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Review [Cancer immunology and canine malignant melanoma: a Paper](#)

[Cancer immunology and canine malignant melanoma: A comparative review](#)

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[McMaster Immunology Research Centre, Department of Pathology and Molecular Medicine, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada, L8S 4K1. Tel.: +1 905 525 9140x22272; fax: +1 905 522 6750.](#) **Abstract**

Oral canine malignant melanoma (CMM) is a spontaneously occurring aggressive tumour with relatively few medical treatment options, which provides a suitable model for the disease in humans. Historically, multiple immunotherapeutic strategies aimed at provoking both innate and adaptive anti-tumour immune responses have been published with varying levels of activity against CMM. Recently, a plasmid DNA vaccine expressing human tyrosinase has been licensed for the adjunct treatment of oral CMM. This article reviews the immunological similarities between CMM and the human counterpart; mechanisms by which tumours evade the immune system; reasons why melanoma is an attractive target for immunotherapy; the premise of whole cell, dendritic cell (DC), viral and DNA vaccination strategies alongside preliminary clinical results in dogs. Current “gold standard” treatments for advanced human malignant melanoma are evolving quickly with remarkable results being achieved following the introduction of immune checkpoint blockade and adoptively transferred cell therapies. The rapidly expanding field of cancer immunology and immunotherapeutics means that rational targeting of this disease in both species should enhance treatment outcomes in veterinary and human clinics.

Keywords: Canine malignant melanoma; [Cancer immunology and immunotherapy](#)

1 Canine melanoma as a model of human melanoma

For at least two decades, veterinary oncologists have advocated using spontaneously occurring tumours in companion animals as models for human cancer ([Knapp and Waters, 1997](#); [MacEwen, 1990](#)). Several recent reviews have re-addressed this approach identifying osteosarcoma, mammary tumours, head and neck cancers, bladder carcinomas, non-Hodgkin lymphoma, prostatic carcinoma, lung cancer and pertinently oral canine malignant melanoma (CMM) as good models for human neoplasms ([Hansen and Khanna, 2004](#); [Khanna and Hunter, 2005](#); [Paoloni and Khanna, 2008](#)). The major benefits of dogs as tumour models include the ability to study genetically outbred and immunologically intact animals in which cancers develop spontaneously and thus, more likely reflect the process of tumourigenesis compared to experimentally induced neoplasms. As pets and owners share the same environment, they may be exposed to the same carcinogens, which, in part, drive tumour development. Regarding disease modelling, animal tumours often have similar clinical presentation, tumour biology and histopathological appearance to their human counterparts and usually progress more rapidly, thereby shortening data maturation times. In addition, few “standard of care” therapies exist for dogs meaning that within reason, trial therapeutics can be instigated at any point. Given these benefits, companion animal tumour models more accurately reflect the features of human cancers compared to rodent models with CMM being a desirable example of one such neoplasm. Conversely, the opportunity to translate the potential value of state of the art human therapeutics to the veterinary clinic also exists.

Human melanoma and CMM share multiple molecular similarities and signalling pathways reflecting their comparability at a subcellular level. These include activation of the AKT mTOR pathway ([Hay, 2005](#); [Kent et al., 2009](#); [Turri-Zanoni et al., 2013](#)), aberrations of the receptor tyrosine kinase KIT ([Murakami et al., 2011](#); [Newman et al., 2012](#); [Rivera et al., 2008](#); [Tsao et al., 2012](#); [Turri-Zanoni et al., 2013](#)), up regulation of cyclooxygenase-2 (COX-2) expression

(Becker et al., 2009; Martínez et al., 2011), dysregulation of the Wnt/ β -catenin pathway (Han et al., 2013; Larue and Delmas, 2006) and expression of chondroitin sulphate proteoglycan 4 (CSPG4) (Mayayo et al., 2011; Price et al., 2011). Some molecular differences also occur between the species, for example mutation of codon 599 in BRAF, a member of the mitogen-activated protein kinase (MAPK) cascade is a common feature of human cutaneous malignant melanoma and results in elevated kinase activity (Davies et al., 2002), this was not identified in oral CMM samples (Shelly et al., 2005). Interestingly these mutations are similarly rare in human mucosal melanoma (Maldonado et al., 2003). Moreover, a recent study utilising array comparative genomic hybridisation (aCGH) and fluorescent *in situ* hybridisation (FISH) revealed genome wide cytogenetic similarities between human and canine mucosal melanoma (Poorman et al., 2014). The comparative biologic aspects of malignant melanoma with particular emphasis on molecular lesions has been reviewed in depth elsewhere (Sulaimon and Kitchell, 2003).

Both CMM and human malignant melanoma have a propensity to behave in a biologically aggressive manner (Abbas et al., 2014; Simpson et al., 2014) with oral CMM, in particular, being highly metastatic and usually associated with a poor prognosis (Smith et al., 2002). Human primary mucosal melanomas also follow a clinically aggressive course with five-year survival rates (25%) being much lower than the cutaneous (80.8%) and ocular (74.6%) counterparts (Mihajlovic et al., 2012). Survival times quoted for conventionally treated stage II or III oral CMM (Table 1.) range from 3- to 12 months with metastasis being a significant cause of mortality (Bateman et al., 1994; Freeman et al., 2003; MacEwen et al., 1986; Murphy et al., 2005; Proulx et al., 2003). It is, however, worth noting that not all oral CMMs are clinically aggressive and some early stage canine patients as well as patients with histologically benign lesions can have extended survivals (MacEwen et al., 1986; Smedley et al., 2011).

Table 1 World Health Organisation (WHO) stage of oral CMM.

<i>T: Primary Tumour</i> T1 Tumour \leq 2 cm diameter <i>tumour</i>	
T1	Tumour \leq 2 cm diameter
T2	Tumour 2–4 cm diameter
T3 Tumour $>$ 4 cm diameter N: Regional Lymph Nodes T3	Tumour \geq 4 cm diameter
<i>N: Regional lymph nodes</i>	
N0	No evidence of regional node involvement
N1	Histologic/cytologic evidence of regional node involvement
N2 Fixed nodes M: Distant Metastasis N2	Fixed nodes
<i>M: Distant metastasis</i>	
M0	No evidence of distant metastasis
M1	Evidence of distant metastasis

Stage I: T1 N0 M0.

