Review Article

**Devil facial tumours: Towards a vaccine**

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**Abstract**

The Tasmanian devil is the only mammalian species to harbour two independent lineages of contagious cancer. Devil Facial Tumour 1 (DFT1) emerged in the 1990s and has caused significant population declines. Devil Facial Tumour 2 (DFT2) was identified in 2014 and evidence indicates that this new tumour has emerged independently of DFT1. Whilst DFT1 is widespread across Tasmania, DFT2 is currently found only on the Channel Peninsula in south east Tasmania. Allograft transmission of cancer cells should be prevented by Major Histocompatibility Complex (MHC) molecules. DFT1 avoids immune detection by downregulating MHC class I expression, which can be reversed by treatment with interferon-gamma (IFNγ), whilst DFT2 currently circulates in hosts with a similar MHC class I genotype to the tumour. Wild Tasmanian devil numbers have not recovered from the emergence of DFT1, and it is feared that widespread transmission of DFT2 will be devastating to the remaining wild population. A preventative solution for the management of the disease is needed. Here, we review the current research on immune responses to DFTs and vaccine strategies against DFT1 and outline our plans moving forward to develop a specific, effective vaccine to support the wild Tasmanian devil population against the threat of these two transmissible tumours.

**Devil facial tumours: Towards a vaccine**

***The Tasmanian devil and its tumours***

The Tasmanian devil (*Sarcophilus harrisii*) is the world’s largest extant carnivorous marsupial and is endemic to the Australian island state of Tasmania (Hawkins *et al.* 2008). Over the last twenty years, the wild Tasmanian devil population has been in steep decline (Hawkins *et al.* 2006, McCallum *et al.* 2007, McCallum *et al.* 2009), resulting in the reclassification of the Tasmanian devil from “Least concern” to “Endangered” by the IUCN red list in 2008 (Hawkins *et al.* 2008). This dramatic fall in devil numbers is primarily due to the emergence of a contagious cancer, Devil Facial Tumour 1 (DFT1) (McCallum et al. 2007). More recently, a second independent linage of contagious cancer termed Devil Facial Tumour 2 (DFT2) has also emerged (Pye et al. 2016a). Both tumours are spread as allografts during social biting behaviours, and form large, necrotic tumours primarily on mucosal surfaces around the face, neck and oral cavity (Loh et al. 2006, Pearse & Swift, 2006, Pye, *et al.*, 2016a).

The first photographs of a wild Tasmanian devil with large, disfiguring tumours on the face were captured in 1996, and these are thought to be the first images of DFT1 (Hawkins *et al.* 2006, Loh et al. 2006). The disease has rapidly spread and now persists in devil populations covering over 80% of mainland Tasmania, causing significant local population declines averaging 77% in affected populations (McCallum *et al.* 2009, Lazenby et al. 2018). DFT1 tumours are estimated to cause almost 100% mortality among devils (Loh *et al.* 2006, Tovar *et al.* 2011), although rare cases of tumour regression have been reported (Pye et al. 2016b).

In 2014, Pye et al. (2016a) identified two devils in south east Tasmania whose tumours demonstrated abnormal histopathology and were negative for the specific DFT1 marker periaxin. The genotype and karyotype of these tumours are distinct from DFT1, but conserved between tumours from different animals, indicating a new, independent lineage of transmissible tumour which has been termed DFT2. At the time of writing, DFT2 has been formally identified in at least 19 devils in the Channel region of south east Tasmania, and remains geographically restricted to this region (Pye *et al.* 2016a; Kwon *et al.* 2018, Hamede 2019).

There is evidence that devils are rapidly evolving in response to the DFT1 threat, some devils are living longer with the tumour and raising immune responses against it (Epstein et al. 2016, Pye et al. 2016b, Ruiz-Aravena et al. 2018). Modelling of the long term effects of DFT1 indicates that only 21% of predicted scenarios result in the extinction of the Tasmanian devil, and the tumour itself is over twice as likely to disappear entirely from the wild population (Wells et al. 2019). However, these models do not factor in the potential effects of DFT2 if the new tumour spreads beyond its current range and it is not clear whether the emerging adaptations against DFT1 in the devil population will also be beneficial against DFT2. Were DFT2 to follow a similar trajectory to DFT1, it has the potential to cause irreparable damage to the remaining wild devil populations, reigniting concerns that this vulnerable species may struggle to survive in the wild.

Several strategies have been implemented to stabilise the wild population with varying degrees of success. Attempts have been made to remove diseased animals from the population to keep regions of the island disease free, but these have been unsuccessful at stopping the spread of DFT1 (Lachish *et al.* 2010, Beeton and McCallum 2011, Save the Tasmanian Devil Programme 2011). Captive breeding programmes and the establishment of disease-free insurance populations have been more successful (Hogg *et al.* 2017), generating a population of over 700 individuals held in zoos, semi-wild fenced enclosures and on isolated islands and peninsulas around Tasmania (Biggs, 2018). Despite this success, there are problems associated with the captive breeding and re-release of these animals, including ensuring genetic diversity is maintained in the captive population (Hogg *et al.* 2015, McLennan *et al.* 2018), local effects on other wildlife populations (Jones and McCallum, 2007) and increased risk of road strike incidents following the release of captive bred animals, as well as difficulties with tracking and monitoring the released individuals (Grueber *et al.* 2017).

