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INVITED REVIEW

Two Decades of the Impact of Tasmanian Devil Facial Tumor Disease

Gregory M. Woods,[1,*] Samantha Fox,[†,‡] Andrew S. Flies,[*] Cesar D. Tovar,[*,§] Menna Jones,[¶] Rodrigo Hamede,[¶] David Pemberton,[†] A. Bruce Lyons[§] and Silvana S. Bettiol[§]

*Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania 7005, Australia; [†]Save the Tasmanian Devil Program, DPIPWE, GPO Box 44, Hobart, Tasmania 7001, Australia; [‡]Toledo Zoo, 2605 Broadway, Toledo, OH 43609, USA; [§]School of Medicine, College of Health and Medicine, University of Tasmania, Hobart, Tasmania 7005, Australia; [¶]School of Natural Sciences, University of Tasmania, Private Bag 55, Hobart, Tasmania 7001, Australia

[1]E-mail: G.M.Woods@utas.edu.au

Synopsis The Tasmanian devil, a marsupial carnivore, has been restricted to the island state of Tasmania since its extinction on the Australian mainland about 3000 years ago. In the past two decades, this species has experienced severe population decline due to the emergence of devil facial tumor disease (DFTD), a transmissible cancer. During these 20 years, scientists have puzzled over the immunological and evolutionary responses by the Tasmanian devil to this transmissible cancer. Targeted strategies in population management and disease control have been developed as well as comparative processes to identify variation in tumor and host genetics. A multi-disciplinary approach with multi-institutional teams has produced considerable advances over the last decade. This has led to a greater understanding of the molecular pathogenesis and genomic classification of this cancer. New and promising developments in the Tasmanian devil's story include evidence that most immunized, and some wild devils, can produce an immune response to DFTD. Furthermore, epidemiology combined with genomic studies suggest a rapid evolution to the disease and that DFTD will become an endemic disease. Since 1998 there have been more than 350 publications, distributed over 37 Web of Science categories. A unique endemic island species has become an international curiosity that is in the spotlight of integrative and comparative biology research.

Introduction to the Tasmanian devil and devil facial tumor disease

The Tasmanian devil (*Sarcophilus harrisii*), is a member of the Dasyuridae family and is the world's largest carnivorous marsupial, now endemic to the island of Tasmania, Australia. The devil is most commonly found along Tasmania's eastern half, and northern and northwest coastline in dry forests, woodlands, and coastal scrub (Jones and Barmuta 2000). Devils are the top predator in Tasmanian ecosystems (Hollings et al. 2016) and the dominant scavenger with morphological specializations for consuming bone (Jones 2003). Devils are primarily nocturnal, with a home range in the order of 4–27 km^2 and a mean daily movement of 9 km (Pemberton 1990). The social behavior of sexual contact and competition for food leads to fighting which is characterized by biting, with many of the bites occurring on the face (Hamede et al. 2008).

Tasmanian devils have been isolated in Tasmania for approximately 14,000 years after sea level rise created Bass Strait (Lambeck and Chappell 2001). Devils are now considered endemic to Tasmania following their extinction on the Australian mainland about 3000 years ago (Bruniche-Olsen et al. 2018; White et al. 2018). The extinction was likely driven by El Niño Southern Oscillation (ENSO)-related climate change with additional direct and indirect pressures from human intensification (Johnson and Wroe 2003; Prowse et al. 2014) and the introduction of dingoes (Letnic et al. 2012). Guiler (1982) reported very low populations of devils in the first half of the 1900s, coinciding with the population crash of the thylacine.

