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Cancer immunotherapy

Tumor vaccination, IL-12 gene therapy and metronomic chemotherapy

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Table of Contents

List of Abbreviations

APC = antigen presenting cell

CPX = cyclophosphamide

CR = complete response

CTLs = cytotoxic T lymphocyte

DC = dendritic cell

DTH = delayed type hypersensitivity test

FMO = fluorescence minus one

hGM-CSF = human granulocyte macro-phage-colony stimulating factor

HSA = hemangiosarcoma

hTyr = human tyrosinase

ID = intradermal

IFN-gamma = inteferon-gamma

IL-10 = interleukin-10

IL-12 = interleukin-12

IL-6 = interleukin-6

IM = intramuscular

IT-EGT = intratumoral electro-gene transfer

LF2000 = lipofectamine 2000

MDSCs = myeolid derived suppressor cells

MHC = major histocompatibility complex

MSC = mesenchymal stem cells

MST = median survival time

MVD = microvessel density

NED = no evidence of disease

NK cells = natural killer cells

NSAIDS = non steroidal anti-inflammatory drugs

PBMC = peripheral blood mononuclear cells

PBS = phosphate buffered saline

PD-1 = programmed cell death-1

PDL-1 = programmed death ligand-1

pDNA = plasmid DNA

PR = partial response

PRRs = pattern recoginition receptors

SC = subcutaneous

SD = stable disease

SEM = standard error of the mean

TERT = telomerase reverse transcriptase

TGF-β = transforming growth factor beta

TLR = toll like receptor

TMZ = temozolomide

Tregs = regulatory T cells

TSP-1 = thrombospondin-1

TVT = transmissible venereal tumor

VEGF = vascular endothelial growth factor

VEGFR-2 = vascular endothelial growth factor-2

GENERAL INTRODUCTION

This chapter is partly based on:

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And

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1. Introduction

The first proof of concept for cancer immunotherapy dates back to the late eighteen hundreds when William Coley demonstrated that tumor regression occurred in patients after local injection of *Streptococci* or a mixture of *Streptococcus pyogenes* and *Serratia marcescens* (Coley's toxins). However, Coley's work was criticized by many colleagues and his concept did not break through. Now, more than 100 years later, cancer immunotherapy is on its revival and it is considered a new cornerstone in cancer treatment.[1] This is for instance evidenced by Science's selection of cancer immunotherapy as Breakthrough of the Year in 2013 and by recent multi-million to billion deals in the pharmaceutical industry concerning immunotherapeutic drugs.[2-4]

The long road to success for immunotherapy can be explained by the enormous complexity of interactions between tumor cells and the immune system. These interactions are summarized by the term tumor immunoediting and can lead to three possible outcomes; elimination, equilibrium or escape.[5] The importance of immunoediting was first recognized in experimental models, where genetic modification of immune pathways greatly influenced tumor development. Swann and Smyth describe evidence for the three phases of tumor immunoediting in humans.[5] Maybe the ultimate confirmation of the ability of the immune system to control tumor growth is the multitude of immune suppressive mechanisms universally present in tumors, apparently necessary for their existence. Indeed, it is well established that the capability to evade immune control is an essential hallmark for tumor growth.[6]

If we look at the most important immunological players in tumor control, dendritic cells (DCs) can be identified as the master regulators of the immune response. DCs present tumor antigens to the adaptive immune system and depending on their interaction with T cells this will lead to tolerance or immunity.[7] DCs interact with T cells on three levels. The first level involves the binding of an antigen-major histocompatibility complex (MHC) to an antigen specific T cell receptor. On a second level, costimulatory signals (e.g. CD80, CD86, CD40 ligand) bind to stimulatory (e.g. CD28) or inhibitory (e.g. CTLA-4) receptors on T cells. Lastly, cytokines secreted by DCs form the third level of interaction of DCs with T cells.[8] No one signal can be categorized as tolerogenic or immunogenic at its own, the result on the

immune response depends on the integration of several signals from DCs and the differentiation state of the interacting T cells.[8] Interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) 1 are major inducers of tolerogenic DCs, whereas immunogenic DCs are most classically induced by toll like receptor (TLR) signaling but also TLR-independent activation is described.[9,10] CD8 cytotoxic lymphocytes (CTLs) are considered the main effector cells in tumor immunity. Their effector functions require binding of the T cell receptor to MHC-peptide complex on the tumor cells and include interferon-gamma (IFN-gamma) secretion, the death receptor pathway and the granule exocytosis pathway. IFN-gamma has diverse antineoplastic effects, including direct anti-proliferating and pro-apoptotic effects on tumor cells, inhibition of angiogenesis and stimulation of the innate immune system.[11] The death receptor pathway involves the binding between the death receptor ligand on the surface of CTLs and a death receptor on the tumor cell, resulting in apoptosis of the latter (the FAS-ligand/receptor pathway).[12] In the granule exocytosis pathway, perforins and granzymes are secreted by exocytosis toward the target cell. They work cooperatively in inducing apoptosis of the target cell, perforin as membrane disrupting protein facilitating the delivery of the apoptosis triggering granzymes. Effector cells themselves are not affected because during the secretion of these granules, cathespin B is sequestered on the cell membrane which inactivates perforin that would diffuse back to the effector cell.[13] Apart from CTLs, also natural killer cells (NK cells) can contribute to immune-mediated cell killing, with similar effector mechanisms as CTLs but they do not require priming by DCs for activation or MHC-binding with the target cell.[14] Different factors present in tumors can lead to a cytotoxic response of NK cells, including MHC-I downregulation and the expression of NK2GD ligands on tumor cells.[15]

Despite the potential of the immune system to eliminate cancer cells, tumors develop because of cancer induced immunosuppression. Tumors use multiple mechanisms to survive even in the presence of tumor reactive CTLs, either naturally present or induced by immunotherapy. Tumor cells downregulate MHC-I and/or immunogenic antigens, thereby avoiding CTLs recognition.[16] More downstream, resistance against immune cell induced apoptosis has also been demonstrated, by blocking the death receptor pathway or by interfering with the perforin/granzyme pathway.[12] Tumor cells can even turn the tables and cause apoptosis of CTLs. For example, some tumor cells express the FAS-ligand, resulting in T cell apoptosis when binding to the FAS-receptor on T cells.[17] Tumor cells can also expose programmed death-ligand 1 (PDL-1) on their surface. Binding of PDL-1 to programmed cell death 1 (PD-

1), a death receptor present on activated T cells, causes inhibition and apoptosis of tumor-infiltrating T cells.[18] Additionally, tumor cells that express the tryptophan degrading enzyme indoleamine 2,3-dioxygenase will cause apoptosis of CTLs, as CTLs are very sensitive to tryptophan depletion.[19] Secretion of immune suppressive cytokines by tumor cells can also have a direct effect on effector immune cells.[20]

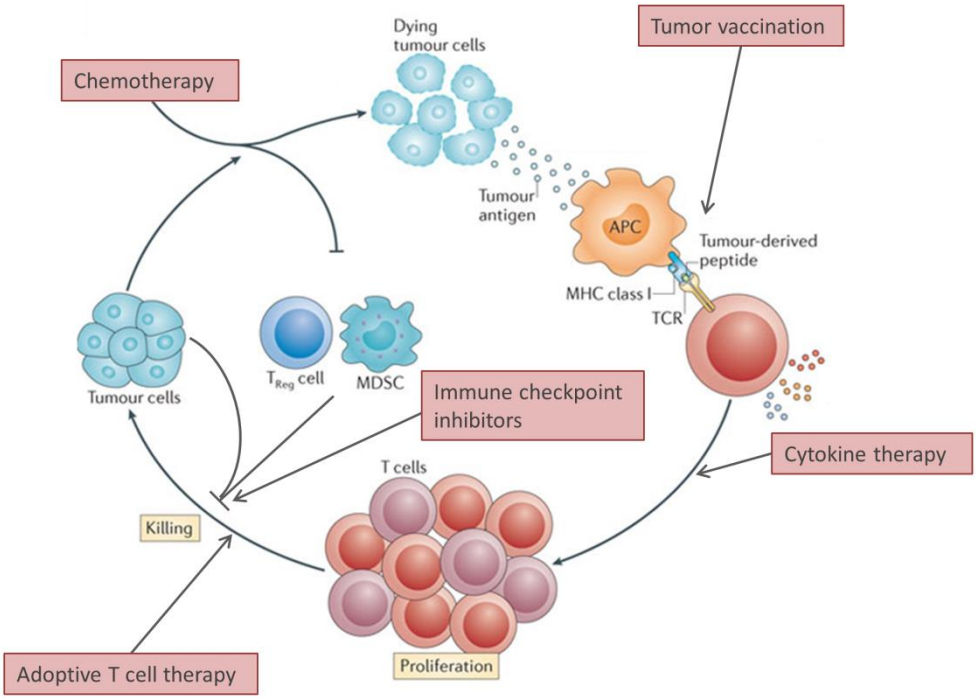

Fig. 1:Interactions defining an anti-tumor immune response and potential immunotherapeutic interventions. Adapted from Restifo et al.[21]

Apart from these direct interactions between tumor cells and immune effector cells, a major mechanism of immune evasion involves the accumulation of immune suppressive regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) in the tumor microenvironment. The most important cytokines involved in Treg induction are TGF-β and IL-10.[22] Tregs suppress CTLs as well as NK cell-mediated cytotoxicity and also have an effect on B cells, CD4 cells and DCs.[23] This Treg mediated immune suppression ranges from cell-to-cell contact dependent mechanisms (e.g. CTLA-4) to the secretion of cytokines (e.g. IL-10, TGF-β). MDSCs are a population of immature myeloid cells, induced by several cytokines of which VEGF and G(M)-CSF are important examples. MDSCs have the potential to inhibit CTLs, DCs, and NK cells as well as to expand Tregs. They exert their immune suppressive effect by secretion of enzymes (e.g. arginase 1, nitric oxidase 2) and cytokines (e.g. IL-10, TGF-β) as well as by contact dependent mechanisms through e.g. the exposure of PDL-1 on their surface.[24]

It is clear that, in order to be effective, cancer immunotherapy has to be a multimodal treatment. Potential immunotherapeutic interventions, acting on different levels of the immune response are illustrated in figure 1. Treatment modalities evaluated in this thesis include genetic tumor vaccination, interleukin-12 (IL-12) gene therapy and metronomic chemotherapy. Components were selected based on their immunological synergistic action and practical feasibility in veterinary medicine, since a fast transition to clinical trials in veterinary patients was one of the goals of this thesis.

2. Tumor vaccination

Although tumor specific precursors of CTLs are naturally present in cancer patients, they are not efficiently activated.[25] Tumor cells lack costimulatory signals and thus cannot activate T cells directly. Dendritic cells are responsible for tumor antigen presentation to T cells, but are not activated in a tumor environment because of a lack of stimulatory signals and the presence of tolerogenic signals induced by the tumor.[26] Tumor vaccination can replace the natural antigen presentation pathway and thus induce the generation of tumor-specific CTLs, a first important step towards effective immunotherapy.

2.1. Tumor antigens

Based on their expression pattern, antigens used in tumor vaccination can be divided into five major categories: differentiation antigens, cancer/testis antigens, mutational antigens, ubiquitous antigens and viral antigens.[27] Differentiation antigens are overexpressed in a given type of cancer but they are also expressed in the corresponding normal tissue. Examples include prostate specific antigen and tyrosinase. They are suboptimal targets for vaccination due to self-tolerance mechanisms and the risk of autoimmune disease. However, these issues are of less importance for differentiation antigens that are only expressed during the earlier stages of differentiation. Differentiation antigens have been broadly used because they were first identified. Cancer/testis antigens form the second category of antigens and are expressed in germ line tissue and different types of cancers. Germ line tissue lacks MHC expression, therefore these antigens are considered tumor-specific. Hence, tolerance and autoimmunity is not a major issue. An overview of the different cancer/testis antigens can be found in the review of Caballero *et al*.[28] The third category of antigen comprises mutational antigens which result from mutations and are strictly tumor-specific. Strong immune responses can be

elicited against these antigens as tolerance is not an issue. Unfortunately their usage is limited because it is also patient specific or at least restricted to certain patients. One way to ensure that unknown mutational antigens are included into tumor vaccines is by using whole, autologous tumor cell vaccines.[29] Ubiquitous antigens, the last category of tumor antigens, are expressed in many normal tissues and overexpressed in tumors. Tolerance and autoimmunity are two important concerns.[29] The rationale behind their capacity to be effective without causing autoimmunity is that overexpression in tumor cells can reach the threshold for T cell recognition, breaking immune tolerance.[27] A well-known antigen belonging to this group is Her2/neu.[29] Lastly, for tumors with a viral etiology, vaccination against viral antigens is an attractive option because the foreign nature of the antigen limits problems with tolerance and autoimmunity. Different cancers are associated with viral infection; the most common human papilloma virus (HVP) induced cervical cancer.[30] Two HPV vaccines for the prevention of cervical cancer have been approved and first clinical successes with therapeutic vaccines have been achieved.[31,32]

Based on the origin of the tumor antigens one can distinguish three classes of antigens: autologous, allogeneic and xenogeneic antigens. Autologous antigens are isolated from the patient's own tumor cells and thus represent a personalized therapy. Moreover, knowledge of the antigens expressed by the tumor is not necessary. The use of autologous antigens is often the only way to ensure that tumor specific mutational antigens are included in the vaccine. Autologous antigens can be included in tumor cell vaccines, DC vaccines (by loading DCs with tumor cell lysate, killed tumor cells or whole tumor RNA), peptide vaccines (by extraction of tumor associated peptides) and DC-tumor fusion vaccines. As a second class, allogeneic antigens are antigens from the same species as the patient, but from a different individual. Allogeneic antigens can be utilized in all tumor vaccine platforms (see point 2.2 below) and mass production is feasible when they are used as a peptide/protein or genetic vaccine. A third class of tumor antigens are xenogeneic antigens which are derived from another species than the patient. By utilizing xenogeneic antigens it is possible to break immune tolerance to self-antigens and to induce a much stronger immune response.[33] Xenogeneic antigens can be administered as a recombinant peptide/protein or genetic vaccine or can be included in tumor cell or DC vaccines.

2.2. Vaccine platforms

Tumor vaccines can be administered via different platforms. Tumor cell vaccines consist of lysed or killed tumor cells obtained from the patient (i.e. autologous tumor cell vaccines) or from tumor cell lines derived from another patient (i.e. allogeneic tumor cell vaccines). Because tumor cells have acquired strategies to escape immune surveillance their preparation for vaccination purposes is critically important.[34] A great advantage of tumor cell vaccines is that the tumor antigens do not need to be identified and that it evokes an immune response against a broad range of antigens. Moreover, they contain epitopes for both $CD8^+$ and $CD4^+$ T cells resulting in a stronger anti-tumor response and greatly diminishing the chance of tumor escape compared to single epitope vaccines.[34] Autologous tumor cell vaccines are a completely patient-specific therapy. Disadvantages include the need of a sufficient amount of tumor cells and the problems concerning standardization, quality control and large-scale production. Allogeneic tumor cell vaccines pose less problems concerning manufacturing than autologous vaccines, but the advantage of a patient-specific therapy is lost.

Cancer vaccines based on DCs that are loaded with tumor antigens have gained much attention in the field of immunotherapy. The first step in the production of these DC vaccines is the isolation of dendritic precursor cells from the patient's blood or bone marrow. These precursor cells are subsequently differentiated into DCs and loaded with tumor antigens. The source of the tumor antigen can be (synthetic) tumor peptides, (recombinant) tumor proteins, whole tumor cells, plasmid DNA or mRNA encoding a tumor antigen. There have been promising reports of efficacy with DC vaccines; however large scale application of these vaccines is limited due to their labor-intensive production.[35] Nevertheless, Provenge®, the first approved therapeutic tumor vaccine for human use, is a DC vaccine.[36]

Genetic vaccines are frequently used in cancer vaccination, because the intracellular synthesis of the target antigen has the capability of inducing robust T cell responses.[37] DCs may acquire and present the antigen when the nucleic acids are directly delivered into the DCs after injection. Alternatively, they may acquire the tumor antigens indirectly via cross-priming, i.e. from keratinocytes or myocytes that have taken up and express the tumor antigen encoded by the nucleic acids.[33] Genetic vaccines can be divided into two major groups: vaccines based on recombinant viruses and vaccines based on nucleic acids (plasmid DNA (pDNA) or mRNA). Bacterial plasmids are the first and most extensively used nucleic acid vectors. More recently,

in vitro transcribed mRNA is explored as an alternative.[38] The major advantage of DNA and mRNA vaccines is that they can be easily designed and produced *in vitro.* However, transfection efficiency of naked nucleic acids is low. There are three main methods to enhance transfection efficiency; physical methods, chemical vectors and biological vectors.[39,40] Viral vectored vaccines can induce good immune responses via the attraction and infection of antigen presenting cells (APC), but a major disadvantage is the induction of an immune response against the viral vector, making further boosts less effective.[29] Moreover, antigenic competition can occur, where anti-vector responses dominate over vaccine-specific responses.[41] Issues concerning large-scale production, stability and stringent safety requirements are other disadvantages of genetic vaccines based on viral vectors.[42] Genetic vaccines are much more applicable for commercial use than tumor or DC based vaccines, but highly immunogenic tumor antigens have to be known and the immune response will be directed to only the antigen(s) encoded by the vaccine, allowing the development of escape mutants. The major advantage of genetic vaccines compared to peptide vaccines is that they are not restricted by MHC-haplotype.[33]

MHC-restricted peptides are widely used as tumor vaccines. These vaccines are relatively easy to produce. However, the immune response is limited to the selected epitope(s), which may lead to the emergence of escape variants and hence insufficient clinical effects.[43] Furthermore, vaccines based on short peptides (8 to 10 amino acids long), which can be directly loaded into MHC molecules, are only applicable in patients that have the right MHC molecules for presenting these peptides. Vaccination with longer peptides containing multiple epitopes or full length proteins circumvents MHC restrictions, immune escape and induces also $CD4^+$ T helper cells.[43] These longer peptides and proteins are not able to bind directly to MHC molecules, but have to be taken up and processed by APC. This leads to the presentation of different class I and class II epitopes by the same APC, which is more efficient than independent presentation by different APCs. Therefore, long peptides or proteins are more effective than single peptide mixtures.[41] However, the manufacturing of longer peptides or full length proteins is, compared to single-epitope peptides, more difficult.