A protective vaccine against DFTs is an attractive solution to preserve devils in the wild. This would allow for the release of vaccinated devils from captive insurance populations into areas affected by DFTs, and also enable the vaccination of wild devils in these populations to help establish herd immunity. Here, we review the current status of research into vaccination programmes against DFTs in the broader context of current cancer vaccine research and indicate possible next steps in developing a successful vaccine programme for use in the wild population.

***Cancer vaccine development***

The use of the immune system to treat cancer can be traced as far back as the late 1800’s, when William B. Coley began intentionally infecting sarcoma patients with streptococcal organisms to induce an immune response, often resulting in cancer regression (McCarthy, 2006). As cancer research has developed, so too has our understanding of how the immune system can be exploited to treat malignancies. We now know that this link between tumour cells and an immune response is reliant on the presence of immunogenic antigens on the surface of the cancer cell. One major mechanism of tumour immunosurveillance is through CD8+ T-cells, which become activated by the presence of mutated or “non-self” tumour antigens presented by Major Histocompatibility Complex (MHC) Class I molecules (Burnett, 1970; Töpfer *et al.*, 2011, Vitale *et al.* 2019). A wealth of research has been undertaken to identify methods for strategies to exploit MHC class I restricted tumour antigens and induce an immune response against tumours. These have included vaccinations using whole tumour cell preparations (Berd *et al.* 2004, Small *et al.* 2007) which doesn’t require knowledge of specific tumour antigens but is more vulnerable to the general effects of self-antigen immune tolerance, as well as more targeted approaches using single tumour antigens as the vaccine target. These antigen targets can be neoantigens generated from proteins mutated in the cancer mutanome, which are tumour specific and should not cause off target effects. However, despite an abundance of mutations in the exome of any given tumour, very few of these translate to immunogenic targets presented by MHC class I on the cell surface (Yadav *et al.* 2014). This lack of highly specific antigens on the tumour surface, coupled with the degenerate nature of T-cell receptor (TCR) - MHC class I interactions (Mason, 1998; Wilson *et al.* 2004), have proven challenging for researchers in the area of cancer immunotherapy and in some cases has initiated toxicity against healthy tissue (for example the MAGE-A3 antigens) (Linette *et al.* 2013, Morgan *et al.* 2013), demonstrating the importance of understanding peptide binding in the context of both healthy and diseased cells. Vaccines may also be targeted towards antigens which are aberrantly expressed on the tumour cells, such as antigens from early development or antigens which are overexpressed compared to healthy cells. These are known as tumour associated antigens, and while not as specific as neoantigens, there is evidence that patients can harbour antibodies against these antigens that form an effective immune response without inducing autoimmunity (Vella *et al.* 2009, Marijt *et al.* 2018).

Despite these difficulties, two cancer vaccines are currently approved for use in patients; Oncophage, a patient specific heat shock protein-peptide complex vaccine approved for use in Russia to treat renal cell carcinoma, and in clinical trials for a number of other tumour types (Reviewed by Wood and Mulders (2009)); and Sipuleucel-T, a cell based immunostimulant therapy that involves stimulation of the patient dendritic cells to respond to an antigen from prostatic acid phosphatase, approved by the FDA for treatment of hormone refractory prostate cancer (Reviewed by Wei *et al.* (2015)). These vaccines are therapeutic rather than prophylactic, however prophylactic vaccines against human tumours are under development (Keenan *et al.* 2014, Bautz *et al.* 2017).

The development of cancer vaccines, particularly prophylactic vaccines, is a complex process, and at present there remains several gaps in knowledge preventing the production of an effective vaccine against DFTs, however the field is making strides towards filling these gaps.

***Devil facial tumours: What we know***

Genetic analysis of DFT1 and host polymorphic microsatellite markers has demonstrated that DFT1 is a non-self cell type derived from a common progenitor (Siddle *et al.* 2007b, Murchison *et al.* 2012) and there is no evidence to suggest that DFT1 was the result of a viral transformation (Murchison *et al.* 2012). DFT1 lacks cytogenetically identifiable sex chromosomes, but work by both Murchison *et al.* (2012) and Deakin *et al.* (2012) has identified two copies of an X chromosome in the DFT1 genome, and no evidence of the Y chromosome gene *SRY,* indicating that DFT1 emerged in a female devil. A transcriptomic analysis of DFT1 by Murchison *et al.* (2010) revealed that the expression of miRNAs and coding genes in DFT1 is highly similar to that seen in nervous tissue, and demonstrated that DFT1 cell lines and tumours express components of the myelination pathway, indicating that DFT1 is most likely of Schwann cell origin. Indeed, it has been previously demonstrated that Schwann cell specific protein PRX is a highly specific marker for DFT1 tumour cells (Tovar et al. 2011). Additionally, the DFT1 genetic profile clusters closely with that of devils from the same geographical location that DFT1 was first sighted, indicating the tumour emerged in a devil in the north east of the island (Stammnitz *et al.* 2018).

