8 and applied to Ni-NTA agarose with rotation for 1h at 4°C. Ni-NTA resin was collected by centrifugation, washed twice with MT-PBS containing 10mM imidazole twice, and then twice with MT-PBS containing 35mM imidazole twice, before elution in MT-PBS containing 180mM NaCl and 250mM imidazole pH 8. Protein was concentrated by centrifugal ultrafiltration and applied to a Superdex-75 gel filtration column with elution in MT-PBS. Protein was concentrated to 0.5mg/mL by centrifugal ultrafiltration (as estimated by absorbance at 280nm), before aliquots were snap frozen in liquid nitrogen and stored at -80°C until required. (Supplementary table shows size-exclusion chromatography analysis of the recombinant IL2 and a representative functional assay with different IL2 dilutions.)

Antibody measurement by flow cytometry

Washed DFT1 tumour 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 (CLS3799, Corning, New York, USA) and placed on ice. Serum samples were diluted 1:50 (final dilution 1:100) with washing buffer, mixed with the DFT1 tumour cells, incubated on ice for 30 minutes, washed twice with washing buffer, and incubated with 50 µL of 2 µg/mL of a monoclonal mouse anti-devil IgG [37] for 30 minutes. They were again washed and incubated with 50 µL of 0.2 µg/mL of AlexaFluor 488-conjugated goat anti-mouse IgG antibody for 30 minutes (Molecular Probes, Leiden, The Netherlands), washed again and 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). Control DFT1 cells (labeled with the secondary and tertiary antibodies, but no devil serum) did not show background fluorescence. Evidence for a positive response was regarded as median fluorescence intensity of at least 1.5 times the average median fluorescence intensity of the captive, control, non-DFT1 exposed devils. For accurate comparison, all serum samples were tested simultaneously.

Cytotoxicity assays

Radioactive ^{51}Cr cytotoxicity assays were performed using the method specified by Brown *et al.* [15]. Briefly, assays were performed in V-bottomed 96 well plates (CLS4894, Corning, New York, USA) on triplicate samples containing 10^4 DFT1 cells with a range of PBMC:target ratios. Negative and positive controls for ^{51}Cr release contained RPMI-10FCS culture medium and 1% Triton X100 detergent in water, respectively. C5065 DFT1 cells were labelled for 2 h with 100 mCi of

radioactive ^{51}Cr solution (Perkin-Elmer, Massachusetts, USA) then washed 3 times in RPMI-10FCS and resuspended for assay use. The assays were incubated for 4 or 18 h at 37°C in a humidified atmosphere of 5% CO2/95% air. The plates were centrifuged for 5 minutes at 170 g before 100 µl aliquots of supernatant were harvested into individual polystyrene tubes and analysed for γ radioactivity (LKB Wallac, Turku, Finland).

Cytotoxicity was calculated from CPM values in comparison to positive and negative control samples using the formula:

Percent killing =

((sample CPM - mean negative control CPM) / (mean maximum control CPM - mean negative control CPM)) ×100 (%)

The effect of Poly I:C was assessed at concentrations of 10, 5, 1 or 0.1 µg/ml, with the optimal response observed at 5 µg/ml (data not shown). 5 µg/ml Con A had previously been determined as the optimal concentration for stimulation of Tasmanian devil PBMC [21]. For these assays PBMC were stimulated for 48 h with either poly I:C or Con A, the cells were washed and then used in an 18 h cytotoxicity assay. The cytotoxicity assays that were testing 'conditioned medium' or IL-2 were performed by adding 25% vol/vol 'conditioned medium' or 1% vol/vol recombinant Tasmanian devil IL-2 during the cytotoxicity assays, without the need for prior activation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1: Antibody and cytotoxic responses against DFTD cells from healthy and diseased Tasmanian devils.

(a) Serum antibody levels of one captive immunized devil as a positive control and six naïve captive devils (Cd). DFTD- specific IgG levels were 4.7-fold higher in the immunised devil (median fluorescence intensity = 461) than the average of the healthy captive devils (average median fluorescence intensity = 98). For comparison, fluorescence intensity has been normalised to mode. Experiments were performed twice with similar results.

