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Immunology of a Transmissible Cancer Spreading among Tasmanian Devils

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Devil facial tumor disease (DFTD) is a transmissible cancer that has killed most of the Tasmanian devil (Sarcophilus harrisii) population. Since the first case appeared in the mid-1990s, it has spread relentlessly across the Tasmanian devil’s geographic range. As Tasmanian devils only exist in Tasmania, Australia, DFTD has the potential to cause extinction of this species. The origin of DFTD was a Schwann cell from a female devil. The disease is transmitted when devils bite each other around the facial areas, a behavior synonymous with this species. Every devil that is ‘infected’ with DFTD dies from the cancer. Once the DFTD cells have been transmitted, they appear to develop into a cancer without inducing an immune response. The DFTD cancer cells avoid allogeneic recognition because they do not express MHC class I molecules on the cell surface. A reduced genetic diversity and the production of immunosuppressive cytokines may also contribute. The Journal of Immunology, 2015, 195: 23–29.

Transmissible cancers are exceedingly rare, as allore cognition of tumor cells should be the final barrier should tumor cell transfer occur (1). A number of obligatory events are required for any cancer to be transmissible. The primary “index” tumor must occur in an accessible site, a mode of transmission is essential, the tumor cells must avoid allore cognition, and they require the capacity for infinite growth. In combination, these simultaneous requirements make the development of transmissible tumors extremely unlikely. Examples in humans are limited, but include maternal–fetal transfer of melanoma in utero (2), as well as those resulting from a medical procedure such as transplantation where the recipient is immunosuppressed (3) or closely related to the donor (4). In human cases, transmission to tertiary individuals has not been documented. Devil facial tumor disease (DFTD) is a current example of a naturally occurring transmissible cancer that meets the above criteria.

Understanding the immunology and immune escape mechanisms of this cancer has implications for tumor transmissibility and potential relevance for human cancers.

In the mid-1990s in the far northeast corner of Tasmania, the island state to the south of mainland Australia (Fig. 1A), a single Schwann cell from a female Tasmanian devil (Sarcophilus harrisii) developed into a cancer (5, 6). It is still growing today. This is because devils bite each other, facilitating continuous transmission of the cancer cells (7). Because the cancer occurs in the facial region, it was designated as DFTD (8). This cancer is always fatal. In <20 y DFTD has spread across most of Tasmania (Fig. 1B–D), causing a catastrophic crash in devil numbers (9). The decline is so severe that the devil is listed as endangered at international (International Union for Conservation of Nature “Red List of Threatened Species”), national, and state levels.

DFTD is one of only two currently known transmissible tumors. The other is canine transmissible venereal tumor (CTVT). For >10,000 y, and usually during coitus, these tumor cells have been transmitted between dogs (10). CTVT does not metastasize and does not usually cause the death of the host (11). There are many excellent articles on CTVT (12–14) and reviews comparing CTVT and DFTD (15, 16). This review concentrates on DFTD.

The Tasmanian devil and DFTD

Tasmanian devils are the world’s largest marsupial carnivore and exist only in Tasmania. They are generally nocturnal scavengers that feed on carrion, but sometimes hunt for small mammals and birds (7). They usually have black fur with distinctive white markings on the rump and chest (Fig. 2A) and weigh between 4.5 and 13 kg (7). Devils have extremely powerful jaws and teeth that enable them to completely devour their prey. Since the extinction of the Tasmanian tiger (Thylacinus cynocephalus), the Tasmanian devil is now the principal native carnivore in Tasmania (7). It therefore has a vital role in the ecosystem.

DFTD usually presents as large, solid, soft tissue masses with flattened, centrally ulcerated, and exudative surfaces (Fig. 2B).
It is commonly found in the oral, facial, or neck region or inside the mouth. Necrosis, ulceration, exudation, and bacterial contamination are frequent. It often metastasizes to regional lymph nodes, lungs, and spleen (17).

Histologically, the cancer cells in DFTD appear as pleomorphic round cells with a high nuclear to cytoplasmic ratio, fibrillar, eosinophilic cytoplasm, and often indistinct cell borders (Fig. 2C). DFTD cancers are well vascularized and display high mitotic indices. Lymphoid cell infiltration is usually poor (17). Transcriptome and miRNAome studies formally identified a Schwann cell origin for DFTD (6). Expression of periaxin, a myelin protein, has proven to be a reliable diagnostic marker for DFTD (Fig. 2D) (18).

