Transmission of a fatal clonal tumor by biting occurs due to depleted MHC diversity in a threatened carnivorous marsupial

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A fatal transmissible tumor spread between individuals by biting has emerged in the Tasmanian devil (*Sarcophilus harrisii*), a carnivorous marsupial. Here we provide genetic evidence establishing that the tumor is clonal and therefore foreign to host devils. Thus, the disease is highly unusual because it is not just a tumor but also a tissue graft, passed between individuals without invoking an immune response. The MHC plays a key role in immune responses to both tumors and grafts. The most common mechanism of immune evasion by tumors is down-regulation of classical cell surface MHC molecules. Here we show that this mode of immune escape does not occur. However, because the tumor is a graft, it should still be recognized and rejected by the host's immune system due to foreign cell surface antigens. Mixed lymphocyte responses showed a lack of alloreactivity between lymphocytes of different individuals in the affected population, indicating a paucity of MHC diversity. This result was verified by genotyping, providing a conclusive link between a loss of MHC diversity and spread of a disease through a wild population. This novel disease arose as a direct result of loss of genetic diversity and the aggressive behavior of the host species. The neoplastic clone continues to spread although the population, and, without active disease control by removal of affected animals and the isolation of disease-free animals, the Tasmanian devil faces extinction.

conservation genetics | Tasmanian devil | wildlife disease | immune evasion

The largest remaining marsupial carnivore, the Tasmanian devil (*Sarcophilus harrisii*), is currently under threat of extinction due to a newly emerged wildlife disease (1). Devil facial tumor disease (DFTD) is a contagious tumor that is spread between individuals as a rogue cell line through biting (2). Tumors occur predominantly around the face and neck (3) and are believed to be of neuroendocrine origin (4). During disease progression, the tumor ulcerates, becomes friable, and affected devils usually die within 3–6 months after the first appearance of lesions (3), with no documented immune response. DFTD has decreased devil numbers by 50% since its appearance in 1996, with some populations declining by 90% (5). Although DFTD is widespread across eastern Tasmania, it has not yet been observed in northwestern populations (5).

Pearse and Swift (2) proposed that DFTD cells are transferred between individuals as allografts, because tumor cells taken from different individuals contain identical, complex chromosomal rearrangements. The immune response of devils is proficient (43), and therefore transmission of cells from one individual to another should lead to rapid rejection of the cells by the host immune system, due to recognition of foreign cell surface MHC antigens.

MHC antigens are encoded by the most polymorphic set of genes in the vertebrate genome (6) and are important for pathogen, tumor, and graft recognition. There are two types of antigen-presenting MHC molecules, class I and class II. Class I molecules consist of an α-chain and an associated β2-microglobulin and present endogenous peptide antigens to cytotoxic T cells. Class II molecules consist of an α- and a β-chain and bind exogenously derived peptides for antigen presentation (7).

Class I and class II MHC genes have a highly polymorphic peptide binding region (PBR) that enables recognition of a range of antigenic peptides within a population (8). MHC genes are characterized by stretches of highly conserved amino acids, which maintain the structural integrity of the molecule, and pockets of highly polymorphic residues in the PBR, which are subject to positive selection (8, 9). In a population with high class I polymorphism, grafts between unrelated individuals will be rejected rapidly due to differences between class I alleles expressed on the surface of the donor cells and host cells.

In the presence of a functioning immune system, the failure to recognize and target DFTD could be a consequence of two possible genetic explanations. First, the tumor may "escape" the immune system by modulating expression of MHC genes during tumor growth. Second, devils may lack diversity at MHC loci, resulting in an immune system failure to recognize the tumor as "foreign." Support for these hypotheses comes from studies into other transmissible tumors. The canine transmissible venereal tumor (CTVT) is passed between individuals through coitus. Although it has now evolved into two subtypes, it originated from a single neoplastic clone >200 years ago (10). CTVT passes across MHC barriers by

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Abbreviations: DFTD, devil facial tumor disease; PBR, peptide binding region; CTVT, canine transmissible venereal tumor; SSCP, single-strand conformation polymorphism.

