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Opportunities in immunotherapy for human and canine melanoma

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Abstract

Both in humans and dogs (metastatic) melanoma is a common and aggressive disease for which established treatment methods have not been proven curative. The treatment for human melanoma patients has seen incredible changes over the past few years due to the development and approval of targeted agents, such as BRAF and MEK inhibitors, and new methods of immunotherapy. Over the last few years a large number of immunotherapy options have been proposed and trialed. The present literature study describes these promising immune-based therapies for human melanoma, namely cytokines, monoclonal antibodies (anti-CTLA4 and anti-PD1), cancer vaccines, and adoptive cell therapy, and their most important results. At this point several of these immune-based drugs, like CTLA-4 antibodies and PD-1 antibodies, have demonstrated to induce long term responses in a section of patients and have been approved, while several others are in different stages of development. Challenges that can accompany immunotherapy include a low response rate, a delay in effects, adverse effects, and high costs.

An overview of the clinical trials conducted with immune-based drugs for canine melanoma is also given. To date only the DNA-based canine melanoma vaccine has been approved and many other immunotherapy methods using (non)viral vectors to deliver gene products or cytokine producing cells have been studied. In general, adverse events were minor or absent and treated patients often showed an improved course of disease and survival times. This suggests their usefulness as adjuvant treatment options for canine melanoma.

Outcomes obtained in canine melanoma clinical trials can serve as a good preclinical proof of concept and evaluate safety for human immunotherapy trials. The major strengths of a canine model are the significant similarities in melanoma biology and the spontaneous occurring of the disease in a species that is immune competent, outbred and lives in an environment that is similar to humans.

Contents

Introduction

Melanoma is a relatively frequently seen naturally occurring, often aggressive neoplasm in dogs. It is commonly seen on mucosal surfaces in the oral cavity, acral sites (foot pad and nail bed) and haired skin. Last of which is much less likely to be malignant. In dogs it is the most commonly seen oral neoplasm (Bergman et al., 2003). Some breeds seem to have a higher incidence of melanoma, like dachshunds, Scottish terriers, golden retrievers, and poodles (Withrow et al., 2013). Furthermore, dogs with heavily pigmented mucosa seem to have a higher risk (Bergman, 2007). Besides these primary tumor sites melanomas often metastasize, mainly to the local lymph nodes and lungs, but also the brain, heart, and spleen (Kim et al., 2009). Canine melanomas stem from melanocytes, but the etiology is not yet completely understood, however since it is commonly seen in the oral cavity, sunlight does not seem to play a part (Withrow et al., 2013). As specific breeds seem to have higher risks, this suggests genetic factors could be involved in the pathogenesis (Westberg et al., 2013). Different molecular and genetic factors seem to be associated with melanoma in dogs (including metallothionein, PTEN, p53, RB-1, N-ras, some angiogenic factors, and cyclin kinase inhibitors) (Guldberg et al., 1997; Modiano et al., 1999). Some of these factors have also been shown to be involved in the human melanoma pathogenesis. This supports the use of a comparative medicine approach in melanoma research (van Elsas et al., 1996; Guldberg et al., 1997; Ross & Wilson, 1997).

Treatment for dogs with malignant melanoma consists of several parts. The primary tumor will be removed, preferably with margins. In addition, radiation therapy can be considered if the primary tumor could not be removed completely, the edges of the removal site are not clean or when there is metastasis to local lymph nodes (Bergman, 2007; Manley, 2011). Even with this treatment, the average survival time of melanoma in stage I is often between 12 to 19 months. In stage II it is 5 to 6 months and a mere 3 months in stage III (Bergman, 2007). In humane medicine the occurrence and mortality of melanoma are on the rise. Historically, the diagnosis of metastasized melanoma meant a poor prognosis, with mean survival rates of around 15% after 5 years (Saraceni et al., 2014). Human patients with metastases (stage IV) have a median survival time of less than 10 months (Sirott et al., 1993). Radiation alone or chemotherapy unfortunately seems to have little effect on the cancer both in human and canine patients and for a long time no drug or treatment regimen had been shown to significantly improve overall survival in advanced and metastatic melanoma in humans (Saraceni et al., 2014). In 1998 based on the results of a phase II trial the first immune-based drug, IL-2 was FDA approved on the basis of ‘an unmet medical need’ and this drug gave long-term complete remission in a small part of patients. However, since then even though immunotherapy was regarded promising, it remained quiet in this novel field of melanoma treatment and it was only in 2010 when the next results were published. In the last few years the treatment of metastatic melanoma in human medicine has greatly evolved with the development of several novel forms of immunotherapy and improvements in survival outcomes have been seen with different immune-based drugs (Haanen, 2014). Some of these medications have already been approved and others still in development (Saraceni et al., 2014).

With a focus on immunotherapy, this literary research seeks to summarize the immunotherapeutic treatment options currently available for metastatic melanoma in human medicine and to examine those on the near horizon. It will look into the possibilities of these drugs in the therapy of canine malignant melanoma and previously conducted canine immunotherapy clinical trials will be discussed.

Material and methods

Currently, research into immunotherapy for human melanoma is booming. Conventional treatment methods have proven ineffective in advanced and metastasized melanoma in both humans and dogs. Immunotherapy holds great promise as different types of immune-based drugs seem effective at least in a percentage of patients. Interest in these novel immune-based therapies comprises both veterinary and human medicine and most research takes place in the latter. These drugs are in different stages of development and some have already been FDA approved.

The primary goal of this literature study is to explore the different immune-based therapies for melanoma in both human and veterinary medicine, with the emphasis on the most promising approaches. Considering the incidence of melanoma, the number of clinical trials in this species and the fact that this literature study was done in combination with a clinical trial for a canine melanoma vaccine (see addendum), dogs were chosen as the specific target group alongside humans. This study will also try to determine if the different immunotherapy approaches studied in both species create possibilities for the other species and evaluate what veterinary medicine can gain from the results in human medicine and vice versa.

Research questions:

- What types of immunotherapy are being used and/or studied in human and veterinary research?
- What are the different mechanisms of action of these immune-based therapies?
- Which melanoma immunotherapies hold the most promise?
    - In which developmental phase are these immune-based therapies?
    - In which population has it been tested?
    - What are the results?
- Can the results be extrapolated from one species to another?
- What are the challenges for these immunotherapies?

Retrieval of literature

The literature search has taken place between April 2015 and June 2015. Keywords were immunotherapy, immune-based therapy, treatment, therapy, clinical trial, targeted therapy, melanoma, mucosal melanoma, metastatic melanoma, human, canine, dog, veterinary, ipilimumab, nivolumab, pembrolizumab, CTLA-4, vaccines, adoptive cell therapy, BRAF inhibitor, dabrafenib, vemurafenib, dendritic, animal model, comparative study, tumor immunotherapy, antitumor immunity, cancer immunology, DNA vaccine, monoclonal, antibody, adoptive immunotherapy, anti PD-1, anti PD-L1, and checkpoint inhibitor in a variety of combinations.

Additionally, the references of the retrieved literature were viewed and, if useful, also retrieved. The same applied for clinical trials described in the literature. The databases used for the literature study were PubMed, CAB Abstracts, Scopus and Google Scholar. Information considering ongoing clinical trials was found on Clinicaltrials.gov.

Background into cancer immunology

There are two parts of an immune system, the very specific, but relatively slow acquired or adaptive response, and the faster but less specific innate response. The innate response includes barriers (skin and mucosa), antigen presenting cells (APCs) such as dendritic cells (DC), macrophages and natural killer cells (NK), and cytokines, the coordinators and regulators of cells in the innate immune response. Adaptive immunity allows for specificity, a memory of previously seen pathogens and the possibility to differentiate nonself from self. It also gives the possibility to respond heavier on repeat exposure to the previously seen pathogen. T and B lymphocytes make up the adaptive immunity. T lymphocytes are divided by their MHC (major histocompatibility complex) and CD (cluster of differentiation) into T cytotoxic cells (MHC class I & CD8) and helper T cells (MHC class II & CD4) and Besides these T helper cells and T cytotoxic cells there are regulatory T cells (Treg) and natural killer (NK) cells. Antibodies are produced by B lymphocytes, which is the humoral system. Antibodies can enhance phagocytosis of target cells, activate complement and produce antibody dependent cellular cytotoxicity. The innate and adaptive immune responses are linked by the innate response's ability to influence the adaptive response, and the sharing of effector mechanisms between both immune responses. Immunity can be further separated by whether the response is induced by exposure to a foreign antigen (which is the active response) or whether they are transferred from an immunized individual (which is the passive response). The most important components of both immune systems are lymphocytes, effector and antigen-presenting cells. Responses can also be divided by whether they react to a specific antigen or a nonspecific response, in which immunity is attempted to be conferred by activating the immune system without a specific antigen (Bergman, 2014).