(b) Serum antibody levels of 12 wild Tasmanian devils with DFTD (Dd). Only one devil (Dd13) was regarded as positive, with a median fluorescence intensity of 192, which was at least 1.5 times the average median fluorescence intensity of the six captive, control, non-DFTD exposed devils. For comparison, fluorescence intensity has been normalised to mode. Experiments were performed twice with similar results.

(c) I*n vitro* cytotoxicity responses of PBMC from 11 Tasmanian devils against DFTD tumour cells. Chromium release cytotoxicity assays were performed using PBMC from eight wild devils with DFTD (Dd), one healthy wild devil (Wd1), one healthy captive devil (Cd1) and one immunized captive devil (Cd3) as an example of a positive cytotoxicity response. Cytotoxicity assays were performed once with each of the 11 Tasmanian devils.

Figure 2: Cell mediated cytotoxicity responses by mitogen activated PBMC from healthy and diseased devils.

(a) Cytotoxicity responses of PBMC from two captive devils (Cd) when Con A was incorporated during the 18 h cytotoxicity assay.

(b) Cytotoxicity responses of PBMC from three captive (Cd) and one healthy wild devil (Wd7) against DFTD tumour cells following stimulation with Con A (5ug/ml) for 48 hs prior to an 18 h chromium release assay.

The statistical difference in cytotoxicity between the stimulated and non-stimulated cells was assessed using 2 way ANOVA with a value of $P<0.05$ classified as significant with an asterisk (*).

Figure 3: Effect of conditioned medium and IL-2 on PBMC cytotoxicity responses.

(a) Cell mediated cytotoxicity responses of PBMC from two healthy wild devils (Wd) against DFTD tumour cells in a 4 h chromium release assay performed in the presence of conditioned medium (CM).

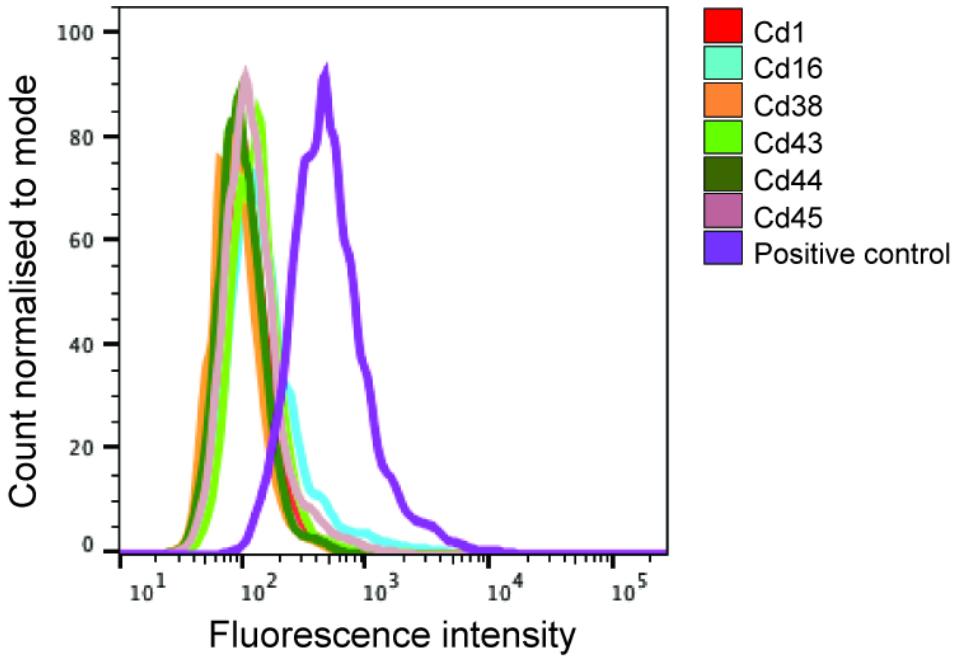
(b) Cell mediated cytotoxicity responses of PBMC from two healthy wild devils (Wd) and two captive devils (Cd) against DFTD tumour cells in an 18 h chromium release assay performed in the presence of conditioned medium (CM).

(c) Cell mediated cytotoxicity responses of PBMC from two captive devils (Cd) in an 18 h chromium release assays supplemented with 1% vol/vol recombinant Tasmanian devil IL-2. The statistical difference between the treated and untreated cells was assessed using 2 way ANOVA with a value of $P<0.05$ classified as significant with an asterisk (*).