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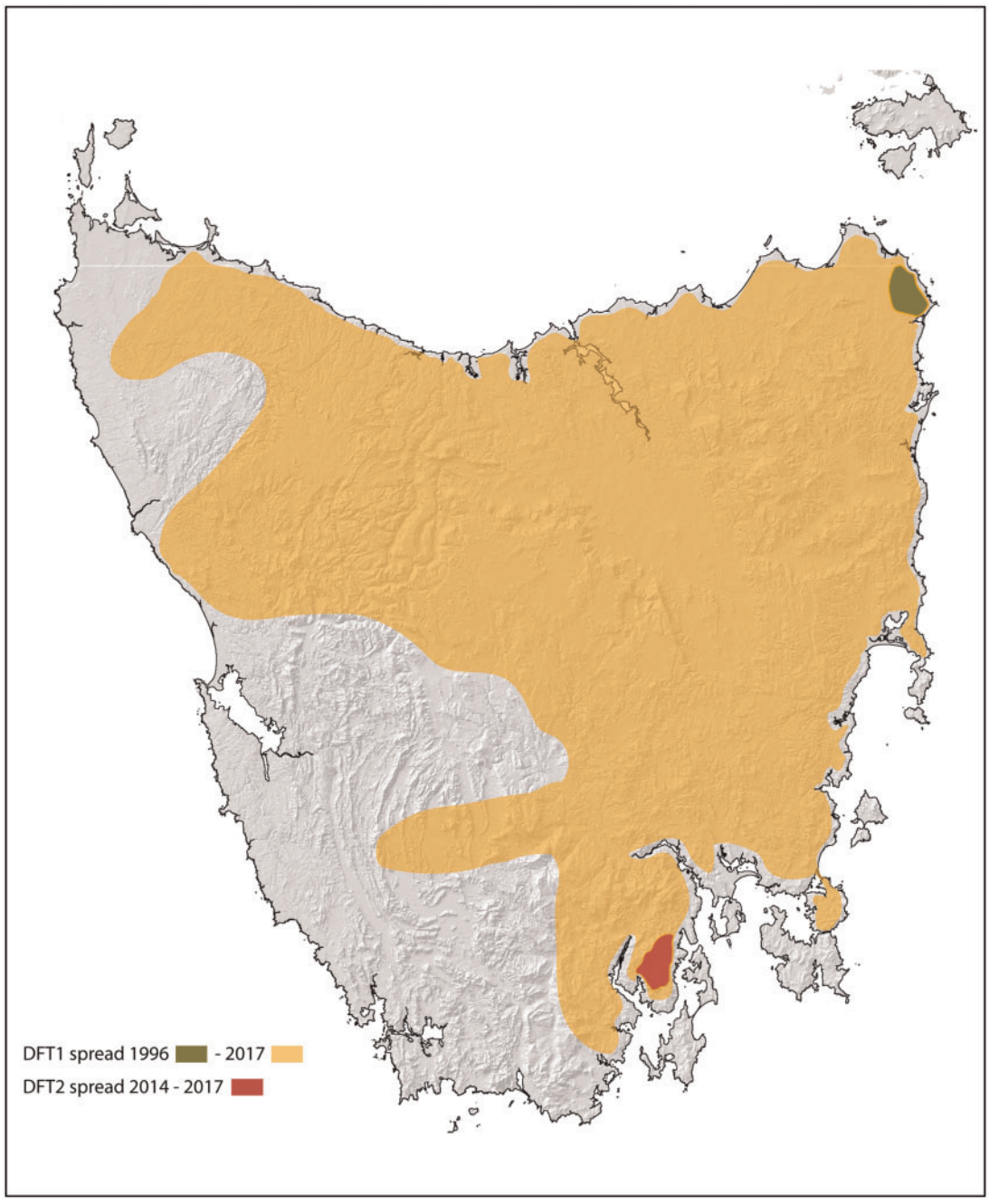

Fig. 1 Spread of DFTD. DFT1 was first recorded in the north-east Tasmania (green) in 1996. The first recorded case of DFT2 was separated by distance and time to DFT1 as the first case of DFT2 (red) was in 2014 in south east Tasmania. DFT1 has spread over most of the island, whereas DFT2 is contained to a small area.

The emergence of the fatal transmissible cancer, DFTD, in 1996 at Waterhouse Point in north-east Tasmania has caused an overall decline in the devil population by approximately 80% (Lazenby et al. 2018). Local declines of up to 95% have been recorded (McCallum et al. 2007; Lazenby et al. 2018). The first case of a second, and independent, transmissible cancer was detected in south-east Tasmania in 2014 (Pye et al. 2016b). Consequently, DFTD comprises two independent transmissible cancers, DFT1 (first identified in 1996) and DFT2 (first identified in 2014). DFT2 is currently restricted to the southeast of the island whereas DFT1 is spread across most of mainland Tasmania (Fig. 1). Both DFTs cause tumors primarily on the face, neck, and oral cavity of the Tasmanian devil, and are first visible as small nodules on mucosal and dermal surfaces. DFT1 originated in a Schwann cell of a female

devil (Murchison et al. 2010) whereas DFT2 arose in a male devil, presumably of a similar cellular origin to DFT1 (Pye et al. 2016b; Stammnitz et al. 2018). Nearly all animals diagnosed with the tumor have been recorded to succumb to the disease. Throughout this review DFTD refers to the disease caused by either DFT1 or DFT2. The emphasis will be on DFT1. Tasmanian devils have been formally listed as an endangered species since 2008 by the International Union for Conservation of Nature (IUCN) and they are also protected by both State and Federal legislation (IUCN Red List).

Comparative biological approaches to identify DFTD

A primary limitation to understanding the interaction between the devil immune system and DFTD cells has been the lack of species-specific tools, a problem that continues to pervade comparative immunology research on non-traditional study species (Brock et al. 2014). White blood cell (WBC) counts can be done without species-specific reagents and thus are often used as a diagnostic test or general measure of immune function in many species (Nunn et al. 2003; Bordes and Morand 2009; Cooper et al. 2012). Analyses of hematological and serum biochemical data are also generalized methods to probe basic immunological parameters. Such methods have been used for marsupials, including Tasmanian devils (Parsons et al. 1971; Peck et al. 2015).

