Allorecognition in the Tasmanian Devil (*Sarcophilus harrisii*), an Endangered Marsupial Species with Limited Genetic Diversity

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**Abstract**

Tasmanian devils (*Sarcophilus harrisii*) are on the verge of extinction due to a transmissible cancer, devil facial tumour disease (DFTD). This tumour is an allograft that is transmitted between individuals without immune recognition of the tumour cells. The mechanism to explain this lack of immune recognition and acceptance is not well understood. It has been hypothesized that lack of genetic diversity at the Major Histocompatibility Complex (MHC) allowed the tumour cells to grow in genetically similar hosts without evoking an immune response to alloantigens. We conducted mixed lymphocyte reactions and skin grafts to measure functional MHC diversity in the Tasmanian devil population. The limited MHC diversity was sufficient to produce measurable mixed lymphocyte reactions. There was a wide range of responses, from low or no reaction to relatively strong responses. The highest responses occurred when lymphocytes from devils from the east of Tasmania were mixed with lymphocytes from devils from the west of Tasmania. All of the five successful skin allografts were rejected within 14 days after surgery, even though little or no MHC I and II mismatches were found. Extensive T-cell infiltration characterised the immune rejection. We conclude that Tasmanian devils are capable of allogeneic rejection. Consequently, a lack of functional allorecognition mechanisms in the devil population does not explain the transmission of a contagious cancer.

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**Introduction**

Devil facial tumour disease (DFTD) is a transmissible Schwann cell cancer of Tasmanian devils (*Sarcophilus harrisii*) [1,2,3]. The cancer cells are passed from animal to animal during the process of biting, which most frequently occurs on the head and neck. Metastases to distant organs are common and affected devils die within months after tumours become apparent [2,4]. This disease was noted in 1996 in the far northeast of Tasmania, and has since spread to over half of the devil’s range [5,6]. The far northwestern areas of Tasmania still remain disease free, although it is predicted that DFTD will affect all devil habitat within the decade. It is possible that extinction of the species will occur within 20 years [5].

Despite being a cell allograft, DFTD tumours do not evoke an immune response [7], and there is no evidence of lymphocyte infiltration into the tumour masses [8]. In vertebrates, organ, tissue or cell transplants are rejected by the immune system, unless the recipient is severely immunocompromised or both recipient and donor share identical Major Histocompatibility Complex (MHC) genes (e.g. homozygous twins) [9]. MHC genes were originally identified in mammalian cells, encoding specialised glycoproteins expressed on the cell surface of almost all nucleated cells. These genes were first related to foreign tissue (allograft) transplantation, but are now known to be essential in the immune recognition of pathogens and tumour cells [10,11]. There are two main MHC subgroups that have different immunological functions, MHC I and MHC II. MHC I molecules are composed of a polymorphic α-chain associated with a non-polymorphic β2-microglobulin. Its role is to present intracellular antigens to CD8+ T-cells. These molecules are present on all nucleated cells. MHC II molecules are composed of a polymorphic α-chain attached to a polymorphic β-chain, and present extracellular antigens to CD4+ T-cells. Under normal conditions, MHC II is only located on the cell surface of professional antigen presenting cells, such as dendritic cells and macrophages (reviewed in [12,13]). Tasmanian devils have a competent immune system [14,15], but the devil population has been through genetic bottlenecks and lacks MHC diversity [16,17].

MHC molecules regulate the immunological mechanisms of tissue graft rejection and provoke vigorous T-cell responses against incompatible cells [18,19]. Different effector processes may reject the allografts, but the main mediators are CD8+ T-cells and CD4+ T-cells. Allopeptides presented to CD8+ T-cells by MHC I molecules stimulate the differentiation of cytotoxic T-cells, which
kill nucleated cells expressing MHC I in the graft. Alloantigens presented by MHC II molecules activate helper T-cells, which differentiate and produce cytokines that damage the tissue graft. Allograft rejection mediated by CD4+ and CD8+ T cells is deemed acute rejection, as it can usually occur within eight to 12 days after the transplant. This form of rejection is primarily due to mismatching of MHC loci [20,21].