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Table 1. Genotypes of matched tumor and blood samples for microsatellite and MHC loci

Tumor samples	Blood samples	Location	Sh2g	Sh2l	Sh3a	Sh3o	Class I α1	Class I α2	Class II β1
T0858	B0858	Bothwell (1)	0,4 (0,0)	2,2 (2,2)	0,0 (0,2)	2,2 (0,2)	1 (1)	1 (1)	1 (2)
T1277	B1277	Forestier (2)	0,4 (0,0)	2,2 (2,2)	0,0 (0,2)	2,2 (0,2)	1 (4)	1 (1)	1 (1)
T1926	B1926	Weegana (3)	0,4 (0,0)	2,2 (—)	0,0 (—)	2,2 (2,2)	1 (2)	1 (1)	1 (—)
TC1926		Weegana (3)	0,4	2,2	0,0	2,2	1	1	1
T2024	B2024	Forestier (2)	0,4 (0,6)	2,2 (2,2)	0,0 (0,2)	2,2 (0,2)	1 (5)	1 (1)	1 (2)
T2027	B2027	Forestier (2)	0,4 (0,0)	2,2 (2,2)	0,0 (0,0)	2,2 (0,2)	1 (4)	1 (3)	1 (4)
T2772	B2772	St. Marys (4)	0,4 (0,2)	2,2 (2,2)	0,0 (0,0)	2,2 (2,2)	1 (1)	1 (1)	1 (3)
T2107	B2107	Forestier (2)	0,4 (0,6)	2,2 (2,2)	0,0 (0,2)	2,2 (2,2)	1 (3)	1 (1)	1 (3)
T2891	B2891	St. Marys (4)	0,4 (0,0)	2,2 (0,2)	0,0 (0,0)	2,2 (0,0)	1 (5)	1 (1)	1 (4)
T1857	B1857	Railton (5)	0,4 (0,6)	2,2 (0,2)	0,0 (2,2)	2,2 (2,2)	1 (1)	1 (1)	1 (4)
T2734	B2734	St. Marys (4)	0,4 (0,0)	2,2 (2,2)	0,0 (0,2)	2,2 (0,0)	1 (4)	1 (2)	1 (4)
T2336	B2336	Narawntapu (6)	0,4 (0,4)	2,2 (2,2)	— (0,2)	2,2 (2,6)	1 (4)	1 (2)	1 (4)
T1363	B1363	Forestier (2)	0,4 (0,0)	2,2 (2,2)	0,0 (0,0)	2,2 (2,2)	— (—)	— (—)	1 (—)
T2021	B2021	Forestier (2)	0,4 (0,0)	2,2 (2,2)	0,0 (0,2)	2,2 (0,2)	1 (4)	1 (1)	1 (2)
T2671	B2671	Buckland (7)	0,4 (0,4)	2,2 (2,2)	0,0 (0,2)	2,2 (2,2)	1 (2)	1 (1)	1 (1)
T2673	B2673	Buckland (7)	0,4 (0,4)	2,2 (2,2)	0,0 (—)	2,2 (0,2)	1 (5)	1 (1)	— (2)
TC3089		Bronte Park (11)	0,4	2,2	0,0	2,2	1	1	1
TC385		Forestier (2)	0,4	2,2	0,0	2,2	1	1	1
T1440		Forestier (2)	0,4	2,2	0,0	2,2	1	1	1
T416		Forestier (2)	0,4	2,2	0,0	2,2	1	1	1

Genotypes of 15 matched tumor/blood samples, 2 additional tumor samples, and 2 DNA samples derived from tumor culture for microsatellite, as well as MHC class I and class II loci. T, tumor biopsy; TC, cultured tumor cells; B, blood sample; —, data not available. For each microsatellite locus, the smallest allele was designated 0, with additional alleles numbered according to their increasing base pair length. SSCP on MHC class I α1 and α2 and class II β1 were scored according to banding patterns from 1 to 6. The values for tumor and DNA data are given, with the values for the blood samples shown in parentheses, except under Location, where the numerals in parentheses correspond to the geographic distribution of collection localities indicated in SI Fig. 5. Additional genotypes of animals that were not diseased are located in SI Table 3.

down-regulating MHC class I and class II expression and up-regulating nonclassical class I expression to avoid the natural killer cell response (10). A contagious tumor has also been reported in the Syrian hamster (11) and is thought to spread between individuals due to a lack of MHC diversity (12).