Immunoglobulins play an important role in many aspects of immune function, but their role in cancer remains ambiguous even in species where isotypes, Fc receptors, and functions have been thoroughly characterized (Nimmerjahn and Ravetch 2007). Defining the role of immunoglobulins in DFTD, and in wild animals in general, is a critical task. Few studies have found strong correlations between survival and pathogen-specific antibodies (Nussey et al. 2014), but in general immunoglobulin levels in serum remain a blunt tool for predicting infection status.

Basic lymphoid tissue anatomy has provided solid, although relatively basic, morphological information. Immunohistochemistry has provided a valuable tool in advancing knowledge of lymphoid architecture. Cross-reacting antibodies developed to investigate other species (e.g., human and mouse) have yielded information about immune–pathogen interplay in non-traditional species (Jurd 1994; Coutinho et al. 1995; Hemsley et al. 1995; Canfield and Hemsley 2000).

Histology and immunohistochemistry using cross-reactive antibodies have been useful tools to investigate the DFTD tumor and the major immunological structural components of the Tasmanian devil (Loh et al. 2006a, 2006b). Initial studies mapped DFTD (DFT1) as an undifferentiated soft tissue neoplasm (Loh et al. 2006a). Immunohistochemistry, with cross-reacting antibodies suggested that DFTD (DFT1) was of neuroectodermal origin (Loh et al. 2006b). Transcriptome sequencing, which is relevant for any species, revealed the original DFTD (DFT1) to have been derived from a Schwann cell (Murchison et al. 2012). Chromosome painting and karyotyping, techniques adaptable to all species, determined that chromosomes 1 and X contain most of the chromosomal rearrangements (Deakin et al. 2012). A major diagnostic breakthrough that provided a valuable research tool became available when a cross reactive anti-periaxin antibody was found to be a reliable marker to identify DFT1 cells (Tovar et al. 2011).

Monitoring the spread of DFTD

Although it was recognized that Dasyurids, and specifically Tasmanian devils, commonly develop a variety of neoplasms (Griner 1979; Canfield and Cunningham 1993) data indicated that the earliest report of a facial tumor was in 1996 (Hawkins et al. 2006). Targeted monitoring for the original DFTD (DFT1) was first performed in 2003 to determine how much of Tasmania was affected by this devastating but little known disease. An informal examination of trapped devils in a state-wide "snapshot" survey showed DFTD (DFT1) to be present in approximately one-third of devil populations surveyed (N. Mooney, personal communication). By 2005, histological confirmation of DFTD (DFT1) in 41 sites covered 51% of the area of devil occurrence in Tasmania (Hawkins et al. 2006). Monitoring of devil populations over the following 13 years showed DFTD (DFT1) to have spread in a south-westerly direction to cover approximately 80% of the state. The rate of spread was affected by geography as well as movement patterns and abundance of devils (Lazenby et al. 2018). In one relatively abundant population in the north-west of the state, where DFTD (DFT1) had arrived relatively recently, repeated monitoring over 3 years showed DFTD (DFT1) to be spreading at approximately 5–7 km per year (Save the Tasmanian Devil Program [STDP], Tasmanian State Government, unpublished data).

Two principle methods have been employed to monitor the spread of DFTD (DFT1). Devils were trapped in specially designed tubular traps made from PVC pipe (diameter 315 mm × length 875 mm; N. Mooney and D. Ralph, unpublished data). The trapping was performed at the most western edge of the known location of DFTD (DFT1), and was carried out annually (2009–2013). Several teams were used to cover a large area in the northwest of the state. As cases of DFTD (DFT1) were located at the disease "front," the trapping teams steadily moved their trap lines west until they no longer recorded any cases of disease. This method relied on histological confirmation of DFTD (DFT1), recorded the change in age distribution and abundance of the populations following disease arrival, and mapped the rate of spread of the disease. For many of these infected areas, the annual monitoring precluded specific time of disease arrival. This was particularly relevant when trapping teams moved into areas of very recent DFTD (DFT1) arrival. With very low disease prevalence, finding cases of DFT1 was either a matter of enormous resourcing or serendipity.