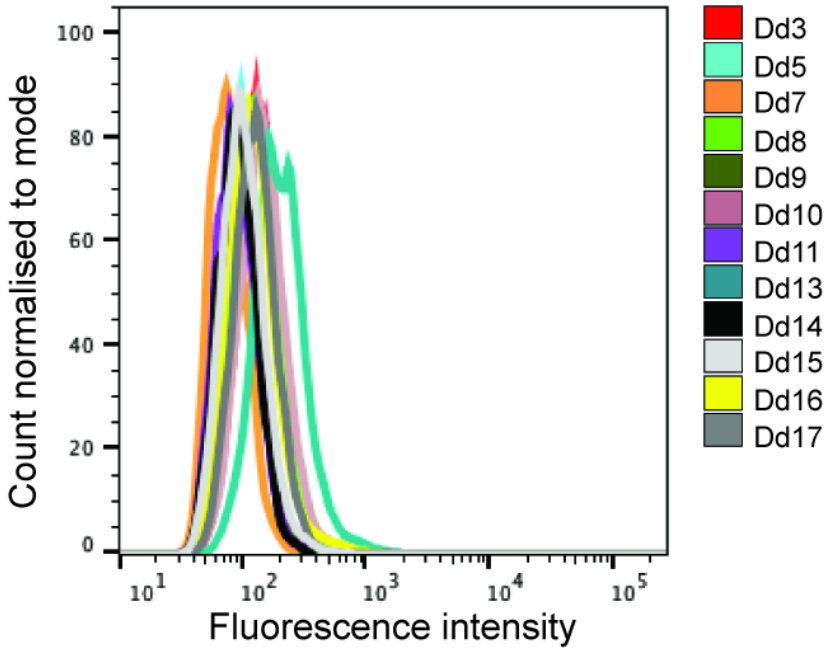
Figure 4: Effect of the Toll-like receptor (TLR) 3 agonist poly I:C on PBMC cytotoxicity responses.

Cell mediated cytotoxicity responses of PBMC from two captive devils (Cd) against DFTD tumour cells in an 18 h chromium release assay supplemented with poly I:C (5 μg/ml). The statistical difference between the two populations was assessed using 2 way ANOVA with a value of $P<0.05$ classified as significant with an asterisk (*).

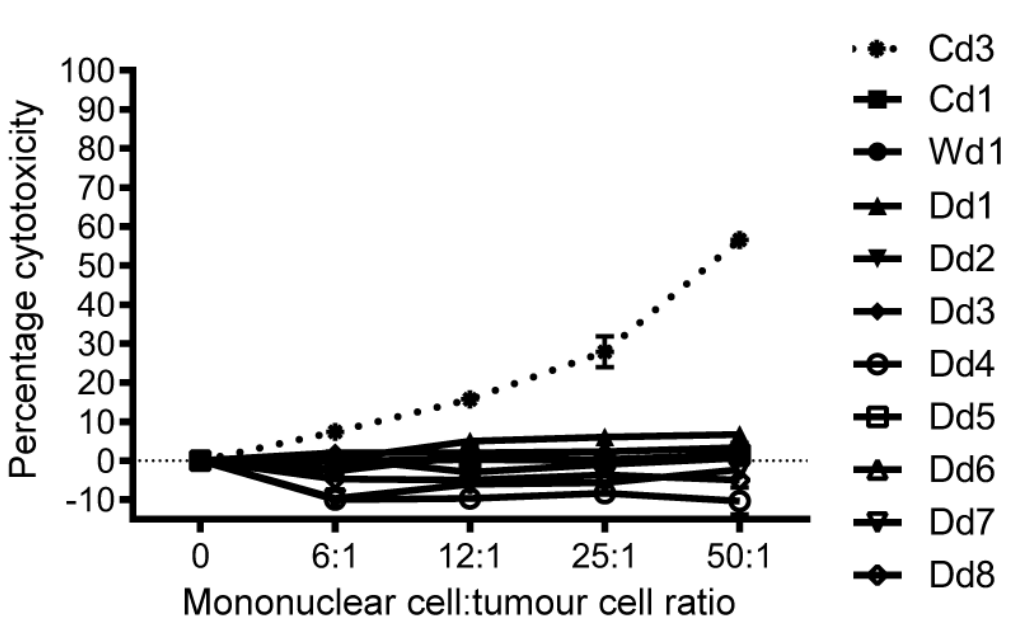
a


Count normalised to mode
Fluorescence intensity
Cd1
Cd16
Cd38
Cd43
Cd44
Cd45
Positive control