To test the allograft theory of DFTD transmission and to investigate the lack of immune response, we genotyped matched tumor and host samples at microsatellite and MHC loci. We tested the hypothesis that DFTD cells down-regulate MHC gene expression using RT-PCR on matched tumor and host samples and examined levels of MHC class I diversity.

Results

Microsatellite and MHC Genotyping: Proof of the Allograft Theory of Transmission. Fifteen matched tumor and host blood samples (Table 1) and 11 blood samples from unaffected individuals [supporting information (SI) Table 3] were genotyped at four previously described polymorphic microsatellite loci (13) and MHC class I and class II loci. Tumors sampled from eight locations throughout eastern Tasmania (SI Fig. 5) had identical microsatellite genotypes at all four microsatellite loci, as well as at MHC class I and class II loci (Table 1). In contrast, 88% (22 of 25) of examined devils had a unique multilocus genotype, and in all infected devils the tumor had a genotype different from the host (Table 1).

Tumor Cells Express Functional MHC Class I and Class II Genes. RT-PCR experiments used RNA from matched tumor cells, spleen, liver, and kidney samples from two affected individuals and four additional tumor samples. These experiments demonstrated that tumor cells express MHC class I and class II genes (Fig. 1). The MHC class I and class II sequences expressed by tumors were aligned with classical class I and class II sequences from eutherian mammals, marsupials, and previously described Tasmanian devil sequences (SI Fig. 6).

Six unique class I sequences were amplified from tumor cells using a single primer set. These sequences show between 91% and 99% amino acid identity to previously described devil classical class I sequences (e.g., SahaI*01 and EF591089 from ref. 14). Nine sites identified as important for peptide binding by classical class I molecules (9) are present in the tumor class I sequences, indicating a classical function (SI Fig. 6*A*). Cysteine residues in the α1 and α2 domains, which form disulphide bridges to stabilize the class I molecule, also are present in the tumor sequences. The 25 residues that interact with the β2-microglobulin are well conserved between the tumor sequences and marsupial class I genes. We have previously shown that the Tasmanian devil has at least five classical and two nonclassical class I genes (14). Nonclassical class I sequences exhibiting tissue-specific expression patterns, a lack of polymorphism, and phylogenetic distinctness from classical devil class I genes (14) were not expressed by the tumor. The six amplified alleles probably account for the five classical loci, with only one locus being heterozygous.

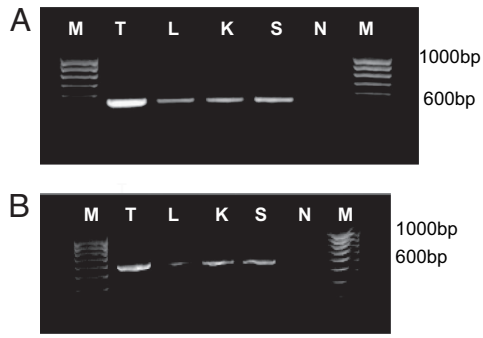

Fig. 1. RT-PCR showing expression of MHC class I (*A*) and class II (*B*) genes by matched tumor, liver, spleen, and kidney samples. T, tumor biopsy; S, spleen; L, liver; K, kidney; N, negative control; M, marker.

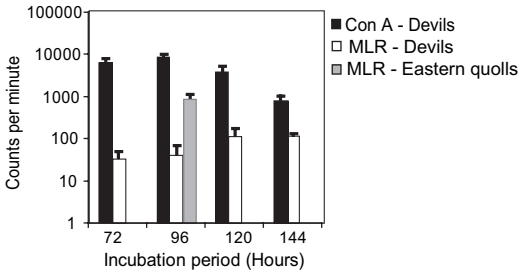

Fig. 2. Mixed lymphocyte reaction and Con A stimulation of lymphocytes from 30 devils and 2 eastern quolls obtained from the same region of Tasmania. Due to the limited amount of blood that could be collected from the quolls, only a single (96 h) time point was possible. To confirm that the devil lymphocytes could proliferate, Con A was added at a final concentration of 50 mg/ml.