The most unusual characteristic of DFTD is its transmissible nature. Early epidemiological observations suggested that DFTD is transmissible, as the disease spread geographically over time (Fig. 1B–D), but the infectious agent was unknown (7). That the cancer cells themselves are the infectious agent was confirmed by independent studies. The original suggestion was based on chromosomal analysis, as different DFTD tumor isolates shared similar complex chromosomal rearrangements. One diseased devil had a pericentric inversion of chromosome 5 in its constitutional karyotype, but this inversion was not observed in chromosome 5 of the tumor it bore, demonstrating that it was not endogenously derived (19). DNA sequencing showed that the DNA of the cancer cells and the host devil are different (6). Whole-genome analysis indicated that all DFTDs share point variants, chromosomal alterations, and copy number changes, which are distinct from their hosts (5, 20). Molecular studies on the MHC genes of DFTD tumors showed that the tumors had identical genotypes at multiple microsatellite and MHC loci (21). All of this research highlights that the karyotype and genotype are consistent between DFTD tumors and distinct from those of their hosts and supports the transmissible nature of the tumor.

**DFTD transmission and immunology of Tasmanian devils**

Devils bite each other on the face and neck, and the penetrating bites cause substantial wounds (22). The transfer of DFTD cells occurs when diseased devils bite healthy devils or when healthy devils bite diseased devils (23). Males and females are equally susceptible to DFTD. There is no evidence for vertical transmission, as anecdotal reports indicate that DFTD diseased mothers do not transfer the cancer to their offspring. Transmission occurs only between devils that display biting behaviors (24).

The biting accounts for the transmission, but to become established the “transplanted” cancer cells must avoid allogeneic recognition. But how does this occur? One possibility is that devils have a compromised or rudimentary immune system. Studies on a range of marsupials have provided evidence suggesting that they have an immune system that is less effective than that of placentals. This was originally based on the slower immune response of marsupials compared with placentals (25). A weak secondary response often follows the prolonged primary response (26). Mitogen-induced transformation (PHA, Con A, and PWM) and MLR responses (allogeneic and xenogeneic) in Matschie’s tree kangaroos (*Dendrolagus matschiei*) were up to 6-fold lower than in placentals (27). Low Ag-specific responses for humoral and cell-mediated immunity led to the suggestion that the koala is “immunologically lazy” (28), and weak cell-mediated responses have been observed with captive marsupials (29, 30). These initial investigations into marsupial immunology were hindered by a lack of marsupial-specific reagents and molecular
knowledge of the marsupial immune system. It is likely that the marsupial immune system is different from, rather than inferior to, the immune system of placental mammals, especially considering that marsupials are born at a very immature stage of development (25). As a model for the marsupial immune system, lymphocytes from the tammar wallaby were stimulated with mitogens and analyzed for proliferation and cytokine production. The results prompted the authors to conclude that the marsupial’s responses had “the hallmarks of T cell activation displayed by placental mammals” (31).

In the absence of specific reagents, it was necessary to undertake classical immunological experiments to investigate the devil’s immune system. This included basic hematology, histology, and functional assays such as mitogen-induced proliferative assays and hemagglutination assays for measurement of Ab production. Differential WBC counts did not reveal anything abnormal, as WBCs were present and were of similar size and appearance to those of other placental mammals (32). Lymphoid organs, including the thymus, spleen, and lymph nodes, were identified in all animals, including juveniles, and all structures were similar in appearance to those in placental mammals (32, 33). T lymphocytes expressed cell surface CD3, monocytes expressed MHC class II (MHC-II), and B lymphocytes expressed CD79 (34). Identification of lymphocyte subsets required the development of specific mAbs (35). This allowed the identification of CD4+ and CD8+ T cells in the thymus, adult lymph nodes, spleen, BALT, and GALT. Similar to mammalian lymphoid tissues, there were more CD4+ than CD8+ cells, specifically in the lymph nodes and splenic white pulp. Their anatomical distribution was also consistent with mammalian lymphoid tissue organization. Lymph nodes, spleen, BALt, and GALT contained IgM+ and IgG+ B cells. Based on the expression of CD1a, CD83, and MHC-II, dendritic cells were identified in the lymph node, spleen, and skin (35). Consequently, Tasmanian devils have the appropriate immune system components for effective immunity.

At a functional level there was no evidence for any defects in immunity. The neutrophils of Tasmanian devils effectively phagocytose bacteria (32). When PBMCs were challenged with the mitogens, strong proliferative responses occurred with PHA, Con A, and PWM, but not with LPS (32). Devils immunized with horse RBCs and analyzed by hemagglutination assays provided evidence for strong Ab responses (36). Xenogeneic immunization with cells from the human erythroleukemia line K562 confirmed that Tasmanian devils are capable of forming humoral responses and can respond to cellular Ags (37).