The second method of monitoring takes advantage of the more recent advances in motion sensing cameras (Thalmann et al. 2014; Meek et al. 2015). Cameras are set to cover a certain area to give year-round monitoring of disease presence. Cameras are set with meat baits and lure canisters, which along with memory cards and batteries, need to be changed every 2 months. Visitation by devils to the cameras reduces as the meat is consumed and the lure canisters dry out and lose their smell. The biggest challenge in analyzing images from the cameras is distinguishing between scar tissue from old wounds, fresh lumpy wounds, and a DFTD tumor. Sometimes this can be impossible to discern, especially if the tumor is inside the oral cavity. Without having the animal in hand there is no ability to feel the lump, which if DFT1 will have a very hard base to it. The advantage of cameras over trapping is the likelihood of seeing more devils. Reports to the STDP through roadkill sightings or the hotline used by the general public have proved effective in tracking occurrences of DFTD. New cases of DFT1 were located further west of the disease-front than previously recorded and the first case of DFT2 was a result of the hotline.

Movement of diseased devils is the principal determinant of the rate of spread of DFTD, and a number of factors including geography, abundance of devils, and food availability are likely to play important roles in determining movement. Rough estimates of disease spread can be based on the decline in spotlight counts of devils combined with confirmed cases of the disease. The decline in spotlight counts provides an indication of loss of devils and the need to confirm cases validates that the population decline was due to DFTD (DFT1). The spread from the north to the south of the state has been more rapid than that from the east to the west (McCallum et al. 2007). Accurate estimates of the rate of disease spread are challenging because only devils with visible symptoms of DFTD can be diagnosed. Not all devils within a given area are sampled during any one survey (Bode et al. 2009), although the recapture probability of devils is high (in the order of 80%) (Lachish et al. 2007). Imperfect detection is a challenge common to surveys of wild animals (Williams et al. 2002). The rate of spread of the disease is variable as different barriers (rivers, mountains, dense bush) affect devil movements (Storfer et al. 2017). The rate of spread appears to vary between 7 and 52 km/year (McCallum et al. 2007).

Evidence that DFTD (DFT1) is transmissible (contagious)

A remarkable feature of DFTD (DFT1) is that the cancer cell itself is transmitted between unrelated devils as an infectious "pathogen." As a contagious cancer, the malignant cells are transferred between individuals as a natural allograft, forming new clonal tumors reviewed in Woods et al. (2015). The original proposal for the "allograft theory of transmission" came from basic studies of the karyotype of the DFTD (DFT1) cells (Pearse and Swift 2006). Chromosomes of the cancer cells had complex chromosomal rearrangements. However, the cancer cells from devils from different locations showed an identical karyotype, which in every case was different from the host. More detailed karyotypic studies confirmed that DFTD (DFT1) cells from different devils have similar complex chromosomal rearrangement (Deakin and Belov 2012; Murchison et al. 2012; Pearse et al. 2012). Genetic studies of multiple DFT1 tumors at the DNA, microsatellite, and major histocompatibility complex loci all provide support for the clonal nature of the cancer cells and therefore the contagious nature of the tumor (Siddle et al. 2007; Murchison et al. 2010, 2012). The genomes of DFTD (DFT1) tumors on two devils isolated in distance and time shared an almost identical genetic variation (Murchison et al. 2012). Genome sequencing of DFTD (DFT1) tumors identified that all DFT1s share point variants, structural variants, and

copy number changes, which are distinct from their hosts (Miller et al. 2011; Murchison et al. 2012). Although there was evidence of karyotypic evolution, the DFTD (DFT1) genome appeared stable, hence the long term survival of this transmissible cancer. DFTD (DFT1) distinguishes itself from other cancers, not only just due to transmissibility, but also genomic stability which is not found in most solid tumors (Hanahan and Weinberg 2000, 2011). The canine transmissible venereal tumor has also demonstrated relative genomic stability across 11,000 years of inter-individual transmission (Strakova and Murchison 2015), suggesting the selection pressure maintains key functional genes across time and individuals.

Immune system of the Tasmanian devil

As allografts produce strong immune responses, "grafted" DFTD (DFT1) cancer cells had to overcome immunological barriers of the host (Woods et al. 2007). Either the immune system of the host or the nature of the cancer cells could have contributed to the failure to reject the "grafted" DFT1 cells. At the time of DFTD (DFT1) discovery, very little was known about the immune system of the Tasmanian devil. Consequently, and given the lack of reagents to probe the devil's immune system, initial research relied on basic analyses of blood films and lymphoid organs. This was especially relevant as marsupials were believed to have a "weaker" immune response than that of placental mammals (Jurd 1994).

Differential WBC counts and lymphoid organ analyses were similar in appearance to those in placental mammals (Kreiss et al. 2008, 2009a). An analysis of serum biochemistry and hematology against DFTD (DFT1) status revealed that total WBC counts and neutrophils were higher in diseased devils, whereas lymphocytes were decreased; this suggests that the devil immune system is affected by DFTD in a manner similar to an acute infection and that WBC levels are predictive of disease status (Peck et al. 2016). Interestingly, this study found no association between disease status and serum immunoglobulin levels, another routine diagnostic test that can be predictive of acute infection status.