Four unique class II DAB sequences, representing at least two loci, are expressed by the tumor cells and show between 96% and 100% identity to previously characterized DAB sequences (SI Fig. 6*B*) (14). The class II sequences amplified from the tumors contain a cysteine bridge, an RFDS motif for CD4 binding and an NGT glycosylation site in the β1 domain (15). Prior studies have shown that a tryptophan residue and an asparagine residue, located in the β1 domain, are important for peptide binding (SI Fig. 6*B*) (15), yet all tumor samples express sequences in which the tryptophan residue is substituted for a lysine residue. Although, a substitution at this position is rare, it has been observed in other marsupials (16), and its effects on antigen binding are unknown.

Low Polymorphism at MHC Class I Loci. Two approaches were taken to investigate levels of polymorphism in devil MHC genes. First, allogeneic responses were analyzed *in vitro* by using mixed lymphocyte reactions. Lymphocytes from 30 eastern Tasmanian devils were pooled and tested against each other as well as serving as target cells for lymphocytes from a range of eastern devils, including a northern and an island population (SI Fig. 5). No mixed lymphocyte responses were observed with any of these cultures (Fig. 2 and SI Table 4). Strong proliferative responses were observed when Con A was used as a mitogen, providing an effective positive control confirming that the cells could proliferate when provided with an appropriate stimulus. Because marsupials demonstrate low allogeneic responses (17, 18), two-way mixed lymphocyte reactions were performed by using the lymphocytes from two unrelated eastern quolls (*Dasyurus viverrinus*). Like the Tasmanian devil, the eastern quoll is a dasyurid marsupial and shares a similar habitat. When compared with the devil and assessed after 96 h of culture, the two-way mixed lymphocyte responses of the quoll lymphocytes was >20 times more effective than the mixed lymphocyte reaction of the pooled devil lymphocytes (Fig. 2). However, these responses were still not equivalent to mitogen stimulation.

Molecular typing at MHC loci of 21 devils and 19 tumors using single-strand conformation polymorphism (SSCP) identified six class I α2 domain types, six class I α1 domain types, and five class II β1 domain types (Table 1 and SI Table 3). SSCP was performed on the class I α1 and α2 domains separately, these domains were chosen for analysis because they encompass the PBR of the molecule. Of host blood samples, 78% had an identical SSCP pattern for the class I α2 domain as the tumor, 25% were identical to the tumor at the α1 domain, and 14% had an identical SSCP pattern for class II β1 as the tumor (Table 1); however, no hosts had an identical MHC type to the tumor at both class I and class II loci. Individuals with unique MHC types were selected for sequencing. Allele sequencing identified 26

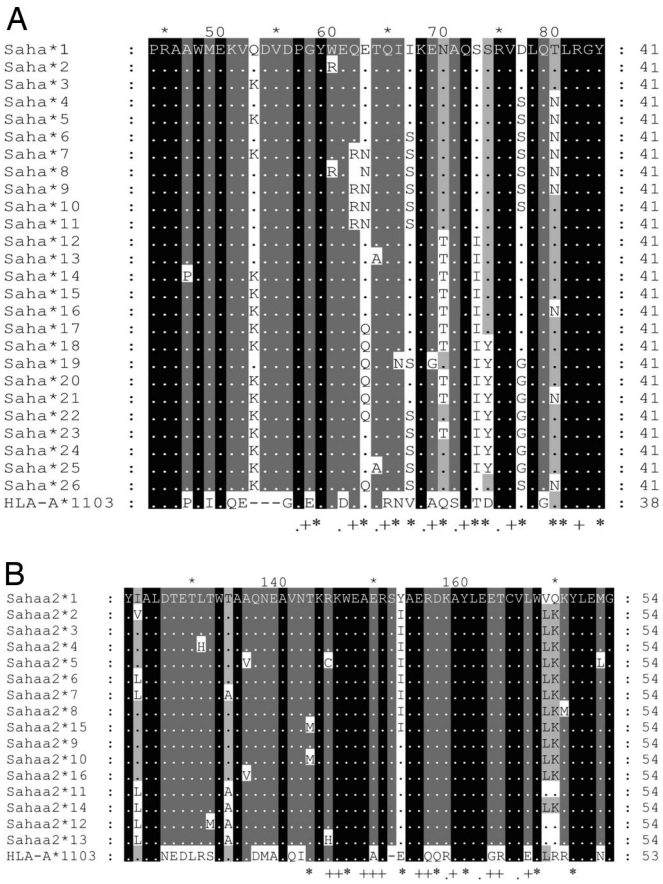

Fig. 3. Amino acid alignments of class I α1 and α2 domains from devils. (*A*) Amino acid alignment of unique class I α1 domain sequences from devils. Orientation of the residues in the molecule is indicated below the sequence as follows; asterisks, residue points toward the peptide binding site; plus sign, residue is on the α-helix pointing upward toward the binding site; dot, residue is on the α-helix pointing away from the peptide binding site. (*B*) Amino acid alignment of unique class I α2 sequences from devils. Orientation of the residues in the molecule is indicated below the sequence as in *A*.