2.3. Tumor vaccination in veterinary medicine

Preclinical vaccination studies in purpose bred laboratory dogs

The immune response and possible adverse effects of veterinary tumor vaccines have been evaluated in healthy Beagles.[44-53] Additionally, the clinical response of DNA and DC vaccines has also been studied in a dog cancer model, i.e. Beagles inoculated with transmissible venereal tumor (TVT) cells.[54,55]

These preclinical studies resulted in three important conclusions. It is possible to evoke an anti-tumor immune response in healthy dogs and dogs with TVT, proving that there is a good foundation for the use of tumor vaccines in the battle against cancer. Apart from autologous antigens, chicken HSP70[54], human tyrosinase[44], canine telomerase reverse transcriptase (TERT)[45,56], human her-2/neu[45,56], human carcinoembryonic antigen[48,52] and bovine disialoganglioside GD3[50] were also examined as tumor antigens. The second conclusion concerns the safety of tumor vaccines. The dogs were monitored very closely, by means of clinical investigations and blood examinations.[45,48-51,53-56] In some studies the presence of anti-nuclear antibodies has been checked and in others histological analysis of the organs has been performed.[48,50] No signs of autoimmunity or other abnormalities were detected. Two studies reported mild adverse effects. In one study, a DC vaccine was given in the popliteal lymph node and the authors noticed transient pyrogenic effects and slight lameness at the site of injection, which resolved without further treatment within 48 hours post injection.[48] In another study, a mild and transient inflammatory reaction was seen at the site of injection after vaccination with tumor cells transfected with human granulocyte macrophage-colony stimulating factor (hGM-CSF).[51] This was due to the localized production of hGM-CSF, which was intended to attract inflammatory cells to the injection site. In all of the other studies, no adverse effects or signs of autoimmunity were detected. These conclusions about immunogenicity and safety are true for tumor cell vaccines[51], DC vaccines[46-50,55] and genetic vaccines.[45,48,52,54,56] Finally, these preclinical studies showed that, at least with TVT, a clinical response can be achieved using DNA and DC vaccines as sole therapy.[54,55] It has to be mentioned that TVT is an immunogenic tumor that is quite unique in its behavior.[57] Therefore, TVT is not of great value as model for other tumor types.

Purpose bred laboratory dogs have also been used to compare different delivery routes for DNA vaccines. These studies showed that DNA vaccination combined with electroporation is

superior in evoking a potent immune response in comparison to injection of DNA without electroporation. This is partly due to the fact that electroporation facilitates the cellular uptake of the DNA. Another explanation is the adjuvant effect of electroporation. Indeed, electroporation induces minimal and transient tissue damage resulting in local inflammation at the injection site.[58] Without electroporation, both the intramuscular (IM) and intradermal (ID) administration of naked DNA induce an absent to minimal cellular immune response, with the ID route slightly better than the IM.[44] However, significant humoral immune responses have been obtained after IM and ID administration of naked DNA and this response was higher after IM injection than after ID injection.[48] Interestingly, needle-free injection systems, like Bioinjector® 2000 and Vitajet®, which use high pressure to force the dissolved DNA vaccine into the dermis or muscle result in a far better immune response than the traditional IM or ID route of administration.[44] However, the needle-free transdermal route is less potent in evoking an immune response than the electroporation-based DNA delivery method.[54] Nevertheless, a crucial advantage of the former technique is that it can be used on non-anesthetized veterinary patients.

Clinical trials with canine tumor vaccines

Trials assessing efficacy that included a statistical survival analysis

The studies summarized in table 1 and 2 were designed to assess efficacy. However, before discussing these trials we want to draw the attention to some sore points. A limitation of many studies is the number of patients. The results of such small-scale studies should be considered as preliminary and need confirmation in larger studies. Mostly the authors emphasize this in their discussion but often no further research is done. Moreover, historical control groups are frequently used. The high risk of false positive results associated with single-arm trials makes them highly ineffective in predicting efficacy.[59,60] Due to improved supportive care, earlier detection, better equipment and technology, expected outcomes can change over time. Moreover, interinstitutional variability in outcome is often large and selection bias can never be completely excluded.[59-61] Therefore, the following paragraphs should be read with these considerations in mind.

Table 1: Clinical trials with tumor vaccines that resulted in a survival benefit for the vaccinated dogs

Tumor type	Vaccine type	Number of patients	Control group	Variable	Effect	Reference
Oral melanoma	DNA vaccine	58	Published data (1999)	25% lower percentile MST	464 vs 156 days	[62]
Digital melanoma	DNA vaccine	58	Published data (1995)	MST	476 vs 365 days	[63]
Melanoma	Tumor cell vaccine	283	Concurrent	MST	610 vs 91 days (CS) /201 vs 65.5 days (PS)	[64]
Lymphoma	Tumor cell vaccine	11	Concurrent, placebo controlled	MST	>348 vs 197.5 days	[65]
Lymphoma	DNA vaccine	14	Recent historical cases from same institution	Lower 95% CI MST	57.6 vs 26 weeks	[66]
Hemangiosarcoma	Tumor cell vaccine	28	Recent historical cases from same institution	MST	182 vs 133 days	[67]

MST = median survival time, CI = confidence interval, CS = complete resection of primary tumor, PS = partial resection of primary tumor

Vaccines with a significant survival benefit

Melanoma

An important breakthrough in the treatment of canine melanoma and the field of tumor vaccination was achieved with a DNA vaccine encoding the human tyrosinase for the treatment of oral melanoma. Merial has commercialized this vaccine under the name Oncept™. It is the only veterinary therapeutic tumor vaccine licensed by the USDA. The licensing followed after a successful clinical trial that resulted in prolonged survival compared to historical control dogs.[68-70] Recently, its potency was proven in digital malignant melanoma as well.[63] Vaccination with a plasmid encoding murine tyrosinase and murine gp75 generated similar results.[63,68] In all these studies, the pDNA encoding the xenogeneic tyrosinase was administered IM in a needle-free manner without electroporation, using the Bioinjector 2000® or the Vitajet® device. The great advantage of this administration method is that, in contrast to electroporation, no general anesthesia is required. In early trials, it was administered four times weekly.[68,69] This protocol was replaced by four biweekly injections followed by boosts every six months in more recent trials.[63,70] An antibody response against human tyrosinase was present in three out of nine tested patients, with two of them also positive for antibodies against canine tyrosinase.[71] A correlation between the antibody

response and the clinical response was observed, but this did not reach statistical significance. This correlation was not expected as tyrosinase is considered to be an intracellular protein and hence one would not expect that it can be recognized and targeted by antibodies. The authors speculated that tyrosinase is expressed at a low-level on the cell-surface of melanoma cells. Recently, it has been discovered that a peptide fragment of tyrosinase is indeed presented by MHC I class molecules at the surface of melanoma cells.[72] Cellular immune responses were not investigated. Early preliminary trials with a heterogeneous patient population of 170 dogs suggested longer survival times than commonly found for oral melanoma.[68,69] This was confirmed with a statistical survival analysis comparing 58 vaccinated dogs with a historical control group collected from published data. Only patients with stage II or III oral melanoma with no macroscopic evidence of the primary tumor at the start of vaccination were included in this study.[70] As median survival time (MST) was not reached for vaccinated dogs, the 25% lower percentiles of the two groups were compared. This showed that 75% of vaccinated dogs were expected to live beyond 464 days versus 156 days for the control group. However, a very important weakness of this study is that survival data found in an article published in 1999 were used to establish this historical control group.[70,73] Nevertheless, the observed effect was considered large enough by the authors to exceed the confounding factors of a non-randomized trial. Strictly speaking, to unambiguously prove the clinical efficacy of Oncept™ a randomized, placebo-controlled study should be performed. The same vaccine prolonged MST of 58 patients suffering from digital melanoma as well, although the effect is less spectacular: a MST of 476 days in comparison with 365 days for a historical control group found in the literature.[63] The authors indicated a long interval between diagnosis and start of the vaccination for most patients as an important explanation for the lower efficacy of the vaccine in this study.

A spectacular survival benefit for melanoma patients has also been seen in two trials with a tumor cell vaccine.[64,74] Depending on the availability, autologous or allogeneic formolized tumor cells were injected together with xenogeneic cells genetically engineered to express human interleukin-2 (IL-2) and hGM-CSF. The vaccine was given subcutaneously (SC), five times weekly, followed by five times biweekly, five times monthly, five times every three months and then every six months until death or relapse. Before vaccination, all patients were treated with surgery and thymidine kinase suicide gene therapy, with the latter repeated in unresectable/incompletely removed or relapsed tumors. Immune responses were not monitored. An initial trial with 31 treated dogs reports a MST time of 370 days, significantly

higher than 76 days for concurrent non-randomized control dogs.[74] More recently, results after 9 years of follow-up have been published for 283 patients receiving the combination treatment and 135 concurrent non-randomized control dogs.[64] Median survival was significantly higher for dogs treated with the combination treatment, i.e. 610 versus 91 days for dogs with complete resection of the primary tumor and 201 versus 65.5 days for dogs with partial resection of the primary tumor. However, control dogs were treated with surgery alone, whereas vaccinated patients were treated with surgery, the adjuvanted vaccine and suicide gene therapy. It is therefore impossible to determine if and how extensive the vaccine contributed to the survival benefit.

Lymphoma

Lymphoma was the first tumor type to be explored in the field of veterinary tumor vaccination. A tumor cell vaccine proved, already in 1976, to be efficient in a small placebo controlled trial.[65] This trial tested a tumor cell lysate, admixed with Freund's complete adjuvant, in 11 patients with lymphoma. Dogs in complete remission after induction chemotherapy received the vaccine IM, two times with one week interval, followed by two injections with two weeks interval. The induction of an anti-tumor immune response was not investigated. A MST of > 348 days, which was significantly higher than 197.5 days in the placebo control group, was observed. Median remission period was also significantly higher in the vaccinated group, i.e. 133.5 versus 30.2 days. Surprisingly, no further studies with this vaccine have been reported.

A gene-based vaccination approach consisting of a pDNA and an adenoviral vector encoding canine TERT has been tested for the treatment of lymphoma.[66] Fourteen dogs with B cell lymphoma participated in this trial. Due to relapse soon after the interruption of the induction chemotherapy, maintenance chemotherapy was given during the vaccination period. TERT expression has been reported in the majority of canine tumors.[75-78] In this study, all of the analyzed lymphoma samples were positive for TERT mRNA, confirming that TERT is a suitable target for immunotherapy in lymphoma.[66] The vaccine regimen consisted of two injections of the adenoviral vector at a two-week interval followed by five successive pDNA vaccinations every two weeks. The vaccines were injected IM and electroporation was used directly after pDNA injection to facilitate its intracellular uptake. Routine application of this treatment by veterinary practitioners will be hampered by the need of an electroporation device and the fact that it requires general anesthesia of the dogs. Immune responses were

measured with an IFN-gamma ELIspot and were positive in the majority of patients. Median survival time could not be calculated for the vaccination group. The lower limit of the 95% confidence interval for overall survival for vaccinated dogs was 57.6 weeks, which is higher than 26 weeks for the historical control group. The latter consisted of recent cases treated in the same institute by the same veterinarians with the same chemotherapy protocol as the vaccinated patients. Such a historical control group minimizes the unwanted variability associated with a single-arm study. An increased remission period of 26 weeks compared to 14 weeks in the historical control group was also observed. These encouraging results and the broad applicability across tumor types make this vaccine a very promising candidate for further clinical studies.

Hemangiosarcoma

Hemangiosarcoma has been evaluated as a possible target for tumor vaccination in one clinical trial.[67] Twenty-eight dogs were immunized with an allogeneic tumor cell lysate vaccine with liposome-DNA complexes (lipoplexes) as adjuvant. The DNA in the lipoplexes did not code for an antigen. The dogs also received doxorubicin chemotherapy and the vaccine was administered intraperitoneally five times with two weeks interval followed by three injections once a month. Strong humoral responses were detected against the allogeneic tumor cells used in the vaccine. A control group was compiled from historical cases treated with doxorubicin in the same institutes that participated in this study. The MST in this control group was 133 days, while the vaccinated group had a statistically significant higher MST of 182 days. Adverse effects were limited to those caused by the doxorubicin treatment. Although the prolongation of the MST with seven weeks reached statistical significance, it may however not be sufficient to convince many owners to pay for this treatment, on top of the chemotherapy.

Vaccines that failed to obtain a significant survival benefit

In the trials summarized in table 2 a statistical analysis was performed, but no survival benefit could be demonstrated. However, in these trials, spectacular results were sometimes seen in a small subset of patients, which is typical for tumor vaccination studies. An understanding why the same vaccine works in some patients but not in others is of great importance. Therefore the development of predictive biomarkers that allow identification of patients who might benefit from cancer vaccination would be of great help and would avoid premature abandonment of useful vaccines.[61]

Table 2: Clinical trials with tumor vaccines that did not result in a survival benefit for the vaccinated dogs

Tumor type	Vaccine type	Number of patients	Control group	Variable	Reference
Lymphoma	Tumor cell vaccine	58	Concurrent, randomized	Survival	[79]
Lymphoma	Tumor cell vaccine	26	Concurrent, randomized, placebo controlled	Survival, duration first remission	[80]
Lymphoma	Tumor antigen coated microbeads	15	Recent published data	Disease free interval	[81]
Lymphoma	CD40-B cell vaccine	19	Recent historical cases from same institution	Survival, time to progression	[82]

Irradiated whole tumor cells, administered by intralymphatic injection, have been explored as a tumor vaccine for the treatment of lymphoma by Jeglum *et al*.[79,83] The vaccination schedule consisted of two injections with two weeks interval, followed by another injection after one month and after six weeks. As with the other trials with lymphoma, only patients in complete remission after induction chemotherapy were included. The rationale behind intralymphatic injection was that the antigen is delivered where an immune response is initiated. The consequence is that the dogs had to be anesthetized for vaccination, which makes it less suitable for clinical use. Immune responses were not measured. A preliminary trial with 30 patients suggested a longer MST and remission duration than documented for lymphoma in the literature.[83] Later on the same vaccine was tested in more patients (n=58) and after a comparison with the randomized control group a trend towards longer survival was again observed. However, this trend did not reach statistical significance.[79]

In a study of Turek *et al.* 26 patients with B cell lymphoma that were in complete remission received a vaccine consisting of tumor cells transfected with hGM-CSF.[84] The vaccine was administered by an ID injection; four times weekly followed by four injections biweekly. Tumor specific immune responses were seen in every patient, measured with a delayed-type hypersensitivity (DTH) skin test. This placebo-controlled trial found no improvement in first remission duration and survival for dogs receiving the vaccine. There was no correlation between the result of the DTH skin test and the clinical response.

Henson *et al.* came up with a new type of tumor cell vaccine that consists out of microbeads on which cell membrane fragments of autologous tumor cells are loaded.[81] The authors chose this approach based on previously published data showing that presenting antigens on these

cell-sized silica microbeads is superior in eliciting a CTLs response. Dogs in complete remission after induction chemotherapy received three weekly SC injections of the vaccine together with SC injections of IL-2 and GM-CSF as adjuvants. This protocol was implemented in 15 patients with B cell lymphoma. Half of the patients showed evidence of an immune response detected with a DTH skin test. Vaccination resulted in similar survival times as historical control dogs collected from published data. No correlation between the DTH skin test and clinical response existed.

Sorenmo *et al*. evaluated a vaccine that used CD40-activated autologous B lymphocytes (CD40-B cells) instead of DCs as APC.[85] In a previous study, this group had established that canine CD40-B cells can act as APC and they described an efficient protocol for the generation of CD40-B cells from the peripheral blood of dogs.[86] A great advantage over DCs is that a large number of CD40-B cells can be obtained from a small number of precursors. This requires less blood volume to procure sufficient numbers of APC for recurrent vaccinations. The CD40-B cells were loaded with autologous tumor RNA by means of electroporation.[85] The vaccine was given to 19 patients suffering from lymphoma in complete remission, by three ID injections with two to three weeks interval. Immune response was evident in half of the patients when analyzed with an IFN-gamma ELIspot. It did not result in longer survival times when compared with matched historical control dogs, recently treated in the same institution. However, in the vaccinated group a greater percentage of dogs achieved secondary remission (7.7% versus 40%) and a prolonged lymphoma-specific survival after relapse.