Stage II: T2 N0 M0.

Stage III: T2 N1 M0 or T3 N0 M0.

Stage IV: Any T, any N and M1.

Although loco-regional control of oral CMM using radiation or surgery may be successful, the prevention or treatment of disseminated disease using cytotoxic drugs has previously been considered unrewarding with no real extension of survival time (Bergman, 2007; Veil, 2012; Bergman, 2013). Multiple different chemotherapy protocols have been described for CMM but responses can be described as modest at best (Boria et al., 2004; Page et al., 1991; Rassnick et al., 2001). A recent study suggested adjunct post-operative carboplatin might be of use in CMM; however, as there was no control group in this study a definitive conclusion cannot be made (Dank et al., 2014). The role of conventional chemotherapy in human mucosal melanoma is also unclear (Mihajlovic et al., 2012). Since neither oral CMM nor human mucosal melanoma patients have prolonged survival times with existing conventional therapies, spontaneously occurring CMM is an attractive platform for developing much-needed novel and efficacious treatment strategies for both species. Targeted therapies for deranged pathways in CMM are being developed *in vitro* and *in vivo* (Borrego et al., 2014; Gil-Cardesa et al., 2010; Kent et al., 2009; Moriyama et al., 2010; Watanabe et al., 2010), however much of the recent research activity involves immunomodulatory therapies.

2 Cancer immunology

A recognised hallmark of malignancy is tumour immunoevasion where neoplasms utilise multiple mechanisms to avoid destruction by the host's immune response (Hanahan and Weinberg, 2011). Spontaneous development of a tumour such as CMM in an animal possessing an intact immune system offers an excellent opportunity to analyse the pathways of cancer immune escape. Furthermore, such a model offers scope to devise and apply innovative immunotherapeutics possessing potent anti-neoplastic effects.

Both innate (rapid and non-specific) and adaptive (specific with memory) immunity are required to prevent neoplastic development (Vesely et al., 2011). The failure of immunosurveillance during tumourigenesis implies that the immune system is ignorant of and/or rendered impotent to the impending danger of tumour formation. Using studies in knockout mice, Shankaran et al. demonstrated that either defective innate or adaptive immunity alone or a combination of both, increase tumour formation (Shankaran et al., 2001). Both recombination activating gene (RAG2) deficient mice $+/-$ in which the development of lymphocytes required for an adaptive response is prevented, and signal transducers and activators of transcription (STAT)-1 deficient mice $+/-$ in which the STAT defect disables interferon signalling pathways that are crucial for innate immunity, are at higher risk for cancer development (Shankaran et al., 2001). Furthermore, these investigations suggest that tumours developing in immunologically intact animals proceed through immunoselection, which facilitates tumour progression in an immunocompetent host. Thus cancers that are able to develop in the face of a functional immune system are shaped by the immune response and become more able to survive, leading these authors to coin the phrase "cancer immunoeediting" (Shankaran et al., 2001). The immune system is therefore involved not only in surveillance to try and prevent tumour formation but also subsequently in shaping the progressive immunoevasive tumour cell phenotype.

Tumours are able to reduce antineoplastic immune responses (immunoevasion) in a variety of ways. So called "anti-inflammatory" cytokines that suppress anti-tumour immunity such as interleukin-10 (IL-10) and transforming growth factor- β (TGF- β) can be produced by tumour cells and also by regulatory T-cells (Tregs) (Jarnicki et al., 2006; Kim et al., 2005). Decreased antigen presentation and perturbed antigen presenting cell (APC) function also impede the development of protective immunity (Kerker and Restifo, 2012). Central and peripheral tolerance (i.e. prevention of an effector response against tumour antigens) play a role in allowing tumour survival and proliferation in hosts with functioning immune systems, an observation that is not unexpected as tumours are derived from self tissues (Mapara and Sykes, 2004). Recently, the roles of two cell populations, Tregs and myeloid-derived suppressor cells (MDSCs) have been implicated in carcinogenesis by favouring peripheral tolerance towards tumours (Ostrand-Rosenberg and Sinha, 2009; Wang and Wang, 2007). Tregs were found in increased numbers compared to healthy controls in two oral CMM studies (Horiuchi et al., 2010; Tominaga et al., 2010). Further analysis by Horiuchi and co-workers revealed a positive correlation between Treg numbers and clinical stage (Horiuchi et al., 2010). MDSCs are potent inhibitors of both innate and adaptive immunity and are therefore also a significant problem for cancer immunotherapy (Sinha et al., 2007). Taken together, these studies imply that for an immunotherapeutic strategy to be effective in cancer therapy, it must be able to overcome or eliminate the existing tumour tolerance and mechanisms of immune evasion.

3 Immunotherapy for melanoma

As a result of ineffective chemotherapy treatments, multiple immunotherapeutic strategies have been developed to target canine (Table 2) and human malignant melanoma and these can be divided broadly into targeting either the innate or adaptive immune system (Figure 2). Whilst some human vaccines are now used prophylactically against virally-induced tumours such as human papilloma virus (HPV)-induced carcinomas, these are outside the scope of this communication and have been reviewed elsewhere (Lu et al., 2011; Schiller et al., 2012; Villa, 2011). Presently, throughout the field of veterinary oncology, no widely available prophylactic anti-cancer vaccines exist for dogs.

Table 2 Summary of Immunotherapeutic Trials for CMM.