Due to its recent emergence, little is known about the cellular origins of DFT2. Initial work by Pye at al. (2016a) identified the presence of a Y chromosome in the DFT2 karyotype, which is confirmed by the presence of the *SRY* gene (Stammnitz *et al.* 2018), indicating that a single clonal origin for DFT1 and DFT2 was unlikely. Recent work by Stammnitz *et al.* (2018) found that DFT2 biopsies, like DFT1, are positive for neuroectodermal markers S100 and Neuron-Specific Enolase, but are negative for periaxin. DFT2 is also negative for markers for epithelia and smooth muscle. This suggests that DFT2 likely emerged from the neuroectoderm. As with DFT1, DFT2’s genetic profile clusters most closely with devils from the geographical location where DFT2 was discovered. This is currently the only location that DFT2 has been observed, indicating that the tumour emerged in a devil from this area, likely very recently. DFT2 also shows no evidence of viral DNA in its genome.

***The immune response to Devil facial tumours***

Both DFT1 and DFT2 transmit as allografts and should induce a strong anti-graft immune response in host devils due to differences in MHC molecules of the tumour and the host. The DFT1 and DFT2 tumours will still carry the MHC genotype of the founder devil which initially developed each cancer, and thus should initiate a strong anti-graft immune response in the host devil. This should occur through direct recognition of mismatched MHC molecules present on the tumour cell surface by host CD8+ T-cells, initiating rapid graft rejection. Additionally, a slower immune response can occur even where the MHC molecules of the donor and host are matched if the bound peptides are ‘non-self’ (Sebille *et al.* 2001). In the case of DFTs, we would expect this to be mediated by host antigen presenting cells (APCs) taking up and presenting graft derived peptides to T-cells in the lymph node (Harper *et al.* 2015). B-cells can also initiate anti-graft immune responses, both directly within the grafted tissue by cytokine production or by the production of alloantibodies which can recognise antigens on donor cells (Thaunat *et al.* 2005, Haririan *et al.* 2009). Combined, these mechanisms of foreign cell rejection form a robust immune response which should prevent the continuous transmission of DFT1 and DFT2 between genetically distinct hosts.

In light of the ability of DFTs to avoid the anti-graft immune response, the devil immune system has been studied and it has been established that the Tasmanian devil has competent cellular and humoral immune systems (Woods *et al.* 2007, Kreiss *et al.* 2008, Brown *et al.* 2011). It has recently been demonstrated that there is a significant reduction in T-cell diversity between 11 months and 2 years of age, which would affect the ability to respond to pathogens in older animals (Cheng et al. 2019) and may explain the age distribution of DFT1 cases skewing towards older animals (Jones et al. 2008, McCallum et al. 2009). Kreiss *et al.* (2011) have also demonstrated that Tasmanian devils can reject skin grafts following transplantation, indicating that Tasmanian devils should be capable of raising an anti-graft immune response to DFTs. Despite this, very few devils have been identified which can mount an effective immune response to DFT1 (Pye et al. 2016b). Siddle *et al.* (2013) have demonstrated that DFT1 cells have downregulated components of the antigen presentation pathway, including β2-m and TAP1/TAP2, with the ultimate end of removing functional MHC class I from the cell surface. This is likely how DFT1 avoids eliciting a non-self response from alloreactive T-cells between MHC mismatched hosts. The downregulation of this process is epigenetic, and functional MHC class I can be restored to the surface of DFT1 cells *in vitro* by treating cells with the inflammatory cytokine interferon-gamma (IFNγ). It has also been demonstrated that DFT1 infection correlates with a restriction in the T-cell repertoire of adult devils, indicating that the tumour directly impacts the host immune system (Cheng et al. 2019).

In stark contrast, Caldwell *et al.* (2018) have shown that DFT2 cells are still largely positive for surface MHC class I, albeit this expression is highly variable between DFT2 cell lines and tumours, and even within tumour samples. Interestingly, MHC class I expression by DFT2 cells appears to be enriched for a monomorphic, non-classical MHC class I molecule SahaI\*UK. Non-classical MHC class I alleles in humans have been implicated in a number of immunosuppresive and immunoregulatory functions (Rokhafrooz et al. 2018, Ouni et al. 2019), and the overexpression of SahaI\*UK in DFT2 may be aiding the spread of the tumour. Furthermore, DFT2 appears to be circulating in hosts with similar MHC class I genotypes, and there is evidence to suggest the tumour may be losing MHC class I expression as it contacts more genetically distinct host animals, indicating it may be on a similar trajectory to DFT1 and may progress to full MHC class I loss and widespread transmission in the near future (Caldwell *et al.* 2018).