The activation of an immune response usually occurs in the draining lymph nodes in the area of the tumor. With a tumor of a larger size, it can be able to cause priming on its one. With smaller tumors, it is likely that inflammation and the following influx of dendritic cells (coming from the bone marrow) causes a so danger signal that further activates the adaptive immunity. The dendritic cells are activated by antigens of apoptotic or necrotic neoplastic cells and subsequently migrate to the lymph nodes (Beverly et al., 2000; Grolleau et al., 2005). Immune system invaders contain antigens, which are tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) for neoplastic cells. TSAs only exist in neoplastic cells, while TAAs are present in both normal and tumor cells, although they are expressed in different ways. Ideally, TSAs would be used to create cancer therapies, as they are so specific that they can only be used for individual patients and tumors (Aly, 2012).

An effective generation of antitumor immunity by the host has to overcome significant barriers. Many tumors manage to evade these surveillance mechanisms and are able to grow in immunocompetent hosts as is illustrated by the high number of animals and people that succumb to different kinds of cancer. There are many different methods through which tumors manage to trick the immune response. They include Immunosuppressive cytokine production and impaired dendritic cell function through the inhibited dendritic cell maturation or their inactivation through changes in IL-6/IL-10/VEGF/GM-CSF (Morse et al., 2002), as well as induction of regulatory T cells (such as CD4, CD25, and CTLA-4 positive cells), which in turn can suppress tumor-specific CD4/CD8+ T cells and MHC I loss through different pathways such as gene loss (Yamaguchi & Sakaguchi, 2006). Finally, tumors can induce a MHC I antigen presentation reduction through B7-1 attenuation (which is an important costimulatory for MHC and CD28 mediated T-cell receptor stimulation) when the MHC system is still intact (Bergman, 2014).

Immunotherapy for human melanoma

In the past decade there have been an immense number of clinical trials that explored novel immunotherapy options in the treatment of human melanoma. In regards to the scope of this literature study and this large number, the choice has been made to only discuss the results of those that show promise or are in phase III of development.

'Targeted therapy' options BRAF- and MEK-inhibitors

Although not forms of immunotherapy, there are a few other drugs that need to be mentioned in the review of new treatment strategies for metastatic melanoma in order to fully comprehend the results of research into immunotherapy for metastatic melanoma and to have a more complete overview of current possibilities. Melanoma is notorious for the resistance to chemo and radiotherapy, and until a few years ago, high dose interleukin-2 (HD IL-2), and dacarbazine, a DNA alkylating agent, were the only approved drugs available (Rotte et al., 2015). Since 2011, great advances have been made in the treatment of human melanoma with the approval of inhibitors of mitogen-activated protein kinase kinase (MEK), and inhibitors of BRAF (Finn et al., 2012; Saranga-Perry et al., 2014; Rotte et al., 2015). The BRAF-MEK-ERK pathway is of great importance to cell growth and survival. Of patients with advanced melanoma, 40–60% have mutations in the BRAF gene, which causes aberrant cell growth. The so called V600E-mutation of the BRAF gene is found in over 90 percent of melanomas with the BRAF mutation (Stadler et al., 2015). It has been long known that a large percentage of melanomas has these mutations in the BRAF gene, however studies into inhibitors of the mutated BRAF protein were published first in 2010 (Davies et al., 2002; Bollag et al., 2010; Flaherty et al., 2010). To oncologists, treating many patients with metastatic melanoma, effects of this inhibitor (vemurafenib) were certainly very impressive. Over 85% of patients showed clinical benefit and half of them had an objective decrease in metastases. Even brain metastases seemed to respond to this therapy (Chapman et al., 2011). The enthusiasm for these seemingly great effects was soon tempered by the fast and relentless resistance seen after a median of 5-6 months of treatment (Nazarian et al., 2010; Wagle et al., 2011). The research then focused on a combination of 2 different kinase inhibitors, namely the BRAF-inhibitor (Dabrafenib), and the MEK inhibitor (trametinib) (Davies et al., 2002; Flaherty et al., 2010). Based on the results of a randomized phase 2 study the combination gave an even higher chance of an objective improvement in disease load (> 75%) and long-lasting effect, as measured by progression-free survival. This was almost four months longer than with the BRAF inhibitor alone (Haanen, 2014).

Cytokines

In 1998 a recombinant interleukin 2 came onto the market and gave hope for a better treatment. In vitro, this interleukin is generated in immune responses and subsequently stimulates differentiation, growth, and survival of antigen-specific CD8+ and CD4+ T-cells. IL-2 is important for the distinction between nonself and self, and induces T-cell immunologic memory (Cantrell & Smith, 1984; Malek, 2008). The anticancer activity of IL-2 is due to its ability to be a growth factor for T lymphocytes, its ability to stimulate lymphokine-activated killer cells, and antigen-independent NK cells. It also stimulates lymphocytes at the location of malignancy (Waldmann, 2006). Atkins et al. studied the overall toxicity and efficacy of IL-2 as treatment method for metastatic melanoma. In the clinical trials with 270 patients the overall response rate was 16% (6% complete response and 10% partial response). The median response duration in patients with a complete response was not reached at

the time of publication, and of responders 28% remained free of progression at 54 months (Atkins et al., 1999). This shows that patients with a response could remain disease free for years (Saraceni et al., 2014). Adverse reactions are mostly the result of capillary leak syndrome and extreme vasodilation, and treatment with high-dose IL-2 necessitates an intensive care-like setting for managing patients. It also necessitates a very careful patient selection. Although it is currently known what phenotype is appropriate for IL-2 use, research is taking place into identifying the genotype for which this therapy would be most appropriate (Saraceni et al., 2014). Currently, sold under the name 'Proleukin', interleukin 2 is recommended as a first-line therapy for the stage IV melanoma that is unresectable and where the patient has a 'good performance status' (which means a patient is functionally relatively good) and as second therapy for BRAF positive patients with 'poor performance status' (the patient is not doing well functionally) (Kaufman et al., 2013).

Interferons (IFNs), which are pleiotropic cytokines that have antiviral and immunomodulatory effects, have been studied for their effectiveness in activating immune cells and their ability to upregulate the presentation of antigens to T cells in human melanoma settings (Kirkwood & Ernstoff, 1984). Pegylated interferon α-2b (p-IFN α-2b; which is IFN α-2b that is conjugated to polyethylene in order to reduce absorption and clearance, and therefor increase overall immunogenicity), has since 2011 been FDA approved as an adjuvant therapy for patients with surgically treated melanoma (Herndon et al., 2012; Tarhini et al., 2012). It is mostly recommended as an adjuvant for high-risk stage II and III melanomas. However, it appears that there is limited consensus about the use of p-IFN α-2b (Kaufman et al., 2013).

Future cytokine therapies

The shown benefits of interleukin 2 in (metastatic) melanoma patients have inspired research to look further into other cytokines that could possibly give positive results. Granulocyte macrophage colony stimulating factor (GM-CSF, a hematopoietic cytokine) is known to stimulate multipotent progenitor cells (Kaufman et al., 2014). It stimulates the growth of macrophage progenitors first, followed by, granulocyte, eosinophil, erythroid, and multipotent progenitors (Shi et al., 2006). Results of preclinical trials that used mice models showed GM-CSF to induce inhibition of neoplastic growth and caused regression of the tumor. GM-CSF was then tested as an intratumor monotherapy, together with chemotherapy, and also as an adjuvant treatment in melanoma patients with a varying success rate (Kaufman et al., 2014). One randomized, multicenter phase III clinical trial with stage 2 resected melanoma patients using GM-CSF alone or in combination with gp-100 (a tumor-associated antigen; TAA), tyrosinase peptides, and MART-1 antigen, has been completed and results are being published in the near future (Rotte et al., 2015). First results however are given and show an overall survival time gain of 10.3 months with GM-CSF treatment (69.6 with GM-CSF vs. 59.3 with placebo) and the median recurrence free time was 2,5 months longer (11.4 with GM-CSF vs. 8.8 with placebo). The combination of GM-CSF with the peptide vaccine gave the highest 5-year survival, but differences with placebo groups were very small (4%) (Clinicaltrials.gov: NCT01989572).