unique class I α1 sequences (Fig. 3*A* and SI Fig. 7*A*) and 16 unique class I α2 sequences (Fig. 3*B* and SI Fig. 7*B*). These alleles were amplified with multilocus primers (14). Because of the high level of sequence similarity between the devil class I loci, it was impossible to design locus-specific primers, and estimates of class I variation are based on multiple loci. All predicted molecules appear to be capable of peptide binding. Very low amino acid diversities are found in the α2 domain (15% maximum and 14% average amino acid variation) and α1 domain (21% maximum and 16% average amino acid variation).

We used residues in the PBR, identified as highly polymorphic in human and mouse (6, 19), to compare polymorphism in devils, humans, and Gir lions (*Panthera leo persica*) (n = 25), a wild population that has undergone severe historical population reduction. The devil sequences showed far fewer substitutions at polymorphic residues than has been found in similar studies on lion and human (Fig. 4 and SI Table 5). Within the class I sequences, only 13 of a possible 38 PBR residues are polymorphic, and only two sites in the PBR have more than two substitutions.

Z tests for positive selection were performed on the α1 and α2 domains of the class I sequences at PBR sites and non-PBR sites (Table 2). In the α2 domain, there was no significant difference in the number of nonsynonymous to synonymous substitutions, and there was no evidence of positive selection on PBR or

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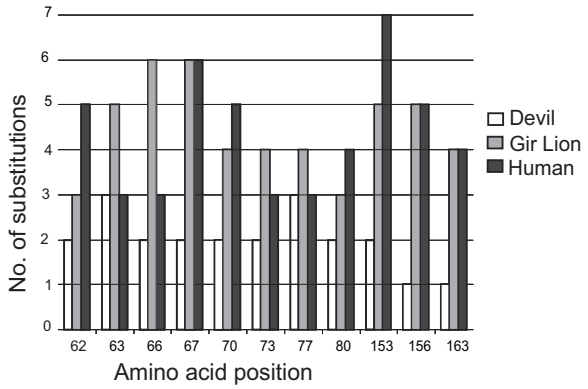

Fig. 4. Graphical representation of polymorphism in the PBR of class I sequences from humans (HLA-A, HLA-B, and HLA-C) (19), wild Gir lions (multiple loci) ($n = 25$), and Tasmanian devils (multiple loci) ($n = 25$). The y axis shows the number of substitutions at polymorphic residues involved in peptide binding. The x axis shows the amino acid position according to Bjorkman and Parham (19).

non-PBR sites. The PBR sites in α1 domain had an excess of nonsynonymous to synonymous substitutions, and a Z test indicated positive selection at PBR sites.

The class I α1 and α2 sequences amplified from the hosts were compared with the class I sequences expressed by the tumor cells. Expressed class I sequences spanning multiple domains were not amplified from the hosts, so comparisons were made by using the α1 domain and α2 domain separately. Five of the six tumor class I α1 sequences were found in one or more devil hosts; indeed, the α1 domain of tumor allele 3297T*4 was found in every devil examined. All tumor α2 domain sequences were found in one or more devil, which is unsurprising, because this domain had extremely low variation.

Discussion

Here we provide conclusive multilocus genetic evidence for the allograft theory of DFTD transmission, confirming that this disease is a clonal rogue cell line. We demonstrate that altered MHC expression, a common cause of immune evasion by tumors (20), is not responsible for a lack of immune response to DFTD, and we suggest that low MHC diversity in the devil has enabled natural transmission of tumor cells between individuals.