Thus far, no prophylactic vaccine exists against either DFT1 or DFT2, and contraction of either tumour usually results in the death of the animal. However, evidence is emerging that wild devils are able to raise an immune response to DFT1 in rare cases (Pye *et al.* (2016b) and that captive devils can be induced to raise an effective immune response against DFT1 (Tovar *et al.* 2017). Pye *et al.* (2016b) have identified antibodies against DFT1 cells in the sera of 6 wild devils captured between 2011 and 2015 in West Pencil Pine, central North Tasmania, which correlated with tumour regression in at least four animals. In one animal without observed tumour regression, the production of serum antibodies corresponded with the infiltration of T-lymphocytes into the tumour mass. Interestingly, the antibodies present are specific for MHC class I positive DFT1 cells, indicating that this may be an MHC restricted immune response against the tumours. This is supported by evidence from Siddle *et al.* (2013) that in rare cases, β2-m positive cells on the outside of the tumour mass in biopsies correlate with the infiltration of CD3+ lymphocytes.

At present very little is known about how wild devils respond to DFT2. Caldwell *et al.* (2018) have identified infiltrating CD3+ lymphocytes in multiple DFT2 tumours, indicating that devils can respond to DFT2 tumours. Strikingly, infiltrating lymphocytes do not seem to correlate with MHC class I expression in the tumours, and an MHC class I negative tumour contains infiltrating immune cells. How DFT2 avoids activating an alloresponse from host T-cells in MHC mismatched hosts is not understood, and the mechanisms behind immune evasion in DFT2 need to be further studied.

***Initiating an immune response against DFT1***

The ability of wild host devils to raise an immune response to DFT1 (albeit infrequently) and the potential for an MHC class I restricted response has informed several vaccination trials, and the data being generated is promising. It is worth noting that these studies have primarily used a single DFT1 cell line that was established in culture and is defined by Pearse et al (2012) as strain 3 (DFTD1-C5065, RRID:CVCL\_LB79). In the studies discussed below this is the cell line used for all experiments unless otherwise stated. The vaccine preparations and associated immune responses used in devils from the studies discussed in this section are summarised in Table 1.

Preliminary vaccination studies in Tasmanian devils using irradiated DFT1 cells failed to induce cytotoxic or humoral immune responses (Brown *et al.* 2011). However, it has been demonstrated that DFT1 cells can be used to induce an immune response in mice when DFT1 cells are inoculated as a xenograft (Pinfold *et al.* 2014). Subsequently, Kreiss *et al.* (2015) trialled different combinations of inactive DFT1 cell preparations and adjuvants to assess devil immune responses to vaccination (summarised in Table 1). Of the six devils used in this study three (TD2, TD4 and TD5) produced anti-DFT1 antibodies in response to immunisation. TD2 and TD4 produced antibodies in response to two different vaccine preparations and demonstrated significant cytotoxicity against DFT1 cells following multiple immunisations. Two devils (TD1 and TD3) produced low titres of antibody against DFT1 following immunisation, which were only detectable by ELISA, did not persist for long following final immunisation and resulted in only low levels of cytotoxicity. TD2 was challenged with the same DFT1 cells used in the vaccine preparation and remained disease free for 189 days. When re-challenged with a different strain of DFT1, the devil remained disease free for a further 154 days, before developing small tumours at both challenge sites which were curatively surgically resected. This study demonstrated that whilst variable, devils can produce specific antibodies against DFT1 following immunisation with inactive cell preparations in the presence of an adjuvant. Crucially, this study also demonstrated that antibody production following vaccination can be protective and can increase disease free survival following DFT1 challenge. It has also been demonstrated that mitogen-activated devil mononuclear cells, which may be Natural Killer (NK) cells, are able to kill DFT1 cells *in vitro*, indicating that it is possible to induce devil immune cells to target the tumour (Brown *et al.* 2016).

Further vaccination and immunotherapy studies by Tovar *et al.* (2017) have shown that immunisation with inactive DFT1 cell preparations can result in antibody production and associated tumour regression or lack of tumour engraftment. This study has also demonstrated that vaccination using MHC class I positive DFT1 cells (MHC-I+) is more effective than vaccination with MHC class I negative DFT1 cells (MHC-I-), and that sonicated or irradiated whole cell lysates are more effective at initiating an immune response than protein extract alone. A total of 7 devils were immunised using different DFT1 cell preparations, including MHC-I+ cells and both whole cell and protein extract, combined with different adjuvant and booster immunisations (Table 1). 6 devils immunised with inactivated MHC-I+ DFT1 cells induced higher levels of serum antibodies against both MHC-I+ and MHC-I- DFT1 cells than the devils immunised with MHC-I- cell preparations or adjuvant only controls. However, this alone was not enough to initiate effective anti-tumour responses after DFT1 challenge. Remarkably, one devil (TD4-Mm) immunised with sonicated and irradiated MHC-I+ DFT1 cells produced a strong antibody response against MHC-I+ and MHC-I+ DFT1 cells and remained tumour free after DFT1 challenge for 189 days before being euthanised for age related reasons, indicating the immunisation protocol may have prevented or at least delayed tumour engraftment.