The development of specific monoclonal antibodies was required for the identification of lymphocyte subsets. CD4+ and CD8+ T cells were identified in lymphoid organs and as seen in lymphoid tissues of other mammals, there were more CD4+ than CD8+ cells (Howson et al. 2014). Functional assessment of lymphocyte proliferation, phagocytosis, skin graft rejections, antibody responses to immunizations with sheep red blood cells, and cytotoxicity assays for innate killer cells such as NK-like cells demonstrate that there was no evidence for immune defects (Kreiss et al. 2008, 2009b, 2011a, 2015; Brown et al. 2011, 2016). As Tasmanian devils scavenge on carcasses that would have high microbial loads, the ability to recognize pathogens would be paramount to survival. Consequently, innate cell receptors such as toll like receptors (TLRs) would need to be present and functional. This was indeed the case as the innate cells of the Tasmanian devil expressed a comprehensive range of functional TLRs (Patchett et al. 2015, 2017).

Immune response to DFTD (DFT1)

It is clear that healthy devils are not immunosuppressed and should respond to DFTD (DFT1) "allografts." Tasmanian devils have a reduced genetic diversity, potentially due to a "founder effect" or a "bottleneck" in the population (Jones et al. 2004). This reduced diversity was particularly evident at the MHC gene loci, and this was the primary focus of most early studies to explain the lack of allogenic recognition of DFTD (Siddle et al. 2007). However, as skin grafts are rejected, limited MHC diversity may contribute to, but cannot fully explain, the lack of rejection of DFTD "grafts." As cancer cells can downregulate MHC-I to make them evade the immune system (Fassati and Mitchison 2010) the expression of MHC-I was investigated. It was discovered that the DFTD (DFT1) cells do not express MHC-I antigens on their cell surfaces (Siddle et al. 2013). The absence of MHC expression is most likely the major cause for the failure to induce an allogeneic response thereby making the cancer cells effectively "invisible" to the devil's immune system. Other contributing factors might include cytokines (Morris and Belov 2013), the loss of the original dendritic cells from the index case preventing direct recognition, down regulation of tumor-related molecules (e.g., DAMPS, TSTAs), up-regulation of inhibitory cell surface signaling molecules (e.g., PD-L1 (Flies et al. 2017); and the tumor microenvironment (Hui and Chen 2015).

A mouse xenograft model has also been developed for understanding tumor biology in a live host (Kreiss et al. 2011b; Pinfold et al. 2014). These studies provided the initial *in vivo* evidence that an immune response can be generated against the DFTD cells. The use of immunodeficient mice has provided researchers with a model that extends *in vitro* studies, without the need to use the endangered species

under investigation. Although most of the studies of human diseases using immunodeficient mice has led to "humanized mice" (Ito et al. 2018), such a model could be adapted to non-classical species.

Epidemiology of DFTD and alteration of population structure

DFTD is almost 100% fatal, usually resulting in death within 9–12 months of the presentation of a tumor (Hamede et al. 2012) and potentially at least 2 years for some devils after initial inoculation (Wells et al. 2017). Unlike most infectious diseases, DFTD (DFT1) has its greatest effect on the "fittest" population, including devils with the highest reproductive output (Wells et al. 2017). Once DFTD (DFT1) has invaded a population, an important indicative sign of disease presence is the loss of the older age classes. In eastern Tasmania, where the disease originated, population growth rate declined by 50% per year during the first 6 years following disease outbreak (Lachish et al. 2007). The typical pattern is population decline with most of the remaining animals falling into the 1–2 year age class (Lazenby et al. 2018). Mature breeding individuals (2–4 years old; devils usually live for 5–6 years in the wild and rarely breed beyond the age of 5 years) disappear first from a population, followed by the 2 year olds (the usual age of sexual maturity in a DFTD-free population) (Lachish et al. 2009). This primary reduction in age class is probably due to disease transmission occurring during the mating season between sexually mature devils. As DFTD is spread by injurious contact when individuals bite each other, transmission is likely greatest among adults of breeding age as injuries from intraspecific bites peak during the mating season (Hamede et al. 2008). These seasonal and demographic patterns of biting injuries underlie the demographic changes of progressive reduction in age structure that follow disease outbreak (Lachish et al. 2009).

A consequence of the reduction in local population density following DFTD outbreak is that growth rates of sub-adult devils increase. A greater prey availability and nutrition enable a greater proportion of younger females to attain a sufficient body mass to breed in their sub-adult year (age 1 year) (Jones et al. 2008a; Lachish et al. 2009). This reduced competition has facilitated precocial breeding with females mating at the age of 1 year, rather than the usual 2 year sexual maturity point (Lachish et al. 2007; Jones et al. 2008b). Females from diseased populations appear to have more pouch young than females from non-diseased areas (Lazenby et al. 2018). Precocial breeding provides reproductive compensation to counter severe disease-caused mortality rates but is not sufficient to significantly slow the decline in population growth rates (Lachish et al. 2009). Precocial breeding may result in rapid evolution of traits leading to a species that is more resilient to DFTD (Jones et al. 2008a).