The production of Abs following challenge with cellular immunogens requires T cell help, implicating the involvement of the cell-mediated arm of the immune system. Evidence for cell-mediated immunity was also provided in the form of cytotoxicity following immunization with K562 cells. PBMCs from Tasmanian devils immunized with K562 cells killed these target cells when cocultured in vitro. It was most likely that the cytotoxicity was mediated by NK cells (37). These results provide support for a competent immune system and suggest
that inherent immunodeficiency is unlikely to be responsible for the transmission of DFTD. Consequently, other explanations were required to account for the establishment of transmitted DFTD cells. Potential explanations to account for the lack of allogeneic recognition of DFTD tumor cells include a lack of genetic (MHC) diversity or immune evasion strategies.

Potential DFTD immune escape mechanisms: reduced genetic diversity

A proposal to account for how DFTD cells could establish in unrelated hosts was that devils lack genetic diversity and the DFTD cells were not recognized as “foreign” by the host devil’s immune system (21). It was assumed knowledge that Tasmanian devils lack genetic diversity, potentially due to a “founder effect,” whereby the current population was established from low numbers of devils (38). Microsatellite analysis across a range of Tasmanian devils revealed moderate genetic variability (39). Based on genome sequencing of Tasmanian devils, it is clear that devils have a modest genetic diversity (20). Indeed, historical data indicate that Tasmanian devils have existed for many centuries with low genetic diversity (40). The low genetic diversity is also apparent in the MHC gene loci, and at one stage provided the best explanation to account for the lack of allogeneic recognition of DFTD (21). A precedent could be found in the cheetah (Acinonyx jubatus) population studied by O’Brien et al. (41). These animals had a lack of MHC diversity, most likely due to a combination of a genetic bottleneck and inbreeding (42). Skin grafts between unrelated cheetahs did not induce an acute graft rejection response, providing functional evidence for limited MHC diversity.

A similar situation in Tasmanian devils might explain the transmission of DFTD “allografts.” Acceptance of skin allografts between Tasmanian devils would be an important test of the theory that limited MHC diversity is responsible for a lack of tumor allograft recognition (21). However, the low genetic diversity does not appear to account for the lack of graft recognition, as skin allografts between devils were immunologically rejected. The rejection was clearly apparent macroscopically and microscopically within 14 d after the skin graft, with evidence of T lymphocyte infiltration (43).

MLRs provide an in vitro assessment of MHC differences and allogeneic reactions (44). Initial studies using pooled devil lymphocytes as stimulators showed low MLR responses (21). When two-way MLRs were performed, there was a range of responses. The greatest responses were found between geographically separated devils (43). These devils had substantial differences in the MHC nucleotide sequences (45). The combination of skin grafts and MLR results suggests that devils should be able to mount an effective, allogeneic immune response.

Potential DFTD immune escape mechanisms: immunosuppressive cytokines

Cancer cells have developed various immune escape mechanisms to avoid immunological destruction. Immunosuppressive cytokines have been used by tumors of Schwann cell origin as an immune escape mechanism (46). There is evidence for this in DFTD, as tumor transcriptome analysis identified transcripts for IL-10, IL-6, vascular endothelial growth factor-α, and TGF-β. However, there did not appear to be any significant upregulation of any of these cytokines in tumor tissue compared to control tissue (47). It was unknown whether these transcripts were translated to protein, as this could have direct implications in the immune response.

Abs that cross-reacted with Tasmanian devil IL-10 and TGF-β were used to analyze their presence in the tumor microenvironment. IL-10 was detected in the tumor cells in 19 of the 21 devil facial tumor samples analyzed, and TGF-β was detected in 10 of 18 devil facial tumors (L.J. Howson, unpublished data). As this preliminary screen involved one sample per tumor, it cannot be ruled out that the negative samples may have been due to sampling. However, this is unlikely because when the cytokines were detected, the staining was homogeneous throughout the tumor.

In studies of humans and mice, IL-10 production by tumor and host cells is one of the most important mediators of immune suppression in cancer (48). Its presence in DFTD samples implies an immunosuppressive role, potentially due to the potent inhibitory effects of IL-10 on APCs, interfering with the Ag processing and maturation capabilities of these cells (49) as well as the maintenance of tolerogenic immune responses (50). These immunosuppressive biological actions of IL-10 have also been described in studies of marsupial IL-10 (51) and are likely to be similar with the Tasmanian devil.