This study also demonstrated that MHC-I+ DFT1 cells can be used as an effective immunotherapy in devils. Three devils (TD1-My, TD6-Tp and TD7-Sy) were injected with live MHC-I+ DFT1 cells following the engraftment of DFT1 tumours after challenge. This immunotherapy resulted in elevated serum levels of antibodies against MHC-I+ andMHC-I- DFT1 cells, increased immune cell infiltrate into the tumour mass, and regression of the tumours. This therapy was not effective at treating the non-immunised control devil, and immunotherapy using inactivated MHC-I+ DFT1 cells was not effective at treating any of the immunised devils. Crucially, these vaccination trials have also demonstrated that MHC class I increases the immunogenicity of DFT1 cells, indicating the immune response is MHC class I restricted. However, immunotherapy using live MHC class I positive DFT1 cells is a high risk treatment, and in two cases (one immunised devil and the non-immunised control) resulted in the formation of tumours at the immunotherapy site.

The strongest induction of antibody responses seen in the study by Tovar *et al.* (2017) included the adjuvants PolyI:C and CpG oligonucleotides (Table 1), two ligands of Toll-like receptors (TLR), targets which has been heavily utilised by adjuvants in human vaccination studies (Galluzzi *et al.* 2012) and have been demonstrated to be functional in the Tasmanian devil (Patchett et al. 2015). ISCOMATRIX™ with Poly I:C and CpG oligonucleotides has proved to be a highly effective adjuvant combination in human studies (Silva *et al.* 2015), however the efficacy of this combination in devils has not been fully elucidated. A study by Patchett *et al.* (2017) assessed the effect of stimulating the TLR on immune activation in the Tasmanian devil, to inform further improvement of adjuvant combinations in the vaccination programme. *In vitro* stimulation of Tasmanian devil peripheral blood mononuclear cells (PBMCs) with the TLR ligands PolyICLC (similar biological function to PolyI:C but more stable) and imiquimod and IL2 induced IFNγ production from predominantly CD3+CD4- cells, indicating a cell mediated immune response. The effect of these adjuvants on devil immune responses *in vivo* was then assessed by vaccine with PolyICLC and imiquimod as adjuvants using a model antigen (Keyhole limpet haemocyanin (KLH)) in place of DFT1 cells to reduce antigen mediated immune modulation (Table 1). Devils immunised with KLH alone or KLH and imiquimod showed very little response to vaccination. Devils immunised with PolyICLC as an adjuvant, with and without imiquimod, exhibited a strong antibody response which was comparable across both preparations, indicating that PolyICLC alone is sufficient to induce an antibody response in Tasmanian devils. This antibody response decreased in the four devils immunised with PolyICLC adjuvant and was at primary response levels by 6 months post immunisation. Exposure of these devils to KLH without adjuvants 10 months post immunisation resulted in a rapid antibody response in all four devils, indicating that PolyICLC induces long term immunological memory in Tasmanian devils. This study has demonstrated that TLR ligands, particularly TLR3 ligands like PolyI:C and PolyICLC, are able to induce significant long term immune responses when used as vaccination adjuvants in the Tasmanian devil.

[Insert Table 1 here]

Vaccination studies in the Tasmanian devil have been limited by small sample sizes due to the restricted use of devils in research, and the frequent use of older animals, which may need to be euthanised due to age related health problems before the trials are complete. The implementation of the Wild Devil Recovery Project by the Tasmanian Government in 2015 presented an opportunity to trial vaccination strategies on a younger, larger cohort of devils. Pye *et al.* (2018) immunised a total of 52 devils from the disease free insurance populations prior to their wild release in two separate trials. The details of both immunisation and monitoring programmes are summarised in Table 2. It is worth noting that due to the nature of trapping wild devils, not all animals received all immunisations, and this has been accounted for during data analysis. 19 devils released in the first trial in Narawntapu National Park (NNP) received two to four monthly immunisations consisting of sonicated and irradiated MHC-I+ DFT1 cells, followed by a final booster immunisation four months later prior to wild release. 33 devils released in the second trial at Stony Head (SH) a year later received only two immunisations followed by a 5 month booster immunisation post wild release. Sera was collected from immunised devils at regular intervals throughout the primary vaccination course (See Table 2 for detail) and assessed for antibodies against MHC-I+ and MHC-I- DFT1 cells. In both studies there was no significant difference in the levels of antibodies detected between the two cell types, and average serum levels of anti-DFT1 antibodies increased after each immunisation.

The differing primary immunisation protocols in the NNP trial had no significant impact on levels of antibody in the sera, but both age and sex significantly affected the outcome in the NNP cohort, with juvenile and female devils producing higher levels of antibody. Interestingly, sex did not have a significant impact on the antibody response of the SH devil cohort. Of the total 52 devils vaccinated, 50 were producing antibodies by the end of the primary immunisation course. Post release trends in the NNP population indicated antibody levels reverted to baseline levels 12 weeks post booster, whereas in the SH cohort many devils retained the antibody levels seen after their second primary immunisation 5 months later, prior to their booster vaccine.

This study also performed MHC microsatellite analysis to determine if MHC diversity is linked to vaccination outcomes. Three MHC class I linked loci were found to have a significant association with vaccination outcome. The MHC class I alleles associated with highest antibody responses were not present in the DFT1 cell line used in the immunisations, indicating that MHC diversity results in a stronger immune response against DFT1, although this has never been demonstrated in unvaccinated devils. The antibody production against MHC-I- cells indicates that whilst the immune responses arising from vaccination are MHC linked, the immune system can also respond to non-MHC linked markers on the DFT1 cells, a promising finding for generating a vaccine against a tumour that normally does not express MHC.