A predictive in vitro test for T-cell recognition of allogeneic MHC molecules is the mixed lymphocyte reaction (MLR) [22]. This experiment has been used in clinical analyses of allogeneic impact of MHC I and II mismatches between live donors (e.g. kidney donors) and recipients. Donors with poor MLR responses against the recipient have lower incidence of graft rejection [23,24]. MLR experiments can be performed with cultured lymphocytes from both donor and recipient and the resultant proliferation will correspond to differences in the MHC alleles of both individuals (two-way MLR). By inactivating the mononuclear cells from the donor (by X-irradiation or chemical mitotic inhibition), only the recipient response is evaluated (one-way MLR).

Skin grafting between unrelated individuals has been performed to test MHC disparity in a wild mammal, the cheetah (Acynonyx jubatus). Captive cheetahs were unable to quickly recognize alloantigens as foreign and reject the skin grafts [25]. More recently, wild-living Namibian cheetahs were shown to have low levels of MHC variation, although this did not appear to impair their immune response against infectious diseases [26,27]. To date, a lack of MHC diversity has been the primary reason given for the lack of rejection of allograft tumours by Tasmanian devils. It has been proposed that devils have a similar range of MHC antigens as the tumours [16,17], and therefore do not see the tumours as foreign. Acceptance, or delayed rejection, of skin allografts would indicate the lack of MHC diversity has been the primary reason given for the lack of rejection of allograft tumours by Tasmanian devils. It has been proposed that devils have a similar range of MHC antigens as the tumours [16,17], and therefore do not see the tumours as foreign. Acceptance, or delayed rejection, of skin allografts would help to confirm the theory that impoverished MHC diversity is responsible for a lack of tumour allograft recognition [28,29]. To determine whether this limited MHC diversity is sufficiently low to permit allograft transplantation, we characterised levels of functional MHC variation by two-way MLR and skin grafts experiments.

Materials and Methods

Ethics statement

All experiments describing the use of animals were undertaken with approval and under inspection of the Animal Ethics Committee of University of Tasmania, permit numbers A9491 and A11052. Captive devils were housed in groups of two or three in 100 m² enclosures. Animals were fed possum or wallaby meat once a day and water ad libitum. All procedures (blood collection, skin graft surgery, biopsy and bandage removal) were performed with the animals under general anaesthesia and all efforts were made to minimise pain or discomfort.

Two-way mixed lymphocyte reaction

Fifteen Tasmanian devils from five areas in eastern Tasmania (Nugent, Epping, Mount William National Park, Forestier Peninsula and Bronte Park) and nine devils from four regions in western Tasmania (Woolnorth, Temma, Milkshake Hills and Granville Harbour) were used. Figure 1 shows a map of Tasmania with the geographical locations of all devils used for MLR and skin graft experiments. The DFTD-affected area is also illustrated. Whole blood was collected into lithium heparin tubes from the jugular vein while under general anaesthesia. Mononuclear cells were harvested using density gradient centrifugation as described previously [14]. Cells were diluted to a concentration of 10^6 cells/mL in RPMI 1640 incomplete medium (CSL Limited 05182301) supplemented with 10% pooled devild plasma, 2 mM of glutamine (Sigma G7513) and 15 mg of gentamicin (Pfizer 6102210).

MLR experiments were conducted in U-bottomed 96 well plates (Iwaki 3870-096), mixing 100 µL of a mononuclear cell suspension from one devil with 100 µL of a mononuclear cell suspension from another devil. Background controls consisted of 200 µL of mononuclear cells from individual devils incubated alone and positive controls consisted of mononuclear cells of individual devils incubated with 50 µg/mL of Concanavalin A (Con A, Sigma C7275). Mixed lymphocyte cultures were incubated at 37°C and 5% CO2 for 144 hours. Mitogen controls were incubated under the same conditions for 96 hours. Eighteen hours prior the harvesting, cultures were pulsed with 1 µCi tritiated thymidine (Amersham Pharmacia Biotech TKA 310) to assess proliferation. Cells were harvested onto a filter paper and dried overnight at room temperature. Uptake of thymidine by proliferating cells was measured by radiation emittance using a scintillation counter (Pharmacia, 1214 Rackbeta). Results were obtained in counts per minute (CPM) and stimulation indices (SI) were calculated with the formula: SI = Average CPM of mixed culture/Average CPM of control cultures. Mitogen stimulated control cultures were calculated by dividing the average of incorporated thymidine in stimulated cultures by non-stimulated cultures. All SI controls were considered to be one in the MLR experiments, as no proliferation was characterised in autologous cultures. All experiments were conducted in triplicates. MLR results were considered strong when SI >10, moderate when...
5<SI<10 and low or no reaction when SI<5. To compare differences in MLR stimulation indices between groups and between ‘eastern versus eastern’, ‘western versus western’ and ‘eastern versus western’, results were log transformed and analysed with nonparametric Mann Whitney test with non-Gaussian distribution.