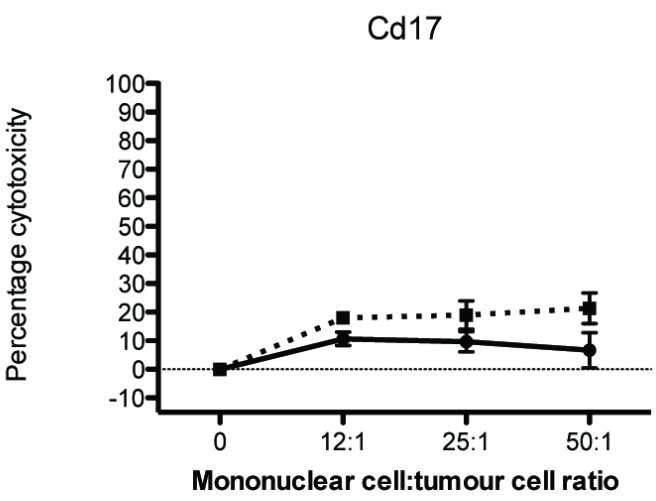
b

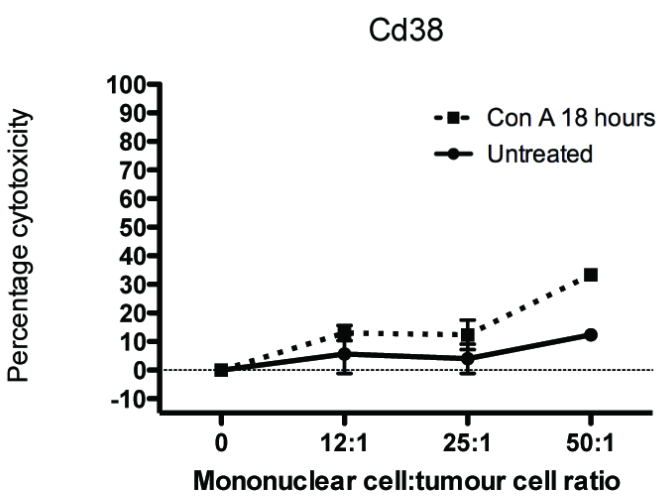

Count normalised to mode
Fluorescence intensity
Dd3
Dd5
Dd7
Dd8
Dd9
Dd10
Dd11
Dd13
Dd14
Dd15
Dd16
Dd17

c

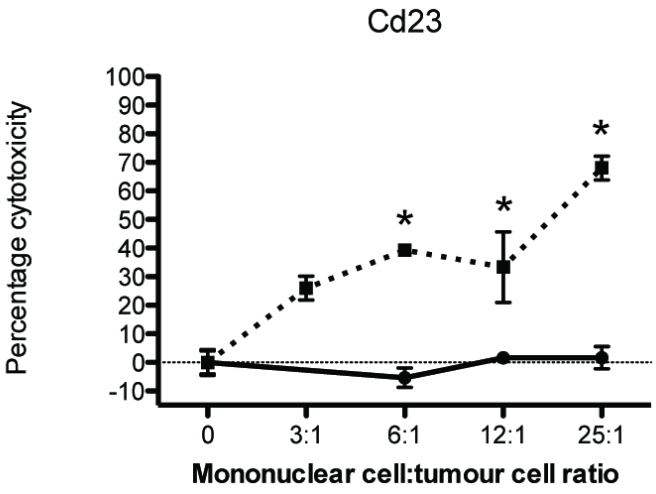

Percentage cytotoxicity
Mononuclear cell:tumour cell ratio
0
6:1
12:1
25:1
50:1
Cd3
Cd1
Wd1
Dd1
Dd2
Dd3
Dd4
Dd5
Dd6
Dd7
Dd8