Trials assessing efficacy without a control group

Lastly, there are also clinical trials with tumor vaccines that did not include a control group. These trials are summed up in table 3. Mostly, it is only stated if a clinical response was achieved. A clinical response is a complete response (CR; the disappearance of all lesions) or a partial response (PR; a decrease of greater than 30% in the sum of the greatest dimension of all lesions). Many authors consider stable disease (SD; a decrease less than 30% or an increase less than 20%) also as a positive effect.[87] However, these criteria, developed for cytotoxic drugs that are expected to cause tumor shrinkage early in the treatment, are inadequate for the evaluation of tumor vaccines.[61,88-90] Overall survival is the golden standard, but for exploratory trials this is not always a feasible endpoint. Progression free survival is a good alternative as a significantly shorter follow-up time is required.[61] From these

preliminary trials, without any statistical comparison with a control group, no definite conclusions can be drawn.

Hogge *et al.* evaluated an autologous tumor cell vaccine in patients with melanoma, osteosarcoma and fibrosarcoma.[91] The tumor cells were genetically engineered to express hGM-CSF. Experiments in laboratory Beagles demonstrated the efficacy of this approach to attract inflammatory cells to the site of injection.[51] Three ID vaccinations were given at 12 day intervals to ten melanoma patients. Some patients had no macroscopic disease at the start of vaccination, others entered the trial with macroscopic tumor present. Immune responses were not evaluated. Three of the ten melanoma patients had a clinical response or stable disease, two dogs with no evidence of disease at the start of vaccination remained that way. Although the survival times are not statistically compared with a control group, the authors interpret their data as a suggestion that the vaccine prolongs survival time. Three cases of fibrosarcoma were also included. One patient had a partial response, another remained free of detectable disease and in the third patient the tumor progressed without response. The same vaccine is mentioned in a case report of a dog with a vertebral fibrosarcoma. After vertebrectomy and subsequent vaccination the dog was monitored for two years and no relapse occurred.[92] Two dogs with osteosarcoma were treated with this vaccine, but no beneficial effects were observed.[91]

A preliminary study with only three melanoma patients explored the use of autologous DCs that were transfected with an adenoviral vector encoding human glycoprotein 100 (gp100) as xenogeneic tumor antigen.[93] The transfected DCs were injected back into the patient by the SC route. This was repeated three times monthly. Dogs had undergone surgery before vaccination and were treated with radiation therapy before and during the vaccination period. One of the three patients had a strong CTL response, which was associated with a long term clinical response.

Table 3: Preliminary trials with canine tumor vaccines without a control group

Tumor type	Vaccine type	Number of patients	Variable	Frequency of responding patients; MST	Reference
OSA	Tumor cell	2	CR, PR, SD, NED	0/2	[91]
Melanoma	vaccine	10		4/10	
FSA		3		2/3	
Melanoma	DC- vaccine	3	CR	1/3	[93]
FSA	DC-vaccine	1	CR, PR	1/1	[94]
MGT		4		4/4	
Melanoma	Tumor cell vaccine	34	Tumor control rate	35.3%	[95]
PNST	Peptide vaccine	9	Tumor control rate	5/9	[96]
Lymphoma	Tumor cell vaccine	7	Survival attributable to vaccine	7/7	[97]
FSA	Tumor cell	11	MST	1051 days	[98]
OSA	vaccine	5	PR, NED	3/5	
MGT	DNA vaccine	7	PR	6/7	[99]

CR = complete response, PR = partial response, SD = stable disease, tumor control rate = CR + PR + SD, NED = no evidence of disease, OSA = osteosarcoma, FSA = fibrosarcoma, MGT = mammary gland tumor, PNST = peripheral nerve sheath tumor, MST = median survival time

In the study of Mito *et al.* four dogs with breast cancer and one dog with fibrosarcomas at two sites received an intratumoral injection of autologous DCs in combination with an intratumoral injection of recombinant canine IFN-gamma.[100] The DCs were not loaded with antigen *in vitro*. It was considered that the DCs injected into the tumor took up antigen from tumor cells and were matured and activated *in situ* upon stimulation by IFN-gamma. The DC were given eight times at weekly intervals. The IFN-gamma injection happened together with the DCs and was repeated the second and the fifth day afterwards. One of the two tumors of the fibrosarcoma patient showed a partial response. Unfortunately, the dog died of disseminated intravascular coagulation due to massive necrosis of tumor tissue. Three of the four dogs with breast cancer showed a complete response and one a partial response. Again, one patient died as a consequence of massive necrosis of the tumor. An anti-tumor immune response of three responding patients was assessed with a radioactive peripheral blood mononuclear cells (PBMC) proliferation assay and was present in all three of them.

For the treatment of melanoma, the use of an allogeneic tumor cell vaccine was also explored. The tumor cells were transfected with human gp100, thereby exploiting the advantages of xenogeneic vaccination.[95] Transfection of the cells occurred via gene gun particle-bombardment. Thirty-four patients, with and without macroscopic tumor present at the time of first vaccination entered the trial. The vaccination schedule consisted of eight ID immunizations, four weekly followed by another four biweekly. The immune response was well characterized. Antibodies against human gp100 were detected in half of the tested patients. There was no correlation between the extent of gp100 antibodies and the clinical response. Cellular immune responses were evaluated with the DTH skin test and a CTLs assay using the allogeneic tumor cells of the vaccine as targets. The DTH test and the CTLs assay were positive in half of the tested samples. The DTH test correlated with the clinical response, however the CTLs assay did not. A tumor control rate (CR, PR, SD for minimum six weeks) of 35.3% is reported. Median survival time was 153 days, no statistical analysis with a control group or published data was performed. Dogs experiencing tumor control had a significantly longer MST than dogs having no response (337 versus 95 days), suggesting that this is a valid criterion for the evaluation of the efficacy of tumor vaccines.

One protein vaccine has been evaluated in veterinary oncology. Recombinant human vascular endothelial growth factor (VEGF) together with liposome-DNA complexes as adjuvant was administered ID in nine patients with peripheral nerve sheath tumors.[101] The vaccination scheme consisted of three biweekly vaccinations, followed by three monthly vaccinations. Three out of four dogs who received three or more vaccinations developed antibodies against human VEGF. None of the dogs had detectable antibodies against canine VEGF. The authors suggest that they were elicited but not detected due to rapid elimination after complexing with circulating canine VEGF. In two of these dogs there was indeed a significant reduction in circulating canine VEGF concentrations. The same was observed in two dogs without antibodies against human VEGF, which may suggest that also a cellular immune response is involved in the reduction of circulating canine VEGF. Three patients had a PR, two patients a SD. Reduction in circulating VEGF was correlated with tumor response.

Morphogenesis together with Novartis Animal Health developed ImmuneFx™, a vaccine with tumor cells transfected with Emm55, a bacterial antigen.[102] These cells have gained foreign characteristics for the immune system, and all of the antigens of the tumor cells are processed and presented as such. Veterinary physicians can send autologous tumor cells and

the company provides the vaccine in one to two weeks. The vaccine is administered intravenously four times weekly followed by monthly boosts. A published trial with seven lymphoma patients reported anti-tumor immune responses in every patient when assessed with a CTLs assay and an increased survival in the vaccinated dogs. However, we have to remark that they considered increased survival as days that the dog lived beyond the outer expectation limit of 42 days, with a correction if dogs were also treated with chemotherapy. The authors selected this expectation limit of 42 days based on data from the literature claiming that without treatment dogs with lymphoma die within four to six weeks. Consequently, in view of the low number of patients and the absence of a real control group in this study we can only consider this increase in survival as a promising result that should be confirmed in a randomized, placebo-controlled study. This is the only trial with lymphoma where complete remission was not an inclusion criterion. Additionally, in this study some patients also received chemotherapy concomitant with the vaccination.[103]

As already discussed, the combination of surgery, tumor cell vaccination and thymidine kinase suicide gene therapy was very effective in melanoma patients. In a recent report Finocchiaro *et al.* evaluate the efficacy of this treatment in patients with fibrosarcoma and osteosarcoma.[98] The study included eight fibrosarcoma patients. Five patients had no macroscopic tumor present at the start of the vaccination. A MST of 1051 days was documented, which was higher than the 236-532 days the authors found in the literature for fibrosarcoma. The conclusion that the vaccine improved the survival time was not based on a statistical comparison with a control group. The same protocol was used to treat five osteosarcoma patients. Tumors in four dogs were surgically removed before vaccination. In one dog this was not possible because the contralateral limb was amputated in the past for a previous osteosarcoma. The latter dog did exhibit a partial response, but was euthanized after three months because of poor quality of life. In two dogs no evidence of disease was present for over six months. One dog had a reappearance of the tumor within five weeks and was euthanized after six months. The fifth dog had signs of an affected lymph node after five months and was euthanized.

A case report of a patient with astrocytoma treated with the combination of surgery, IFN-gamma gene transfer and tumor cell lysate vaccine has been published.[104] Five ID immunizations were given biweekly. The first contained autologous tumor cell lysate. Because of the slow growth of the autologous cells in culture an allogeneic lysate had to be

used for the subsequent vaccinations. The vaccine contained CpG oligonucleotides as adjuvant. The IFN-gamma gene transfer was performed using an adenoviral vector in the tumor tissue that remained after surgical tumor debulking. The dog experienced transiently focal neurological symptoms. An antibody response and a cellular response were identified. Cellular immunity was assessed by flow cytometric analysis of CD8 IFN-gamma positive T cells. After one year, the dog was still tumor free.

A recent study evaluated genetic vaccination against p62 in seven dogs with mammary gland tumors.[99] Vaccination schedule consisted of weekly IM injections of a plasmid encoding human p62, with the dose ranging from 0.75 to 2.5 mg and number of vaccinations from 3 to 10. Although initially an increase in tumor volume was apparent, the vaccine resulted ultimately in a partial response in six out of seven patients, ranging from 23 to 78% decrease in tumor volume.

Discussion

Trial design: The wide range of vaccination protocols used in the different clinical trials is striking. This high variability of protocols was also noticed in studies that used the same type of tumor vaccine. The absence of a standardized protocol is related to the fact that the optimal administration route, dose and schedule for tumor vaccines are unknown at the moment. Apart from the vaccination protocol, also the selection of patients can affect the outcome. For example the outcome of a tumor vaccine may be better in patients that underwent tumor resection before vaccination. This wide range of vaccination protocols makes it very difficult to compare the efficiency of tumor vaccines. When a tested vaccine is ineffective, this may be due to a truly ineffective vaccine formulation, but also to suboptimal selection of the administration route, dose, schedule or patient population. The development of biomarkers identifying patients that could potentially benefit from cancer vaccination would decrease the number of false negative trials. The lack of a standardized trial design is a problem inherent to the field of tumor vaccination and is an equally important problem in human oncology.[90] Other concerns about the discussed vaccination studies are the low number of patients and the rare inclusion of a valid control group. Objections to a randomized trial are related to the increased sample size and ethical concerns. Randomized trials will allow more meaningful conclusions and will decrease the number of unnecessary trials. In the end, less patients, resources and time will have to be used. It may seem unethical to withhold promising treatments from patients. It is reasonable to argue that it is also unethical that a non-effective

treatment is given to many patients based upon a false positive trial.[59,60] In conclusion, although single-armed trials remain appropriate in selected circumstances, trials that investigate clinical efficacy should be randomized trials.

Tumor types: Melanoma and lymphoma are by far the most popular targets in veterinary cancer vaccination studies. Also in human medicine, these two forms of cancer were the first and most extensively studied candidates for cancer vaccination. Both tumors are known to be immunogenic and especially for melanoma extended knowledge exists about tumor antigens.[90] In human oncology, virtually all tumor types have become targets for tumor vaccination.[105-115]

Vaccine types: The majority of veterinary trials used tumor cell based vaccines. DC vaccines have up till now only been administered to a small number of canine patients. Due to the high cost price, this vaccine type will be difficult to introduce in veterinary medicine. To give an idea, the cost price of Provenge® in human oncology, which is a DC vaccine, is about 93 000 dollars per course of treatment.[116] Only two DNA vaccines were tested in veterinary oncology, but both with great success. Besides for canine melanoma, little is known about important immunogenic tumor antigens in veterinary oncology. This has hampered the development of other DNA vaccines. The recently developed TERT DNA vaccine can become an elegant solution for this problem. TERT is a non-specific tumor antigen, overexpressed in most canine tumors.[75-78] This vaccine, already tested in canine patients with lymphoma, has potential to be effective in a broad range of tumor types. At present, the good results of DNA vaccination in animals, against tumors as well as infectious agents, cannot be achieved in human trials. This is however most likely a matter of optimizing the delivery method for human subjects, and not because this approach would not work in humans.[117]

Immunological response: Tumor vaccination leads to an immune response in the majority of patients, with an appearance four to eight weeks after the first vaccination. This is also true for patients who simultaneously receive chemotherapy, confirming that this combination is not contraindicated. Some of the clinical trials investigated a correlation between the clinical response and the immune response.[71,81,84,93,95] However, conflicting results have been published. Nevertheless, presence of an immune response reflects a mode of action and therefore kits that can detect a specific immune response can contribute greatly to the development of a new product. Also in human oncology immunological assays that can

predict the clinical response of tumor vaccines are urgently needed. More research to validate such immune assays is needed, comparable to human oncology where an international research project called 'MIATA' is started to resolve this problem.[118]

Clinical response: Of the ten trials where a statistical survival analysis was performed, six could demonstrate a significant survival benefit for vaccinated dogs. However, since the majority of these trials are not randomized, for definite prove that tumor vaccination has potential as a complementary treatment modality for cancer, more randomized placebo controlled trials are necessary. With tumor vaccination being the sole therapy added to the standard treatment, the genetic vaccines for the treatment of lymphoma and melanoma give the best results.[66,70] If we consider all trials, the tumor cell vaccine in combination with surgery and suicide gene therapy for the treatment of melanoma is superior.[64]

Safety: Maybe the most consistent and trustworthy finding of all vaccination trials is the safety of tumor vaccines. In all the preclinical and clinical trials dogs were monitored closely, with no significant adverse effects in all but two trials. In the trial were DC vaccination was combined with IFN-gamma gene therapy there were two fatalities due to massive necrosis of the tumor.[100] This can be prevented by surgically debulking the tumor before vaccination, thus this treatment still has clinical potential. The second reported adverse effects consisted of transient neurological symptoms in a patient with astrocytoma.[104] In this dog a tumor cell lysate vaccine was combined with intracranial surgery and IFN-gamma gene therapy. The symptoms disappeared without treatment, so again they are not a reason to discontinue the use of this vaccine. In human medicine, tumor vaccination for the treatment of brain tumors has already been done in multiple trials and is considered safe.[115]

Conclusion: In conclusion, veterinary tumor vaccination trials prove that tumor vaccination is safe and can be effective, even in combination with chemotherapy. Collaboration between human and veterinary medicine is of mutual interest and can lead to great results. For example the success of the canine melanoma vaccine, with 1500 canine patients treated in 2008, had led to the use of this vaccine in human clinical trials.[70,119,120] It has to be stressed that all the effective vaccines are adjuvant therapies, either after or concomitant with surgery or chemotherapy. Hence, the total costs of a combination treatment can become very high. Therefore, in veterinary oncology tumor vaccination will, at least in the near future, be limited to motivated owners. Tumor vaccination trials suffer from a high likelihood of false positive

results due to lack of randomization and significant risk of false negative results due to insufficient knowledge of optimal patient selection, administration route, dose and vaccination schedule. As long as this is not solved, evaluation of tumor vaccines will be suboptimal at best.

3. Cytokine therapy

Cytokines are a heterogeneous group of small signaling (glyco)proteins with more than 100 known members. Despite their heterogeneity they also have some common characteristics. Cytokines are pleiotropic in every aspect; the same cytokine can be produced by a vast diversity of cell types, have an effect on many different target cells and have very different biological effects which can be autocrine, paracrine or endocrine.[121] Cytokines are bioactive at very low concentrations and have a short half-life.[122] Many cytokines play a crucial role in tumor immunology and are thus explored as anticancer agents.[121,123,124] However, the biological characteristics of cytokines pose serious challenges for their therapeutic use. Their pleiotropic effects often lead to serious adverse effects, which is aggravated by the necessity of administering high doses because of their short half-life. However, in the last decade researchers have been able to diminish the toxicity of cytokines via molecular engineering of cytokines with prolonged half-life, enhanced specificity or localized activity. This enhanced the clinical applicability of cytokine therapy.[123] Currently, IL-2, interferon-alpha (IFN-alpha) and tumor necrosis factor-alpha (TNF-alpha) are approved for clinical use in human oncology. All three are associated with serious adverse effects when given systemically.[124,125] For IL-2 and IFN-alpha, engineered formulations are now available and TNF-alpha is only administered via isolated limb perfusion.[123,125]

IL-12 is the most potent anti-tumoral cytokine studied to date.[126] However, because of severe toxicity with even fatalities in early clinical trials, clinical development was halted. Now, with new possibilities for delivery, IL-12 is again intensively studied in clinical trials. .[127] In these trials IL-12 gene therapy is mostly used to express IL-12 in the tumor. IL-12 is a critical factor that can tip the balance towards immunity instead of tolerance.[128] It is a very potent third level activation signal for T cells.[129] IL-12 also induces clonal expansion and cytolytic function in activated CTLs as well as NK cells.[129-131] Myeloid derived suppressor cells decrease their immune suppressive function in response to IL-12 and can be even converted to immune cells that help to establish an anti-tumor response.[126,132] These strong immune

stimulatory effects are complemented by inhibition of tumor angiogenesis.[133] In this PhD thesis we combined intratumoral IL-12 gene therapy with a DNA cancer vaccine. In this combination, IL-12 will synergize with tumor vaccination by providing an adequate intratumoral cytokine environment for the systemically generated CTLs to fully exercise their cytotoxic functions. Moreover, IL-12 will complement tumor vaccination by stimulating NK cells as additional effector cells next to CTLs.