[Insert Table 2 here]

These studies have provided a wealth of evidence that it is possible to stimulate an effective immune response *in vivo* against DFT1. However, responses in devils are variable and the antigens on DFT1 cells that are targets for the immune system are not understood. While there is some evidence that these are MHC restricted, there is also evidence that other antigens such as heat shock proteins are immunogenic in the tumours (Tovar et al. 2018) and these may also be playing a role in whole cell vaccine responses. Whilst in the same way that humans have variable responses to vaccination, some of this variability derives from normal differences in the host devil immune systems (Kimman *et al.* 2007). Current vaccination strategies in humans are moving towards specific epitope targets rather than whole organism preparations in an attempt to vaccinate against pathogens which have resisted traditional vaccination methods, and to reduce antigenic load and adverse effects (Chauhan *et al.* 2019, Jain and Baranwal 2019, Ahmad *et al.* 2019). Similarly, there is a drive to develop vaccines against cancer based on single peptide targets to generate a strong and specific immune response against malignant cells (Zilberberg *et al.* 2015). A similar, more targeted approach may help solve the current difficulties in DFT vaccination studies.

***Towards a DFT vaccine***

As DFTs are genetically distinct allografts, we would expect MHC to play a role in immune rejection of the tumours, and indeed evidence indicates that MHC class I is involved in successful immune responses against DFT1. Given this, we hypothesise that vaccine strategies targeting MHC class I may induce an immune response against the tumours, which is more specific and effective than those raised by whole cell preparations. Before peptide vaccine targets can be identified, some gaps exist in our current knowledge of the induced immune response to DFTs that must be filled.

Firstly, the cellular origins of the tumours need to be well understood. This would allow for identification of tumour specific antigens by comparison of antigens between healthy and diseased cell states. Whilst tumour-specific mutated antigens would be ideal vaccine targets due to their specificity, it is rare for mutated proteins to end up presented by MHC class I on the cell surface (Yadav *et al.* 2014), and tumour associated antigens, which are present in some healthy cells but are upregulated on tumour cells may present a more viable option (Vella *et al.* 2009, Marijt *et al.* 2018). Identification of both tumour specific and tumour associated antigens requires an understanding of antigen presentation in the tumour progenitor cells. We currently know that DFT1 expresses components of the myelination pathway and likely emerged from a Schwann cell (Murchison *et al.* 2010), therefore isolation and full characterisation of Tasmanian devil Schwann cells and their immunopeptidome is required. Currently, the cellular origins of DFT2 are not well defined, though evidence indicates it may be of neuroectodermal origin (Stammnitz *et al.* 2018).

Secondly, if we intend to target MHC class I restricted peptides, we need a good understanding of MHC class I allele expression throughout the devil population and in both DFT1 and DFT2. Peptide vaccines exploit the host antigen presentation system to elicit an immune response, and for a peptide vaccine to be effective it must be possible for host antigen presenting cells to present the peptide on their own MHC molecules to T-cells. This means that peptide vaccines are restricted to certain MHC haplotypes (Bartnik *et al.* 2012). Ironically, the diminished genetic diversity of the Tasmanian devil at the MHC locus, which is likely to be a factor in the emergence of DFT1 and DFT2, may prove useful for the development of a peptide vaccine for broad population use (Siddle *et al.* 2007a, Siddle et al. 2007b, Siddle *et al.* 2010, Cheng *et al.* 2012, Cheng and Belov 2014). A study by Caldwell et al, (2018) has identified a classical MHC class I allele (SahaI\*27) which is shared by DFT1 and DFT2, appears to be present in host devils and may be common within the wider devil population (Lane *et al.* 2012). Another classical allele (SahaI\*27-1) is present in both tumours and in two out of three hosts. SahaI\*27-1 differs from SahaI\*27 by only one non-synonymous mutation not predicted to affect peptide binding, and is expected to bind the same peptide sequences as SahaI\*27. Additionally, two non-classical MHC class I alleles (Saha-UK and Saha-UD) are present in DFT1, DFT2 and all sequenced hosts. Saha-UK is a monomorphic allele which should bind the same peptide sequences between individuals and is highly expressed in DFT2. Saha-UD demonstrates low polymorphism and likely binds similar sequences between individuals, but is only expressed at trace levels in DFT2 (Caldwell *et al.* 2018). These data indicate that vaccine targets restricted to any of these four alleles are likely to be recognised by a large number of animals. The prevalence of these specific alleles in the wider population needs to be fully assessed to predict the effectiveness of restricted peptides in a large scale vaccination programme.

