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.

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

<table>
<thead>
<tr>
<th>Tumor samples</th>
<th>Blood samples</th>
<th>Location</th>
<th>Sh2g</th>
<th>Sh2l</th>
<th>Sh3a</th>
<th>Sh3o</th>
<th>Class I α1</th>
<th>Class I α2</th>
<th>Class II β1</th>
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<td>2.2</td>
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<td>1 (1)</td>
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<td>1 (4)</td>
</tr>
<tr>
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<td>B2672</td>
<td>Buckland</td>
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<td>2.2</td>
<td>0.0</td>
<td>2.2</td>
<td>1 (5)</td>
<td>1 (1)</td>
<td>1 (4)</td>
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<td>Bronte Park</td>
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<td>1 (2)</td>
<td>1 (4)</td>
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<td>1 (2)</td>
<td>1 (4)</td>
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<tr>
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<td>1 (2)</td>
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</table>

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 and class II loci. 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).

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.
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. 6B) (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. 6B) (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 α1 domain types, six class I α2 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 unique class I α1 sequences (Fig. 3A and SI Fig. 7A) and 16 unique class I α2 sequences (Fig. 3B and SI Fig. 7B). 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 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
and Parham (19). Tide binding. The

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

<table>
<thead>
<tr>
<th>Domain</th>
<th>N</th>
<th>dN</th>
<th>dS</th>
<th>Z statistic</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>α1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBR sites</td>
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<td>0.044</td>
<td>1.925</td>
<td>0.028</td>
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<td>Non-PBR sites</td>
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<td>0.014</td>
<td>0.004</td>
<td>0.952</td>
<td>0.172</td>
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<tr>
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<td>0.903</td>
<td>0.184</td>
</tr>
<tr>
<td>Non-PBR sites</td>
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<td>0.029</td>
<td>0.021</td>
<td>0.399</td>
<td>0.345</td>
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</table>

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).

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 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.

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
Materials and Methods

ongoing removal of affected animals from the population. In light of the devils inability to recognize the clonal facial when designing conservation strategies. For devils, ensuring tion as well as disease resistance. These findings reinforce the (35), including disease (36). This study provides a frightening belived to be more vulnerable to novel environmental stresses at microsatellite and MHC loci (13). Populations restricted to islands generally have reduced genetic variation (34) and are a population due to a lack of histocompatibility barriers. The 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). Murga 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 isoflurane 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.

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 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 1x buffer, 2 mM MgCl2, 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°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 1x buffer, 2 mM MgCl2, 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-
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% CO₂. A pool of plasma from 10 healthy 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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