Immunotherapeutic strategy	Trial design	Arm of immune system activated	Clinical outcome	References
<i>Corynebacterium parvum</i> used as an adjunct to surgical resection of oral CMM	Phase II prospective controlled trial, stratification attempted based on stage and signalment. Surgery alone used as a control	Innate	MST of dogs with stage II/III disease significantly longer ($p = 0.01$) when treated with <i>C. parvum</i> (288 d) vs. surgery alone (121 d) vs. surgery alone (121 d). Stage I dogs significantly better prognosis than other stages irrespective of treatment	MacEwen et al. 1986 (1986)
L-MTP-PE used either alone or in combination with GM-CSF as an adjunct to surgical excision of oral CMM	Phase II prospective trial with randomised treatment groups and mock treated control. Study subdivided into two trials	Innate	MST of dogs with stage I significantly longer ($p = 0.045$) when treated with L-MTP-PE (not reached) compared with mock control treatment (414 d). Stage I dogs significantly better prognosis than other stages irrespective of treatment. No benefit conferred by the addition of GM-CSF	MacEwen et al. 1999 (1999)
PEGylated TNF α administered to dogs with measurable tumours where standard therapy had failed or been declined	Phase I dose escalating prospective clinical trial for various solid tumours	Innate	Dose limiting toxicity consisting of vascular leak in one dog and coagulopathy/hypotension seen at 30 μ g/kg. Transient partial tumour responses seen in 13% of dogs. Lymphocytic and neutrophilic tumour infiltrate as well as tumour necrosis observed	Thamm et al. 2010 (2010)

Lipid complexed human FasL DNA administered intra-tumourally to oral CMM	Prospective phase I toxicity study in 5 dogs with CMM	Mimics adaptive effector function	No local or systemic toxicity observed. Two dogs achieved CR and died at 24 and 44 weeks of unrelated disease; two dogs achieved PR; one one died later of an unrelated illness at 13 weeks with SD and the other died at 82 weeks due to PD; 5th dog died at 3 weeks due to PD	Bianco et al. 2003 (2003)
Replicate-deficient adenovirus expressing CD40L administered intra-tumourally	Pilot study of 2 dogs with CMM	Innate and adaptive	Minimal signs of local toxicity. One dog had CR and died of unrelated disease at 401 d, other dog had PR with no signs of progression at 120 d	Von Euler et al. (2008)
Replicate-deficient adenovirus expressing CD40L administered intra-tumourally	Extension of above pilot study to include 19 dogs with CMM	Innate and adaptive	MST 160 d; 5 CR, 8 PR, 4 SD and 2 PD. Tumour infiltration with T and B lymphocytes observed	Westberg et al. 2013 Intra-tumoural injection of lipid- (2013)
Intra-tumoural injection of lipid-complexed plasmid DNA encoding a bacterial superantigen (staphylococcal enterotoxin B) and either canine GM-CSF or canine IL-2	Phase I/II prospective trial without controls in CMM	Innate and adaptive	Minimal local toxicity and no systemic toxicity reported. Objective response rate (CR + PR) of 41%, MST for stage I, II and III dogs was 61w, 57w and 24 w, 57 w and 24 w respectively with stage IV not determined	Dow et al., 1998 Dow et al. (1998)
Intra-tumoural combination treatment via injection of lipid-complexed herpes simplex thymidine kinase with ganciclovir (i.e. via injection of lipid-complexed herpes simplex thymidine kinase with ganciclovir (i.e. suicide gene therapy) alongside irradiated transgenic xenogeneic cells secreting human GM-CSF and IL-2	Phase I/II prospective non-randomised controlled trial in CMM. Controls included untreated, surgery alone and suicide gene therapy alone	Innate and adaptive	Minimal toxicity seen with combination therapy. Objective response rate (CR + PR) of 47%, overall median survival for combination therapy (160d) significantly greater than untreated controls (69d, p d) significantly greater than untreated controls (69 d, P < 0.001), surgery alone (82 d, P \leq 0.02) and suicide gene alone (94d, P d, P < 0.05)	Finocchiaro et al. 2008 (2008)
Allogeneic tumour cell vaccine expressing xenogeneic melanoma antigen human gp100	Prospective phase II clinical trial without control group in CMM	Adaptive	Objective response (CR + PR) rate of 17%. Overall MST 153d Alexander et al., 2006 d	Alexander et al. (2006)
Post-operative treatment using tumour bed injections of lipid-complexed herpes simplex thymidine kinase with ganciclovir (i.e. complexed herpes simplex thymidine kinase with ganciclovir (i.e. suicide gene therapy). Subsequent subcutaneous injections of irradiated transgenic xenogeneic cells secreting human GM-CSF and IL-2 in combination with autologous/allogeneic formalised tumour cell vaccine	Phase I/II prospective non-randomised trial using surgery alone as control group in CMM	Innate and adaptive	Minimal toxicity reported. Combination treated median survival (370 d) significantly longer (P < 0.00001) than surgically treated controls (76 d)	Finocchiaro et al. 2007 (2007)
Subcutaneous injection of autologous DCs expanded ex vivo and transduced with human gp100 given as adjuvant to radiation therapy	Pilot study of three dogs with oral CMM	Adaptive	No adverse events recorded. One dog in CR at 48 months, second dog died at 22 months of other causes with unknown remission status and third dog died of PD at 7 months	Gyorffy et al. 2005 (2005)
Intramuscular administration of human tyrosinase plasmid DNA	Phase I prospective trial with three dose escalating cohorts of dogs with advanced stage CMM with or without local tumour control	Adaptive	Mild injection site reactions, no significant systemic toxicities. MST overall 389 d. One dog with stage IV disease had complete resolution of pulmonary metastases for 329 d, and another four dogs (two with stage IV, and one each with stage II and III) had long term survivals (421 = 588 + d)	Bergman et al. 2003 (2003)
Intramuscular administration of plasmid DNA encoding either human tyrosinase, murine gp75, murine tyrosinase, human GM-CSF or murine tyrosinase + human GM-CSF	Phase I prospective trial with multiple cohorts of escalating doses	Adaptive	Mild local injection reactions observed and one dog developed vitiligo. MSTs: human tyrosinase 389d; murine gp75-153d; murine tyrosinase-242d (low dose) and 224 - 389 d; murine gp75 - 153 d; murine tyrosinase - 242 d (low dose) and 224 d (higher doses); human GM-CSF 148 d and murine tyrosinase combined with human GM-CSF >402d. Overall MST of 569 d. Overall MST of 569 d for stage II/III dogs with loco-regionally controlled disease receiving any of the xenogeneic vaccines	Bergman et al. 2006 (2006)
Intramuscular administration of plasmid DNA encoding human tyrosinase used adjunctively following loco-regional control of oral CMM	Phase II clinical trial with vaccinated CMM dogs enrolled prospectively compared to historic control group with loco-regionally controlled disease	Adaptive	MST of vaccinated group (not reached) significantly longer (pP < 0.001) than historic controls (324 d). MST of vaccinated dogs could not be determined as more than 50% were censored from survival analysis	Grosenbaugh et al. 2011 (2011)