Some preliminary studies with cytokine therapy have been initiated in veterinary oncology. Intratumoral delivery of lipid complexed pDNA encoding GM-SCF or IL-2, in combination with a bacterial superantigen, was evaluated in dogs with melanoma.[134] Intravenous administration of lipid complexed pDNA encoding IL-12 was evaluated in dogs with lung metastases after resection of osteosarcoma.[135] IL-12 plasmid, delivered by electroporation, was administered to dogs with mast cell tumors, squamous cell carcinoma and melanoma.[136,137] Moreover, intratumoral IL-12 gene therapy has been combined with electrochemotherapy in dogs.[138,139] In cats with fibrosarcoma, intratumoral injection of recombinant interferon-gamma, intratumoral viral gene therapy with IL-2, intratumoral gene therapy with IL-2, IFN-gamma and GM-SCF by magnetofection and intratumoral adenoviral IL-12 gene therapy have been evaluated.[140-143] In horses, intratumoral injection of IL-12 plasmid for the treatment of melanoma, as well as intratumoral injection of recombinant IL-2 in combination with cisplatin for the treatment of sarcoid, was explored.[144,145] Although these trials demonstrate proof-of concepts of efficacy, safety and feasibility, the low number of patients and lack of a control group precludes any definite conclusions.

4. Metronomic chemotherapy

The regular administration of chemotherapeutics at low doses was called metronomic chemotherapy by Hanahan *et al.* in 2000 and is characterized by immune stimulatory and anti-angiogenic modes of action.[146] Why these mechanisms of action are only evident with low and not high doses of chemotherapy can be explained by two reasons. Firstly, it can be a truly dose-dependent effect, when selective sensitivity of certain cell types to the cytotoxic action of the drug is the determining factor. This is for instance the case for the immune stimulatory action of metronomic cyclophosphamide by depleting Tregs. Regulatory T cells are more sensitive to cyclophosphamide than effector T cells. Low doses will only affect

Tregs and thus result in immune stimulation. Higher doses will also affect effector T cells resulting in immunosuppression.[147] Other mechanisms of action rely on the different timing of metronomic versus traditional dosing. Also high doses of chemotherapy are antiangiogenic during treatment, but during the forced drug-free breaks because of toxicity a rebound proangiogenic effect negates the initial antiangiogenic effect of chemotherapy at high dose.[148] Because of the different mode of action, metronomic chemotherapy can be used in patients with tumors resistant to high dose of the same drug. As there are indications that metronomic chemotherapy can be equally effective as standard dose chemotherapy, but with a better quality of life, clinical trials directly comparing both treatment schedules are being conducted. Metronomic chemotherapy can also be combined with standard dose chemotherapy to avoid drug-free breaks or in combination with other treatment modalities for its synergistic biological effects.[149]

The immune stimulatory actions of metronomic chemotherapy are diverse and dependent on the drug, comprehensively reviewed in the article of Hao *et al*.[150] The immune effects of metronomic cyclophosphamide are the most extensively characterized and include but are not limited to the depletion of Tregs, maturation of DC, induction of MHC-I and NK2GD ligands.[150] Of special interest for our combination therapy is the depletion of Tregs, considered major obstacles for immunotherapy.[151] Drugs that have proven to decrease Tregs in rodent models and human patients and are easily orally administered are cyclophosphamide and temozolomide. The selective effect of metronomic cyclophosphamide towards Tregs is explained by reduced intracellular ATP levels in these cells, leading to decreased glutathione synthesis and thus reduced detoxification of cyclophosphamide.[147] For temozolomide, the mechanism of selective toxicity towards Tregs is not yet elucidated.

4.1. Metronomic cyclophosphamide in veterinary medicine

Two trials investigated the effect of metronomic cyclophosphamide as an adjuvant therapy after surgery (table 4). The first clinical trial with metronomic chemotherapy in veterinary medicine consisted of nine dogs with stage II hemangiosarcoma that were treated with a combination treatment of metronomic cyclophosphamide (12.5-25 mg/m²) alternating with metronomic etoposide (50 mg/m²) and combined with continuous piroxicam administration (0.3 mg/kg) after splenectomy. This group was compared with three randomized control dogs, supplemented by 21 historical control dogs, treated with doxorubicin after splenectomy. Median survival time of dogs treated with metronomic chemotherapy was significantly higher

than control dogs (178 vs 133 days). Treatment was well tolerated, although two dogs developed hemorrhagic cystitis which led to discontinuation of the cyclophosphamide treatment.[152] Another trial investigating the efficacy of metronomic cyclophosphamide in an adjuvant setting included incomplete resected soft tissue sarcomas. Thirty dogs were treated with cyclophosphamide at 10 mg/m² in combination with piroxicam at 0.3 mg/kg and compared to 55 contemporary, non-randomized control dogs without additional treatment.[153] Disease free interval was significantly higher in the treated dogs. Again, hemorrhagic cystitis was observed in three dogs, which necessitated euthanasia in one dog.

Table 4: Clinical studies with metronomic chemotherapy in dogs

Tumor type	Patients	Control group	Treatment protocol[1]	Outcome	Reference
Stage II splenic hemangiosarcoma	9	3 randomized + 21 historical doxorubicin treated dogs	Surgery followed by CPX 12.5-25 mg/m²+etoposide 50 mg/m²+piroxicam 0.3 mg/kg	MST 133 days vs 178 days	[152]
Incomplete resected soft tissue sarcoma	30	55 non-randomized dogs	CPX 10 mg/m²+piroxicam 0.3 mg/kg	Median DFI 211 vs minimal 410 days	[153]
Various metastasized cancers	15	/	CPX 25 mg/m²+celexocib 2 mg/kg	1 CR, 5 SD (MST 3.39m)	[154]
Various metastasized cancers	10	/	CPX 12.5 mg/m^2+piroxicam 0.3 mg/kg	1 CR, 1 PR, 2 NED after surgery	[155]
Soft tissue sarcoma	11	/	CPX 12.5 mg/m² or CPX 15 mg/m²	MVD and Tregs decreased (15mg/m²)	[156]
Various advanced stage cancers	15	/	Toceranib 2.75 mg/m²+CPX 15mg/m²	Tregs decreased, serum IFN-gamma increased	[157]
Various cancers	81	/	Lomustine 2.84 mg/m²	6%PR, 30%SD	[158]
Various cancers	36	/	Chlorambucil 4 mg/m²	1 PR, 17 SD	[159]
Transitional cell carcinoma	31	/	Chlorambucil 4 mg/m²	1 PR, 20 SD (MST 221 days)	[160]

1: all dosages are per day

CPX=cyclophosphamide; MST=median survival time; DFI=disease free interval; CR=complete response; SD=stable disease; PR=partial response; NED=no evidence of disease; MVD=microvessel density; Tregs=regulatory T cells

Four studies describe the use of metronomic cyclophosphamide in advanced stages of disease (table 4). The first report documents clinical response of metronomic chemotherapy in 15 dogs with metastasized cancer.[154] Cyclophosphamide was given at 25 mg/m² and combined with celecoxib at 2 mg/kg. One dog with malignant melanoma experienced a CR, five other dogs had SD. Median overall survival was 3.39 months. No hemorrhagic cystitis or other toxicities were observed. Baseline serum levels of VEGF and interleukin-6 (IL-6) were measured. Responders (dogs with CR and SD) had lower baseline VEGF levels, suggesting that VEGF levels may serve as a predictor of efficacy. In the overall patient population, there was a strong correlation between baseline VEGF levels and poor prognosis. IL-6 was not correlated with therapy response or prognosis. A second study investigated the biological activity of metronomic cyclophosphamide in dogs with soft tissue sarcomas.[156] Eleven dogs were either treated with 12.5 mg/m² or 15 mg/m² for four weeks and the effect on Tregs and microvessel density (MVD) were examined after two and four weeks. Only the dosage of 15 mg/m² had a significant effect on the percentage of Tregs and MVD. At a dosage of 12.5 mg/m², only the absolute number of Tregs was decreased. However, the authors state that the limited number of patients warrants careful conclusions about the dose dependent effect on Tregs. After four weeks of treatment, tumors were surgically removed and no further clinical follow-up was performed. The third study evaluated immune effects and safety of toceranib (2.75 mg/m²) given every other day and complemented after 14 days by daily cyclophosphamide (15 mg/m²) in 15 dogs with advanced disease, of which 12 had metastases.[157] Treatment was well tolerated, with only grade 1 to 2 adverse effect, mainly during the first two weeks on toceranib treatment. Regulatory T cells were significantly decreased after toceranib treatment. After addition of cyclophosphamide, Tregs did not decrease further, however interferon-gamma levels in serum were only increased at time points when dogs received the combination treatment. This suggests either a further improvement in immune function after addition of cyclophosphamide or prolonged exposure to toceranib. In the fourth and last study; dogs treated with metronomic cyclophosphamide (12.5 mg/m^2) and piroxicam (0.3 mg/kg) serve as a historical control group for the evaluation of a combination of this regimen with proton-pump inhibitors.[155] For the group only receiving metronomic chemotherapy, ten dogs with various metastatic cancers are retrospectively described, with some of them treated by surgery before start of treatment. One dog experienced a CR, one dog a PR and two dogs showed no evidence of disease after surgical removal of the tumor.

4.2. Metronomic regimens of other drugs in veterinary medicine

One study with metronomic lomustine (2.84 mg/m²) is published including a heterogeneous patient population of 81 dogs (table 4).[158] Different tumor types were treated, with some patients receiving prior or concomitant surgery, standard chemotherapy or radiotherapy. In 22 patients, therapy was discontinued due to toxicity (mainly gastrointestinal) and one dog died of treatment-related nephrotoxicity. Of dogs receiving only metronomic lomustine, 6% experienced a partial response and 30% stable disease. Also chlorambucil at 4 mg/m² is explored as metronomic chemotherapy in dogs (table 4).[159] In 36 dogs with various cancers, 3 dogs experienced a CR, one dog a PR (remission rate: 11%) and 17 dogs (47%) had SD. This led to a MST of 153 days. Treatment was very well tolerated, with adverse effects limited to grade 1 to 2 gastrointestinal effects in 4 patients. The same treatment regimen was applied in 31 dogs with transitional cell carcinoma.[160] One PR and SD in 67% of the patients was observed, leading to a MST of 221 days.

5. Canine cancer patients as model for human oncology

Cancer is an important disease in dogs, with a recent study in the UK identifying it as the most common cause of death.[161] Therefore, exploring new treatment options in dogs is of great importance for veterinary medicine. However, these trials have also great value for human oncology, as cancer in dogs represents an excellent model for cancer in human patients.

Tumors result from malignant transformation of a small number of the patient's own cells and develop in close interaction with the tumor microenvironment and the immune system. In rodent models, tumors are either artificially induced or a result of inoculation of a great number of cultured tumor cells. Inbred rodents are not fully immune competent and in nude mice the adaptive immune system is evidently completely absent. Therefore, the complexity of spontaneously arising tumors in humans cannot be approximated with these models. This results in very low predictive power of rodent models, with a tremendous amount of negative phase I trials in human patients.[162,163]

Spontaneously arising tumors in companion animals can be a very valuable intermediate model. In these pets, tumors develop in a syngeneic environment and in the presence of an

intact immune system. Although there are some concerns about inbred dog populations, it has been demonstrated that the genetic diversity in a population of dogs with a certain tumor type is similar to the diversity in a human population.[163] Many tumors in dogs share biological, histological and clinical characteristics with their human counterparts. Moreover, responses to treatment are very similar. Genetic research has revealed nearly identical genetic changes in both species.[162,163] This is especially true for lymphoma, osteosarcoma, melanoma and soft tissue sarcoma.[164,165] Hemangiosarcoma is a highly metastatic, prevalent tumor that is very suitable as a model for anti-metastatic and anti-angiogenic therapies. Some tumor types, like osteosarcoma and brain tumors, are much more common in dogs than in humans, so it is more feasible to conduct clinical trials with sufficient statistical power.[164,165] Progression of cancer in companion animals is slow enough to be able to compare responses to therapies and fast enough to obtain results within a reasonable period of time.[162,164] In human medicine, only patients who are refractory to the standard treatment options can enter a phase I trial. In veterinary medicine no such restrictions exist, any patient can participate as long as this is based on an informed owner's consent, approved by an ethical committee. Therefore, new medicines can be evaluated in early stages of disease and in the setting of minimal residual disease after e.g. surgery or chemotherapy.[164] An additional advantage of dogs is their body size, that allows surgical interventions, medical imaging and tissue/blood sampling much like in human patients.[162,164]

The present climate is very favorable for implementing clinical trials with dogs as treatment models for humans. The availability of species-specific research tools, which was an important impediment in the past, is increasing rapidly.[162,164,166] Veterinary infrastructure today meets the standards of good clinical practice.[166] Limited treatment options exist in veterinary oncology and dog owners want the best care possible for their pet. The benefit of their participation for human medicine is generally considered as a plus. For these reasons, compliance with treatment, control visits and autopsy are exceptional.[164]

It can be concluded that dogs are excellent treatment models for human oncology in general. Evidently this applies even more to treatments dependent on the patient's immune system. Many immunotherapy studies in dogs are conducted in close cooperation with human medicine.[66,74,81,84,85,93,98,100] A good example of how this approach can benefit both veterinary and human medicine is the melanoma vaccine Oncept™. Clinical trials with this vaccine led to its licensing and widespread use in veterinary oncology and urged clinical trials with DNA

vaccines encoding murine/human tyrosinase in humans.[120] Another example is a vaccine consisting of an adenoviral vector targeted to DCs, first studied in laboratory Beagles followed by a clinical trial in humans.[52,167] Although not related to tumor vaccination, a last prime example of how canine trials can accelerate the development of new treatments for humans has been given by the work of McEwen *et al.*, who tested a new immunotherapeutic treatment which was subsequently approved for the treatment of pediatric osteosarcoma.[73,168,169]

SCIENTIFIC AIMS

Scientific aims

Scientific aims

The general aim of this PhD thesis was the evaluation of a multimodal immunotherapeutic approach to treat cancer, with as ultimate future goal the evaluation of this treatment in companion dogs afflicted with cancer.

The first objective was a proof-of-concept study in mice that the combination of tumor vaccination, interleukin-12 (IL-12) gene therapy and metronomic cyclophosphamide was safe and each component resulted in biological and clinical benefits (chapter 1). For this study, the B16-F10 melanoma model was used, as the individual components are well characterized in that model and thus needed no further optimization.

After the confirmation that the proposed combination therapy indeed has potential because of safety and complementary mode of action, the individual components were further prepared for the evaluation of the combination therapy in dogs. In that context, a second aim was to characterize the effects of metronomic cyclophosphamide and temozolomide on regulatory T cells (Tregs) in a clinical trial with canine cancer patients (chapter 2). The major goal of this chapter was to investigate the potential value of this treatment for our immunotherapeutic combination approach. However, given the excellent applicability of metronomic chemotherapy as stand-alone therapy in veterinary medicine, some general considerations regarding the evaluation of this treatment modality outside the scope of immunotherapy are also given.

A third aim was the development of a universal tumor vaccine. In chapter 1 a tyrosinase vaccine was used, which is evidently only applicable in melanoma patients, not very common in veterinary medicine. To be able to evaluate the combination treatment in a sufficient number of patients, a tumor vaccine potentially effective in many tumor types was deemed necessary. For this reason, vascular-endothelial growth factor receptor-2 (VEGFR-2) was selected as antigen. Although different VEGFR-2 vaccines have been evaluated in the past, none of the formulations were feasible or available for evaluation in dogs. Therefore, the immunogenicity and safety of a new vaccine, consisting of a commercially available plasmid encoding human VEGFR-2, was tested in healthy mice

and laboratory dogs (chapter 3). Furthermore, the efficacy of this VEGFR-2 DNA vaccine was studied in two tumor mice models (chapter 4).

The fourth and final aim consisted of enhancing the applicability of gene therapy in oncology in general, and of IL-12 gene therapy specifically. The approach used in chapter 1 is limited to tumors that can be reached externally. Tumor specific delivery of IL-12 after systemic infusion of transfected cells that preferentially home to tumor tissue could circumvent this issue. In chapter 5, the first steps towards evaluation of this form of gene therapy are set. A murine cell line with the theoretical potential to migrate towards tumor tissue was tested for tumor specific migration *in vitro*. Furthermore, methods to transfect these cells with reporter plasmids were evaluated. Lastly, transfection with IL-12 plasmid DNA was performed to quantify IL-12 production capacity and to gather preliminary *in vitro* data on the capability of this approach to modulate anti-tumor immune response.