Much of transmission likely occurs during the mating season, when injurious contacts peak in both males and females (Hamede et al. 2008, 2013b). Patterns of biting injuries and subsequent infections suggest that it is the dominant individuals that are responsible for a large proportion of transmission. As devils with fewer bite injuries had a higher incidence of DFTD (DFT1) it suggests that dominant devils are more likely to acquire DFTD (DFT1) than submissive devils. As the initial tumors are more likely to be inside the oral cavity, it is feasible that the dominant individuals are biting into the tumors of diseased devils (Hamede et al. 2013b). This concords with observations that the most reproductively fit devils are those more likely to become infected (Wells et al. 2017). The diseased devils could then transmit DFTD when they are bitten on the face.

Heterogeneity in epidemic patterns has been observed across Tasmania. For example there was a reduced impact at one site in north-western Tasmania, at one site in north-western Tasmania (West Pencil Pine) (Hamede et al. 2012). At this site disease prevalence remained low, there was no population decline and age structure remained unaltered for up to 5 years after the epidemic outbreak (Hamede et al. 2012). Three plausible explanations have been postulated to explain these differences in epidemic outcomes among affected populations: (1) lower host susceptibility to infection, (2) lower intraspecific bite rates, and (3) differences in virulence among tumor genetic sublineages. To date, no differences have been found in MHC copy number variation between infected and healthy individuals (Lane et al. 2012) and bite rates among populations that differ in epidemic outcomes are not significantly different from each other (Hamede et al. 2013a), suggesting that tumor genetic sublineages may have a role in driving transmission dynamics and epidemic outcome. This has been recently documented at West Pencil Pine where a sudden localized replacement of tumor genetic sublineages resulted in a significant increase in DFTD prevalence, sudden population decline, and mortality in adult devils (Hamede et al. 2015). The competition between tumor sublineages and the changes in epidemiological parameters suggests that the tumor sub lineages and

susceptibility to infection are under strong selective pressure.

Risk factors and prevention strategies

The Tasmanian State Government provided the first level of protection for the species by estabishing an Insurance Population within Tasmania. This metapopulation now contains over 700 animals housed in 44 institutions throughout Australia, America, Europe, and New Zealand. Fortunately, Tasmanian devils are amenable to captive breeding and the Insurance Population has been a resounding success. However, protection for wild devils is not so easily secured. Removal of DFTD from wild populations was trialled between 2004 and 2010, when a disease suppression program was carried out on the Forestier peninsula in south east Tasmania. Animals with external signs of DFTD (DFT1) were euthanized to determine whether this conferred an advantage to the remaining population. Analysis of results showed that this methodology was not resulting in a significant difference when compared with an unmanaged site (Lachish et al. 2010). Further analysis proposed that without access to a pre-diagnostic test (the ability to determine whether a devil had contracted DFTD before showing any outward signs) total removal would be impossible. However, this methodology would do no more than remove animals that DFTD would remove several months later and therefore culling is not an effective option (Beeton and McCallum 2011).

Methods for securing healthy populations of devils in the wild either involve translocations of healthy devils to islands, or involve isolating populations behind barriers. Both of these methods have been adopted by the STDP in trying to return devils to the wild. In 2012 and 2013 a total of 28 devils were released onto Maria Island, a National Park 4 km off the coast of mainland Tasmania. In the following years the devils settled and reproduced, creating an abundant and healthy population of approximately 100 animals (DPIPWE, unpublished data).

Also, in 2012, all devils were removed from the Forestier peninsula to create an isolated area. Barriers were installed to prevent diseased devils from moving into the newly created isolated area. Following years of monitoring to ensure no devils, or disease, remained on the Forestier peninsular, in 2015/16 a population of 49 healthy Tasmanian devils was reintroduced into this protected area, to live wild while still protected from DFTD (DPIPWE, unpublished data).

The key to a successful conservation outcome for the devil and the Tasmanian ecosystem, however, is to augment diminished wild populations. The aim is to return population sizes to a level of ecological functionality, even in areas of disease presence. Releasing healthy endangered animals into an area with a continuing threat faces its own challenges. To this end, trial augmentations of devils to three sites on mainland Tasmania occurred between 2015 and 2017 to determine the best protocol to ensure the greatest chance of survival of devils released back into the wild. Questions posed during the trial included: do captive or wild devils survive better; how far do devils travel on release; how long does it take devils to settle following release; does release directly into the wild (hard release) or indirectly via large enclosures (soft release) confer a greater survival advantage? Results from the releases are still being analyzed but results suggest that wild born devils survive better than captive born devils, which appear to lose their innate wariness of vehicles (Grueber et al. 2017). Additionally, all devils released as part of these trials were given a trial immunization. To establish efficacy would require enough test subjects, in a real life test scenario. These three trial releases provided an opportunity to test this vaccine.