The antitumor abilities of other cytokines, like IL-12, IL-15, IL- 18, and IL-21, have been studied as well. However there is currently there limited data available on the safety of IL-15 and IL-21 and different formulations of cytokines are in the phase I stage of research. IL-12 and IL-18 seem to be safe for melanoma patients as these cytokines are in phase II stage of development (Rotte et al., 2015).

Anti-Ctla-4

Another approved immune-based drug for melanoma is Ipilimumab. This is a monoclonal antibody against cytotoxic T lymphocyte antigen-4 (CTLA-4). CTLA-4 is expressed on T cells next to costimulatory receptor CD28. While these CD28 receptors only activate T cells when connected to ligands of antigen presenting cells (APC), CTLA-4 interferes with IL-2 production and expression, stops the cell cycle progression of T cells, and antagonizes the activation of T cells (Karandikar et al., 1996; Phan et al., 2003; Weber, 2007; Rotte et al., 2015). Inhibition of the receptors of CTLA-4 using ipilimumab gave an increased T cell activity and subsequently led to regression of the melanoma (Phan et al., 2003). A randomized, double blind clinical trial with 676 patients showed survival benefits in ipilimumab treated patients when compared to patients treated with a peptide-based (gp100) vaccine (overall survival 10.1 vs 6.4 months). After these results, ipilimumab was approved in 2011 for the treatment of unresectable melanoma. The patients were followed for 55 months, and resistance to the drug was not reported in patients that responded to treatment (Hodi et al., 2010).

In another large study, 502 untreated melanoma patients were randomized to receive either ipilimumab combined with dacarbazine or only dacarbazine. Patients that were responsive or had stable disease received ipilimumab or placebo every 12 weeks as maintenance therapy. Adding ipilimumab to the treatment significantly improved overall survival compared with only dacarbazine (11.2 months vs 9.1 months). Survival rates in the combination group were also significantly higher at 1 year(47.3% vs 36.3%), and remained that way at 3 years (20.8% vs 12.2%). However grade 3 or 4 adverse reactions were seen in 56.3% of patients treated with both ipilimumab and dacarbazine, in the group with only dacarbazine adverse events were seen in 27.5% of patients (Robert et al., 2011; Saraceni et al., 2014).

The long-term results confirmed the responses and their durability as seen in the first clinical trials for ipilimumab. Prieto et al. studied the long term survival data and the follow-up of 177 metastatic melanoma patients. To test the duration of response, patients who were enrolled in three different studies were included. Of the total group of patients, 56 were treated with ipilimumab in combination with a gp100 vaccine (first protocol), 36 patients were treated with ipilimumab in combination with IL-2 (second protocol), and 85 patients were treated with ipilimumab with intrapatient dose-escalation and were also randomized to be treated either with a gp100 vaccine or a placebo (third protocol). For protocols 1, 2, and 3, median survival times were respectively 14, 16, and 13 months and the percentages of patients still alive after 5 years were respectively 13%, 25%, and 23% (Prieto et al., 2012). In 2013 an evaluation of long-term survival data was presented for ipilimumab. The analysis included 1861 patients treated with ipilimumab from 12 phase II and III trials, which were followed up to 10 years. It showed the median overall survival was 11.4 months. After 3 years 254 of patients (22%) were still alive and after 7 years the survival rate was 17%, of patients that survived those years no deaths occurred (Schadendorf et al., 2013). The observed ipilimumab overall survival data demonstrated a significantly more durable survival in relation to the historical data in the era before the BRAF inhibitors (Saraceni et al., 2014).

Ipilimumab is now recommended as a first-line therapy in BRAF negative unresectable stage IV melanoma patients that have a ‘poor performance status’ and as second-line therapy in melanoma patients with ‘good performance status’ independent of whether they have a BRAF mutation (Kaufman et al., 2013; Rotte et al., 2015). The most important limitation of ipilimumab therapy is the

low response rate in a large percentage of patients, as is shown by the nearly 50–60 % of unresponsive patients (Hodi et al., 2010; Robert et al., 2011). However, even though responses are lower, they are much more durable when compared to targeted therapies, such as BRAF or MEK inhibitors. Another important limitation of the treatment is the fact that many patients suffer from severe adverse events like autoimmune colitis, which can drastically limit the duration of therapy and can even cause death. A last downside clinicians should keep in mind is the fact that responses may take a long time, with an average time to complete response of 30 months and sometimes progression is seen before regression (Postow, 2012; Saraceni et al., 2014). Therefore several combining treatments like ipilimumab with BRAF inhibitors (Dabrafenib), human monoclonal anti-PD1 (nivolumab), and/or radiotherapy have been mentioned to lower adverse events and increase the response rate. These therapy combinations are in various stages of clinical testing (Wolchok et al., 2013; Rotte et al., 2015).

Programmed cell death receptor (PD-1) and its ligands

Another important immune checkpoint receptor is Programmed death-1, which is expressed on T cells and is known to downregulate the activity of T cells. PD-1 expression is induced when T cells are activated and it limits their inflammatory response (McDermott & Atkins, 2013; Kyi & Postow, 2014; Merelli et al., 2014). PD-L1 is one of the ligands of PD-1 and is expressed on non-hematopoietic and hematopoietic tissues. The expression of both PD-1 and its ligand in tumor microenvironment shows a role for this type of immune evasion by the melanoma (Zou & Chen, 2008; Kyi & Postow, 2014). Monoclonal antibodies against PD-1 and its ligand are being tested and are currently in different phases of development (Rotte et al., 2015). Two anti PD-1 antibodies, pembrolizumab and nivolumab, were approved by the FDA in 2014 for the therapy of metastatic melanoma after progression of disease during ipilimumab (anti Ctla-4) treatment, as well as in BRAF positive melanoma patients, after progression while being treated with a BRAF inhibitor (Larkin et al., 2015). These PD-1 antibodies were shown to induce an objective response in 30 to 40% of treated patients, with most responses being durable. Several phase III trials have demonstrated a significantly better effect of nivolumab when compared to chemotherapy, in previously untreated patients or patients after progression during treatment with ipilimumab and in patients with melanomas positive for BRAF mutation, after progression during BRAF inhibitor therapy (Robert et al., 2015a; Weber et al., 2015). Weber et al. (2015) showed an objective response rate that was significantly higher in the nivolumab group (32%) when compared to the chemotherapy group (10%). After a follow up period of 6 months, 36 of the 38 responding patients still had ongoing responses. Robert et al. (2015a) treated previously untreated patients without BRAF mutations with cytotoxic chemotherapy (dacarbazine) or nivolumab. Nivolumab was highly superior in terms of response rate (40% for nivolumab vs. 13.9% for dacarbazine), median progression free survival (5.1 months for nivolumab vs. 2.2 months for dacarbazine), and 1-year overall survival (72.9% for nivulomab vs. 42.1% for dacarbazine). One study conducted long-term follow up of patients treated with nivolumab, including 107 patients with melanoma. At 1 year the survival rates were 62% and 43% at two years, with a median overall survival of 16.8 months. At 2 years 27% of patients remained progression free, which made the updated response similar to the original publication where they reported a response rate of 31%. The long-term safety assay was also comparable to the original analysis, 22% of patients experienced grade 3/4 treatment-related adverse events and 5% showed grade 3/4 immune-related adverse events (Topalian et al., 2014). Recently, a phase III clinical trial with patients with advanced melanoma showed that pembrolizumab had a significantly longer progression-free interval, overall

survival time and response rate than seen with ipilimumab. Significantly more serious adverse events (grade 3 and 4) were seen with ipilimumab than with pembrolizumab (Robert et al., 2015b). A phase II trial that compared ipilimumab alone and in combination with nivolumab in BRAF negative patients showed response rates of 61% with the combined treatment and 11% with just ipilimumab. The combined therapy had a complete response in 22%, for the monotherapy this was 0%. Adverse effects of grade 3 of 4 were seen in 54% of patients that were treated with both drugs and in 24% of patients in the monotherapy group (Postow et al., 2015). Another phase III trial with 945 previously untreated patients showed a median progression-free survival of 11.5 months with a combination therapy of nivolumab and ipilimumab, as compared to 2.9 months with ipilimumab, and 6.9 months with nivolumab (Larkin et al., 2015).