RESEARCH STUDIES

CHAPTER 1

Combination of interleukin-12 gene therapy, metronomic cyclophosphamide and DNA cancer vaccination directs all arms of the immune system towards tumor eradication

This chapter is based on

Denies S., Cicchelero L., Van Audenhove I., Sanders N.N. (2014). Combination of interleukin-12 gene therapy, metronomic cyclophosphamide and DNA cancer vaccination directs all arms of the immune system towards tumor eradication. J Control Release 10;187:175-82.

1. Abstract

In this work a combination therapy that acts upon the immune suppressive, the innate and specific arm of the immune system is proposed. This combination therapy, which consists out of intratumoral interleukin-12 (IL-12) gene therapy, human tyrosinase (hTyr) DNA vaccination and metronomic cyclophosphamide (CPX), was evaluated in a B16-F10 mouse model. Following groups were compared: (1) no treatment, (2) control vector, (3) intratumoral IL-12 gene therapy, (4) intratumoral IL-12 gene therapy + metronomic CPX, (5) intratumoral IL-12 gene therapy + metronomic CPX + hTyr DNA vaccination. Next to clinical efficacy and safety, we characterized acute effects of IL-12 and anti-tumor immune response after a second tumor challenge. All treatment groups showed increased survival and higher cure rates than control groups. Survival of non-cured mice was increased when metronomic CPX was combined with IL-12 gene therapy. Furthermore, mice that received metronomic CPX had significantly lower percentages of regulatory T cells (Tregs). Addition of the hTyr DNA vaccine increased cure rate and resulted in increased survival compared to other treatment groups. We also demonstrated that the manifest necrosis within days after IL-12 gene therapy is at least partly due to IL-12 mediated activation of natural killer (NK) cells. All cured mice were resistant to a second challenge. A humoral memory response against the tumor cells was observed in all groups that received IL-12 gene therapy, while a cellular memory response was only observed in the vaccinated mice. In conclusion, every component of this combination treatment contributed a unique immunologic trait with associated clinical benefits.

2. Introduction

Active immunotherapy has earned its place in oncology research today, mainly due to its unique capacity to induce long-term protection, in the shape of a memory immune response. However, a specific antitumor response takes time to develop. Consequently, a sore point of many active specific immunotherapeutic agents is that they lack an acute effect, which renders them ineffective in controlling a rapidly growing tumor on their own. Therefore, it is often combined with non-immunological treatments that control gross tumor growth.[170]

However, targeting the innate immune system can be a valuable alternative, as this arm of the immune system is able to react almost immediately. A further advantage is that the innate immune system can play a beneficial role in influencing the adaptive immune response towards protective antitumor immunity.[171] The combination of an adequate stimulation of both the innate and the adaptive immune response can therefore aid in the development of a robust long term antitumor immune response. This antitumor immune response is counteracted by immune-suppressive mechanisms, induced by the tumor thereby protecting itself against immune attack. A treatment to decrease these suppressive cells is indispensable for the success of any immunotherapeutic approach in the treatment against cancer.[172] Therefore, we believe that stimulation of the innate immune systems together with a cancer vaccination approach and an agent that tackles the immunosuppressive tumor environment may result in better responses.

Of the various ways to modulate the innate immune system we have chosen in this study interleukin-12 (IL-12) for the following reasons. IL-12 is a cytokine that very strongly stimulates the innate immune system by acting as a growth factor for natural killer (NK) cells, inducing cytokine production in these cells and enhancing their cytotoxicity.[173] NK cells can react very quickly upon stimulation, as they can exert their cytotoxicity without prior sensitization, antibody binding or protein presentation.[174] Ensuring rapid tumor control, IL-12 meets the first prerequisite of our innate immune arm of the therapy. Moreover, IL-12 favors cytotoxic T lymphocyte generation by inducing a Th1 response, functioning as a link between innate and adaptive immunity.[173] Lastly, the immune effects of IL-12 are complemented by its capacity to decrease the suppressive function of myeloid derived suppressor cells (MDSCs) and by its antiangiogenic activity.[175,176] Altogether, next to induction of acute effector functions, IL-12 therapy will create an ideal environment for the development of an effective specific antitumor immune response. This makes this cytokine exceptionally suitable to combine with specific immunotherapy. Although the therapeutic potential of IL-12 has been known for over a decade, its use has long been impeded by unacceptable toxicity when administered systemically.[133,177] However, current technologies for local gene delivery have renewed interest in this cytokine. We have selected plasmid injection followed by electroporation for this experiment based on the impressive results reported with this technique.[178-180]

Tumor vaccination can be achieved by tumor cell vaccines, dendritic cell (DC) vaccines, genetic vaccines, protein or peptide vaccines. Tumor cell vaccines have the key advantage of including a wide variety of patient specific antigens. DC vaccines circumvent the difficulty of obtaining adequate tumor antigen presentation *in vivo*. Despite these advantages, labor intensive production and problems with quality control impede large-scale application of cell based vaccines.[35] Genetic vaccination has the advantage to induce, next to a humoral immune response, a strong cellular immune response because the antigen is endogenously synthetized inside cells.[181] Therefore, genetic vaccination was chosen over protein or peptide vaccination. Plasmid injection is safer than viral gene therapy and delivery with electroporation has the advantage of providing an inherent adjuvant effect.[182] Hence, to obtain a specific antitumor response we delivered, via electroporation, a DNA vaccine encoding hTyr. A xenogeneic vaccination strategy was used as this can overcome immune tolerance to self-antigens, which comprise most tumor antigens.[183] Moreover, this vaccination protocol is already approved by the USDA for clinical use in dogs with melanoma and phase I human clinical trials have recently been completed, making a fluent transition to further evaluation of this treatment in the clinical setting possible.[70,184,185]

MDSCs and Tregs are induced by the tumor to obtain an immunologically permissive tumor microenvironment. MDSCs are a population of immature myeloid cells and are characterized by CD11b and Gr1 expression in mice. In humans, phenotypic markers are not yet conclusively established, although $CD11b^{+}CD33^{+}HLA\text{-}DR^{low}$ cells are commonly classified as MDSCs. Strategies targeting these cells include MDSCs deactivation, inhibition of MDSCs formation, MDSCs depletion and forced differentiation in mature cells.[24,186] IL-12 belongs to the latter category, resulting not only in a decrease in MDSCs, but also an increase in antigen presenting cells.[176] Regulatory T cells are $CD4^{+}CD25^{+}FoxP3^{+}$ cells. Low doses of chemotherapy, tyrosinase kinase inhibitors and monoclonal antibodies to Tregs receptors can decrease the number or lower the suppressive capacities of Tregs.[187] Metronomic cyclophosphamide (CPX), the most extensively studied method to suppress Tregs, is selected for this experiment, due to its excellent safety profile, ease of administration and low cost price. Low doses of CPX selectively kill Tregs because these cells have low levels of ATP, resulting in decreased glutathione synthesis, which is an important detoxification pathway for CPX.[147,188]

In summary, in this paper we investigated in a B16-F10 melanoma model whether metronomic CPX and DNA vaccination against hTyr could improve intratumoral IL-12 gene therapy. To answer these questions different treatment groups were included. To monitor the clinical efficacy of these treatments the tumor growth, the response rate and survival was determined. Additionally, the safety, the humoral and cellular immune responses as well as the role of NK cells in the acute anti-tumor effects of intratumoral IL-12 gene therapy were studied. Importantly, the immune response was measured after a second tumor challenge, reflecting a memory immune response and hence long term protection. The latter is very important as it distinguishes immunotherapy from all other oncology treatments.

3. Materials and Methods

Plasmids and tumor cells

The B16-F10 tumor cell line was generously provided by Johan Grooten (Department of Biomedical Molecular Biology, Ghent University, Belgium). Transduction with luciferase was performed as described earlier.[189] Briefly, retrovirus was produced in HEK293T cells by $CaPO_4$ transfection, harvested after 48 and 72h, filtered and concentrated by ultracentrifugation. Transduction was performed in the presence of 8 µg/ml polybrene and efficiency was evaluated by bioluminescence. These cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, 100 IU/ml penicillin and 1 mmol/ml L-glutamine (Invitrogen, Carlsbad, USA).

Murine IL-12 plasmid (pORF-mIL-12, indicated as IL-12 pDNA in this study) was purchased from Invivogen (Toulouse, France). A detailed description of the plasmid can be found on the website of the manufacturer.[190] Human tyrosinase plasmid was generously provided by Alan N. Houghton and Jedd D. Wolchok (Sloan-Kettering Institute for Cancer Research, NY, USA) and has been described elsewhere.[191] As control pDNA we used a plasmid encoding for green fluorescent protein. Purification of the plasmids was done with the EndoFree Giga kit, following the instructions of the manufacturer (Qiagen, Valencia, USA).

Study design

For tumor inoculation, luciferase positive B16-F10 cells were harvested by trypsinization, washed twice with calcium and magnesium free Dulbecco's phosphate buffered saline (PBS, Invitrogen) and 10^5 tumor cells in PBS were injected subcutaneously in 8 weeks old C57BL/6JRj mice (Janvier Breeding Center, Le Genest St. Isle, France). The treatment scheme (Fig. 1) was started when the smallest diameter of the tumor reached 3-5 mm, which was 10-14 days after inoculation. We marked the first day of treatment as day 0. The mice were randomly divided in 5 groups of 12 mice and the following treatments were compared: (1) no treatment, (2) intratumoral electro-gene transfer (IT-EGT) of control pDNA, (3) IT-EGT of IL-12 pDNA, (4) IT-EGT of IL-12 pDNA combined with low dose CPX (metronomic CPX), (5) IT-EGT of IL-12 pDNA combined with metronomic CPX and hTyr pDNA vaccination. IT-EGT of control pDNA and IL-12 pDNA was performed on day 0, day 4 and day 7. The IT-EGT protocol consisted out of an intratumoral injection of 50 µg of pDNA in 40 µl PBS with a hypodermic needle. This was followed by electroporation with the BTX ECM 830 device (Harvard apparatus, Holliston, USA) using plate electrodes (3 pulses of 75 V with a pulse duration of 40 ms and a 100 ms interval). Cyclophosphamide (Endoxan®; Baxter, Deerfield, USA) was administered via the drinking water from day 0 until day 28. Continuous administration of 20 mg/kg/d of CPX was based on an average intake of 3 mL of drinking water per day by 20 g mice. The drinking water with CPX was changed twice weekly.[192] On day 0 and day 14 hTyr pDNA (15 µg in 20 µl PBS) was injected intradermally and followed by electroporation with the BTX Agilepulse device (Harvard apparatus) using 4 mm gap needle electrodes (2 pulses of 450 V with a pulse duration of 0.05 ms and a 300 ms interval, followed by 8 pulses of 100 V with a pulse duration of 10 ms and a 300 ms interval).

To assess the concentration of IL-12 in tumor and serum samples, and the percentage of Tregs we sacrificed 3 mice in each group five days after the last IT-EGT of IL-12 or control pDNA (i.e. at day 12). The remaining mice were followed for tumor growth and survival and were euthanized when humane endpoints were reached. These humane endpoints included tumor size (> $1cm^3$), significant weight loss (>20%) or self-mutilation. Cured mice, i.e. mice that were tumor-free for 100 days, were subjected to a new subcutaneous tumor challenge. Ten days after this challenge, three mice per group were euthanized for assessment of

immune parameters. The other mice were monitored for over one year. This experiment was approved by the ethical committee of the Faculty of Veterinary Medicine (Ghent University; EC-DI 2012-072)

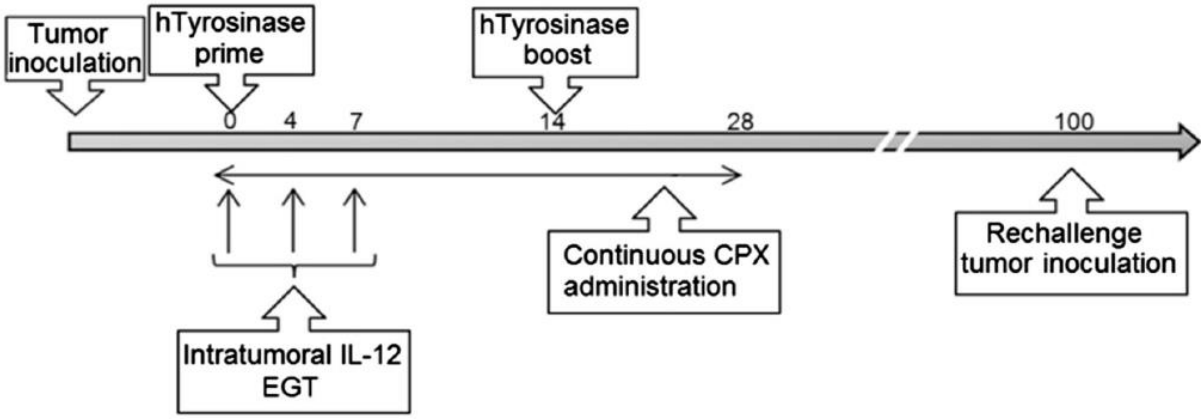

Fig. 1: Schematic representation of the experimental set-up: Treatment began when the smallest diameter of the tumor reached 3-5 mm, and consisted of three intratumoral IL-12 electro-gene treatments (EGT), two vaccinations with human tyrosinase plasmid (hTyrosinase) and metronomic cyclophosphamide (CPX). After 100 days, cured mice were rechallenged.

Determination of the concentration of IL-12 in tumor and serum

Serum and tumor tissue from 3 mice per group were collected at day 12. Tumor tissue was lysed by homogenization with Tissue Disruptor in Tissue Extraction Reagent I (Invitrogen) supplemented with protease inhibitors (Roche, Basel, Switzerland). The solution was centrifuged in a Universal 320R centrifuge (Hettich, Tuttlingen, Germany) at 10000 rpm during five minutes, after which supernatant was stored at -80 °C until analysis. Blood was taken by cardiac puncture under terminal anesthesia and after centrifugation serum was collected and stored at -80 °C until analysis. IL-12 concentration in tumor and serum was measured with an ELISA-kit (Biolegend, San Diego, USA) following the manufacturer's instructions.

Flow cytometric staining of Tregs

Splenocytes were isolated from 3 mice per group at day 12. One million cells in 100 µl FACS buffer (BD Biosciences, Erembodegem, Belgium) were stained for cell-surface antigens with FITC-conjugated anti-CD25 (Biolegend) and PerCp-conjugated anti-CD4 (Biolegend) antibodies. Fixation and permeabilization of the splenocytes preceded intracellular staining

with the PE-conjugated anti-Foxp3 antibody (FoxP3 staining kit, eBiosciences, San Diego, USA). The cells were analyzed with an Accuri C6 flow cytometer (BD Biosciences) and events in the lymphocyte gate were selected. Color compensation was done based on single staining, threshold for positive events were set based on fluorescence minus one (FMO) controls. The percentage Tregs was determined as the percentage of $CD4^+CD25^+Foxp3^+$ cells within the $CD4^+$ population.

Tumor growth, survival and safety

Tumor size was determined with a digital caliper. Mice were observed daily and weighed twice weekly to detect possible adverse effect of the treatment and recognition of humane endpoints. Survival analysis was performed using euthanasia when humane endpoints were reached as positive events.

Challenge

Mice that were tumor-free for 100 days were again challenged with $1x10^6$ B16-F10 cells subcutaneously. Injection of viable tumor cells was confirmed with *in vivo* bioluminescent imaging. Mice were injected intraperitoneally with 100 µl of D-luciferin (15 mg/ml, Goldbio Technology, St. Louis, USA) and *in vivo* imaging was performed after 10 minutes with an IVIS lumina II (Perkin-Elmer, Zaventem, Belgium). *In vivo* bioluminescent imaging was repeated every week to follow up tumor formation or tumor cell disappearance.

Intracellular cytokine staining

Ten days after rechallenge of the surviving mice, 3 mice were sacrificed and splenocytes were isolated and cultured in the presence of tyrosinase peptides. To assess both immunogenicity and cross-reactivity of the xenogeneic DNA vaccine, human as well as murine tyrosinase peptide mixes were used as stimuli (PepMix™ Mouse Tyrosinase and PepMix™ Human Tyrosinase; JPT, Berlin, Germany). After 2h of incubation, Brefeldin A (Biolegend) was added to block secretion of cytokines. After an additional 4h incubation, splenocytes were harvested, washed and resuspended in FACS buffer (BD Biosciences). Cell-surface staining with FITC-conjugated anti-CD8 antibody (Biolegend) was followed by fixation, permeabilization and intracellular staining with PE-conjugated anti-IL-4 (Biolegend) and APC-conjugated anti-interferon-gamma (IFN-gamma) (Biolegend) antibodies. The cells were analyzed with an Accuri C6 flow cytometer (BD Biosciences) and events in the

lymphocyte gate were selected, color compensation was done based on single staining, threshold for positive events was set based on FMO controls. Controls for specificity of the cytokine response were assessed by including non-stimulated splenocytes in the analysis.

Detection of antibodies

Serum was taken from 3 mice ten days after rechallenge of surviving mice. B16-F10 tumor cells were washed and resuspended in 50 µl of diluted mice serum (1/8 dilution in FACS buffer). Cells were incubated for one hour at 37 °C and 5% CO2. Subsequently the cells were washed and stained with APC-conjugated anti-mouse IgG antibodies (Biolegend) and the number of positive cells was analyzed via flow cytometry. Control for specificity of staining included B16-F10 cells that were not incubated with serum and stained with the secondary antibodies.