Although ≈90% of sampled devils were genetically unique, all examined devil facial tumors had an identical genotype at multiple microsatellite and MHC loci, substantiating the clonal nature of the tumor. In addition, the tumor genotype was different from that of all examined host devils (Table 1), verifying that the tumor cells are not "self," but came from an external source. DFTD is only the second naturally occurring transmissible tumor to be described. The only other naturally occurring transmissible tumor, CTVT, has evolved into genetically distinct subtypes over time and is essentially a nonfatal "parasite" in dogs (10). Although we found no evidence of differences in DFTD genotypes, it is important to note that the CTVT cell line is at least 200 years old (10), whereas the DFTD cell line emerged recently, ≈10 years ago (5). DFTD may, with time, evolve into a less lethal form, but maintaining devil numbers in the meantime will be difficult (1).

Table 2. Summary of synonymous (dS) and nonsynonymous (dN) substitutions for class I α1 and α2 domains

Domain	*N*	dN	dS	*Z* statistic	*P*
α1					
PBR sites	18	0.144	0.044	1.925	0.028
Non-PBR sites	24	0.014	0.004	0.952	0.172
α2					
PBR	20	0.034	0.010	0.903	0.184
Non-PBR sites	36	0.029	0.021	0.399	0.345

Many tumors, including CTVT, avoid immunological recognition by down-regulating classical class I (class Ia) expression and up-regulating nonclassical class I (class Ib) expression (10, 21, 22). By expressing class Ib molecules, tumor cells avoid recognition by natural killer cells and escape immune recognition by T cells (22). Immune evasion by DFTD does not occur due to an alteration in MHC gene expression. We showed that DFTD tumors express at least three, possibly all five, class Ia loci and do not express any class Ib loci. Surprisingly, the tumors, which are believed to be of neuroendocrine origin (4), express class II molecules. Class II molecules are usually only expressed on hematopoietic and thymic cells, including monocytes, macrophages, and B cells (23). Histological analysis of DFTD has clearly shown that T lymphocytes do not infiltrate the tumor or the metastases to draining lymph nodes, despite the expression of MHC class I and MHC class II on the tumor cells (3, 4). Consequently, T cells are not activated by the tumor itself or by tumor cells within the lymph nodes. Expression of functional, classical MHC molecules by DFTD cells, with no evidence of an immune response to the foreign tumor, is highly unusual and suggests that an alternative explanation exists for the ability of DFTD to pass between individuals as a graft.

Cytokines within the microenvironment of the tumor may play a role in tumor evasion. In CTVT, TGF-β1 plays a major role in helping the tumor evade the host response (24). Characterization of cytokine profiles during tumor growth should be conducted; however, it seems unlikely that the tumor secretes a potent suppressive factor that limits lymphocyte activation, because the culture supernatant obtained from DFTD cultures does not inhibit mitogen-induced lymphocyte proliferation (SI Fig. 8).

We therefore propose that a lack of MHC diversity in the devil is the most likely explanation that has enabled natural transmission of tumor cells, with foreign cells seen as "self" rather than "non-self." Pioneering studies by O'Brien and colleagues (25) demonstrated that a lack of graft rejection between unrelated cheetahs (*Acinonyx jubatus*) and the subsequent acceptance of foreign grafts was due to a lack of MHC diversity, the result of an ancient genetic bottleneck followed by inbreeding (25). These samples were not genotyped, although later studies confirmed low levels of class I diversity in cheetahs (26). Similarly, graft acceptance can occur between immunocompetent pocket gophers (*Thomomys bottae*), where MHC diversity is low (27). The combination of MHC genotyping and an absence of alloreactivity against non-self MHC antigens between individuals in mixed lymphocyte reactions indicates that the devil is unable to mount an immune response to DFTD due to depleted MHC diversity.

Devil class I sequences show less variation at class I loci than is found in other species, including humans, mice, and even the inbred Gir lions (Fig. 4) (20). The devil class I α2 sequences have only 14% average amino acid variation across multiple loci compared with 41% in only 10 Gir lions across multiple loci (20). At the α1 domain, the average variation for the devil sequences is 16% compared with 40% in the Gir lions. A lack of variation in the devil class I sequences also extends to the usually polymorphic PBR. The low levels of class I diversity in the devils and the high level of similarity between tumor and host types is further highlighted by the fact that all but one of the sequences expressed by the tumor are found in at least one host and that one class I sequence is found in all of the tumor and hosts samples.