Intramuscular administration of plasmid DNA encoding human tyrosinase used adjunctively following loco-regional control of oral CMM	Retrospective analysis of stage I-III oral CMM patients that had loco-regional control with or without subsequent plasmid DNA vaccination	Adaptive	No significant difference ($p = 0.12$) between MST of vaccinated (485 d) and non-vaccinated (585 d) dogs. For stage II/III dogs no significant difference in PFS of 179.5d for vaccinated vs. 247.5d for non-vaccinated and OST of 477 d for vaccinated vs. 247.5 d for non-vaccinated and OST of 477 d for vaccinated and 491 d for non-vaccinated dogs	Otnod et al. 2013 CR = complete response, MST = median survival time, PD = (2013)
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CR, complete response, MST, median survival time, PD, progressive disease, PR = partial response, SD = partial response, SD, stable disease.

3.1 Innate Immunity

Treatments targeting the innate arm of the immune system aim to activate an anti-tumour immune response non-specifically. Bacillus of Calmette and Guérin (BCG) as well as the cytokine interferon- α (IFN α) have been described to treat human melanoma patients (Garbe et al., 2011). Historically, in the 1970s, BCG was used with limited success for the treatment of canine mammary tumours (Bostock and Gorman, 1978) and for canine osteosarcoma where a single trial demonstrated increased survival times for dogs treated with amputation and adjuvant BCG therapy compared to amputation alone (Owen and Bostock, 1974). More currently, in human oncology clinics BCG is used for the treatment of superficial bladder transitional cell carcinoma since mechanistically this leads to the infiltration of the bladder with a broad range of immune cell types accompanied by the induction of various cytokines (Schenkman and Lamm, 2004). Interferon- α has direct anti-proliferative effects on neoplastic cells as well as indirectly inciting an anti-cancer immune response, and has been used to treat human patients with melanoma, hairy cell leukaemia and renal cell carcinoma (Jonasch and Haluska, 2001), however, the side effects of high dose IFN α are substantial (Jonasch and Haluska, 2001). A case series describes the use of low dose IFN α for the treatment of keratoconjunctivitis sicca (Gilger et al., 1999) in dogs but efficacy against CMM has not been assessed. Given the paucity of canine studies, one can conclude that the above approaches aimed at activating innate anti-tumour immunity are not yet established in CMM.

Slightly more recently, two other agents that provoke a non-specific immune response, *Corynebacterium parvum* and liposome encapsulated muramyl tripeptide-phosphatidylethanolamine (L-MTP-PE) (with the latter being used as sole therapy or in combination with the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF)) have been investigated for the treatment of CMM (MacEwen et al., 1986, 1999). GM-CSF acts as a growth factor for dendritic cells (DCs) and macrophages, both of which are crucial innate cells playing important roles in antigen presentation. Treatment of human patients with a selectively replicative herpes simplex virus designed to express GM-CSF led to durable response rates in recently presented phase III clinical trial data (Andbacka et al., 2013). In another approach, by isolating and activating canine pulmonary macrophages Soergel et al. were able to enhance their *in vitro* killing ability of CMM cells when these tumour cells were pre-treated with monoclonal antibodies specific for the melanoma associated ganglioside antigens GD2 and GD3, thereby demonstrating antibody-dependent cytotoxicity mediated by the activated macrophages (Soergel et al., 1999). It is worth noting, however, that APCs such as DCs are able to induce Tregs as well as cytotoxic T-lymphocytes (CTLs), thereby promoting tumour immunoevasion and thus possible therapeutic failure in certain settings (Curiel, 2007). Tham and colleagues reported an alternative cytokine treatment with tumour necrosis factor- α (TNF α) administered in a PEGylated form to dogs with a variety of advanced tumours (Thamm et al., 2010). The varying responses seen in trials to these non-specific immune-modulators may partly explain, along with cost and availability why these agents are not commonly used clinically for CMM during the present day.

3.2 Adaptive Immunity

Provoking a tumour-specific immune response requires engagement of the adaptive immune system and this can be achieved using specific cytokines, certain monoclonal antibodies, adoptive cell transfer and various vaccination strategies (discussed later).

Cytotoxic (CD8+) T-cells are considered key effector cells in cancer immunotherapy with the majority of therapeutics aimed at provoking specific anti-tumour cell mediated immunity (Rosenberg et al., 2004). Killing by cytotoxic T-cells is facilitated primarily by the perforin/granzyme mediated pathway (Trapani and Smyth, 2002), however expression of Fas-ligand (FasL) by CD8+ T-cells means that this can bind to the Fas receptor (Fas) on the cognate target cell and induce cell death via apoptosis (Lowin et al., 1994). Fas-ligand mediated cell killing was effectively mimicked in canine melanoma patients by delivering FasL DNA intralesionally (Bianco et al., 2003). Although this does not induce an adaptive immune response these results are promising and larger studies are required to validate this treatment for CMM.

Co-stimulatory molecules are found on immune cell surfaces and their binding as well as the binding of antigen receptors to peptide:MHC complexes is required to activate immune effectors (Sharpe, 2009). When one such molecule CD40, a receptor found on the surface of B cells and APCs binds to CD40L expressed on T-cells it activates the CD40 bearing cells and thus enhances humoral as well as cell mediated immunity (Elgueta et al., 2009). Treatment of CMM with a replicate deficient adenovirus expressing CD40L administered intra-tumourally has been piloted (eVon Euler et al., 2008; von Euler et al., 2009) and succeeded by a larger clinical trial of CMM patients (Westberg et al., 2013). The use of viral agents as cancer immunotherapeutics is a rapidly growing field and results from on-going human and veterinary trials are eagerly awaited.