In vitro effect of IL-12 on tumor cell growth and tumor cell lysis by immune cells

B16-F10 tumor cells (10^6 cells in 80 µl PBS) were *in vitro* electroporated, using a BTX ECM 830 (Harvard apparatus), with 5 µg of IL-12 pDNA or control pDNA (2 pulses of 140 V, 5 ms length and 100 ms pulse interval). Subsequently, $5x10^4$ viable electroporated cells were seeded in a black 96 well cell culture plate. Growth of cells electroporated with IL-12 pDNA versus control pDNA was compared using a previously described *in vitro* luciferase assay.[193] Briefly, 10 µl of D-luciferin (15 mg/ml) was added to each well and after 10 minutes incubation (37 °C, 95% relative humidity, 5% CO2) bioluminescent imaging was performed using an IVIS lumina II (Perkin-Elmer). Measurements were performed after 0, 24 and 48 hours.

To evaluate the effect of IL-12 on tumor cell lysis by immune cells we co-incubated IL-12 electroporated B16-F10 cells and non-electroporated B16F-10 cells with purified NK cells and purified $CD8^+$ splenocytes, harvested from five C57BL/6JRj mice. The effector/target ratio in these mixtures was 15:1. Purification of immune subsets was achieved by magnetic beads separation (Dynabeads, Invitrogen). Tumor cell death of non-electroporated and IL-12 electroporated tumor cells by immune cells was determined by using respectively non-electroporated and IL-12 electroporated tumor cells without immune cells as reference. Tumor cell death was determined by an *in vitro* luciferase assay, as described above.

Statistical analysis

The statistical software package R was used. Because of the high cure rate observed in treatment groups and thus high proportion of censored cases, Kaplan Meier analysis of survival is not appropriate.[194] An alternative approach, based on the mixed cure model, was used. For each time point the overall probability of survival for different groups was calculated as the proportion of mice alive at that time point. These probabilities across all time points were summed to come to a cumulative probability of overall survival for each group. A relative overall survival ratio of this cumulative probability was calculated for pairwise comparisons between treatment groups. Significance of this finding was calculated by 10.000 random permutations on the observed data. Analogue calculations were performed for a relative cure ratio and a relative non-cured survival ratio. Differences in immunological parameters were analyzed with one-way ANOVA with Bonferroni correction for pairwise comparisons. Differences between *in vitro* tumor cell death after incubation with splenocytes from the same mouse with and without IL-12 were compared with a paired t-test.

4. Results

Clinical response and cure rate

Clinical response was followed in 9 mice in every group. Characterization of clinical response is subdivided by occurrence of complete responses and tumor growth in remaining tumor bearing mice (Fig. 2). We observed no complete responses in the control groups. In the IL-12 IT-EGT group and IL-12 IT-EGT + metronomic CPX group share a similar pattern of complete responses, i.e. rapid rise in number of complete responses, with no additional complete responses after 30 days (Fig. 2A). The fast response to IL-12 gene therapy is also illustrated in figure 2C. This leads to a cure rate for IL-12 IT-EGT of 67% and for IL-12 IT-EGT+ CPX of 55%. Interestingly, a different pattern is visible in the vaccinated group, where additional complete responses were observed far after 30 days, suggesting that this is due to action of the vaccine and not of IL-12. Cure rate for this group reached 89%. Mice that received no treatment or intratumoral electroporation of a control plasmid are characterized by fast tumor growth (Fig. 2B). This is translated in a short mean survival (22.7 ± 1.4 days and 22.3 ± 1.3 days respectively) for these control mice. If we look at differences in tumor

growth of non-cured mice between treatment groups, tumor growth is markedly slower for the IL-12 IT-EGT + CPX group compared to IL-12 IT-EGT alone. As a consequence, mean survival for the non-cured mice in this combination group is higher compared to IL-12 IT EGT alone (73.75 ± 7.67 days versus 50 ± 8 days). Only one mouse in the vaccinated group died, after 75 days.

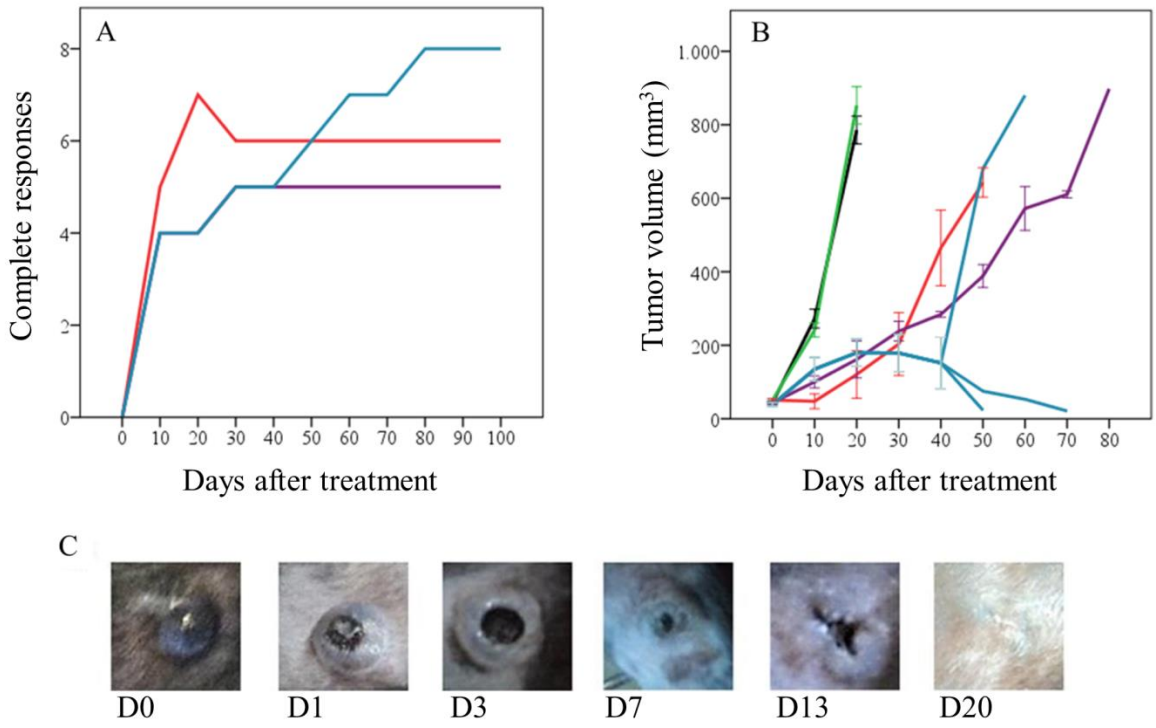

Fig. 2: Number of mice with complete response (panel A) and tumor growth curve of tumor bearing mice (panel B) in function of time after treatment. Representative photos showing short term tumor shrinkage after intratumoral IL-12 pDNA electro-gene transfer are shown in panel C. Mice that were cured were not included in the tumor growth curves. Error bars represent standard error of the mean. Because of the low number of tumor bearing mice after 40 days in the group receiving IT-EGT with IL-12 pDNA, metronomic cyclophosphamide and human tyrosinase vaccination, tumor volume is displayed individually. The number of mice in each group was 9. ▬ = no treatment; ▬ = intratumoral electro-gene transfer (IT-EGT) with control vector ; ▬ = IT-EGT with IL-12 pDNA; ▬ = IT-EGT with IL-12 pDNA and metronomic cyclophosphamide; ▬ = IT-EGT with IL-12 pDNA, metronomic cyclophosphamide and human tyrosinase vaccination.

Relative overall survival ratios between groups (group in row/group in column) are listed in table 1. Thus, values significantly higher than 1 represent a higher chance of survival for mice in the group listed in rows compared to the group listed in columns. All treated mice

have a significant 3 to 4 time higher chance of survival compared to control groups. Between treatment groups, we see no difference between IL-12 IT-ETG and IL-12 IT-EGT + CPX. However, the vaccinated mice have a moderate higher chance of survival compared to the other treatment groups, i.e. 17% compared to IL-12 IT-EGT and 10% compared to IL-12 IT-EGT+ CPX (relative overall survival ratio of 1.17 and 1.10 respectively).

Table 1: Relative ratios of overall survival, cure and non-cured survival between different treatment groups

	Control	Control pDNA	IL-12 pDNA	IL-12 pDNA + CPX
Relative overall survival ratio				
IL-12 pDNA	3.57***	3.46***	/	/
IL-12 pDNA + CPX	3.76***	3.64***	1.05	/
IL-12 pDNA+ CPX + hTyr pDNA	4.13***	4,00***	1.17*	1.10*
Relative cure ratio				
IL-12 pDNA	NA	NA	/	/
IL-12 pDNA + CPX	NA	NA	0.83	/
IL-12 pDNA+ CPX + hTyr pDNA	NA	NA	1.30	1.60*
Relative non-cured survival ratio				
IL-12 pDNA	2.22***	2.13***	/	/
IL-12 pDNA + CPX	3.13***	3,01***	1.41*	/
IL-12 pDNA+ CPX + hTyr pDNA	3.52***	3.38***	1.41	1

Control = no treatment; Control pDNA = intratumoral electro-gene transfer (IT-EGT) with control vector; IL-12 pDNA = IT-EGT with IL-12 pDNA; IL-12 pDNA + CPX = IT-EGT with IL-12 pDNA and metronomic cyclophosphamide; IL-12 pDNA + CPX + hTyr = IT-EGT with IL-12 pDNA, metronomic cyclophosphamide and human tyrosinase vaccination. (*** $p<0.001$, * $p<0.05$)

For the calculation of relative overall survival ratio both the chance of cure and the chance of survival if not cured are considered (table 1). If we separate these factors to gain insight in the contribution of treatment components we can draw following conclusions. Cyclophosphamide does not increase cure rate, but increases chances of survival for non-cured mice by 41% (relative non-cured survival ratio of 1.41). However, this is not sufficient to significantly enhance overall survival compared to IL-12 IT-EGT alone. When the vaccine is added to the treatment, a 60% increase in cure rate is observed (relative cure ratio of 1.6).

For vaccinated mice, the combined effect of increased cure rate and increased survival of non-cured mice sum up to the 17% higher chance of overall survival compared to IL-12 IT-EGT alone. Increased cure rate results in a 10% higher chance of overall survival compared to IL-12 IT-EGT + CPX.

Regulatory T cells and memory immune responses

On day 12, the percentage of Tregs was determined. There was no difference in percentage of Tregs between control mice (6.57 ± 1.48%) and mice treated with IL-12 IT-EGT (5.93 ± 0.75%). Mice that received metronomic CPX had significant reduced Tregs compared to mice that not received metronomic CPX (Fig. 3). In detail, the Treg percentages in mice that received IL-12 IT-EGT plus metronomic CPX or IL-12 IT-EGT plus metronomic CPX and hTyr vaccine were respectively 4.25 ± 0.47% (p=0.02) and 4.05 ± 0.13% (p=0.01). No differences in percentage of $CD4^+$ cells were observed (data not shown).

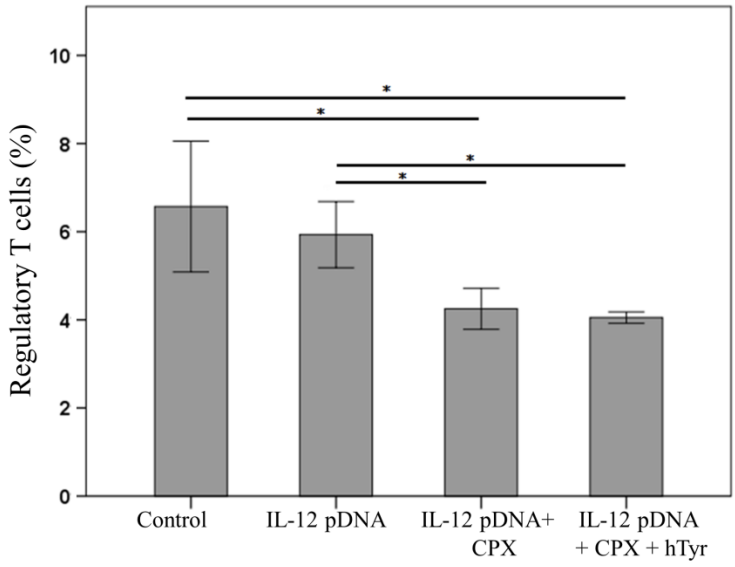

Fig. 3: Metronomic cyclophosphamide (CPX) significantly reduced the percentage of regulatory T cells. Twelve days after the start of the treatment the average percentage of regulatory T cells in the spleen of 3 mice per group was determined. Mice that received CPX had a significant reduction in the percentage of regulatory T cells compared to the control group and IL-12 gene therapy group. IL-12 gene therapy alone was not able to reduce Tregs. Control = no treatment; IL-12 pDNA = intratumoral electro-gene transfer (IT-EGT) with IL-12 pDNA; IL-12 pDNA + CPX = IT-EGT with IL-12 pDNA and metronomic cyclophosphamide; IL-12 pDNA + CPX + hTyr = IT-EGT with IL-12 pDNA, metronomic cyclophosphamide and human tyrosinase vaccination. Error bars represent standard deviation. (* $p<0.05$)

Ten days after rechallenge, the percentage of IFN-gamma positive lymphocytes after incubation with murine tyrosinase peptides was determined. There was no difference in IFN-gamma positive lymphocytes between the control groups (no treatment group: 0.03 ± 0.03%; control pDNA group: 0.03 ± 0.03%), the IL-12 IT-EGT group (0.05 ± 0.04%), and the IL-12 IT-EGT plus metronomic CPX group (0.05 ± 0.02%). However, mice that received the hTyr pDNA vaccine had a statistically significant higher percentage of IFN-gamma positive lymphocytes (0.17 ± 0.04%) compared to the control groups ($p<0.001$) and the other IL-12 IT-ETG groups ($p<0.001$). Similar percentages of IFN-gamma positive lymphocytes were observed after incubation of the splenocytes of hTyr vaccinated mice with human (0.16 ± 0.05%) or murine tyrosinase peptides (0.17 ± 0.04%).

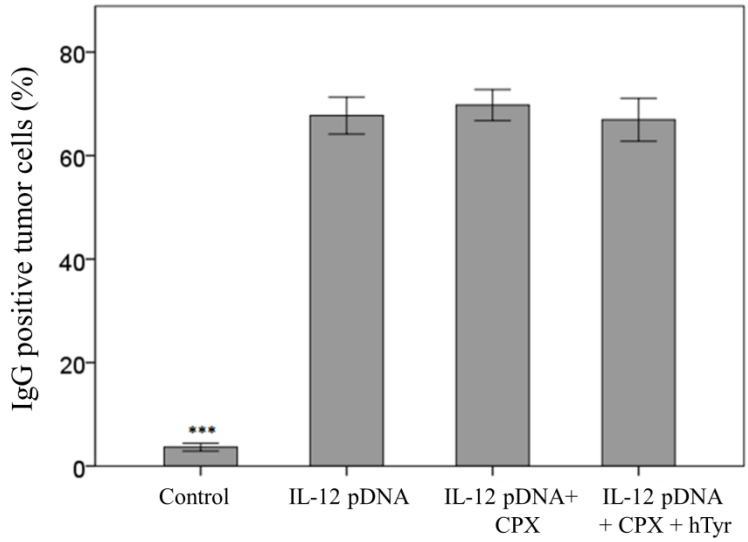

Fig. 4: A humoral memory response is established after intratumoral electro-gene transfer of IL-12 pDNA. To determine the presence of antibodies against the tumor cells we incubated diluted sera of each mouse (3 mice per group) with a fixed amount of B16-F0 melanoma cells and determined via flow cytometry the percentage of tumor cells that became bound with IgG antibodies present in the mice's serum. Control = no treatment; IL-12 pDNA = intratumoral electro-gene transfer (IT-EGT) with IL-12 pDNA; IL-12 pDNA + CPX = IT-EGT with IL-12 pDNA and metronomic cyclophosphamide; IL-12 pDNA + CPX + hTyr = IT-EGT with IL-12 pDNA, metronomic cyclophosphamide and human tyrosinase vaccination. Error bars represent standard deviation. (*** $p<0.001$ compared to all other groups)

Additionally, also a similar percentage of IFN-gamma producing T cells was present in the CD8 positive (0.10 ± 0.05%) and CD8 negative lymphocyte population (0.07 ± 0.06%) of mice that received the hTyr DNA vaccine. There were no differences in IL-4 response

between the different groups (data not shown). Ten days after the rechallenge also serum samples were taken to determine the presence of antibodies against the tumor cells. All treatment groups had, compared to the control groups, significantly higher levels of IgG antibody directed against B16-F10 cells ($p<0.001$). However, between the treatment groups, no significant differences could be detected (Fig. 4).

Adverse effects and IL-12 concentration in tumor and serum

No weight loss was observed during the study period in any group. We noticed that one mouse in the IL-12 IT-EGT plus metronomic CPX group and one mouse in the group that received IL-12 IT-EGT combined with metronomic CPX and hTyr pDNA vaccination showed lethargy and decreased interaction with the investigator. This occurred during the week of IL-12 treatments, and resolved spontaneously within two days. All mice in the vaccinated group exhibited alopecia in the weeks following second tumor challenge. Hair loss started in the area surrounding tumor inoculation and progressed in some mice, ranging from focal alopecia to almost generalized flank alopecia. This alopecia remained stable for over 12 months. None of the mice in the other treatment groups exhibited alopecia.