Finally, in order to identify MHC class I restricted vaccine targets, we have to understand the peptide binding repertoire of MHC class I in the Tasmanian devil. For a peptide to be a suitable vaccine target it must be bound and presented on MHC class I in tumour cells, but not in healthy cells, as vaccines targeted against peptides which are present on healthy tissues can cause significant immune mediated damage to healthy tissues (Morgan et al. 2013). This means a detailed understanding of the peptides being presented on the cell surface of healthy and diseased cells, the “immunopeptidome”, is required. To generate an immunopeptidome, MHC class I molecules are purified from whole cell lysate by immunoaffinity purification, often using an antibody specific to the MHC class I heavy chain of interest. The peptide fractions can then be isolated and sequenced by mass spectrometry. This is becoming an increasingly robust workflow in humans and mice, although low affinity peptides are often lost throughout the process (Bassani-Stenberg, 2018). These methods have also been adapted to effectively generate immunopeptidome data from bovine, porcine and bat samples (Pedersen *et al.* 2011, Wynne *et al.* 2016, Nielsen *et al.* 2018), generating invaluable information on antigen presentation across multiple species.

A barrier against defining the immunopeptidome of a given cell type and species is the availability of specific antibodies to isolate MHC class I molecules. Our lab has generated the first pan-MHC class I immunopeptidomes from DFT1, DFT2 and fibroblast cell lines using an antibody against native devil β2-m (Gastaldello *et al.* in preparation). Whilst these datasets provide interesting insights into general peptide binding by MHC class I in the Tasmanian devil, it provides no information on specific MHC class I allele binding properties, and it is impossible to determine whether any given candidate peptide is likely to be bound by the common MHC class I molecules SahaI\*27/27-1 or SahaI\*UK. We are in the process of generating a workflow, based on work by Wynne *et al.* (2016), whereby allele specific immunopeptidomes can be analysed in non-model species in the absence of specific antibodies. Briefly, this has involved engineering recombinant MHC class I constructs with cytoplasmic protein tags which can be used for immunoaffinity purification of the MHC class I trimer in lieu of allele specific MHC class I antibodies. Once binding motifs are generated, these can be searched against the pan-MHC class I immunopeptidome to identify peptides binding these common MHC class I molecules which are likely to be the most widely useful vaccine targets. Once we have this information, peptide vaccine candidates that will be effective across a wide range of hosts can be confidently selected and their immunogenicity assessed. This assessment would initially be performed *in vitro* using T cell cytotoxicity assays and APCs with the desirable MHC class I genotype and eventually *in vivo* by immunisation of live devils with candidates identified as immunogenic during *in vitro* assays. Any immunogenic candidates can then be fed directly into current vaccination studies and combined with other currently potential therapeutic targets in DFT1 such as PD-L1 (Flies et al. 2016) and heat shock proteins (Tovar et al. 2018) to induce anti-tumour immune responses. While implementation of a large scale vaccination programme in a wild species is problematic, vaccination programmes, including those with multiple immunisations, could be performed on captive animals prior to translocation from insurance populations into the wild. However, this is unlikely to be adequate to establish herd immunity in the wild species, and thus would require a constant replenishment of the wild species from insurance animals. Vaccination may require a prohibitively large and costly trapping programme to fully implement, but there has been previous success vaccinating wild species against the rabies virus using an orally administered vaccine packaged into bait (Maki et al. 2017). A similar approach to vaccination against DFTs which exploits the natural scavenging behaviour of devils may be feasible once the safety of any potential vaccine is determined. An alternative strategy would be to focus vaccine efforts on the currently restricted DFT2, to prevent its further spread.

***A dual vaccine against DFTs?***

Both DFT1 and DFT2 share MHC class I alleles thought to be common in the devil population, and early evidence indicates that DFT2 has emerged from a similar tissue type to DFT1. This raises the possibility that these two tumours may respond to the same vaccination programme.

DFT2 may have emerged from a similar tissue to DFT1 and the two tumours share a similar mutagenic profile (Stammnitz et al. 2018). This indicates that DFT1 and DFT2 may share specific mutations which are visible to the immune system. In malignant peripheral nerve sheath tumours, the closest equivalent human cancer type to DFT1, several common mutations have been identified (Brohl *et al.* 2017) some of which result in aberrant antigen expression on the tumour cell surface (Nobeyama and Nakagawa, 2016), indicating that it may be possible for DFT1 and DFT2 to share antigens, and indeed early analysis of our immunopeptidome data has identified some peptides common to both cancers which may present useful targets (Gastaldello *et al.* unpublished).

Whilst research into vaccinations against DFTs has made great strides in recent years, the variable antibody responses, combined with infrequent correlation of antibody production and tumour regression, means that the wild devil population is still at serious risk. In particular, the emergence of DFT2 has raised concerns that this new tumour may cause irreparable damage to the remaining population, and a protective vaccine is sorely needed to prevent its widespread transmission. We propose that a peptide vaccine is the best way forward for the vaccination programme, as it would allow for the generation of a specific, tumour targeted immune response in host devils, and may open door for a dual vaccination approach to protect devils against both tumours.