Skin graft procedures

Seven unrelated captive devils of eastern Tasmania were used for two-way skin grafting experiments (one of the animals was used for two procedures). All devils were three years old females, except TD 187, which was a three year old male. Table 1 shows the pairing and origin of devils for each procedure. Surgeries were performed in two devils simultaneously. Animals were premedicated with acepromazine (0.2 mg/Kg) and morphine (1 mg/Kg) injected subcutaneously. For further analgesia, xylazine 1% was injected in the subcutaneous tissue around the surgery site (not exceeding 20 mg/Kg). General anaesthesia was induced by isoflurane delivered via an endotracheal tube. The hair of the surgery site (the dorsal area of the animal) was clipped and shaved and the site disinfected with a solution of chlorhexidine and cetrimide. The surgery field was covered with a sterile fenestrated drape.

A square (3×6 cm) was drawn on the skin of the mid-dorsal region of each devil and a dermatome knife with a sterile blade was used to ‘shave’ 1 mm thick dermis and epidermis of the demarcated skin. This loose piece of skin was divided into approximately two equal pieces (approximately 3×4 cm) and one of them slid in to the cranial wound (autograft). The other piece was used to ‘shave’ 1 mm thick dermis and epidermis of the area of each devil and a dermatome knife with a sterile blade was used to ‘shave’ 1 mm thick dermis and epidermis of the cranial wound (allograft). The other piece of skin was divided into an equal part (approximately 3×4 cm), and one of them was left to the cranial wound (allograft). The other part was used to ‘shave’ 1 mm thick dermis and epidermis of the cranial wound.

Monitoring of the grafts

On Days 7, 14 and 21 the grafts were visually inspected for signs of rejection. The grafts were photographed and a punch biopsy (3–4 mm) was taken from the grafts and fixed in 10% buffered formalin solution for one to four weeks. After fixation, skin biopsy punches were processed and paraffin embedded. Four to six sections (3 μm thickness) were cut onto 3-aminotriethoxysilane (Sigma A7222) coated slides for haematoxylin and eosin staining and the same number of sections was prepared for immunohistochemistry.

Immunohistochemistry

Skin biopsies were labelled for T-cells with a rabbit anti-human CD3 antibody (Dako A0452). Isotype controls included non-specific rabbit immunoglobulin fraction (Dako X0903) and negative controls were labelled without the primary antibody (replaced with antibody diluent, Dako S0609). Sections of devil lymph nodes were used as positive controls.

Tissue sections were deparaffinised in xylene and hydrated through graded alcohol solutions to water and boiled in citrate buffer solution (pH 6) in an electric pressure cooker for 10 minutes at medium heat. Slides were left to cool to 35°C and placed in PBS. Endogenous peroxidase activity was quenched by incubating sections with a solution of 3% H2O2 for 15 minutes and non-specific protein binding was blocked with serum-free protein block solution (Dako X0909) for 30 minutes. The antibody was diluted (1:300) with antibody diluent and placed onto the slides for 60 minutes. Antibody binding was detected by placing a biotinylated link universal followed by streptavidin and horseradish peroxidase (30 minutes each) (LSAB kit, Dako K0690). A solution of dianisobenzidine (Dako K3466) was placed onto the slides for ten minutes to allow for brown colour development of positive cells and then washed with distilled water. Sections were briefly counterstained with haematoxylin for 40 seconds, dehydrated through graded alcohol solutions to xylene and coverslipped.

Histology and CD3-labelled sections were examined for signs of immunological rejection and a pathological score adapted from a previous study in human hand transplantation [30] was given to each section. Table S1 describes the criteria used to determine immune rejection.