Cytokine therapy is able to promote adaptive immune responses as well as the previously described innate induction. High dose interleukin-2 (IL-2), a T-cell growth factor has documented efficacy against human melanoma; however, its toxicity can be significant and response rates only modest (Rosenberg et al., 1994). Exposure of canine peripheral blood lymphocytes to low dose recombinant human IL-2 enhanced their killing activity against a canine melanoma cell line *in vitro* (Helfand et al., 1994b).

Treatment of CMM cell lines *in vitro* with IL-2 activated peripheral blood lymphocytes and monoclonal antibodies targeting GD2 and GD3 gangliosides resulted in additive cytotoxicity compared to using either strategy in isolation (Helfand et al., 1994a). In a subsequent clinical trial, a combination therapy approach was employed which aimed to activate the innate system as well as expanding T lymphocytes utilising an intra-tumoural injection of a lipid-complexed plasmid DNA encoding both a bacterial superantigen and a cytokine (either GM-CSF or IL-2) (Dow et al., 1998). By combining lethally irradiated transgenic xenogeneic cells secreting human IL-2 and human GM-CSF with the herpes simplex thymidine kinase suicide gene and ganciclovir, Finocchiaro and co-workers were able to extend survival times significantly in CMM patients treated with these agents (Finocchiaro et al., 2008). Another cytokine, the pleiotropic cytokine interleukin-12 (IL-12) exerts multiple down stream effects and promotes both innate and adaptive anti-tumour immunity (Tahara and Lotze, 1995). Culturing canine peripheral blood mononuclear cells (PBMCs) with recombinant human IL-12 enhanced their proliferation and also increased their cytotoxicity against allogeneic CMM cells *in vitro* (Phillips et al., 1999). Further clinical trials are needed to assess the potential of these promising CMM therapies.

Two recent, exciting developments to reach the human clinic for melanoma, and which theoretically offer potential future treatment options for CMM are the use of immune checkpoint inhibitors and adoptive cell therapy. Immune checkpoint molecules are expressed on T-cells as an important mechanism to regulate the immune response and maintain self-tolerance (Pardoll, 2012). Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) are both proteins found on the surface of cytotoxic T-lymphocytes. Their binding to relevant ligands expressed on immune cell and tumour cell targets dampens effector T-cell immune responses and leads to immune evasion, which is detrimental to anti-tumour immunity (Nirschl and Drake, 2013). Interestingly checkpoint molecules are also highly expressed on the surface of Tregs where paradoxically they activate this subset of T-cells; checkpoint blockade is therefore able to inhibit immunosuppressive Tregs (Pardoll, 2012). Clinical trials to prevent normal ligand binding by administering ipilimumab and lambrolizumab, monoclonal antibodies against CTLA-4 and PD-1, respectively, revealed remarkable responses against advanced stage human malignant melanoma (Hamid et al., 2013; Hodi et al., 2010). Blockade of the PD-1 receptor pathway can also be accomplished using monoclonal antibodies against its ligand PD-L1 resulting in durable tumour responses in people suffering from advanced stage malignancies including melanoma (Brahmer et al., 2012). As ipilimumab is a fully human antibody and lambrolizumab is a humanised antibody and as both require multiple dosing, neither of these treatment options would be optimal for CMM due to an immune response against these xenogeneic antibodies. However, phage display technology has been used to produce canine single chain variable region fragments (scFvs) (Braganza et al., 2011) and this technique offers significant future potential for the manufacture of therapeutic canine monoclonal antibodies such as the "check point checkpoint inhibitors".

Adoptive cellular therapy in which autologous tumour infiltrating lymphocytes (TILs) are harvested from human melanoma patients, expanded *ex vivo* and then re-infused intravenously post-lymphodepletion has also resulted in some incredible effects in clinical trials with advanced stage melanoma patients (Rosenberg and Dudley, 2009). Engineering of autologous T-cells *ex vivo* to alter the T-cell receptor (TCR) has also been performed enabling T-cells to engage with tumour cells in patients where the unaltered autologous cells might be unable to bind to the appropriate targets (Robbins et al., 2011). Latterly T-cells with chimeric antigen receptors (CAR T-cells) have also been developed in pre-clinical melanoma models to enhance T-cell target recognition and killing (Zhang et al., 2014). CAR T-cells are already in the human clinic for other malignancies such as the CD19 CAR for acute lymphoblastic lymphoma (ALL) (Maude et al., 2014), and it remains to be seen if they prove as useful for melanoma. Whilst such adoptive cell therapy is hopeful to many, this therapy is very expensive and requires sophisticated techniques and is not readily applicable to CMM at the current time.

In addition to the efficacy of immunotherapy against melanoma demonstrated in the above canine and human examples, other evidence supports the rationale behind immunological targeting of this tumour type. One rare but remarkable phenomenon in human melanoma is complete and spontaneous regression of metastases, which is thought to be immune-mediated but is incompletely understood (Bramhall et al., 2014). More definitive evidence for the role of spontaneous anti-tumour immunity in human melanoma is the positive correlation between the presence of tumour infiltrating lymphocytes (TILs) and improved melanoma specific survival (Thomas et al., 2013); moreover decreased TILs in the primary tumour predict sentinel lymph node metastasis in patients with cutaneous melanoma (Taylor et al., 2007). Neither spontaneous remission of CMM nor the effect of TIL on CMM prognosis have been reported therefore one can only speculate as to their biologic relevance in dogs.

4 Melanoma vaccination

The failure of conventional chemotherapy to extend survival combined with evidence of induced and spontaneous positive effects of the immune system in melanoma patients mean there is great interest in targeting advanced malignant melanoma immunologically in both man and dog.