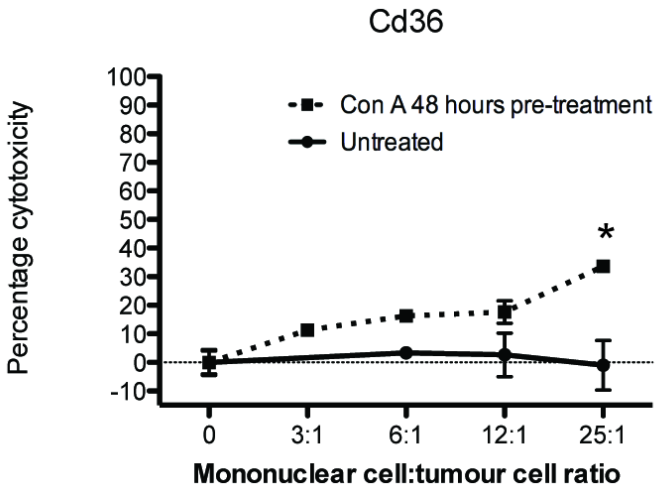
a

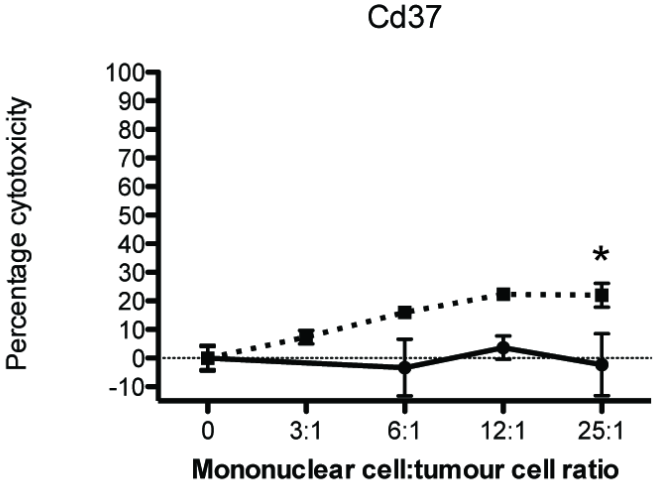

Cd17
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
0
12:1
25:1
50:1
Mononuclear cell:tumour cell ratio

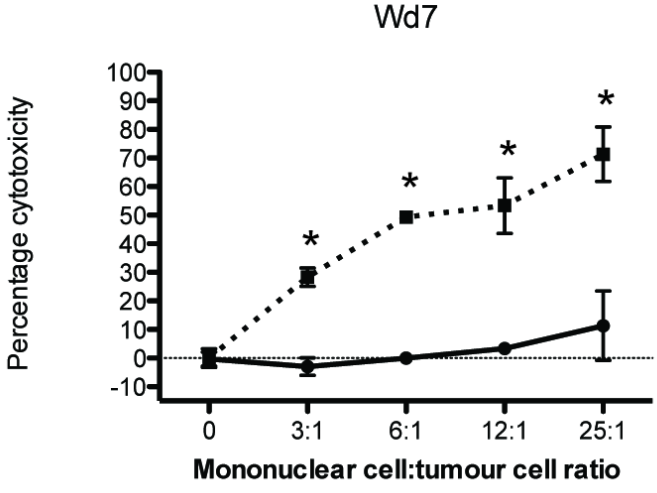

Cd38
Percentage cytotoxicity
Con A 18 hours
Untreated
100
90
80
70
60
50
40
30
20
10
0
-10
0
12:1
25:1
50:1
Mononuclear cell:tumour cell ratio

b

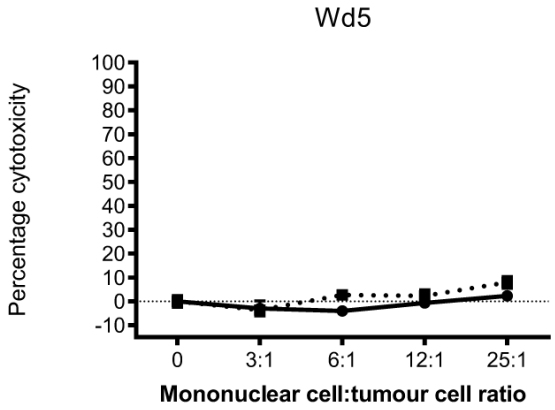

Cd23
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
*
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio

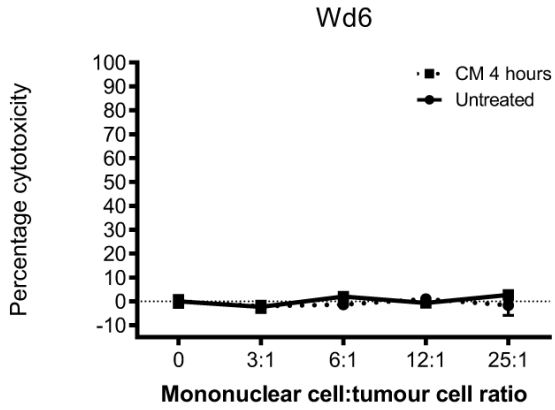

Cd36
Percentage cytotoxicity
Con A 48 hours pre-treatment
Untreated
100
90
80
70
60
50
40
30
20
10
0
-10
*
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio


Cd37
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio

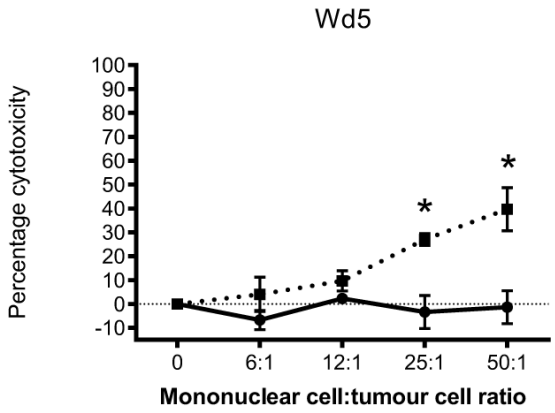

Wd7
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
*
*
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio

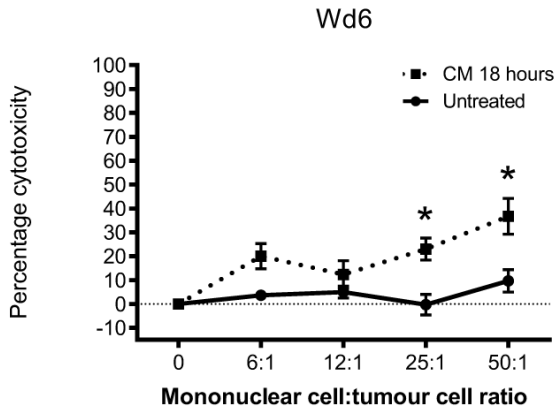
a

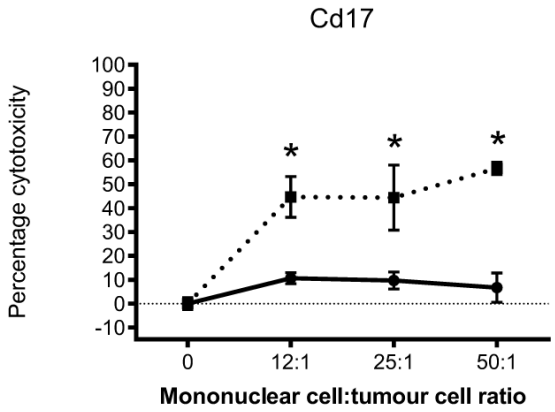

Wd5
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio

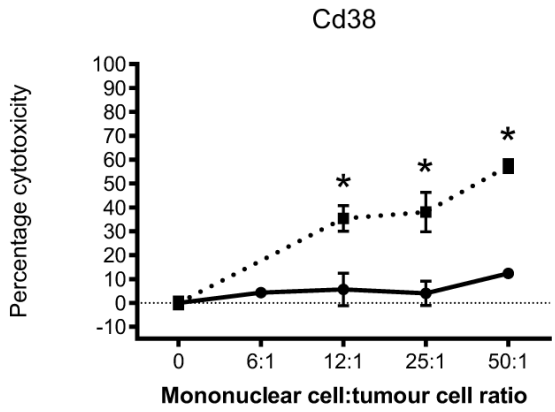

Wd6
CM 4 hours
Untreated
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio

b

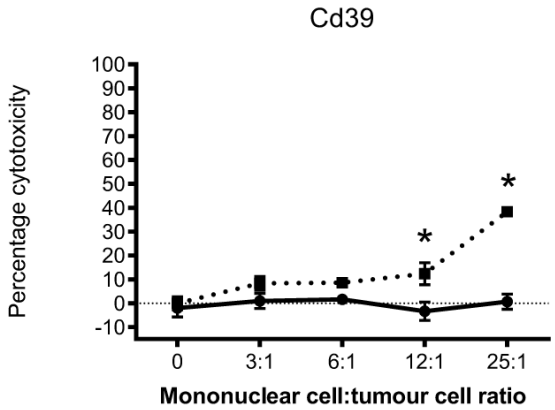

Wd5
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
0
6:1
12:1
25:1
50:1
Mononuclear cell:tumour cell ratio

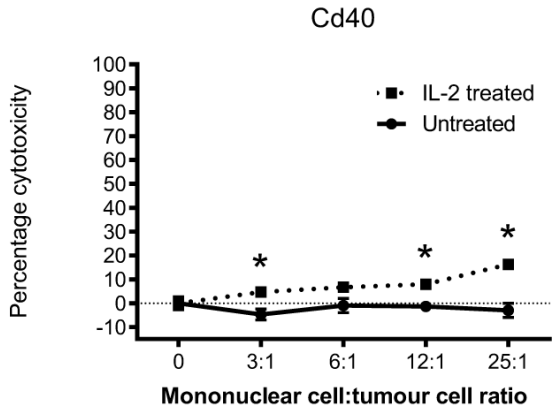

Wd6
CM 18 hours
Untreated
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
0
6:1
12:1
25:1
50:1
Mononuclear cell:tumour cell ratio

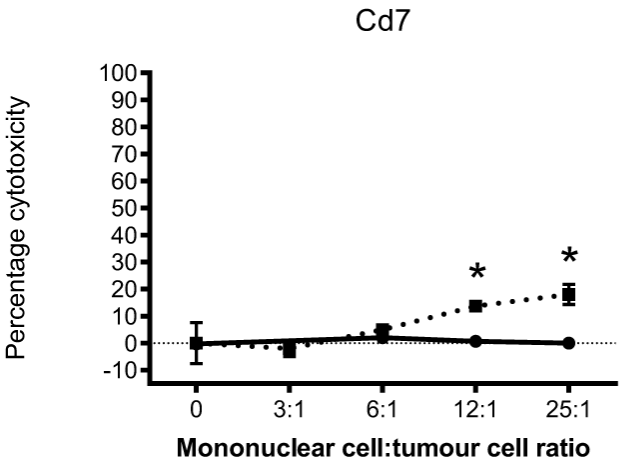

Cd17
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
*
0
12:1
25:1
50:1
Mononuclear cell:tumour cell ratio

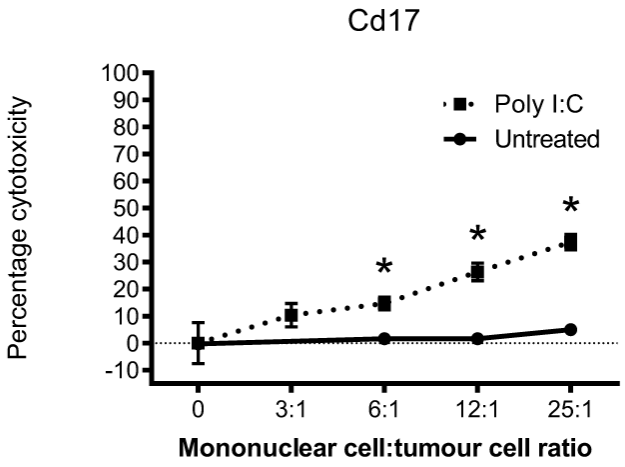

Cd38
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
*
0
6:1
12:1
25:1
50:1
Mononuclear cell:tumour cell ratio

c


Cd39
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio


Cd40
IL-2 treated
Untreated
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
*
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio


Cd7
Percentage cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio


Cd17
Percentage cytotoxicity
Poly I:C
Untreated
100
90
80
70
60
50
40
30
20
10
0
-10
*
*
*
0
3:1
6:1
12:1
25:1
Mononuclear cell:tumour cell ratio