Genotyping of skin graft recipients and donors

The seven individuals used for skin graft experiments were genotyped at MHC I α chain and MHC II β chain loci through nucleotide sequencing. Genomic DNA samples were extracted from fresh or frozen whole blood using MoBio UltraClean BloodSpin Kit. The α1 domain of MHC I genes and β1 domain of MHC II genes were amplified by PCR using previously published primers and PCR conditions [17,31]. PCR amplifications were performed on a Bio-Rad MJ Mini Personal Thermal Cycler and the Platinum Taq DNA Polymerase High Fidelity Kit (Invitrogen 11304-011) was used to ensure the lowest error rate. PCR products were isolated by running 1.8% agarose TBE gels using Bioline HyperLadder IV as size marker and purified from the gel using UltraClean 15 DNA Purification Kit (MoBio 12100-300). Purified DNA fragments were cloned in a pGEM-T Easy Vector (Promega A1360) or JM109 High Efficiency Competent Cells (Promega L1001) cloning system. Twenty-four clones were picked for each sample. Plasmids were extracted using UltraClean 6 Minute Mini Plasmid Prep Kit (MoBio 12300-250) and sequenced in two directions with T7 and SP6 primers at the Australian Genome Research Facility. Two independent PCRs were performed for each individual, and only sequence variants found in more than one PCR amplification were included in the subsequent analyses to minimize nucleotide errors yielded during PCR, cloning and sequencing. The sequencing results were quality-checked in Sequencher 4.1.4 (Gene Codes) and aligned with previously identified devil MHC alleles [17] in BioEdit using the ClustalW alignment tool [32,33].

Results

Tasmanian devils respond in mixed lymphocyte reactions

To confirm that the mononuclear cell suspensions were able to proliferate, all suspensions were incubated with the mitogen Con
A. Each of these controls resulted in high SI, indicating that experiment conditions were optimum (data not shown). There was a wide range of MLR responses among all groups, from SI<1 to SI>100. Figure 2a summarises the MLR proliferations among the eastern groups. The MLRs from devils from Mount William National Park showed the highest SI, whereas the MLRs from devils from Nugent had the lowest responses. Figure 2b illustrates the MLR responses among the western groups. The MLRs from devils from Granville Harbour and Woolnorth had the highest proliferations, whereas MLRs from devils from Milkshake Hills and Temma had the lowest responses. Because DFTD is spreading from eastern to western areas of Tasmania, we investigated the functional MHC diversity between devils from these two areas. Figure 2c shows that the MLR responses from west versus east devils were significantly higher than the MLR responses from east versus east devils and west versus west devils. In order to compare these results more effectively, the SI was characterised as either strong (SI>10), moderate (5<SI<10) or low or no reaction (SI<5). Most MLRs between devils from east and west yielded strong responses. Figure 3 illustrates the intensity of MLR responses among all groups.

Tasmanian devils reject skin allografts

Skin graft surgeries were performed to test whether allogeneic tissue from an unrelated devil would be immunologically accepted by the recipient devil. Five of the eight skin allografts and six of the eight autografts engrafted successfully, as determined by macroscopic and histological assessment. The remaining three allografts and two autografts showed pathological changes characteristic of mechanical trauma, resulting in failure of these tissues to engraft. The main histological alterations in these unsuccessful grafts were necrosis associated with polymorphonuclear cell infiltration, surface parakeratosis and fibrin deposition. All successful grafts appeared indistinguishable from the autografts at Day 7. Four of five engrafted allografts had macroscopic changes associated with immune rejection 14 days following the surgery. These changes consisted of scaly lesions, coagulative exudates and brown to black coloration of the allografted skin. For one allograft, these macroscopic changes were not visible until 17 days after the procedure. Microscopically, immune rejection was characterised as Grade II, III or IV rejection (moderate, severe or very severe, respectively). Typical alterations were moderate to severe perivascular and interstitial CD3 infiltration, dermal and epidermal lymphocytic exocytosis, usually accompanied by apoptotic keratinocytes and epidermal necrosis. Spongiosis was a common finding. Most of these alterations were recognised 14 days after the surgery, and progressed to very severe rejection on Day 21. In three cases, there was total loss of the allografted skin, hence a biopsy could not be taken. Table 2 summarises the results for all surgeries (Table S2 describes the pathological changes of the allografts in detail) and Figure 4 shows the macroscopic and microscopic appearance of a representative result from one experiment.
MHC I and II genotyping

MHC genotyping and MLR results for animals used for the skin graft surgeries are summarized in Table 3 and Table 4. TD 190 and TD 199 have exactly the same set of MHC I and II alleles. TD 188 and TD 189 have three (two MHC I and one MHC II) allelic mismatches, resulting in amino acid variations at two peptide binding sites in MHC I α1 domain. The other two pairs of individuals both have four MHC allele mismatches but no different amino acid substitutions at peptide binding sites were identified.