Adoptive Cell Therapy

Another immune-based approach to treating melanoma is adoptive T cell therapy (ACT). In this therapy allogeneic or autologous lymphocytes that have a strong antitumor activity (tumor-infiltrating lymphocytes; TILs) are identified and selected in vitro. After being cultured and manipulated ex vivo and along with growth factors to stimulate expansion and survival, they are infused back into the patient. Sometimes the patient is also modified before cell transfer, in order to provide an ideal environment for the transferred cells (for example through lymphodepletion) (Rosenberg et al., 2011). Rosenberg et al. studied the efficacy of ACT using autologous TILs (combined with high dose IL-2) to achieve durable complete regression in previously treated metastatic melanoma patients. Objective response rates in 3 trials using lymohodepleting preparative regimens (chemotherapy alone or in combination with 2 or 12 Gy irradiation) were 49, 52 and 72%, respectively. 20 patients of 92 in total (22%) achieved complete regression, of which 19 had ongoing complete regression beyond 3 years. The 3- and 5-year survival rates for the complete group were 36 and 29%, respectively. In the complete response group this was 100 and 93%. Response was not related to previous therapy (Rosenberg et al., 2011; Saraceni et al., 2014).

Although shown to be effective, lymphodepletion through high-dose toxic chemotherapy unfortunately causes unwanted side effects. Therefore a trial was done into the combination of ACT with IFN- α, as this is routinely used in stem cell transplantation as a replacement for the more toxic IL-2. This trial was conducted to test the efficacy and safety of ACT in combination with daily injections of IFN- α in metastatic melanoma patients (Verdegaal et al., 2011). In this trial 10 patients were treated with up to 6 T cell infusions, which consisted of tumor-reactive CD4+ and/ or CD8+ T cells. 7 days prior to infusion patients received low-dose IFN- α on a daily basis for 12 weeks. Of the patients, 5 showed objective response, including 1 complete regression, 1 partial regression and 3 stable diseases. Only one patient observed a serious adverse effect, although all responsive patients showed transient leucopenia (Verdegaal et al., 2011).

Currently several studies are taking place into TIL-based therapies worldwide. Including one that is trying to find out if young TILs, that are less difficult and time-consuming to create, are as effective as selected TILs, which would mean that this kind of therapy could reach more patients (Dudley et al., 2013). Although ACT seems promising, there is not yet enough data on the safety and proof-of-concept details of the treatment (Rotte et al., 2015). All of the 16 clinical trials registered for testing the ACT/TILs in melanoma are still in phase I or II (Clinicaltrials.gov).

Cancer vaccines

At the moment there are many clinical trials into cancer vaccines being carried out, they are based on the hope that attenuated tumor cells or the tumor cell peptides can induce an immune response against the tumor. Different antigens combinations have been studied in order to find out if they evoke a TAA-specific response and therefore have an anti-tumor effect (Aranda et al., 2013; Rotte et al., 2015). In general, these vaccines can be divided into 4 subtypes. The first being peptides of TAAs or full length TAAs, of which the short peptides have to bind to the MHC on APCs and are subsequently presented to T cells. The full length TAAs are processed by APCs after which they are presented to T cells. The second subtype are cancer cell lysates that contain TAAs, they are either given alone or in combination with chaperones that suppress T cells (like cyclophosphamide). The third subtype is TAAs delivered to the target cells using RNA molecules, viral delivery systems or naked DNA. The last type of cancer vaccines is based on dendritic cells, which includes autologous dendritic cells loaded with TAAs ex vivo (Aranda et al., 2013).

Peptides of TAAs

Melan A (also known as MART-1), which binds to MHC type I, GP100, Melanoma-associated antigen genes (MAGE), and NY-ESO (Cancer testis antigen) which binds to MHC type II are the peptides used the most in peptide melanoma vaccines. Clinical trials focus on both nonresectable melanoma and resected melanoma patients. Adjuvants play an important role in the effectiveness of a vaccine in inducing the immune response (Rotte al., 2015). Most of the melanoma vaccine clinical trials are in phase I and II, but a polyvalent allogeneic melanoma vaccine developed is currently in phase III studies and focusses on treating postresection patients with high recurrence risks and the expected completion date is July 2016 (Clinicaltrials.gov: NCT01546571).

Cancer cell lysates

Melanoma vaccines that are based on tumor cell lysates use cultured cancer cells of human origin or autologous melanoma cells as antigens along with different adjuvants. Sometimes, T cell deprivation is used. There are only a few studies that test the cancer cell-based melanoma vaccines, and most of them are in phase I or II (Rotte et al., 2015). One phase III randomized, double-blind, placebo-controlled trial of M-Vax (a melanoma vaccine prepared from autologous cancer cells) plus low dose IL-2, had expected to have results in January 2015, however no results are given to date. (Clinicaltrials.gov NCT00477906)

Gene delivery systems to deliver target TAAs

Gene delivery systems like naked RNA, DNA, or viral vectors are being used to give target gene expression and subsequently make vaccines more effective in creating the right immune response. The DNA-based canine cancer vaccine (Oncept), a xenogeneic vaccine with genes encoding for human tyrosinase (a glycoprotein of importance in the melanin synthesis) is also being tested in human medicine (Bergman 2003; Murakami & Sunada, 2011; Aranda et al., 2013). However, the data on the safety of this system and the others that are being developed for human melanoma patients is not complete, and all the currently registered clinical trials primarily evaluate the safety and tolerance of the respective vaccine, indicating the status of this type of vaccines in the treatment of human melanoma (Rotte et al., 2015).

Dendritic cells

Dendritic cells (DCs) are some of the most important cells in the immune system and are critical in the initiating of immune responses. They capture antigens, subsequently process the proteins into peptides which in turn bind to MHC class I and II molecules. They also play a role in presenting the MHC bound peptides to T cells. After interacting with dendritic cells loaded with antigen, T cells become differentiated and have specific functions (CD8+ cells that become cytotoxic T lymphocytes and CD4+ cells that turn into T helper cells or regulatory T cells) (El Marsafy et al., 2009; Palucka & Banchereau, 2012).

It has been shown that the density of dendritic cells around the melanoma was directly correlated with the thickness of tumor and its prognosis (Simonetti et al., 2007). Because of the importance of dendritic cells in immune responses, several studies have attempted to use their ability to give tumor-specific immune responses. They did so by administering autologous dendritic cells that were infused with tumor-specific antigen (El Marsafy et al., 2009; Palucka & Banchereau, 2012; Anguille et al., 2014; Radford et al., 2014). To date, many clinical trials have been registered that focus on activated autologous dendritic cells ass a possible therapy for melanoma. Half of these clinical trials are in phases II and III, showing that this type of immunotherapy is feasible, tolerated by patients and is able to produce melanoma specific immune responses. Last year, a randomized, multicenter, phase III trial started that aims to evaluate the effectiveness of the adjuvant vaccination of autologous dendritic cells loaded with tumor RNA in patients with resected uveal melanoma and the first results are expected in June 2020 (Clinicaltrials.gov NCT01983748). Another phase III, multicenter, randomized, double-blind trial that aims to compare autologous dendritic cells combined with irradiated autologous neoplastic cells in GM-CSF (granulocyte-macrophage colony stimulating factor; a type of growth factor that stimulates growth of white blood cells), to autologous mononuclear blood cells in GM-CSF as immunotherapy for metastatic melanoma, started in October 2014 and its expected date of completion is 2022 (Clinicaltrials.gov NCT01875653, Rotte et al., 2015).

Other possibilities

Besides the novel immunotherapies described above, many clinical trials are investigating different possibilities. However it is too early to tell if they are promising as these possibilities as they are mostly still in phase I of development. Therefore some of them will be shortly mentioned.

As a costimulatory receptor found on T cells, CD27, is of great importance in the activation and survival of T cells and natural killer cells. CD27 is activated by its ligand, CD70, which is known to be expressed on B cells, T cells and activated dendritic cells (Denoeud & Moser, 2011; Thomas et al., 2014). In preclinical trials a monoclonal antibody, Varlilumab, which targets CD27, showed activity against several types of cancers (Vitale et al., 2012). It is now in phase I trials, to test safety in metastatic melanoma patients (Rotte et al., 2015).

B7-H3 is a receptor found on APCs. Reports on its significance in regulation of T cell activity are however conflicting. While some research showed B7-H3 to have inhibitory effects, other studies reported it as a costimulatory receptor (Chapoval et al., 2001; Leitner et al., 2009). MGA271, an antagonist of B7-H3, was reported to have anti-cancer activity in mice models. It is now in the phase I of development (Loo et al., 2012).

Clinical Trials for Immunotherapy in canine melanoma

Just like in humans, canine malignant melanoma is known for its poor prognosis and to date no treatment protocol has been developed that is effective against this disease. Although not as many as in human medicine, there have been a number of clinical trials aimed at improving the prognosis of canine malignant melanoma using immunotherapy. Some research has looked at similar immune-based drugs as in the human clinical trials, while others have only been tested in dogs.