The concentration of IL-12 in tumor lysate was significantly higher in the tumors electroporated with IL-12 pDNA than in the control groups (5.0 ± 1.2 pg/mg versus 0.07 ± 0.05 pg/mg, $p=0.04$). The IL-12 concentration in serum was not detectable in most mice, except in two mice after IL-12 pDNA therapy, where it reached 35 pg/ml. These mice did not exhibit any signs of toxicity. Overall, there was no significant difference between IL-12 pDNA treated mice and control mice in IL-12 serum concentration.

Challenge of cured mice with a second injection of tumor cells

All cured mice in the different treatment groups (6 in the IL-12 IT-EGT group, 5 in the IL-12 IT-EGT plus metronomic CPX group, and 8 in the group that received IL-12 IT-EGT combined with metronomic CPX and hTyr pDNA vaccination) were rechallenged with tumor cells. Injection of viable tumor cells was confirmed via *in vivo* bioluminescent imaging. No macroscopic tumor developed, but a bioluminescent signal was visible for two to three weeks, after which no signal could be detected, with no difference between treatment groups. One year after the rechallenge all the mice were still alive and tumor free.

The origin of the acute effects after intratumoral IL-12 electro-gene transfer

Intratumoral EGT with IL-12 pDNA caused tumor necrosis within 24h after the first treatment (Fig. 2). Because B16-F10 cells express the IL-12 receptor, a direct influence of IL-12 on tumor growth *in vitro* was investigated. However, no difference in tumor growth between tumor cells electroporated with IL-12 pDNA or control pDNA was observed (data not shown). Another possible explanation for this rapid action of IL-12 *in vivo* is a stimulation of NK cells that subsequently attack tumor cells. There are conflicting *in vivo* studies in the literature concerning the role of NK cells in the antineoplastic action of IL-12 [195,196]. To check if NK cells are responsible for the rapid action of IL-12 we performed an *in vitro* experiment with co-incubation of immune cells and tumor cells electroporated with IL-12 pDNA.

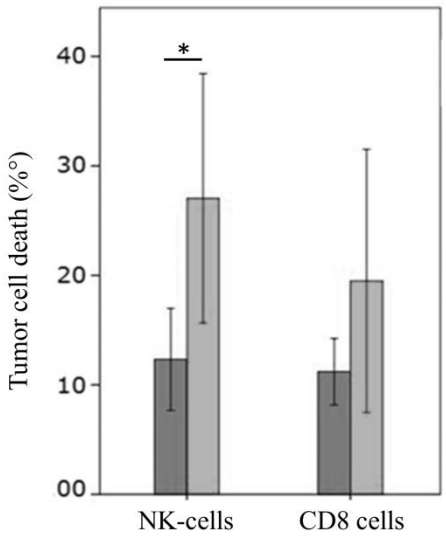

Fig. 5: NK cell mediated tumor cell killing plays an important role in the acute effects of IL-12. Cytotoxic effects of purified NK cells and $CD8^+$ T cells on non-transfected (dark grey) and IL-12 pDNA transfected tumor cells (light gray) are illustrated. Tumor cell death was measured 24h after co-incubation of the immune cells with the tumor cells (n=5). Error bars represent standard deviation. (* $p<0.05$)

When immune cells were co-incubated with IL-12 transfected and non-transfected tumor cells, IL-12 significantly enhanced tumor cell cytotoxicity of purified NK cells (p=0.02). This was evident already after 24h and remained significant after 48h (data not shown). In contrast, IL-12 did not increase the cytotoxicity of $CD8^+$ cells as co-incubation of IL-12-transfected or non-transfected tumor cells with purified $CD8^+$ cells did not result in

significant differences in cytotoxic effects on the tumor cells (Fig. 5). This suggests that in the acute phase, NK cells and not $CD8^+$ cells are the dominant immune cells responding to IL-12.

5. Discussion

In this study we investigated whether metronomic CPX and DNA cancer vaccination could improve intratumoral IL-12 gene therapy. Interleukin-12 is the only agent capable of inducing complete tumor regression on its own.[197-199] Therefore our principle research question was if metronomic chemotherapy and tumor vaccination can enhance the anti-tumor activity of IL-12 gene therapy and not vice versa. Overall survival was significantly enhanced in the vaccinated group compared to IL-12 IT-EGT with 17% and with 10% compared to IL-12 IT-EGT + CPX, due to a 60% increase in cure rate. When compared to IL-12 IT-EGT alone, metronomic CPX significantly enhanced survival of non-cured mice with 40%, but did not increase cure rate. The effect of metronomic CPX alone was insufficient to significantly increase overall survival.

Immune effects during or shortly after IL-12 gene therapy have already been documented, with conflicting results whether immune modulation is important for the clinical effect, and if it are NK cells or $CD8^+$ cells that mainly react in response to IL-12 gene therapy.[195,196,200] In our experiment, mice that were intratumorally electroporated with IL-12 pDNA exhibited local necrosis within one day after treatment, suggesting an important role for the innate immune system. We confirmed this hypothesis in an *in vitro* model, where NK cells were identified as the main effectors of IL-12 induced cytotoxicity. Because B16-F10 cells express the IL-12 receptor, a direct effect of IL-12 on tumor cell growth was investigated.[201] No such direct effects were observed. In this work we have not investigated whether the anti-angiogenic effects of IL-12 were responsible for the rapid onset of tumor necrosis. Although it has been indisputably demonstrated that anti-angiogenic mechanisms contribute significantly to the antineoplastic potency of IL-12, changes in neovascularization only start to occur three days after IL-12 gene therapy.[175] In our study manifest necrosis was observed before this time. Therefore, in agreement with Kim *et al*., we believe that the rapid tumor necrosis after IL-12 gene therapy is mainly due to NK cell stimulation.[200] The electroporation itself is not a causal factor, since necrosis was not observed in the control group subjected to

electroporation of a mock plasmid. Similar to other studies, IL-12 gene therapy had no effect on the percentage of Tregs.[202] Depletion of Tregs only occurred in groups receiving metronomic CPX.

All mice that were cured after IL-12 IT-EGT were resistant to a second tumor challenge. B cell memory was present in mice that received intratumoral IL-12 pDNA. Also Arulanandam *et al*. reported that IL-12 is able to stimulate the induction of B cell memory against viral disease.[203] The massive exposure of tumor antigens after IL-12 mediated tumor necrosis probably assisted the development of a humoral memory immune response against the tumor cells. Previous reports on IL-12 gene therapy for cancer mainly focus on the effect of IL-12 on the acute cellular immune response. Only in the last years the humoral immune response gained interest in cancer immunotherapy, mostly by the success of monoclonal antibodies.[204] Although the anti-tumor effects of endogenously evoked antibodies are not yet sufficiently characterized, there are some hints that they indeed may not be overlooked in cancer immunotherapy. For instance, in tumor vaccination studies humoral immune responses are frequently correlated with clinical efficacy and immunoglobulin kappa chain levels in tumors have proven to be an important prognostic factor in different human cancers.[205,206] Moreover, it has been demonstrated that memory B cells isolated from melanoma patients produce antibodies that are able to kill tumor cells *in vitro*, at least partly by antibody dependent cell-mediated cytotoxicity.[207] More research is needed to determine if a memory B cell immune response is sufficient to protect against a second tumor challenge. Non-vaccinated mice that received intratumoral IL-12 gene therapy did not show a memory cellular immune response. It has been demonstrated that high IL-12 exposure strongly reduces both the quality and quantity of the memory T cell response.[208-210] IL-12 stimulates the cellular immune response very strongly, however, doing so it promotes the differentiation into effector T cells at the expense of the formation of memory T cells. Interestingly, after the combination treatment with IL-12 gene therapy, metronomic CPX and DNA cancer vaccination, a robust cellular and humoral memory immune response was present. The alopecia only seen in vaccinated mice can be explained by this cellular immune response, since alopecia after tyrosinase immunotherapy is mediated by $CD4^+$ and $CD8^+$ lymphocytes.[211,212] Thus, although IL-12 gene therapy is not able to induce a cellular memory immune response as monotherapy, it does not impede its development in combination with tumor vaccination. A possible

explanation for this is that mice were not under influence of IL-12 during boost vaccination. The memory B cell immunity induced by IL-12 gene therapy may explain the resistance of the non-vaccinated mice against a second tumor injection. However, another explanation for resistance against tumor challenge in this experiment is the existence of a NK cell memory. IL-12 has proven to be indispensable for the generation of a NK cell memory, which is independent of B or T cells.[213,214] Additionally, an antibody-dependent NK cell memory has recently been discovered.[215] This is a very attractive explanation for resistance against tumor challenge in this experiment as IL-12 promotes both NK function and a humoral memory immune response.

In the past intratumoral electroporation was only possible for tumors that were reachable from the outside. However, recent developments in electrode design have made it possible to electroporate internal tumors, with already early clinical experience with tumors in the liver, bone, brain and intestines.[216] Another possibility to broaden the applicability of IL-12 gene therapy towards distant tumors is the use of tumor targeting strategies. For example, the use of cells that home to tumors as tumor targeting vehicles for IL-12 is an interesting approach to deliver IL-12 to internal tumors.[217] Additionally, pDNAs that produce IL-12 equipped with a tumor targeting ligand have also been evaluated.[218] To conclude, with the rapid advancement in electroporation and targeted delivery methods, we believe that intratumoral IL-12 (gene) therapy will be applicable in the near future for a broad range of tumor types.

6. Conclusion

In conclusion, every component of this combination treatment contributes a unique immunologic trait: stimulation of the innate immune system via IL-12 gene therapy, depletion of the suppressive Tregs through metronomic CPX, and specific stimulation of the cellular memory immune response via DNA cancer vaccine against tyrosinase. Rapid tumor shrinkage was observed in most mice after intratumoral IL-12 gene therapy. We demonstrated that this was most likely due to stimulation of NK cells. When metronomic CPX was combined with IL-12 gene therapy, survival in non-cured mice was enhanced and addition of the vaccine increased cure rate. This led to a significant increased overall survival

in vaccinated mice compared to the other treatment groups. A memory B cell response was detected in surviving mice that received IL-12 gene therapy alone or in combination with metronomic CPX, while both a memory B and T cell response was only observed in the DNA vaccinated mice. After these encouraging results in mice, we hope to test such combination treatment in spontaneously arising tumors in dogs as a further step towards clinical validation.

7. Acknowledgements

We want to thank dr. Alan N. Houghton and dr. Jedd D. Wolchok (Sloan-Kettering Institute for Cancer Research, NY, USA) for providing the pDNA encoding human tyrosinase. The financial support of Ghent University (BOF) is acknowledged with gratitude.

CHAPTER 2

Immunological and angiogenic markers during metronomic temozolomide and cyclophosphamide in canine cancer patients

This chapter is based on:

S. Denies, L. Cicchelero, H. de Rooster, S. Daminet, I. Polis, I. Van de Maele, N.N. Sanders. Immunological and angiogenic markers during metronomic temozolomide and cyclophosphamide in canine cancer patients.Accepted for publication in Veterinary and Comparative oncology

1. Abstract

Metronomic chemotherapy stimulates the immune response via depletion of regulatory T cells (Tregs) and suppresses angiogenesis by modulating the secretion of thrombospondin-1 (TSP-1) and vascular endothelial growth factor (VEGF). In this study blood was collected from 10 healthy dogs and from 30 canine cancer patients before and two and four weeks after treatment with metronomic temozolomide (6.6 mg/m^2), cyclophosphamide (12.5 mg/m^2), or the combination of cyclophosphamide and temozolomide. The percentage of circulating $CD25^+Foxp3^+CD4^+$ Tregs and the plasma levels of TSP-1 and VEGF were measured. There was a significant difference in the percentage of Tregs between cancer patients and healthy dogs. A significant decrease in Tregs was noted in patients treated with metronomic cyclophosphamide and the combination. Treatment with temozolomide had no effect on the percentage of Tregs. TSP-1 and VEGF levels were respectively significantly lower and higher in cancer patients than in healthy dogs, but they were not influenced by any of the studied metronomic treatment regimens.

2. Introduction

Metronomic chemotherapy is the frequent and continued administration of low doses of chemotherapeutics. Instead of targeting tumor cells directly, metronomic chemotherapy works by slowing the growth of new blood vessels and by selectively eliminating immunosuppressive immune cells.[219] Regulatory T cells are an important class of immunosuppressive immune cells that play a major role in the suppression of an effective anti-tumor immune response in cancer patients. They suppress cytotoxic T cells as well as natural killer cell mediated cytotoxicity and also have an effect on B cells, $CD4^+$ T cells and dendritic cells.[23] Tregs can be depleted by metronomic cyclophosphamide or temozolomide.[220,221] The selective effect of metronomic cyclophosphamide towards Tregs is explained by reduced intracellular ATP levels in these cells, leading to decreased glutathione synthesis and thus reduced detoxification of cyclophosphamide.[147] Also for temozolomide, selective toxicity to Tregs is observed, but the mechanism is not yet elucidated.[221-223]

In human patients suffering from various cancers, intratumoral and circulating Tregs are elevated and this is often correlated with a poor prognosis.[224-227] In some studies depletion of Tregs enhanced tumor-specific immune responses and increased the survival of humans afflicted with cancer.[220,228] In canine cancer patients, higher percentages of Tregs compared to healthy control dogs and a decrease after treatment with metronomic cyclophosphamide have also been reported.[156,229-233] Tregs are best characterized with the surface antigens CD25 and CD4 and the intracellular antigen FoxP3.[234] However, in canine cancer patients Tregs have been monitored using only CD4 and FoxP3 as Tregs markers, because a commercially anti-canine CD25 antibody was not available in the past. This is no longer an impediment and it is proven that also in dogs CD25 is an important marker of a regulatory phenotype.[235-237]

The anti-angiogenic action of metronomic chemotherapy is mainly mediated by the upregulation of the anti-angiogenic protein thrombospondin-1 (TSP-1), as the effect of metronomic chemotherapy is abrogated in TSP-1 deficient mice.[197,238,239] Moreover, it has been proven, *in vitro* and in mouse models, that different metronomic chemotherapy regimens induce TSP-1 production in tumor cells, endothelial cells and stromal cells.[197,239,240] Additionally, increased serum TSP-1 concentrations are found in human patients treated with metronomic chemotherapy.[241-244] TSP-1 binds to the CD36 receptor, which is only present in specific cell types, of which activated endothelial cells are the most important. Binding of TSP-1 to the CD36 receptor on endothelial cells blocks their proliferation and induces apoptosis.[197,239] TSP-1 also binds and sequesters VEGF, thereby also exerting an indirect anti-angiogenic effect.[245] It has been demonstrated that metronomic chemotherapy also directly decreases VEGF secretion in tumor cells and decreased VEGF serum levels are in human patients associated with clinical response to metronomic chemotherapy.[243,246]

The last decade cancer vaccination has gathered much attention in veterinary oncology.[247] However, tumor-induced immunosuppression is a major bottle neck for cancer vaccines, with Tregs playing an important role in this.[151] Therefore, it might be interesting to combine metronomic chemotherapy with cancer vaccination. One study has investigated the effect of metronomic cyclophosphamide on Tregs in dogs at two doses. A dose of 15 mg/m^2 decreased the percentage of Tregs in cancer patients but the lower dose of 12.5 mg/m^2 gave inconclusive results, mainly because of the small sample size of 5 dogs in the latter group.[156] It has been reported that metronomic cyclophosphamide can cause sterile hemorrhagic

cystitis in dogs, which provides a rationale to use the lower dose if this could also result in a decrease in percentage of Tregs.[152,153] Therefore, we reevaluated the effect of cyclophosphamide at 12.5 mg/m^2 on Tregs. For the same reason, the evaluation of other low-dosed chemotherapeutics that have similar effects as low-dose cyclophosphamide is interesting. It has been demonstrated in rodents and humans that metronomic temozolomide can decrease Tregs.[221-223] An important advantage of metronomic temozolomide compared to other Treg depleting chemotherapeutics is that, like cyclophosphamide, it can be given orally, which facilitates its use in canine patients. In this study we evaluated for the first time whether metronomic temozolomide has comparable effects on Tregs in canine patients as metronomic cyclophosphamide. Lastly, also the combination of the two drugs was evaluated, as combinatory treatments can result in synergistic biological effects, requiring lower doses of each drug minimizing the risk of adverse effects. Besides the effect of metronomic temozolomide and cyclophosphamide on Tregs we also measured whether they affect the plasma levels of the angiogenic marker TSP-1 and VEGF in canine cancer patients.

3. Materials and methods

Study population

Ten healthy control dogs and 30 dogs with cancer were included in this study. Control dogs were determined to be healthy by conducting a thorough anamnesis and by physical examination. For dogs with cancer, all patients with a newly diagnosed malignant tumor, for which standard treatment options were not available or declined by owners, were eligible. Patients were diagnosed at the Faculty of Veterinary Medicine of Ghent University. This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University, the Belgian national Deontological Committee and the Federal Agency for Medicines and Health Products.