The discovery of tumour-associated antigens (TAAs) has allowed for the development of techniques to specifically target neoplasms immunologically. Various TAAs, such as tyrosinase have been identified in melanoma (Figure 1) and different immunological approaches exist to invoke responses against these (Buonaguro et al., 2011). Most TAAs are self-antigens and whilst priming an immune response against these is difficult, using closely related paralogues can sometimes facilitate this (Guevara-Patiño et al., 2003).

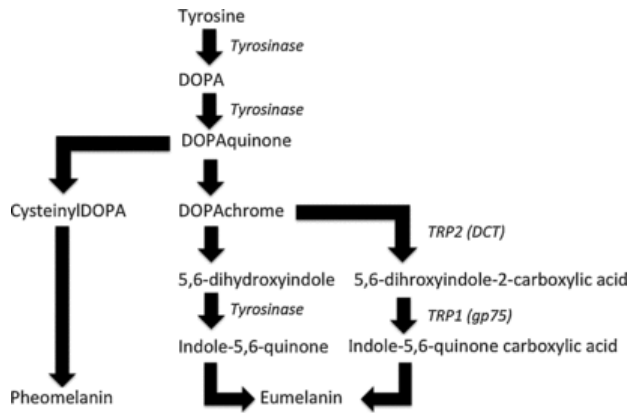


Figure 1 The melanogenesis pathway, enzymes involved in the pathway that act as differentiation antigens are indicated with *italics*. Note the multiple roles of the tyrosinase enzyme.

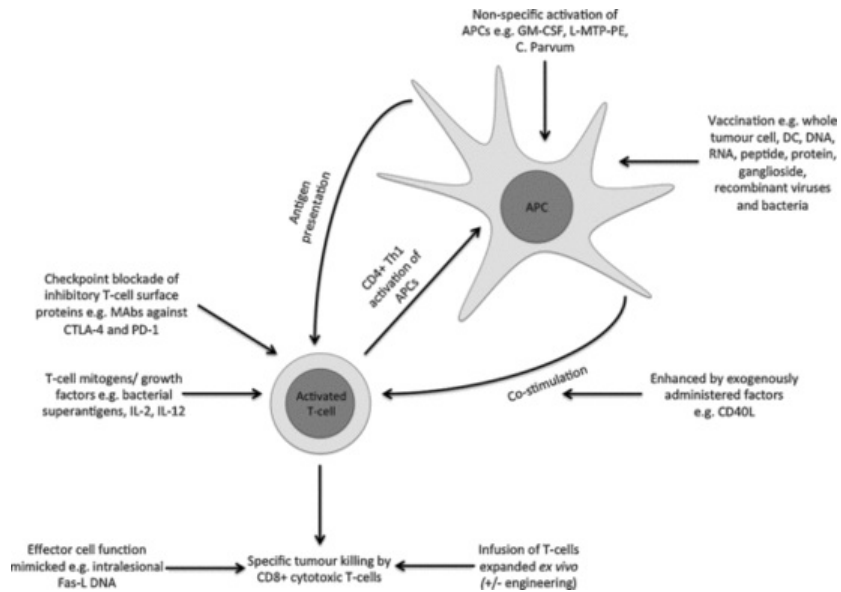


Figure 2 Unleashing the anti-cancer T-cell. Mechanisms and targets of modern cancer immunotherapy. APC: antigen presenting cell; GM-CSF: granulocyte macrophage colony-stimulating factor; L-MTP-PE: liposome encapsulated muramyl tripeptide-phosphatidylethanolamine; C. Parvum:

Corynebacterium parvum; DC: dendritic cell; CD40L: CD40 ligand; CD4+ Th1: CD4+ T-helper type 1 cell; MAb: monoclonal antibody; CTLA-4: cytotoxic T-lymphocyte-associated antigen 4; PD-1: programmed death-1; IL-2: interleukin-2; IL-12: interleukin-12; Fas-L: Fas-ligand.

4.1 Vaccination strategies

Multiple different vaccination strategies have been devised to induce an anti-tumour immune response. These include allogeneic whole cell tumour vaccines, DC vaccination, DNA vaccination, RNA vaccination, peptide vaccination, protein vaccination, as well as utilising recombinant viral and bacterial vectors (Atherton and Lichty, 2013; Lichty et al., 2014; Vergati et al., 2010). Vaccination with the ganglioside TAA, GD3, has also been demonstrated in healthy dogs (Milner et al., 2006). Injection of this weak self-antigen with adjuvants (CpG sequences and RIBI-adjuvant) was able to overcome GD3 tolerance (Milner et al., 2006). Of the other techniques, allogeneic whole cell vaccination, DC vaccination and DNA vaccination have been evaluated for CMM.

4.2 Whole cell vaccination

Various protocols can be used to prepare whole tumour cells for patient vaccination. In a clinical trial using allogeneic whole cell vaccination, the canine melanoma cell line, 17CM98, was transfected with xenogeneic human gp100 (a melanocyte specific trans-membrane protein [Feige](#), a melanoma TAA), killed by irradiation and administered intra-dermally to dogs in an attempt to break tolerance with a combination of self and xenogeneic antigens (Alexander et al., 2006). Hogge and colleagues adopted a

different vaccination strategy and injected healthy dogs with lethally irradiated CMM cells transfected with human GM-CSF resulting in increased numbers of macrophages, which may act as APCs, at the vaccination site compared to non-transfected tumour cell vaccines (Hogge et al., 1999). In a further trial, Finocchiaro and Gilkin combined autologous/allogeneic formalised tumour cells as a vaccine injected concomitantly with lethally irradiated xenogeneic cells producing human IL-2 and human GM-CSF to treat CMM patients (Finocchiaro and Glikin, 2007). Killed whole cell vaccines are able to generate biologically relevant immune responses and exert variable anti-tumour activity.