The first publications date back about 20 years and reported a genetically engineered xenogeneic cell gene treatment. It studied the effects of multiple peritumoral injections of human IL-2 secreting cells after tumor resection and radiotherapy. The therapy showed a significantly higher mean survival of 270 days versus 75 days of the surgery combined with radiation controls (n=16, each group). The treatment did not show any adverse effects (Quintin-Colonna et al., 1996).

After a few years, a new clinical trial individually studied an individually targeted gene therapy for canine melanoma in which human GM-CSF gene transfer was studied (Hogge et al., 1998). It described a method in which, following removal of the tumor, a tumor cell suspension was made which received a gene shot with particles of gold coated with a plasmid with the GM-CSF gene. Subsequently they received ^{137}Cs irradiation, after which cells were injected into the patients. The therapy used in 10 canine patients with melanoma induced 3 objective responses and 1 stable disease. No adverse events were observed. While statistically not significant due to the low case number, the survival time of objective responders was more than 300 days, which is considerably more than the expected survival times for surgery only patients (90–165 days) (Hogge et al., 1998).

Another study used injections in the tumor with a gene for a bacterial superantigen, staphylococcal enterotoxin B, as adjuvant treatment in combination with the immunostimulatory canine GM-CSF gene. The mean survival and objective responses for canine melanoma were: stage I, 427 days and 3/3; stage II, 399 days and 3/5; stage III, 168 days and 4/12; stage IV, 168 days and 0/2. Stage III patients (n=12) had an mean survival of 168 days with this treatment, which was far above the historical surgery controls (105 days).The therapy did not give significant adverse reactions (Dow et al., 1998).

A very different technique was used in a research that used the induction of apoptosis to promote immunologic “priming” in melanoma in dogs. In this approach injections of a plasmid carrying the human Fas ligand gene are made directly into the tumor. The Fas ligand is a type-II transmembrane protein that is part of the tumor necrosis factor family and binding with its receptor induces apoptosis. This therapy resulted in a quick reduction of the tumor in 3 out of 5 treated patients. After that time, each patient was given the standard care therapy as indicated for each melanoma (surgery and radiation), and the final outcome was an objective response rate of 4 out of 5 with mean survival times between 168 and 574 days. The treatment was safe (Bianco et al., 2003).

Research that lead to the first approved canine melanoma vaccine (Oncept) started with a study that injected a plasmid with the human tyrosinase gene through intramuscular needle free injection. Its expression as a specific antigen against melanoma significantly increased the mean survival time to 389 days as compared to a historical control group in which canine melanoma patients had a mean

survival time between 60-150 days (Bergman et al., 2003). At least two of the long-term survivors showed considerable numbers of anti-human tyrosinase antibodies (Liao et al., 2006). A larger study used 58 patients to evaluate the efficacy and safety of this cancer vaccine as adjuvant therapy for oral melanoma in dogs with locoregional disease control (Grosenbaugh et al., 2011). Mean survival was significantly longer (more than 750 days) when compared to a historical control group (324 days). It reported only 14 out of 58 deaths were connected to melanoma, while this number was 34 out of 53 in the historical control group, furthermore no significant adverse events were reported (MacEwen et al., 1999). In a further study human tyrosinase as well as xenogeneic DNAs encoding for murine gp75, murine tyrosinase, and human GM-CSF were evaluated at different doses. Combinations of human GM-CSF and murine tyrosinase were also described. Thirty-three stage II-III melanoma dogs with surgically removed canine melanoma across these xenogeneic vaccine trials showed a mean survival of 569 days, a much higher number than expected for regular treatments. Minimal adverse events were noted, like mild reactions at the vaccination site (Bergman et al., 2006). All these trials supported the approval of the first immunotherapy in canine melanoma, the cancer vaccine with the gene that encodes for human tyrosinase. As a continuation of earlier research, a new one showed the effectiveness and safety of the murine tyrosinase xenogeneic vaccine for canine digit melanoma combined with locoregional disease control (Manley et al., 2011). This retrospective research suggested a longer survival for patients with melanoma treated with xenogeneic DNA vaccine (mean survival time: 476 days) versus 365 days for historical patients treated with only surgery. An additional advantage was seen for those patients that had been vaccinated shortly after diagnosis over those patients that had a longer time between diagnosis and the administration of the vaccinations (Manley et al., 2011).

A different retrospective research of 22 human tyrosinase vaccinated compared to 23 non-vaccinated canine melanoma patients did not give significantly longer progression-free interval, disease-free interval, or mean survival (Ottnod et al., 2013). This outcome strongly suggests the need for controlled trials with a larger amount of patients (Glikin & Finocchiaro, 2014).

One study treated 3 canine melanoma patients with autologous DC's infused with viral vector that encoded for human gp100 as an adjuvant therapy. The median survival (210-1440 days) was higher than expected for canine melanoma patients; however 1 dog passed away sooner as a result of progressive disease. No adverse events were seen (Gyorffy et al., 2005).

In a slightly varying approach where canine melanoma patients were treated through vaccination containing allogeneic melanoma cells that expressed human gp100, the patients with a response had significantly longer mean survival times than patients that had no response (respectively: 337 days and 95 days). Adverse events were mild and only seen in the location of the vaccination (Alexander et al., 2006).

A feasibility trial was done for adenovector CD40 ligand (CD40L can restore the APC function and induce APC maturation) therapy (von Euler et al., 2008). A dog with stage III oral melanoma, treated with intratumor vaccination and subsequently surgery, had no relapse and had a survival time of 401 days. A second dog with conjunctival melanoma, treated only with intratumoral injections, had a remission that lasted 150 days. Adverse events were mild and reversible. As a result of this trial, a pilot study was conducted with local AdCD40L as an adjuvant therapy for canine malignant

melanoma (14 oral, 4 cutaneous, and 1 conjunctival). Up to 6 injections with AdCD40L were administered with intervals of 7 days, which was followed by surgery in 9 dogs. The other 10 dogs just received the immunotherapy. Immune stimulation after treatment was shown by the infiltration with B and T lymphocytes in tumor tissue. Five dogs had a complete response, 8 dogs had partial responses, 4 dogs were stable and 2 dogs had progressive diseases. Mean survival was 160 days (20–1141 days), with 3 dogs still alive at the end of the study. The study showed local AdCD40L therapy can be beneficial in canine malignant melanoma and is a safe form of treatment (Westberg et al., 2013).

Human chondroitin sulfate proteoglycan-4 (hCSPG4) plays a role in stabilizing the interactions between the cell and substratum during early stages of melanoma cell spreading on the endothelial basement membranes. A vaccine based on the DNA of hCSPG4 was evaluated on therapeutic effect and safety. Canine patients with stage II-III oral melanoma that was surgically removed and tested positive for CSPG4 were given monthly plasmid administration. This was followed by electroporation (a technique through which the permeability is increased) 6 to 20 months. Overall survival times (653 versus 220 days) and disease-free intervals (477 versus 180 days) were higher in the 14 vaccinated dogs as compared with the 13 not vaccinated control dogs. No adverse events were noted. The results indicate that this type of treatment could be beneficial in canine malignant melanoma patients (Riccardo et al., 2014).

A very different approach was used in studies that tested suicide gene therapy as an adjuvant treatment in canine melanoma. Suicide gene therapy uses a genetically modified virus (in this case herpes simplex) and injects this into the tumor site. The virus deposits a gene into the cancer cells to produce specific enzymes. One of the most extensively studied genes is herpes simplex virus thymidine kinase (HSV-tk). After production of the enzymes, anti-viral drugs (in this case ganciclovir) are administered, which initiate programmed cell death when it comes into contact with the enzymes and thus specifically targets the cancer cells. In a therapy that combined intratumoral injections with xenogeneic cells that secrete hGM-CSF and hIL-2 with local injections of a plasmid bearing HSV-tk suicide gene plus ganciclovir (n=45), canine melanoma patients that showed local objective responses had a longer survival time than patients that had no local responses (220 days versus 151 days), however both survival times were longer when compared to the surgery controls (82 days) (Finocchiaro et al., 2008).