An absence of mixed lymphocyte responses between devils indicates that individuals from the north, east, and southeast of

Tasmania have functionally identical MHC haplotypes. However, it should be noted that two other marsupials have documented weak mixed lymphocyte responses; the gray short-tailed opossum (*Monodelphis domestica*) (18) and the koala (*Phascolarctos cinereus*) (28). These studies prompted suggestions that the marsupial immune system is "immunologically lazy" or at least different from that of eutherians. To ensure reliability of assay conditions in the current study, positive controls, using a related carnivorous marsupial, the eastern quoll (*Dasyurus viverrinus*), were used. Quolls were found to have allogeneic responses markedly superior to those of devils. Furthermore, devil lymphocytes responded extremely well to mitogen stimulation (43), suggesting that failure to respond in the allogeneic assays was due to a failure of activation rather than an inability to proliferate. The limited genetic diversity at MHC loci and a lack of mixed lymphocyte responses strongly supports our hypothesis that the tumor is able to avoid the immune system due to a lack of diversity in MHC genes. However, conclusive evidence for this will only come from skin-grafting experiments, which are the most accurate measures of genetic immunological identity (25).

This study provides a direct link between disease susceptibility and low MHC class I diversity in a wild population (29). Low MHC class I diversity has been reported for cheetahs (25), the Hawaiian monk seal (*Monachus tropicalis*) (30), and some species of whales (i.e., *Balaenoptera physalus* and *Balaenoptera borealis*) (31); however, links with specific diseases have not been established. DFTD is a transmissible tumor that spreads through a population due to a lack of histocompatibility barriers. The disease has progressed rapidly due to low diversity at MHC loci and the propensity of devils to bite each other around the face and mouth during mating and fights for food (32). Murgia *et al.* (10) suggested that CTVT first arose in an inbred dog or wolf population, because MHC class II loci are homozygous in tumors, whereas the two class I alleles are highly similar. It is possible that CTVT evolved in much the same way as DFTD, yet over time CTVT has developed strategies to regulate cell-surface MHC expression to allow passage to MHC disparate animals.

The Tasmanian devil has undergone several population fluctuations over the last 150 years (33) and has low genetic diversity at microsatellite and MHC loci (13). Populations restricted to islands generally have reduced genetic variation (34) and are believed to be more vulnerable to novel environmental stresses (35), including disease (36). This study provides a frightening example of the potential consequences of loss of genetic diversity in a region of the genome that is vital for self/non-self recognition as well as disease resistance. These findings reinforce the need for conservation biologists to focus on genetic diversity at functionally important loci that play a role in population fitness when designing conservation strategies. For devils, ensuring maximum MHC diversity in insurance populations is paramount. In light of the devils inability to recognize the clonal facial tumors as foreign and mount an immune response, the only course of action is the isolation of unaffected animals and the ongoing removal of affected animals from the population.

Materials and Methods

Sample Collection and Processing. ***Mixed lymphocyte response experiments.*** Animals were anesthetized with isofluorane delivered via mask in an open system, and 8–10 ml of blood was taken from the jugular vein in lithium-heparinized tubes (ethical approval was given by the Animal Ethical Committee of Tasmania's Park and Wildlife Services under no. 33/2004-5 and 32/2005-6). Mononuclear cells were isolated in a gradient (Histopaque 1077; Sigma, St. Louis, MO), centrifuged, washed with RPMI medium 1640 (JRH Biosciences, Lenexa, KS) containing 100 units/ml gentamicin and 2 mM glutamine, and diluted to a concentration of 10^6 cells per milliliter.

RNA and DNA extraction from blood and tissue. DNA extractions were conducted by using the Wizard Genomics DNA purification kit (Promega, Madison, WI) and RNA extractions using TRIzol reagent (Invitrogen, Carlsbad, CA). Blood (300 μl) and $\approx$40 μg of tumor were used in the extraction process. Sample concentrations were measured by using a GeneQuant spectrometer (Amersham Pharmacia Biosciences, Little Chalfont, U.K.).