4.3 Dendritic cell vaccination

Dendritic cell vaccination involves harvesting DCs and then loading these professional APCs with relevant TAAs. Using this strategy, autologous DCs were harvested from bone marrow, expanded *ex vivo*, transduced using an adenovirus expressing human gp100 and administered subcutaneously to treat three dogs suffering from CMM (Gyorffy et al., 2005). Catchpole and colleagues were able to culture and expand DCs from peripheral blood mononuclear cells of CMM patients *ex vivo*, using a cocktail of cytokines thus providing preclinical evidence for the feasibility of autologous DC vaccines (Catchpole et al., 2002). *In vivo* evidence of T-cell mediated immunity was demonstrated by delayed-type hypersensitivity skin testing in healthy dogs following vaccination with autologous DC cells that were pulsed with lysates from the CMM cell line CMM2 (Tamura et al., 2008). Optimisation of these vaccination regimes is needed to cement their place in the veterinary cancer clinic.

4.4 Oncolytic vaccination

Another approach for melanoma vaccination, not yet tried in dogs but which has shown great promise in the pre-clinical setting used two different viral vectors expressing TAAs as a heterologous prime: boost strategy in a mouse model. An adenoviral prime was combined with an oncolytic Vesicular Stomatitis Virus (VSV) boost, both vectors expressed the melanoma TAA human dopachrome tautomerase/tyrosinase-related protein 2 (DCT/TRP2) (Bridle et al., 2010). By utilising heterologous prime: boost vectors in this oncolytic vaccination, marked specific cellular immunity against the TAA was elicited without generating large anti-vector responses; anti-tumour immunity translated into tumour control in aggressive murine models of melanoma (Bridle et al., 2010). These data have been recapitulated in a second study where the VSV boost was replaced with another rhabdovirus exhibiting superior oncolytic properties; namely Maraba MG1 (Pol et al., 2014). The results of on-going trials both in the human and veterinary clinic are eagerly awaited to further appraise such vaccines.

4.5 DNA vaccination

Naked plasmid DNA vaccination encoding TAAs offers an alternative form of therapeutic vaccination. The goal of DNA vaccination is to induce antigen production in host cells after *in vivo* transfection (Fioretti et al., 2010). Naked plasmid DNA vaccines are discussed here; however, plasmid DNA can also be delivered on gold particles resulting in direct transfection of DCs as well as keratinocytes (Porgador et al., 1998). In the case of intramuscular injection of naked plasmid DNA, specific cytotoxic T-cell responses are induced by antigen transfer from transfected myocytes to host professional APCs primarily *via* cross presentation (Fu et al., 1997). Bacterial plasmid DNA can provide the co-stimulation required for T-cell activation as CpG motifs within plasmids drive Toll-like receptor (TLR)-9 dependent stimulation of innate immunity (Hemmi et al., 2000; Klinman et al., 1997). Theoretically the DNA plasmid platform provides a versatile and potent vaccination strategy.

Preliminary preclinical data in mice has generated huge interest in applying DNA vaccines in the cancer clinic. One good example is the xenogeneic vaccination for CMM, which was developed following preclinical investigations in a murine melanoma model (Weber et al., 1998). By vaccinating mice with human cDNA rather than syngeneic DNA encoding the gp75/tyrosinase-related protein 1 (TRP1) differentiation antigen, immune tolerance was broken and led to a protective anti-neoplastic effect (Weber et al., 1998). Although differentiation antigens are shared between normal and tumour tissues, they are recognised as a *bona fide* target of cancer immunotherapy with the caveat that successful vaccination can cause auto-immunity (Buonaguro et al., 2011; Weber et al., 1998). Tyrosinase (a melanosomal glycoprotein) is a differentiation antigen with expression confirmed in canine melanocytic neoplasms (Figure 1) (Brichard et al., 1993; Ramos-Vara and Miller, 2011; Yamaguchi et al., 2007). Its key role in melanogenesis means that tyrosinase represents a valid target for anti-melanoma immunotherapeutics.

5 A new era for veterinary oncology

In February 2010, Oncept™ was the first cancer vaccine to receive full approval from the US Department of Agriculture (USDA) after having previously been granted a conditional license in 2007. Oncept™ is a bacterial plasmid DNA vaccine encoding the human tyrosinase gene and is licensed for the adjuvant treatment of stage II and III oral CMM after loco-regional control. Treatment entails administration of 102 µg of DNA in a volume of 0.4 ml to the semimembranosus/semitendinosus muscles of the hind-leg medially. A purpose-designed, needle free injection device delivers the DNA intramuscularly *via* a transdermal route. The initial course is a series of four doses given two weeks apart with a single booster vaccination every six months, indefinitely thereafter. This vaccine is the only approved therapeutic for *canine* oral CMM.

6 Early evidence supporting Oncept™

Data published in 2003 led to conditional licensing of the human tyrosinase vaccine in dogs. In a phase I trial of dogs with advanced stage melanoma, no significant toxicities were noted and overall median survival time was 389 days (d) (Bergman et al., 2003). Subsequent analyses of the sera revealed antibodies in three dogs against human tyrosinase; these three dogs had good clinical responses (Liao et al., 2006). Two of the three dogs with antibodies against human tyrosinase were also positive for antibodies directed against canine tyrosinase leading to the conclusion that these two dogs were no longer tolerant of this self-antigen (Liao et al., 2006). In a larger follow-up study, dogs with oral CMM were randomised to groups receiving DNA vaccinations of human tyrosinase, murine gp75 or murine tyrosinase (Bergman et al., 2006). Overall median survival times for 33 dogs with loco-regionally controlled stage II/III disease was 569 d for all

xenogeneic vaccines (Bergman et al., 2006). More recently, another study revealed that healthy dogs vaccinated using the needle-free technique with human tyrosinase DNA resulted in T-cell-mediated immunity (Goubier et al., 2008). Specifically, a strong IFN- γ response was elicited post-vaccination when peripheral blood mononuclear cells were re-stimulated with overlapping peptide pools from the human tyrosinase protein (Goubier et al., 2008). Similarly when human melanoma patients were treated with DNA vaccines encoding for murine or human tyrosinase, antigen specific CD8+ T-cells were detected in seven of the eighteen people vaccinated (Wolchok et al., 2007). Taken together, the above data led to full USDA licensing for Oncept™ and was enough for Bergman and Wolchok to conclude that this vaccine “may represent a great leap forward in clinical efficacy” compared with other anti-melanoma immunotherapies (Bergman and Wolchok, 2008).