Vaccination approach to DFTD (DFT1)

The first attempts to induce immune responses were in two devils using irradiated cultured DFTD cells with Montanide (Seppic, France) as an adjuvant. Neither of the devils produced evidence of an immune response (Brown et al. 2011). This study did provide evidence for an NK-like activity mediated in the form of antibody dependent cellular cytotoxicity. Thus providing the initial evidence that the immune response of Tasmanian devils did have the capacity to mount cell mediated cytotoxicity. Later studies using either sonicated or irradiated cells with Montanide, but with the addition of the TLR agonist CpG, resulted in antibody and in some cases *in vitro* cytotoxic responses in 5/6 devils. One of the devils that had seroconverted was challenged with live DFTD (DFT1) cells and appeared to be protected from DFTD. Unfortunately protection was not conferred following a re-challenge with live DFT1 cells. It appeared that the immunization could reduce, rather than prevent, tumor progression (Kreiss et al. 2015).

Allogenic immune responses against MHC-I, that occur in most transplantation rejections, would normally be expected to prevent the engraftment of DFT1 cells. The downregulation of MHC-I

expression appears to make the tumors invisible to the new host's immune system. Although the DFT1 cells do not express the protein for MHC-I, the genes are present and mRNA is produced (Siddle et al. 2007, 2013). Consequently MHC-I expression is under translational or post-translational control. The discovery that MHC-I can be upregulated on DFT1 cells using recombinant IFN-γ (Siddle et al. 2013) provided a new direction for the vaccine research. Upregulation of MHC using IFN-γ in combination with TLR signaling has formed the foundation of ongoing vaccine research. A number of studies also supported an immune based approach to control of DFTD, including the demonstration that activated devil peripheral blood mononuclear cells could kill DFTD cells *in vitro* (Brown et al. 2016). Injection of MHC-upregulated DFTD cells adjacent to experimentally transferred DFT1 tumors in previously immunized devils triggered immune rejection in half of cases (Tovar et al. 2017). Functional activity of devil TLR receptors was confirmed (Patchett et al. 2015) and the efficacy of a stabilized poly-I: C (Hiltonol®) in combination with imiquimod (TLR-3 and -7 agonists, respectively) was established (Patchett et al. 2017) forming the basis for an experimental vaccine candidate.

Evidence of immunologically mediated tumor regression has been identified in a small proportion of wild (non-immunized) devils with DFTD (DFT1) (Pye et al. 2016a). Such evidence provides support that effective immune responses can be generated against DFTD (DFT1) cancer cells and that protection against DFTD is feasible. Armed with evidence of natural tumor regression combined, with observations that DFTD (DFT1) cancer cells are immunogenic and that IFN-γ upregulated MHC-I expression, preliminary vaccine trials were undertaken. The candidate vaccine consisting of killed MHC upregulated DFTI cells (by treating the cells *in vitro* with IFN-γ). The adjuvants included ISCOMATRIX® mixed with stabilized poly I: C (Hiltonol®) and imiquimod. Fifty-two captive bred devils destined for release into sites in mainland Tasmania to bolster local populations were vaccinated. This trial demonstrated a class switched IgG response against DFT1 cells in over 95% of devils, demonstrating specific activation of both B and T lymphocytes (Pye et al. 2018). Some vaccinated devils developed DFTD, and when combined with low recapture rates of vaccinated devils, this limits the ability to make inferences about the protective capacity of these responses. However, leukocyte infiltration in these devil's tumors are more prominent than those in the incumbent population (S. Pye, unpublished data) suggesting immune recognition does occur. Additional signals or immune checkpoint blockade for development of an effective response.

Immunotherapies targeting the immune checkpoint molecules CTLA-4, PD-1, and PD-L1 have recently become the most broadly effective cancer treatment available for humans (Larkin et al. 2015; Tawbi et al. 2018), but this immunotherapy approach is largely untested outside of human medicine. Devil-specific anti-PD-1 and anti-PD-L1 monoclonal antibodies have been developed and used to show that DFT1 and DFT2 cells upregulate the inhibitory molecule PD-L1 in response to IFN-γ stimulation (Flies et al. 2016). However, these anti-PD-1 and anti-PD-L1 monoclonal antibodies were produced in mice, and thus repeated immunotherapy treatment of devils with these antibodies would likely induce an anti-mouse IgG response because the mouse IgG would be viewed as a foreign protein in devils. This response could be ameliorated by "devilizing" the monoclonal antibodies. "Devilizing" in its simplest form would entail producing a recombinant protein that replaces the constant regions of the mouse immunoglobulin heavy and light chains with devil heavy and light chains. Checkpoint molecule immunotherapy on its own is unlikely be used on a large scale, but can be incorporated into vaccines and used to better understand the interaction between DFT cells and the devil immune system. Immunosuppressive cytokines have also been identified as a potential immune evasion mechanism (Morris and Belov 2013), and development and testing of devil cytokines can similarly be used to enhance and inform vaccine development efforts.