Because of these results a new study was set up in which a surgery adjuvant therapy was assayed and had a much better result than the intratumoral approach used above (Finocchiaro & Glikin, 2008; Finocchiaro & Glikin, 2012). The therapy used in the new study was a combination of surgery which was followed by the immunotherapy combination of a subcutaneous vaccine and suicide gene therapy. The subcutaneous vaccine was a mix of neoplastic cells from the patient and xenogeneic cells that produce hGM-CSF and hIL-2. The postsurgical edges were injected with HSV-tk suicide gene plus ganciclovir. Toxicity was minimal. The combination therapy increased the survival time by a sevenfold for complete surgery and threefold for partial surgery. The complete surgery-combination therapy increased overall survival time from 99 days to more than 2848 days. In this study more than 50% of complete surgery patients passed away from causes unrelated to melanoma. A follow up of 9 years was done for a high number of patients (283) and the results showed that the therapy gives the best outcomes when used as an adjuvant therapy for surgery and is most beneficial when the patient

has minimal residual disease (Finocchiaro & Glikin, 2008; Finocchiaro & Glikin, 2012; Glikin & Finocchiaro, 2014). As a result of safety issues and the costs of vector production, viral vectors pose more difficulty in regards to being approved and available for the use in veterinary oncology (Glikin & Finocchiaro, 2014).

Possibilities arising from commonalities in human and canine melanoma

As showed above there have been a large number of clinical trials into novel possibilities of immunotherapy for melanoma in both canine and human medicine. However these trials have often evaluated different immune-based drugs which creates difficulties in extracting the result from one species to another. In order to use one species as a relevant clinical model for the other it is of great importance that the disease shares a number of characteristics. Historically, animal models play an important role in developing new therapeutic strategies to treat human cancer. Genetically engineered mouse models have been used as preclinical models for melanoma research. While most models are helpful in investigating certain aspects of the disease, they don't have the genetic complexity that exists in naturally occurring tumors, where the disease starts with single cells acquiring mutations. Animal models that show the complete spectrum of the disease from benign neoplasms, primary tumors, and metastases are not often found. Moreover, most of current melanoma models are developed with an experimental activation of NRAS and BRAF (Simpson et al., 2014).

Melanomas are sometimes seen in different animals and are more frequent in specific breeds of horses, pigs, and dogs (Goldschmidt & Hendrick, 2002). Canine melanomas have a higher incidence in the Poodle, dachshund, Scottish terrier, golden retriever, cocker spaniel, Chow Chow, Anatolian sheepdog and Gordon setter, although the real incidence in specific breeds of dogs is not well established (Bergman et al., 2013). Canine malignant melanomas are more often seen in the oral cavity and acral sites (nail apparatus and foot pads). They are seen on the haired skin, however less frequent and with less malignancy in those sites (Goldschmidt & Hendrick, 2002). The pathogenesis of melanoma in dogs is not well understood, but the anatomic distribution shows that sunlight is probably not a causative factor. Like in human melanocytic tumors, the differentiation between malignant and benign is sometimes very difficult.
Oral melanomas in dogs are very similar in evolution and clinical progression to the human melanoma that originates from mucosal sites, as it has a similar high tendency to invade surrounding tissue and disseminate (Bergman et al., 2013; Simpson et al., 2014) Both human and canine melanomas are notoriously resistant to chemotherapy and radiation therapy (Bergman & Wolchok, 2008).

Histopathological human and canine melanomas are very similar and the architectural features that are important for the diagnosis and staging of the disease were seen in both species (Simpson et al., 2014).
Both canine and human mucosal melanomas have a wide range of morphologies, consisting of spindloid, epithelioid, mixed epithelioid/spindloid, or small round cells. At the moment of diagnosis, mucosal melanomas in both humans and dogs are usually advanced with significant local invasion, focal necrosis, ulceration, and even metastasis. Similar significant pleomorphism with a considerable variation in nuclear and cell size and shape, as well as in the presence of nucleoli. (Simpson et al., 2014).

The genetic alterations in canine acral and mucosal are not fully documented. Mutations in BRAF exon 15 have not been found in canine mucosal melanomas similar to human mucosal melanoma (Maldonado et al., 2003; Shelly et al., 2005; Fowles et al., 2013). Of human mucosal melanoma about 15 % has activating mutations of c-kit and NRAS, these however appear to be rare in canine mucosal melanomas (Curtin et al., 2006; Fowles et al., 2013). Different molecular and genetic factors seem to be associated with melanoma in dogs (including metallothionein, PTEN, p53, RB-1, N-ras, some angiogenic factors, and cyclin kinase inhibitors) (Guldberg et al., 1997; Modiano et al., 1999). Although published analyses of gene mutations in canine melanomas cover limited gene regions, were done with small numbers of patients and are not conclusive to date, the results suggest that overlapping genetic causes exist (Simpson et al., 2014). Furthermore, similar activation pathways are found in both species. MAPK and AKT signaling pathway activation has been found in primary canine melanomas in different studies (Shelly et al., 2005; Fowles et al., 2013). Corresponding activation of these pathways in human melanomas seems to support the existence of similar signaling pathways (Simpson et al., 2014).

As shown above mucosal melanomas in dogs and humans share significant histopathological, genetic and clinical commonalities, they also share the need for improved treatment options to improve the considerably poor prognosis of metastatic or locally unresectable melanoma. Similar to human melanoma it is likely that there are many distinct melanoma subtypes in the dog, with a variety of molecular and histopathological phenotypes and prognoses. Defining these subtypes, through correlations between mutations, chromosomal, epigenetic, and expression variations between human and canine melanoma, will be an important step when it comes to modeling human melanoma and improving canine melanoma treatment options (Whiteman et al., 2011; Simpson et al., 2014).

In contrast to mouse models, dogs can be a unique opportunity to research specific subtypes, evolution, metastasis rate, and novel therapy options for melanomas in a larger mammal that also develops the disease in an immunocompetent host, which lives in largely the same environment as humans. The advantages that can be gained by using canine models as surrogate clinical study are the possibilities to get serial biopsies of the melanoma during treatment, for pharmacokinetic analysis and identifying biomarkers, as well as multiple medical imaging. Therapeutic effectiveness (and the frequent loss of effectiveness) and optimized schedules can be evaluated much faster in spontaneous melanoma in an immune competent canine setting. It also makes it possible to evaluate the safety of novel therapies in a species that is historically often used in the development of new pharmaceuticals. Furthermore, classical standards of therapy are less common in veterinary oncology, which makes it more acceptable to give patients experimental cancer treatments for untreated disease, rather than to await the failure of previously tested therapies first and having to adhere to many more ethical guidelines in human medicine (Paoloni & Vail, 2013; Simpson et al., 2014). Similarly human studies can also be used as a surrogate clinical trial for the use of novel immune-based drugs in canine malignant melanoma due to the commonalities in the disease in both species. The number of different approaches studied, the number of trials, and money available for clinical studies greatly exceeds that in veterinary medicine and can therefore give the veterinary field clues to which treatment options hold promise for canine patients.

As mentioned clinical trials in canine melanoma patients could give valuable information about new treatment options and influence the phase I and II clinical trials for human melanoma which includes target therapies and promising immunotherapies, of which the evaluation of the latter is of particular interest here. For example, recombinant canine CTLA4-Ig, has been shown to be similar to human CTLA4-Ig in activity, to be safe in dogs and in a clinical trial gave long-lived tolerance and suppression of Th1 cytokines in experimental autoimmune thyroiditis in dogs. This indicates the viability of canine CTLA4-lg in a variety of canine malignant melanoma therapy settings (Choi et al., 2008; Graves et al., 2009; Simpson et al., 2014). Another promising novel immune-based therapy in human melanoma and example of possibility for canine melanoma is the use of PD-1 and PD-L1 antibodies. One study showed that canine oral melanoma also expressed PD-L1 and an anti-PD-L1 antibody therapy induced a higher IFN production from tumor-infiltrating cells, indicating that the PD-1/PD-L1 pathway is also associated with the exhaustion of T-cells in canine tumors. Therefore blocking it could possibly also be an effective treatment strategy for canine malignant melanoma and research is needed to confirm this ability to activate antitumor activity of anti-PD-L1 antibodies in canine malignant melanoma in vivo (Maekawa et al., 2014).

The development, production, and clinical testing of canine immune-based drugs, like anti-PD-1, could aid the optimization of human treatment approaches as well as possibly produce a promising treatment option for canine melanoma (Simpson et al., 2014.)

Drawbacks of immunotherapy

Although the use of immunotherapy shows great potential in the treatment of both human and canine melanoma, there are some significant downsides that need to be mentioned. The most common challenges that are encountered during and after the use of immunotherapy include a nonresponsive or inactive immune system, a delay in effects, adverse effects, and high costs.