DNA vaccines have favourable safety profiles, are relatively cheap and technologically straightforward to manufacture in large batches when compared with some of the previously discussed vaccination technologies (Fioretti et al., 2010). Another theoretical advantage of xenogeneic DNA vaccination is that such vaccines could potentially be employed in multiple species (except the species from which the cDNA originates) in order to break tolerance. At present no publications have assessed the clinical use of Oncept™ in animals other than the dog. Overall, the application of this product in the veterinary clinic was met with eager anticipation.

7 Post-licensing analysis – Is the quality of data sufficient to recommend using Oncept™?

Following FDA approval of Oncept™, two important papers with opposite conclusions were published. Grosenbaugh and colleagues prospectively enrolled 58 dogs with stage II or III oral CMM and treated them with Oncept™ after surgery or irradiation (Grosenbaugh et al., 2011). The melanoma specific median survival time was not reached (Grosenbaugh et al., 2011). In contrast to these findings, Ottnod and co-workers retrospectively analysed 22 vaccinees and compared these to 23 non-vaccinees with oral CMM (Ottnod et al., 2013). When comparing dogs with stage II and III disease, neither progression free survival (PFS) nor overall survival was significantly different for vaccinees compared to non-vaccinees. In a recent editorial, several issues were raised regarding the quality of evidence in both studies (Vail, 2013). A major criticism of these trials was that some of the data are retrospective. Prospective randomised clinical trials are considered “gold standard” for assessing the efficacy of a new pharmaceutical agent, partly as they avoid temporal bias between groups such as changes to primary tumour management, stage migration and grade inflation (Sahora and Khanna, 2010; Vail, 2007). The author of the editorial correctly raised concerns regarding study size for the Ottnod paper (Ottnod et al., 2013) and the level of censoring in the Grosenbaugh study (Grosenbaugh et al., 2011) (it was impossible to derive a melanoma specific median survival time for vaccinees as more than half of these dogs were censored from this analysis) (Vail, 2013). A subsequently published case series of dogs with oral CMM treated with surgery found no survival benefit in a group that received adjuvant vaccination compared to surgery alone (Boston et al., 2014). Based on the results of these trials, it is unclear as to whether a veterinary oncologist should actively advise treatment with Oncept™. Indeed, without a randomised, prospective and double-blinded, clinical trial, veterinary oncologists will not confidently be able to recommend either in favour of or against the use of Oncept™ (Vail, 2013).

8 Possible immunological reasons for lack of efficacy

Whichever way the available efficacy data for Oncept™ are analysed, it is clear that some dogs did not respond to this treatment, as is true with virtually all anti-cancer medications. Pigmentation levels between canine melanomas can vary and dogs with high tumour pigmentation, representative of normal melanogenesis, carry a more favourable prognosis in oral CMM (Bergin et al., 2011). It is possible that in poorly pigmented or amelanotic melanomas, TAA expression may be altered. Perturbation of antigen presentation *via* down regulation of tumour antigens as well as alterations to the major histocompatibility complex class I (MHC I) can both occur within neoplastic cells (Igney and Krammer, 2002). Tyrosinase expression in human melanoma cell lines is variable, thus leading Chen and colleagues to propose that tumours with low levels of tyrosinase expression are poor targets of tyrosinase-specific immunotherapy (Chen et al., 1995). In contrast, a CMM study revealed strong tyrosinase and MHC I mRNA expression in all tumours evaluated irrespective of the degree of tumour pigmentation (Phillips et al., 2012). It should be noted, however, that although these mRNA data are supportive of active gene transcription, one could not conclude that the encoded proteins are properly translated, proteolytically processed and correctly oriented within the cell, which is of key importance to effective antigen presentation (York and Rock, 1996). Another peculiarity of some human melanoma cells is their ability to constitutively express MHC II and this is often associated with a poor prognosis (D'Alessandro et al., 1987; Martins et al., 2009; Ostmeier et al., 2001). Immune escape mediated by this alteration has been proposed as a possible explanation for worsening of the tumour phenotype (Aoudjit et al., 2004). In some circumstances however, CD4+ T-cells are able to kill MHC II positive melanocytes *in vitro* so the full effect of this anomaly is far from understood (Brady et al., 2000) and, currently, no studies evaluating this aberration have been undertaken for CMM.

The early results of DNA vaccines administered to human patients were, on the whole, disappointing which was attributed to their inferred low immunogenicity in the clinical setting (Signori et al., 2010). Mechanisms to increase their potency have been reviewed and include delivery by electroporation and using DNA vaccines in the context of a heterologous prime: boost strategy (Stevenson et al., 2010). Electroporation improves DNA vaccine activity by enhancing antigen expression as well as increasing inflammatory milieu (Best et al., 2009; Low et al., 2009; Roos et al., 2009). Using viral vectors to boost primary plasmid DNA vaccines has proven effective for infectious disease such as malaria (Gilbert et al., 2006) and is thought to focus the immune effector response against the intended target organism as opposed to transfected myocytes or APCs. The value of this type of regimen is yet to be appraised in cancer patients (Stevenson et al., 2010). There are several potential reasons for the inactivity of DNA vaccines generally and, more specifically, within melanoma setting and research is continuing to further enhance their efficacy.

9 Concluding remarks

Recent immunotherapeutic advances have been made against human malignant melanoma including anti-CTLA-4 and anti-PD-1 antibodies as well as adoptive T-cell transfer (Schadendorf et al., 2012). Little is mentioned in the current

human literature regarding the use of plasmid DNA vaccines for this disease so it would appear easy to ignore this treatment modality. Although the data regarding the efficacy of Oncept™ in CMM is inconclusive, a randomised prospective trial is required before passing a final judgement on this therapy. Due to the many similarities between CMM and malignant melanoma in people future collaboration between oncologists and researchers in both fields will be mutually beneficial in the pursuit of finding a cure for this aggressive disease in both species.

Uncited reference

Bergman et al. [2012](#) (2012).

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