Discussion

The presence of a histocompatibility system should prevent the establishment of transmissible cancers in vertebrates [34,35]. Despite this, in addition to DFTD, two other naturally transmissible tumours in mammals exist. Canine transmissible venereal tumour (CTVT) [36], which affects members of the Canidae family. A transmissible sarcoma in captive Syrian hamsters (*Mesocricetus auratus*), which was either artificially or spontaneously transmitted by social interactions [37]. CTVT down-regulates MHC antigens [38] and secretes...
Table 3. MHC genotyping of Tasmanian devils used for skin graft experiments.

| Tasmanian devil ID | MHC I α1 sequence variants | MHC II β1 sequence variants |
|--------------------|-----------------------------|-----------------------------|
| TD 190             | Sahal*27, 28, 32, 35, 49    | SahadAB*01, 03, 05          |
| TD 199             | Sahal*27, 28, 32, 35, 49    | SahadAB*01, 03, 05          |
| TD 187             | Sahal*28, 32, 35, 49, 57    | SahadAB*01, 03, 05, 12      |
| TD 200             | Sahal*27, 28, 32, 35, 49    | SahadAB*01, 03, 05, 11      |
| TD 188             | Sahal*27, 32, 35, 49        | SahadAB*01, 03, 05, 15      |
| TD 189             | Sahal*27, 32, 35, 48        | SahadAB*01, 03, 05, 11, 15  |
| TD 191             | Sahal*28, 32, 34, 48, 49    | SahadAB*01, 03, 05, 13      |

TD 190 and TD 199 shared all MHC I and II alleles. The remaining devil pairs had two to three MHC I allelic mismatches and one to two MHC II allelic mismatches. doi:10.1371/journal.pone.0022402.t003

Table 4. Amino acid difference count at peptide binding sites and MLR results within skin graft devil pairs.

| Tasmanian devil ID | Amino acid difference count at peptide binding sites at MHC I α1 | Amino acid difference count at peptide binding sites at MHC II β1 | Mixed lymphocyte reaction (SI) |
|--------------------|---------------------------------------------------------------|---------------------------------------------------------------|--------------------------------|
| TD 190 and TD 199  | 0                                                             | 0                                                             | 1                              |
| TD 187 and TD 200  | 0                                                             | 0                                                             | 1                              |
| TD 188 and TD 189  | 2                                                             | 0                                                             | 17                             |
| TD 190 and TD 191  | 0                                                             | 0                                                             | 5                              |

TD 188 and TD 189 had two amino acid differences at peptide binding sites at MHC I α1 and had a strong MLR response. The other three pairs did not have amino acid difference count at peptide binding sites at MHC I α1 or MHC II β1 and had low MLR responses.

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indicate that DFTD tumour cells do not express MHC II on the cell surface (unpublished data). It has not been possible to test for MHC I expression, due to the lack of specific antibodies. In tumours with changed MHC profile, NK cells are activated and promote the killing of abnormal cells [54]. Although devils possess the expected range of immune cells [53], NK cell function has still to be explored in the Tasmanian devil. Tumours can utilise other mechanisms to evade immune recognition. Increased resistance to cytotoxic molecules, such as perforin [56], decreased tumour antigen expression [57] and secretion of immunosuppressive factors, such as IL-4, IL-10 and transforming growth factor-β1 [50,59] are alternative (or concurrent) mechanisms of immune escape.

The devil population is under threat because an allogeneic tumour cell clone is being transferred between animals without immune recognition. The host is immunocompetent [14,15], but the population has undergone bottlenecks in the past and lacks genetic and MHC diversity [16,17,60]. This homogeneity in the tumour cell clone is being transferred between animals without escape.

A number of alternative (or concurrent) mechanisms of immune escape are suggested. Factors, such as IL-4, IL-10 and transforming growth factor-β1 significantly affect patterns of disease in free-ranging and captive cheetahs (Acinonyx jubatus) populations. J Wild Dis 41: 542–549.

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Author Contributions

Conceived and designed the experiments: AK KB GMW.Performed the experiments: AK YC FK BW. Analyzed the data: AK YC KB SD GMW. Wrote the paper: AK GMW. Read and commented on the manuscript: AK YC FK BW SD KB GMW.
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