The success of immunotherapy is in part dependent on the immune system of the host, and whether it is able to generate a significant tumorspecific response. While responding patients might have a complete regression of the initial tumor and have a long disease free interval, the part of the patients that respond this well to immunotherapy is usually not very large (Atkins et al., 1999; Hodi et al., 2010; Eggermont et al., 2012). There are a multitude of reasons that could be responsible for resistance to immunotherapy. It could be intrinsic, as seen in transplantation patients, patients with compromised immune functions (like HIV patients), as well as older patients who may not have a fully capable immune system; or it could be an acquired resistance as a consequence of the specific mechanisms within the microenvironment of the tumor that compromise the necessary immune response (Baitsch et al., 2012; Kelderman et al., 2014). In addition, in human medicine different psychological stress factors that the patients could go through after they hear the diagnosis, are found to change several hormone levels, and are known to affect both adoptive and innate adoptive immune responses (Segerstrom & Miller, 2004; Besedovsky, 2012).

Some of the studied immunotherapies seem to take a relatively long time to show their full effectiveness. One study with ipilimumab gave an average time to complete response of 30 months. In addition, some patients might even show progression before regression and even regression of the primary tumor while experiencing progression of new melanoma lesions (Postow et al., 2012; Saraceni et al., 2014). As a consequences of this delay the advice could be to start with other treatment options like BRAF- or MEK-inhibitors that give a fast and significant response in a relatively large percentage of patients, however this is usually temporary after which the immunotherapy can take over. In addition, research is taking place into creating faster responses and higher response rates through combining different approaches and perfecting treatment protocols (Haanen, 2014).

Adverse events differ greatly in occurrence between the different forms of immunotherapy. Overall they are reported in more than 60 % of human patients, while adverse events in the canine clinical trials were mild and occurred in a minority of the patients (Amos et al., 2011; Glikin & Finocchiaro, 2014; Ma & Armstrong, 2014). The more commonly seen adverse reactions include gastrointestinal difficulties like diarrhea, nausea, and colitis, liver abnormalities like hepatitis, dermatitis and local injection site reactions, anorexia, and fatigue (Fellner, 2012; Herndon et al., 2012; Minkis et al., 2013; Lacouture et al., 2014). While Cancer vaccines are seen to induce less adverse effects, however there is a concern that it could induce autoimmunity (Amos et al., 2011). With ipilimumab, there have been reports of autoimmune colitis and vitiligo (Bouwhuis et al., 2011; Lacouture et al., 2014). Only the future will tell us if newer immune treatment forms, like anti-PD1 antibodies, although they seemed to have fewer adverse effects, are indeed safer and give less adverse reactions. It is also suspected that combinations of different approaches will also significantly reduce the occurrence of serious adverse events (Haanen, 2014). Mild and moderate adverse autoimmune effects can often be suppressed with steroidal anti-inflammatory medicine. More severe adverse effects can sometimes be treated using a monoclonal antibody that stops inflammatory cytokines, TNF-α (Weber, 2007;

Rotte et al., 2015). The moderate to serious adverse effects noted in some human clinical trials could possibly be a reason to refrain from using these novel immune-based drugs in veterinary medicine, as it is ethically difficult to use drugs on animals that significantly lower the quality of life of the patient.

The high price of most novel immunotherapies is another big challenge. The development of immune-based drugs uses a number of biotechnological techniques like the synthesis of recombinant peptides, the development of monoclonal antibodies, gene transfection, and ex vivo culture of cells, that makes the costs of some of these drugs enormous. For example, ipilimumab (anti-ctla 4) costs over $100,000 for a single course of therapy, based on the approved regimen for 4 doses. The estimated cost for 1 year would be more than $300.000 with this treatment alone. Although the drug costs in human medicine are covered by the insurance plans and/or sometimes subsidized, there is an ongoing debate about the high costs of immune-based therapy (Fellner, 2012; Jönsson & Wilking, 2012; Rotte et al., 2015). In order to be able to use some of these expensive drugs in veterinary medicine in the future, costs would have to be drastically reduced. Perhaps the possibility of using the immune-based drugs in canine melanoma patients as a surrogate clinical model for human medicine could entice manufacturers to lower prices or subsidize the therapy for this purpose.

Conclusion

Immunotherapy is a booming and exciting relatively new field of cancer management. Especially in human medicine a large amount of studies are taking place in order to find and optimize novel approaches to stimulate a productive antitumor response while at the same time minimizing immunosuppressive factors originating from the melanoma. At this point some promising immune-based drugs, like CTLA-4 antibodies, PD-1 antibodies and cytokines (p-IFN α-2b and IL-2) have been have been approved, while several others are in different stages of development. Figure 1 summarizes the most important approaches currently researched in human melanoma.

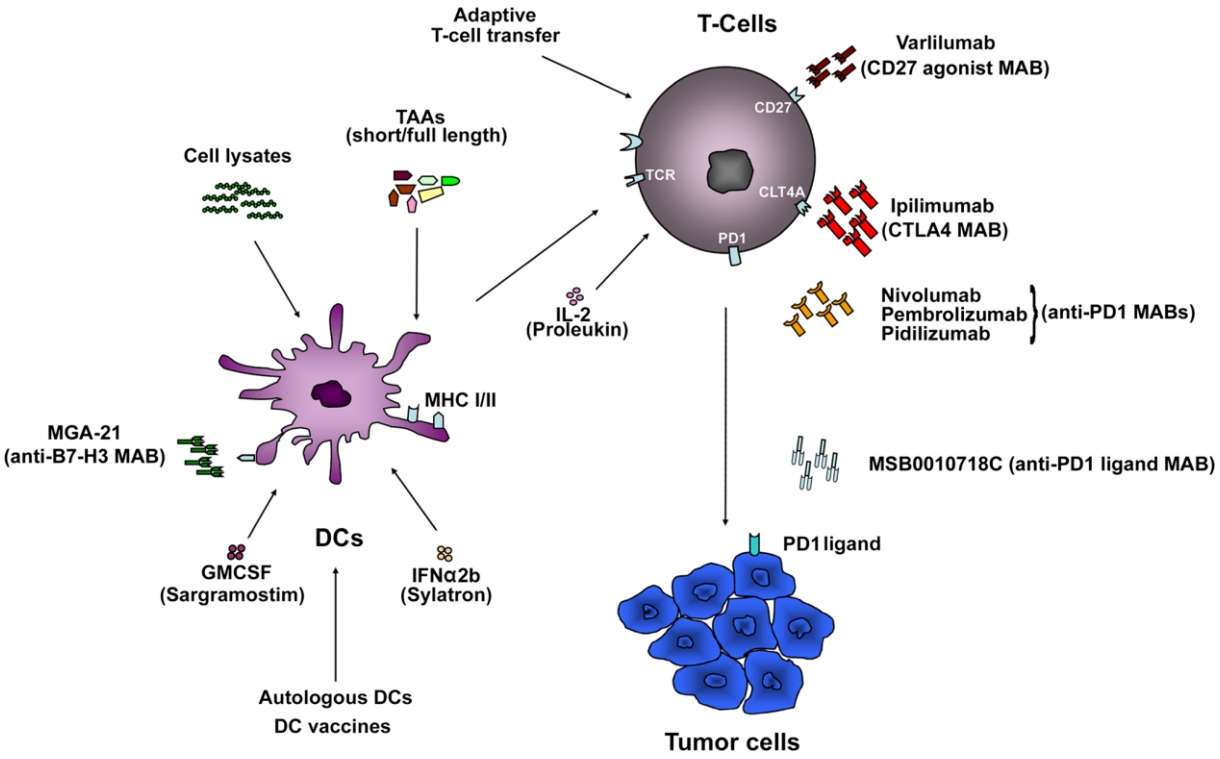

Figure 1: different approaches to human melanoma immunotherapy (Rotte et al., 2015)

Although these exciting novel approaches appear to create a significant survival benefit in human metastatic melanoma, at least in a section of patients, it is unlikely that immunotherapy will become a sole modality in the management of melanoma. The outcome appears to significantly improve when the immune-based drugs are used as an adjuvant therapy in combination with surgery and radiotherapy. Although these immunotherapies seem to have a large potential for greatly increasing patient survival time, response rates can be disappointing and more research is necessary to correctly match different subtypes of melanoma to specific treatment options. Additionally, some of the immune-based drugs can take several months to produce effect and many have been shown to produce serious adverse effects. It is expected that response rates will increase when the right combination of immunotherapies will be used.