In humans and mice, TGF-β suppresses the immune response by directly targeting and downregulating the development and function of T cells, macrophages, and dendritic cells (52). TGF-β can also induce T regulatory cell development, resulting in the induction of immunological tolerance (53). These biological actions have also been described for marsupial TGF-β (51), and they support the utilization of TGF-β by some of the devil facial tumors to escape immune detection. If these cytokines were to play a major role, it would be expected that tumors would be uniformly positive. As this was not observed, the immunosuppressive cytokines may contribute to, rather than cause, suppression.

Potential DFTD immune escape mechanisms: MHC class I downregulation

Cancer cells can downregulate MHC class I (MHC-I) to evade the immune system (54). Epigenetic downregulation of MHC components has also contributed to cancer development (55). This could impair the recognition of allogeneic tumor cells by the adaptive immune system. In the absence of Abs to detect devil MHC-I or β2m-microglobulin (β2m), initial work relied on the detection of MHC-I transcripts. These were identified in tumor tissue (21), indicating that functional MHC genes were present.

MHC-I downregulation can occur via two molecular pathways: disruption to the assembly of MHC-I and β2m in the endoplasmic reticulum and impairment of peptide production and transport into the endoplasmic reticulum. These processes rely on the expression and function of Ag-processing components, with PSMB8 and TAP being commonly targeted (56). Schwann cell tumors, such as the malignant peripheral nerve sheath tumor cell line mentioned above, downregulate Ag-processing components (57). This poses the possibility that Ag-processing machinery may be altered in DFTD. Preliminary analysis of β2m and PSMB8 transcripts in devil facial tumor samples indicated that their expression was lower in devil facial tumor compared with spleen, but higher compared with heart (L.J. Howson, unpublished data). As β2m and PSMB8 represent two separate molecular pathways
for the downregulation of MHC-I, their correlation and relatively low expression suggest that they may be coordinately downregulated. The best evidence for altered MHC-I expression must come from the detection of the proteins. This work has now been completed and shows that the DFTD cells do not express MHC-I Ags on their cell surfaces (58).

The loss of cell surface MHC-I was shown to be due to a coordinated downregulation of Ag-processing genes, including β2m, TAP1, TAP2, and CIITA (58). This occurred by epigenetic regulation rather than structural mutations. We found the expression of β2m, TAP1, and TAP2 genes could be upregulated in vitro with either the histone deactylase inhibitor trichostatin A or with recombinant devil IFN-γ. CIITA expression was upregulated by IFN-γ, but not trichostatin A (58), and therefore IFN-γ was more effective in re-establishing MHC-I expression. As DFTD is of Schwann cell origin, the low expression of Ag-processing genes may be a characteristic inherited from the Schwann cell. In general, human neural cells have relatively low expression of β2m and PSMB8 compared with other tissues (59), and thus it is likely that Tasmanian devil neural cells are also low in their expression.

Loss of MHC-I expression provides an explanation for a lack of an acute allogeneic response to the foreign DFTD cells. As DFTD causes the death of the host relatively quickly, there would be insufficient time for minor histocompatibility Ags to play a role in generating an immune response. It is possible that as the tumor host relationship evolves, eventually some devils may respond against the minor Ags.

**Immune response to DFTD**

Observations that all wild Tasmanian devils infected succumb to DFTD within a short period of time (7) indicate that there is no effective host immune response against the disease. Histology data from DFTD tumor biopsy samples support this, as DFTD progresses with only a limited number of CD3+ T cells infiltrating some devil facial tumors (17, 33). Unexpectedly, when there was CD3+ T cell infiltration, CD8+ T cells were more prevalent than CD4+ T cells. Currently there is no evidence for any devils recovering from DFTD (8, 23), and thus the CD8+ T cells within tumors are not contributing to an effective antitumor immune response. Given the dearth of CD4+ T cells, within or near the tumors, it is unlikely that CD4+ T regulatory cells would be immunosuppressive. With the absence of specific reagents to identify other T cell populations, such as γδ T cells, it is unknown whether these populations are involved. This would appear unlikely, as most of the CD3+ T cells were CD8+.