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**Declaration of interest**

The authors declare no conflicts of interests

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| Study  **Table 1: Summary of the vaccination preparations and protocols and associated immune responses demonstrated in four major studies of immunisation against DFT1.** All vaccine preparations use the C5065 DFT1 cell line unless otherwise stated. NA indicates that this parameter was not assessed. | Vaccine/adjuvant | Vaccine programme | Animals | Antibodies/  Cytotoxicity | Prophylactic |
| Brown *et al.* 2011 | 108 irradiated DFT1 cells(Strain 2, Pearse and Swift 2006) with Montanide adjuvant | Four doses at monthly intervals | TD1  TD2 | None/None  None/none | NA |
| Kreiss *et al.* 2015 | 108 freeze/thawed DFT1 cells (Strain 2: ½ Pea and 2112 (Pearse and Swift, 2006) with Montanide adjuvant | Three doses at weekly intervals with final dose 8 weeks later. TD2 received pre-challenge booster | TD1  TD2 | +/NA  +++/NA | No  Only against strain 2 |
| 108 irradiated DFT1 cells with Montanide adjuvant and CpG 1668 oligonucleotides | Three doses at four weekly intervals | TD3  TD4 | +/++  +++/+++ | NA |
| 108 irradiated DFT1 cells with Montanide adjuvant and CpG 1585 oligonucleotides | Three doses at four weekly intervals | TD5  TD6 | ++/None  None/None | NA |
| 108 sonicated DFT1 cells with Montanide adjuvant and CpG 1668 oligonucleotides | Three doses at four weekly intervals | TD2  TD4 | ++/+++  ++/+++ | NA |
| Tovar *et al.* 2017 | 400 µg protein extract from heat-treated DFT1 cells with ISCOMATRIX™ adjuvant | Three doses at monthly intervals. 1000µg protein booster 6 months post immunisation | TD1-My | None/None | No |
| 2x106 – 1.5x107 freeze/thawed DFT1 cells pretreated with Trichostatin A (TD2-GA) or cytokine enriched medium (TD3-Ty) with ISCOMATRIX™ adjuvant | Two doses at monthly intervals | TD2-GA  TD3-Ty | ++/NA  + (MHC-I+ DFT1 only)/ NA | No  No |
| A) 3x107 sonicated DFT1 cells pretreated with IFNγ  B) 106 irradiated DFT1 cells pretreated with IFNγ  Both preparations included ISCOMATRIX™, Poly I:C and CpG (1585, 2395) oligonucleotide adjuvants | Two doses of A at monthly intervals  Two doses of B at monthly intervals  Booster: 2 doses of B 4 months post immunisation. TD4-Mm received additional double dose of B 7 months post booster 1. | TD4-Mm  TD5-Br | ++/NA  ++/NA | Yes  NA |
| A) 2x106 irradiated DFT1 cells pretreated with IFNγ  B) 2x107 sonicated DFT1 cells pretreated with IFNγ  Both preparations included ISCOMATRIX™, Poly I:C and CpG (1585, 2395) oligonucleotide adjuvants | Three doses of A at fortnightly intervals  One dose of B one month after final dose of A  Booster with A 6 months post final immunisation | TD6-Tp  TD7-Sy | ++/NA  +++/NA | No  No |
| Patchett *et al.* 2017 | 100 µg model antigen KLH | One dose followed by a double dose 42 days later | TD8-Pe  TD9-Au | +/NA  (Anti-KLH)  +/NA | NA  NA |
| 100 µg model antigen KLH with 100 µg imiquimod | One dose followed by a double dose 42 days later | TD10-Ka  TD11-Jo | +/NA  (Anti-KLH)  +/NA | NA  NA |
| 100 µg model antigen KLH with 100 µg PolyICLC | One dose followed by a double dose 42 days later  TD13-Ad received a 100 µg KLH booster immunisation 262 days post second dose | TD12-Gw  TD13-Ad | +++/NA  +++/NA | NA  NA |
| 100 µg model antigen KLH with 100 µg imiquimod and 100 µg PolyICLC | One dose followed by a double dose 42 days later  TD15-Sp received a 100 µg KLH booster immunisation 262 days post second dose | TD14-No  TD15-Sp | +++/NA  +++/NA | NA  NA |

**Table 2: Summary of the vaccination preparations and protocols and serum collection time points from large scale vaccination studies by Pye *et al.* (2018).** All vaccine preparations use the C5065 DFT1 cell line. Not all devils received the full immunisation course and not all devils were recaptured for serum collection at all timepoints.

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| Release site | Number of devils | Primary immunisation course | Booster immunisations | Adjuvants | Serum collection timepoints |
| NNP | 19 | A) 2x107 sonicated MHC-I+ DFT1 cells  B) 2x106 irradiated MHC-I+ DFT1 cells  Two doses of A followed by two doses of B at monthly intervals | 2x106 irradiated MHC-I+ DFT1 cells  Four months post immunisation, immediately pre-release | ISCOMATRIX™ with PolyI:C and CpG oligonucleotides (1585 and 2395) | Fortnightly throughout primary course  2 weeks post primary course  On the day of booster  2 weeks post booster  2, 6 and 12 weeks post release |
| SH | 33 | A) 2x107 sonicated MHC-I+ DFT1 cells  B) 2x106 irradiated MHC-I+ DFT1 cells  One dose of A followed by one dose of B at monthly intervals | 2x106 irradiated MHC-I+ DFT1 cells  Five months post immunisation, four months post-release | ISCOMATRIX™ with PolyICLC and imiquimod | 4 weeks post first immunisation  6 weeks post second immunisation  6 weeks post primary course  Regularly during 4 months post release  5 months post release |