Although less extensive, veterinary medicine has also ventured out into the field of novel immunotherapies in the fight against canine melanoma. Two immunotherapy methods were

supported by a larger number of treated patients: the suicide gene plus allogeneic/autologous vaccine and the xenogeneic vaccine. To date only the latter has been approved and several others methods could hold promise for the future, however it is clear that more research is necessary. Similar to human patients, canine patients that showed objective response had a significantly longer survival time when compared to nonresponders and best results were gained in the presence of minimal residual disease. Overall, only minimal adverse events were noted in the veterinary clinical trials.

Canine medicine has a unique ability to make a large contribution to the progress made in this field. As mouse models offer poor clinical models for human medicine and comparative immunotherapy research in canine models can offer superior predictions of results in human patients. This is largely due to several reasons, including the spontaneous occurring of the disease in a species that is immune competent, outbred and lives in an environment that is the same as humans and the significant similarities in melanoma biology (similar resistance to chemo- and radiotherapy, phenotypes, anatomical preferences, metastatic and clinical behavior, genetic causes and activation pathways and so on.)

Human clinical trials can also be used by the veterinary field as they show the most promising approaches as well as the challenges accompanying these immunotherapies. To extrapolate the human results to canine patients it is important to focus on the results in specific human melanomas (like mucosal melanomas) as they are likely to behave similar to canine melanomas.

Encouraging clinical trials with immune-based therapies for both canine and human melanoma give a sparkle of hope that the previously poor prognosis of this cancer will slowly turn in to a chronic, but manageable disease. However, many more clinical trials and proof-of-principle studies will have to take place in order to further improve different immunotherapies and create the optimal treatment for every individual patient.

Discussion

Research into immunotherapy poses several barriers that make interpreting results difficult. Often there is no true control group as most immune-based therapies are compared to other treatment methods or historical control groups. Regarding the aggressive nature of the disease the lack of true control groups is not surprising, however it does offer statistical difficulties as populations change and standard treatments methods can differ. Randomization is often absent, especially in canine trials.

The enormous variety of tumor types, immunotherapy protocols and treatment combinations in human melanoma immunotherapy research is another reason it is difficult to interpret results and compare different immune-based therapies. Even the definition of response/success differs and several endpoints are used. Further research is desired into specific response criteria, as well as into determining how long it takes for immunotherapies to fully show their effect. As it is known that some immune-based approaches can take months or even years to show their full effect, it is possible that many studies determine effectiveness too early in the trial.

The composition of patient groups may give variations in the results. In human medicine these groups often consist of patients with advanced, sometimes nonresectable and even metastatic melanoma, whereas canine trials have shown that immunotherapy works best against minimal residual disease and therefore effects could be underestimated.
In canine clinical trials the composition of patient groups creates another challenge when interpreting the result as they are often composed of few animals and more importantly, made up of patients with different melanoma locations and stages of disease. These have a very different prognosis to begin with and group composition differences between trials are great, so it is difficult to extrapolate the results to a larger population with a specific site and stage of the disease.

Comparing the histologic, genetically and epidemiological characteristics indicate that canine melanomas are closer to non-sun exposed human melanomas and in particular acral and mucosal human melanomas. This poses challenges in extrapolating the results of canine trials in human medicine and vice versa, as it is important to evaluate the different immunotherapies in these similar groups of melanoma patients. Currently clinical trials in both human and veterinary medicine rarely divide patients into melanoma site of origin and subtype groups and this would be a recommendation for the future. Melanomas in different anatomical locations will likely have a variable response to the different forms of immunotherapy and therefore much can be gained from focusing on these specific locations when comparing results especially between species.
Furthermore, in order to further specify different subtypes of canine melanoma, additional research is of great importance. Relevant subtype-breed combinations could be sequenced, and DNA research on a large number of melanomas can give a better estimate of somatic alterations. Using canine melanoma models to the fullest will necessitate a significant patient subdivision on the basis of genetic, molecular, histopathological and clinical appearance, into different subtypes of canine melanomas. This will enable researchers and clinicians to correctly identify the types that best correlate with their human counterparts and will also create the possibility to match future veterinary immunotherapy options to the best canine candidates and therefore increase response rates.

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Addendum

Research context

The conducted literature study was part of a research internship that also consists of participating in an ongoing clinical trial into Oncept, the canine melanoma vaccine. This clinical trial aims to determine the effects of Oncept on survival time, when administered as an adjuvant therapy. As described in the literature study, the DNA vaccine has shown some promising results in previous studies and this study will try to confirm these outcomes.

As discussed, clinical trials with canine melanoma patients can be used as good preclinical proof of concept and evaluate safety for immunotherapy options in the treatment of human melanoma patients. The hypothesis being tested in this study is: " A xenogenic human tyrosinase DNA vaccine against canine melanoma will significantly prolong the survival expectations of patients." This clinical trial will contribute to the extension of research into the melanoma vaccine in dogs around the world, and possibly also form a contribution to the treatment of human melanoma.

To date 42 dogs have been enrolled in the trial that started in February of 2011, of which 18 dogs are still alive. The group consists of different breeds, sexes and ages as well as different melanoma locations and stages of the disease. Of these dogs, 25 had oral melanomas and 17 had non-oral (e.g. digital or cutaneous) melanomas. The majority of patients were diagnoses by their own veterinarian, however all dogs received a full physical examination and the study attempts to give the dogs the same work-up. All dogs were staged according to the TNM scale founded by the World Health Organization (Table 1).

Table 1: Traditional World Health Organization (WHO) TNM-based staging scheme for canine oral melanoma (Bergman & Wolchok 2008).

T: Primary Tumor	
T1	Tumor diameter: ≤2 cm
T2	Tumor diameter: 2-4 cm
T3	Tumor diameter: >4 cm
N: Regional Lymph Nodes	
N0	No evidence that regional lymph nodes are involved
N1	Histologic/cytologic evidence that regional lymph nodes are involved
N2	Fixed lymph nodes
M: Distant Metastasis	
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

After staging, the patients are treated according to the standard protocol which is formulated by the University Clinic for Companion animals in Utrecht (Table 2). However a portion of the patients did not completely follow this protocol, as the macroscopic tumor was removed by the own veterinarian. Owners are given the possibility to start a new vaccination round six months after the previous round in case there are no signs of metastases, this new round is accompanied by a CT-scan and the same follow-up as the first round.

Table 2: Protocol 'Melanoma Vaccine' by Utrecht University	
Procedure 1	Confirm diagnosis of melanoma with histology or TNAB.
Procedure 2	If indicated a blood test is conducted. Patient owners signs the declaration of consent.
Procedure 3	If indicated a TNAB of lymph nodes is conducted.
Procedure 4	Advanced imaging in the form of a CT scan.
Procedure 5	Surgical removal of macroscopic tumor (in combination with positive lymph nodes).
Procedure 6	Radiation therapy if the tumor is not removed completely (6x6Gy).
Procedure 7	Application of ONCEPT Melanoma Vaccine (4 times, every 2 weeks).
Procedure 8	Follow-up care: Checkup 1, 3 and 6 months after last vaccine (then every 6 months). Checkups at 3 and 6 months include imaging
Procedure 9	Possibility to start a second vaccination round.
Procedure 10	Register data including survival time (ST), recurrence free interval (RFI) metastasis free interval (MFI), and disease free interval (DFI).

Contribution to the study

The clinical part of this thesis consists of following and guiding existing and new patients during treatment and follow-up. New and missing data will be collected and processed into datasheets. To collect missing data on existing patients, owners and veterinarians will be contacted.

I have guided 4 patients, that were still in the phase of receiving their first round of vaccinations, during their treatment and follow up, 1 patient that started a second round of vaccinations and several other patients during their appointments. In addition, I contacted patients that are still alive for follow-up and collected missing data of both living and deceased patients. The patients I guided through their first or second round of vaccinations consisted of the van Basten family with Linda, a 9 year old Rottweiler with stage 3 oral melanoma, the Jochems family with Billy, a 9 year old cross breed with stage 2 cutaneous melanoma, the Peeters family with Henry, a 6 year old Irish Setter with stage 1 oral melanoma and the Chavez family with Pina, a 12 year old Labrador retriever with stage 2 oral melanoma. I also guided the Gijsbers family with Zepp, a 7 year old Riesenschnauzer with stage 1 digital melanoma that received a second round of vaccinations.

In addition, I attended the weekly surgical oncology consultations where, amongst others, canine melanoma patients can be referred to. The information gathered during these consultations concerning the melanoma patients, as well as all email or telephone contact was collected and subsequently processed in their patient cards and the Excel-sheets that will be used for data-analysis.