Another population of cells that could suppress T cell immunity is the myeloid-derived suppressor cell population (60). MHC-II+ cells have been identified in DFTD tumors (36). It is unknown which cell type is expressing MHC-II, but these...
are potentially dendritic cells and macrophages. The macrophages could include myeloid-derived suppressor cells. PBMCs from DFTD-infected Tasmanian devils are not cytotoxic against DFTD cells in vitro. An analysis of serum samples from wild infected devils did not identify Abs to DFTD cells (61). These latter two points, combined with the lack of MHC expression and limited T cell infiltration, suggest that the devil’s immune system is unresponsive to DFTD development. According to the “missing-self hypothesis,” the absence of MHC-I should make DFTD cells targets for NK cells (62). However, this does not appear to be occurring. This lack of spontaneous killing of DFTD cells in vitro is not due to an absence of NK cells, as there is functional evidence for these cells in the peripheral blood of Tasmanian devils (37). It is possible that DFTD cells do not express appropriate activating receptors or express NK inhibitory receptors. These studies collectively suggest that “immunological ignorance” accounts for the failure of DFTD recognition by cells of both the innate and adaptive immune systems (Fig. 3).

If DFTD cells injected into mice induced an immune response, it would indicate that they are immunogenic. DFTD xenografts are accepted in immunocompromised NOD/SCID mice (43), but they are rejected by immunocompetent BALB/c and C57BL/6 mice (63). This rejection correlates with the development of Abs, cytokines, and cytotoxicity. In the mouse environment, DFTD cells are therefore immunogenic, and the ability of the DFTD cells to produce TGF-β and IL-10 does not appear to inhibit this response, at least in the mouse. Immunogenicity of DFTD cells in the devil host/environment, as determined by Abs to DFTD cells, has not generally been observed in wild infected devils (61). One exception appears to be two wild devils that had low levels of Abs (23). This still needs confirmation, as it is unknown whether these Abs were specific for DFTD cells or merely cross-reactive.

The ability to induce an immune response to DFTD cells may require modification of the cells and/or the presence of adjuvants to active innate immune cells, potentially via TLRs. When healthy devils received multiple immunizations with killed DFTD tumor cells in the presence of CpG, there was evidence for immune responses, including cytotoxicity and Ab production, in five of the six devils immunized (64). These responses provide encouragement that devils can produce an immune response against DFTD cells, once tolerance (or ignorance) is broken. One of these devils was challenged with viable DFTD cells and appeared to be protected from DFTD. This protection was short-lived, as rechallenge 1 y later resulted in tumor growth. Refinements of the immunization protocol are now required to optimize the immune response before it could be used to protect Tasmanian devils against DFTD. Such refinements would need to overcome the major immune escape mechanism of DFTD cells, the downregulation of MHC-I (58). As devils can reject skin grafts and PBLs respond in MLRs (43), the ability to re-establish MHC-I expression with IFN-γ should induce an allogeneic response against the tumor cells. Consequently, a cellular vaccine with MHC DFTD cells would be expected to prime devil T cells to DFTD peptides presented by tumor MHC molecules. Our recent results provide convincing evidence that TLRs are functionally expressed on devil leukocytes (A.L. Patchett, R. Latham, K.H. Brettingham-Moore, C. Tovar, A.B. Lyons, and G.M. Woods, submitted for publication). Thus adjuvants that stimulate via TLRs could be included to boost an immune response that would protect against DFTD, forming the basis of a vaccine.

Conclusions
Devil facial tumor disease arose in a female Tasmanian devil sometime before 1996 (7). Since then it has been relentlessly passed on as an allograft, assisted by the biting behavior exhibited during devil feeding and mating. This has resulted in a severe population decline and the listing of the Tasmanian devil as endangered at international (International Union for Conservation of Nature “Red List of Threatened Species”), national, and state levels. A downregulation of MHC-I expression on the tumor is the most likely reason rejection does not occur in newly infected animals (58).

A significant advance has been the demonstration that expression of MHC-I by cultured DFTD cells can be induced using cytokine or drug treatment (58), confirming that DFTD cells retain the structural genes and processing machinery that are necessary for display of MHC-I on the cell surface. A major impediment to developing an immune-based strategy to combat disease transmission has been the restricted number of devils available for research purposes. This highlights the difficulty of working with endangered species. Coupled with the paucity of devil-specific immune reagents, including mAbs, and a lack of information on marsupial immunity, there have been significant challenges to be overcome. However, the ability to re-express MHC-I, along with the demonstration that adjuvanted DFTD preparations can induce humoral and cell-mediated immune responses in Tasmanian devils, suggests that an immunological approach to stop the spread of this disease is feasible. This warrants a significant research effort to produce a protective vaccine to help save this iconic species.

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