Numerous hurdles confront the development of an effective vaccine. The evidence is that the immune response can protect in a minor proportion of wild unvaccinated devils and some vaccinated devils. The immediate challenge is to develop a vaccine that produces a strong response, ideally following a single injection. This would provide a pathway to vaccinate wild devils, in addition to captive devils earmarked for release into the wilderness. The capacity to monitor vaccinated and released devils to test efficacy is also an obstacle to evaluate the efficacy.

What does the future hold concerning DFTD incidence?

In the 20 years since DFTD emerged in Tasmania, there have been no population extinctions but evidence toward the evolution of an endemic disease, coexisting with its host, the Tasmanian devil. DFTD (DFT1, 2, *n*) is now part of the Tasmanian devil's

ecosystem. Devils and DFTD will evolve and while there are incidences of tumor regression, it is not known if this is an evolutionary response or the result of the interplay of phenotypic response by DFTD or Tasmanian devils. Evidence for evolution is that devils are showing geographically widespread responses to the cancer epidemic, with allele shifts indicating evolving immune-modulated resistance to DFTD(DFT1) occurring within four to six generations of local disease outbreak. Long term coexistence of devil and tumor is further supported by tumor regressions and associated natural immune responses (Pye et al. 2016a), variants in the genome associated with tumor regressions (Wright et al. 2017), and increased tolerance to infection with devils surviving more than a year after infection (Wells et al. 2017). Comparable genetics and mutations between DFT1 and DFT2 suggest a similar epidemic outcome for DFT2.

The STDP was implemented in 2003 by the Tasmanian State Government in response to the decline of the Tasmanian devil population resulting principally from the threat of DFTD. Over the past 15 years, the State and Commonwealth Governments have supported the STDP to determine what DFTD is, how it is transmitted, the effect it has on wild populations once it has been present for a period of time, and importantly, provided the funds to create an insurance population of captive devils to protect against extinction of the species. While our knowledge has informed us that extinction in the wild is unlikely, the overarching goal the STDP has always been to maintain an enduring and sustainable population of devils in the wild. The Wild Devil Recovery trials completed between 2015 and 2017 and the creation of source populations on Maria Island and the Forestier peninsula has set a solid foundation toward this goal.

Devils are susceptible to roadkill, especially in DFTD affected areas where roads are within devil habitat. The loss of a couple of breeding females in a subpopulation could exacerbate devil declines. Consequently, the Tasmanian Government has introduced a roadkill project to mitigate the threat on Tasmanian roads to all Tasmanian wildlife, not just its endangered species. This plan for the future of the devil keeps the devil in the Tasmanian environment, while biding time for a vaccine that provides some protection to the devils released into the wild, and/or possible resistance evolving in the devil or changes to the properties of the cancer cells. Boosting wild population numbers through augmentation can help ameliorate this threat and enhance genetic diversity to provide more devils in the environment to perform their ecological function.

Conclusion

It is now two decades since the first case of DFTD(DFT1). The first decade culminated in the 2006 publication that DFTD(DFT1) is a transmissible cancer. The multidisciplinary approach throughout the second decade has produced considerable progress in immunological and ecological aspects. The lack of any population extinctions and the evidence for rapid evolution toward resistance and disease suggests that DFTD(DFT1) will become an endemic disease. As evidence for evolution has occurred within four to six generations of local disease outbreak it is unlikely that devils will become extinct. However, the conservation outcome for the devil is still uncertain. There remains a need to control increasing incidence and subsequent threats to current Tasmanian devil populations. Efforts toward this aim will incorporate effective vaccination programs. This is based on the evidence that devils, exposed to DFTD in the wild, or as part of an immunization protocol, can mount an immune response to DFTD(DFT1). A vaccination protocol would incorporate DFTD cells, induced to express MHC-I, and adjuvants to provide strong innate immune activation as this has produced immune response in most devils. But a rollout of any vaccination would require evidence of protection against DFTD. Other approaches to protect the devil population include early detection techniques, optimization of disease free breeding program, developing management zones, standards and development of efficient primary and secondary prevention strategies, and ongoing interdisciplinary collaborations.

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