RT-PCR on MHC Class I and Class II by Tumor Cells. RNA (1 μg) was reverse transcribed by using the SuperScript III reverse transcription kit (Invitrogen). MHC class I and II sequences were amplified with primer sets 3 and 5 (SI Table 6), respectively, from two matched tumor, liver, kidney, and spleen samples and an additional four tumor samples. Class I and class II sequences also were amplified from tumor cell culture to ensure that there was no contamination of tumor RNA with host RNA. Amplification with primer sets 3 and 5 occurred in 1$\times$ buffer, 2 mM $MgCl_2$, 200 μM dNTP, each primer at 2 μM, and 0.3 μl of *Taq* polymerase (Invitrogen). The PCR cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94.0°C for 30 s, 63°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. β-actin was used as a positive control (data not shown). Positive gel bands were purified (UltraClean DNA purification kit; MO BIO Laboratories, Carlsbad, CA) and cloned into a PGEM T-EASY vector (Promega). Twenty clones were sequenced in both directions from each sample.

Microsatellite Typing of Tumors and Host. Fifteen matched tumor/blood samples, 2 additional tumor samples, 2 DNA samples derived from tumor culture, and 11 blood samples from unaffected individuals were genotyped by using the microsatellite loci Sh2g, Sh2l, Sh3a, and Sh3o (13), following the PCR conditions of Jones *et al.* (13) and visualized as previously described (37).

SSCP on Class I MHC Loci. SSCP was performed on the samples described above by using primer sets 1, 2, and 4 (SI Table 6). Amplification for primer sets 1, 2, and 4 occurred in 1$\times$ buffer, 2 mM $MgCl_2$, 200 μM dNTP, each primer at 2 μM, and 0.3 μl of *Taq* polymerase (Invitrogen). Cycling conditions for primer sets 1 and 2 were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 63°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. Cycling conditions for primer set 4 were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 57°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. SSCP was performed according to the protocol put forth by Sunnucks *et al.* (38).

Sequencing MHC Class I Alleles. Individuals with unique SSCP patterns for MHC class I α1 and α2 domains were identified, and the class I α1 and α2 domains of these individuals were amplified separately by using primer sets 1 and 2 under the conditions described above. Positive gel bands were gel-purified and sequenced as described above.

Sequences were edited and quality-checked with Sequencher 4.1.4 (Gene Codes, Ann Arbor, MI) and BioEdit (39). To estimate PCR and cloning error, we performed independent PCRs on two individuals and sequenced 25 clones from each individual. We estimated that 10% of clones sequenced contained at least 2 bp of cloning error. This figure is more conservative than has previously been reported in the literature (40). Clustal W was used to align sequences, with some manual adjustments (41). The number of synonymous mutations per synonymous site and the number of nonsynonymous substitutions per nonsynonymous site were calculated by using Mega 3.1 (42). Mega 3.1 also was used to test for positive selection in PBR and non-PBR residues separately using the modified Nei–

Gojobori method with Jukes–Cantor adjustment for multiple substitutions at a single site.

Mixed Lymphocyte Responses. Blood from 30 Tasmanian devils from different regions of eastern Tasmania, Australia (SI Fig. 5) was collected, and mononuclear cells were isolated as described above and frozen in a solution of 10% DMSO and 90% FCS at −80°C. When required, cell suspensions were thawed, pooled, and diluted to a concentration of 10^6 cells per milliliter.

Mixed lymphocyte reactions were performed in 96 U-bottomed-well plates in triplicate and incubated from 72 to 168 h at 37°C and 5% CO_2. A pool of plasma from 10 healthy Tasmanian devils was used as a supplement for the cell culture in all experiments. Cells were pulsed with 1 μCi of [methyl-^{3}H]thymidine (Amersham Pharmacia Biosciences) 18 h before harvesting onto a glass-fiber filter paper, and radiation uptake was counted in an automated scintillation counter (LKB–Wallac, Turku, Finland). Results are expressed as cpm or stimulation index, where the stimulation index is the average cpm obtained from the mixed lymphocyte reaction divided by the average background cpm of effector cells alone. As a control for the cell viability and proliferative capacity, cells were incubated with Con A at 50 μg/ml (Con A; Sigma), and the stimulation index was calculated as described above. In addition, a two-way mixed lymphocyte reaction was performed with lymphocytes from two eastern quolls. Eastern quoll pooled plasma was used as a supplement for the cell culture and incubated for 96 h. Other incubation conditions were as described above.

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