Treatment of cancer patients

Dogs were sequentially allocated to one of the following treatment schedules; cyclophosphamide 12.5 mg/m² daily (Endoxan, Baxter, Lessines, Belgium), temozolomide 6.6 mg/m² daily (Temodal, Merck Sharpe & Dohme, Hertfordshire, UK) or cyclophosphamide 12.5 mg/m² plus temozolomide 6.6 mg/m² daily. The dosage of

cyclophosphamide was based on a previous study with metronomic cyclophosphamide in dogs.[156] For temozolomide, it was demonstrated that 1/15 of the cytotoxic dose had a selective effect on Tregs in rodents.[222] No phase I studies with temozolomide are performed in dogs but a study reports 100 mg/m² given in a cytotoxic regimen that demonstrated clinical efficacy.[248] Therefore the metronomic dosage was calculated to be 6.6 mg/m²/day. Reformulation of the drugs was done in collaboration with the Faculty of Pharmaceutical Sciences of Ghent University. Concomitant non-steroidal anti-inflammatory drugs (NSAIDs) were permitted when necessary for pain relief, no other antineoplastic agents were given during the study. As all dogs were in advanced stages of disease and treatment was given in a palliative setting, treatment was continued for as long as the patient lived. As this study was designed to investigate immunological and angiogenic biomarkers during metronomic chemotherapy and not clinical efficacy, no standard follow up of tumor progression was included.

Determination of Tregs

Blood was collected from control dogs and from patients in EDTA tubes one day before treatment and 2 and 4 weeks after the start of metronomic chemotherapy. Blood samples were analyzed the same day. In detail, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation with Ficoll-Paque 1.007 g/ml (GE healthcare Europe, Diegem, Belgium). Subsequently, one million PBMC in a volume of 100 µl phosphate buffered saline were stained with APC-conjugated anti-dog CD4 and FITC-conjugated anti-dog CD25 antibodies (eBioscience, USA, San Diego). Surface staining was done at 4 °C for 30 minutes. Intracellular staining for FoxP3 was done as described previously, with a cross-reactive murine PE-conjugated anti-FoxP3 antibody using the fixation/permeabilization and permeabilization buffer provided by the company.[230] Briefly, cells were washed to remove unbound surface antibodies and subsequently resuspended in fixation/permeabilization buffer and incubated overnight at 4 °C. The next day, cells were washed twice with permeabilization buffer and incubated with the FoxP3 antibody at a concentration of 1 µg per $1x10^6$ cells for 30 minutes at 4 °C. Fluorescence minus one tubes were analyzed as controls to determine thresholds and single staining tubes to perform color compensation. Flow cytometric analysis was done with a BD Accuri C6 flow cytometer and Cflow software (BD, Erembodegem, Belgium). Analysis was performed on the permeabilized lymphocyte

population based on forward and side scatter characteristics. The percentage of Tregs was calculated as $CD25^+FoxP3^+CD4^+$ cells in the overall $CD4^+$ T cell population.

Determination of angiogenic serum markers

Blood was collected from control dogs and from patients one day before treatment and 2 and 4 weeks after initiation of metronomic chemotherapy. After centrifugation with Ficoll-Paque 1.007 mg/ml the plasma fraction was collected and stored at -20 ° C. The concentration of angiogenic markers in these plasma fractions was measured with commercial canine VEGF and TSP-1 ELISA kits following manufacturer's instructions (R&D systems, Minneapolis, USA; Biotang, Lexington, USA).

Statistical analyses

Statistical analysis was done with SPSS software (IBM, Belgium, Brussels). Correlations were calculated with the Pearson correlation coefficient. Differences in baseline values between control dogs and dogs with cancer for Tregs and the angiogenic markers were compared with the independent student t-test and the Mann-Whitney U test respectively. Differences in baseline values between mesenchymal versus epithelial tumors were also investigated, as well as between dogs with and without metastatic disease. Changes in percentage Tregs and angiogenic markers during treatment were analyzed with a repeated measures ANOVA. Flow cytometric data are reported as mean ± standard deviation (SD), and concentration of VEGF and TSP-1 as median and range (minimum-maximum). Significance level was set at alpha = 0.05.

4. Results

Population characteristics

Thirty dogs with cancer were enrolled between 2012 and 2014 at the small animal clinic of the Faculty of Veterinary Medicine of Ghent University. The patient population consisted of 10% mongrels and 90% purebred dogs. The mean age and standard deviation of the dogs was 8.8 ± 2.6 years. Tumor types are listed in table 1. Nineteen dogs (63%) had confirmed metastases, of which 3 dogs underwent complete resection of the primary tumor before start of treatment (2 hemangiosarcoma patients and 1 renal carcinoma patient). Two patients (1

hemangiosarcoma patient and 1 renal carcinoma patient) underwent incomplete resection of the primary tumor without gross metastases present at the time of surgery. Ten healthy aged-matched control dogs (7.6 ± 3.4 years) were also included.

Table 1: Patient population characteristics

	CPX	TMZ	CPX+TMZ
Breed			
Purebred	8	8	10
Mongrel	2	2	0
Sex			
Male	5	7	7
Female	5	3	3
Age (years)	9.0±2.2	8.5±3.1	8.7±2.3
Tumor types			
Heart base tumor	1	1	
Round cell tumor			1
Liver carcinoma		1	1
Soft tissue sarcoma	1	2	1
Histiocytic sarcoma	1	1	
Hemangiosarcoma	2		2
Osteosarcoma		1	
Malignant pilomatricoma	1		
Unknown	1		
Mammary carcinoma		1	1
Nasal adenocarcinoma		1	1
Mesothelioma	1		
Renal carcinoma	1		1
Sinus carcinoma	1		1
Lymphoma			1
Tonsillar carcinoma		1	
Thyroid carcinoma		1	
Metastases			
Present	6	5	8
Absent	4	5	2

TMZ=temozolomide, CPX=cyclophosphamide

Levels of regulatory T cells, VEGF and TSP-1 differ between healthy dogs and dogs with cancer

Regulatory T cells were quantified as the percentage of CD25 and FoxP3 positive T cells in CD4 positive PBMC obtained from healthy dogs and dogs with cancer. In dogs with cancer a

significant higher percentage of Tregs was present than in healthy dogs (4.2 ± 2.1% versus 2.5 ± 0.7%, p<0.001). Although the mean Tregs percentages are significantly different between the two groups, it is clear that not all cancer patients had elevated percentages of Tregs (Fig. 1). There is no association between the percentage of Tregs and the tumor type (epithelial/mesenchymal), the presence of metastases or the use of NSAIDS (data not shown).

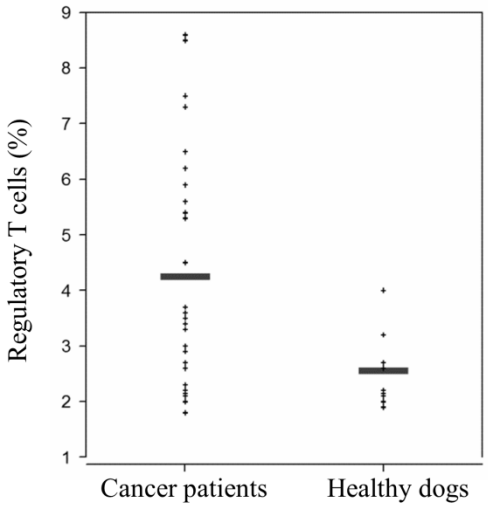

Fig. 1: The percentages of regulatory T cells in PBMC obtained from dogs with cancer patients (n=30) and healthy dogs (n=10). Pluses represent individual dogs, lines represent group mean.

Besides the percentage of Tregs also the plasma concentrations of two angiogenic factors, i.e. VEGF and TSP-1, were compared between dogs with cancer and healthy dogs. In dogs with cancer the concentration of VEGF was significantly higher than in healthy dogs (Fig. 2; 77 pg/ml (range: 56-466) versus 61 pg/ml (range: 31-84), p=0.03). TSP-1 concentration was significantly lower in cancer patients compared to healthy dogs (Fig. 2, 84 pg/ml (range: 26-387) versus 475 pg/ml (range: 284-1945), p=0.001). Patients with metastases had significantly higher VEGF concentrations than patients without metastases (82 pg/ml (range: 65-466) versus 71 pg/ml (range: 56-80), p=0.002), whereas for TSP-1 there was no significant difference. The concentration of both angiogenic markers did not differ between patients with epithelial versus mesenchymal tumors and was not associated with the use of NSAIDs.

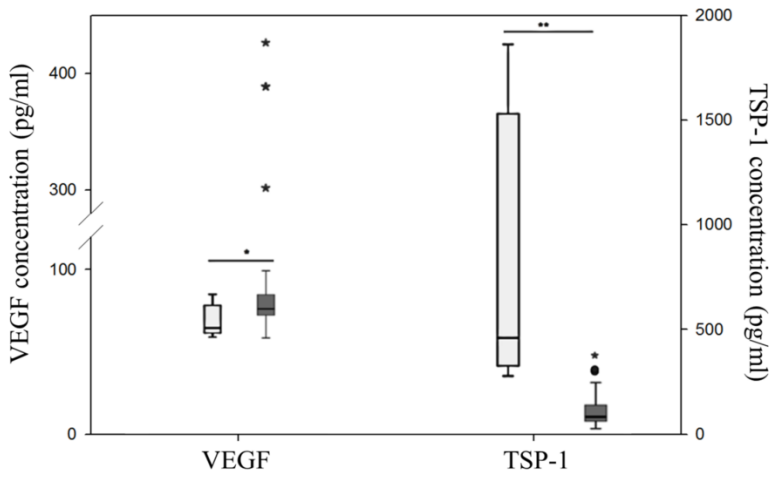

Fig. 2: Boxplots of plasma VEGF and TSP-1 concentrations in healthy dogs (n=10, light gray) and dogs with cancer (n=30, dark gray). VEGF=vascular endothelial growth factor; TSP-1=thrombospondin-1. (*p<0.05, **p<0.01)

Metronomic cyclophosphamide but not temozolomide decreases regulatory T cells, neither influence angiogenic markers

A significant decrease in $CD25^{+}Foxp3^{+}CD4^{+}$ Tregs was noted in patients treated for two weeks with metronomic cyclophosphamide (5.2 ± 2.2% versus 3.8 ± 1.7%, p=0.02) and metronomic cyclophosphamide plus temozolomide (3.7 ± 2.0% versus 2.8 ± 1.0%, p=0.03) (Fig. 3). After four weeks of treatment the percentages of Tregs did not decrease further throughout the treatment period, resulting in a similar Treg percentage after four weeks compared to two weeks. Treatment with metronomic temozolomide had no effect on the percentage of Tregs during the study period (p=0.3). None of the treatment protocols had an effect on the percentages of $CD4^{+}$ cells, demonstrating selective toxicity for Tregs. The concentration of VEGF and TSP-1 did not change during any treatment protocol (data not shown).

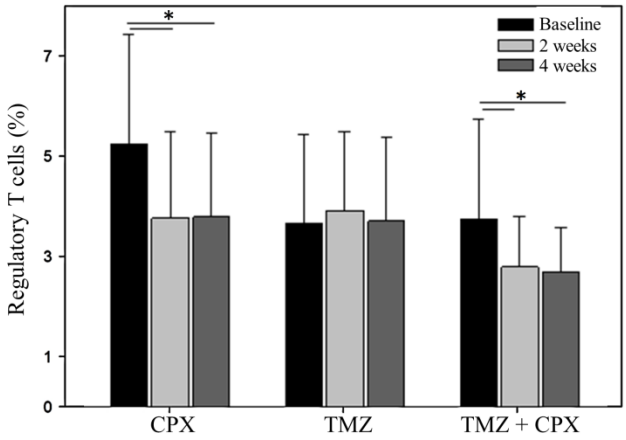

Fig. 3: Effect of the different metronomic chemotherapy protocols on the percentage of regulatory T cells (Tregs) in dogs with cancer. The Tregs were measured one day before the start of the metronomic chemotherapy (baseline) and respectively 2 and 4 weeks after metronomic chemotherapy. Data are represented as means and error bars represent standard errors. CPX=dogs that received metronomic cyclophosphamide (n=10); TMZ=dogs that received metronomic temozolomide (n=10); CPX+TMZ=dogs that received simultaneously metronomic cyclophosphamide and temozolomide (n =10). (*p<0.05)

Evaluation of CD25 expression in CD4$^+$FoxP3$^+$ cells

Because an anti-canine CD25 antibody was not available in the past, previous studies in dogs have characterized Tregs solely based on CD4 and FoxP3 expression.[156,229-233,249] To relate our findings to these studies, the relationship between CD4$^+$FoxP3$^+$ cells and CD25 expression was investigated. True Tregs (defined as CD25$^+$FoxP3$^+$CD4$^+$cells) were on average only 31% of FoxP3$^+$CD4$^+$cells (Fig. 4A). However, if the proportion of CD25 negative FoxP3$^+$ CD4$^+$ cells is relatively stable compared to the CD25$^+$ fraction, changes in FoxP3$^+$ CD4$^+$ cells can still primarily reflect changes in the CD25$^+$ fraction. To investigate this, the correlation between measurements with and without the CD25 marker was calculated. The correlation coefficient between the percentage of FoxP3$^+$CD4$^+$ cells and CD25 positive FoxP3$^+$CD4$^+$ cells was 0.61 ($p<0.001$), corresponding with a coefficient of determination (R^2) of 0.37 (Fig. 4C). This means that only 37% of the variation in FoxP3$^+$CD4$^+$ cells is explained by variation in CD25 positive FoxP3$^+$CD4$^+$ cells, while 88% is explained by variation in CD25 negative FoxP3$^+$CD4$^+$ (correlation coefficient of 0.94, $p<0.001$, Fig. 4B). The percentages of CD25$^-$FoxP3$^+$CD4$^+$ cells were also compared between healthy dogs and dogs with cancer (Fig. 4D). Interestingly, a significant difference in CD25$^-$ FoxP3$^+$CD4$^+$ was found between healthy dogs and dogs with cancer ($p=0.01$). However,

$CD25^{-}FoxP3^{+}CD4^{+}$ cells were not influenced by our metronomic cyclophosphamide regimen (data not shown).

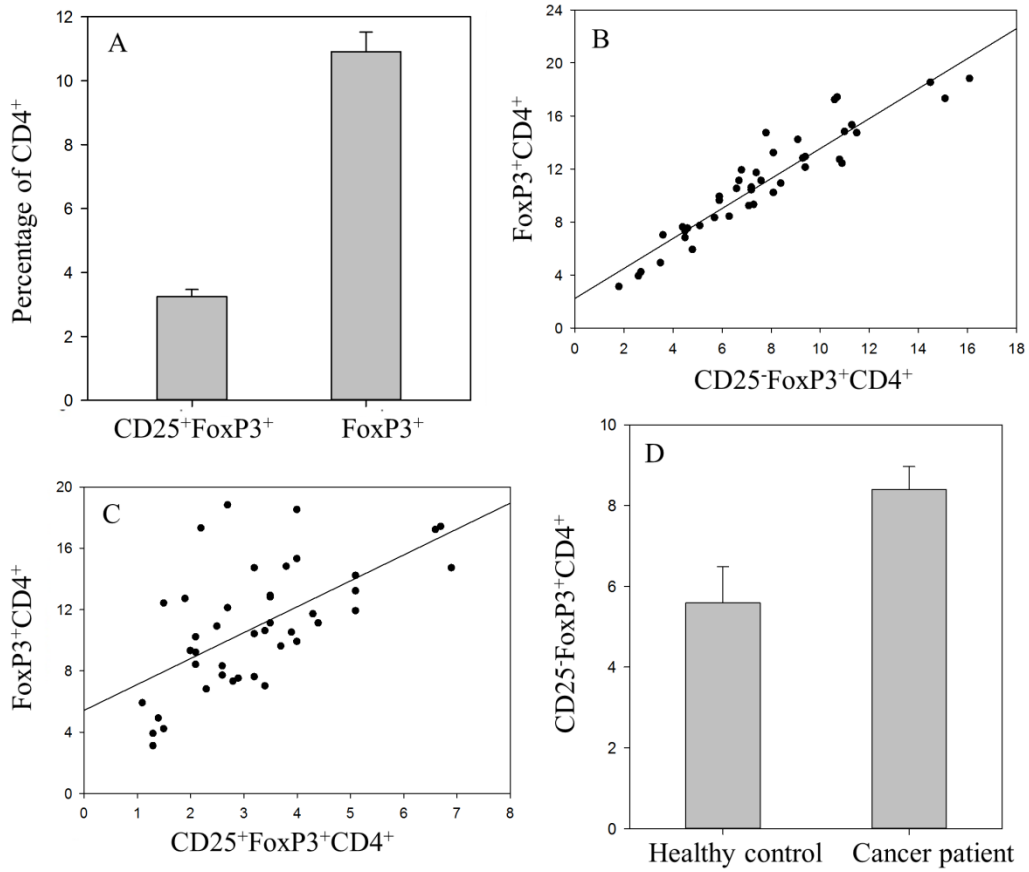

Fig 4: Expression of CD25 in $FoxP3^{+}CD4^{+}$ lymphocytes. (A) $CD25^{+}$ cells are only a minority of the $FoxP3^{+}CD4^{+}$ cells; (B) and (C) $FoxP3^{+}CD4^{+}$ variation is mostly explained by variation in $CD25^{-}$ cells, and not $CD25^{+}$ cells; (D) $CD25^{-}FoxP3^{+}CD4^{+}$ cells differ significantly between healthy control dogs (n=10) and dogs with cancer (n=30). Bars represent mean and error bars standard errors. (*$p<0.05$)

5. Discussion

This study confirmed for the first time with an anti-canine CD25 antibody a significant difference in the percentage of $CD25^{+}Foxp3^{+}CD4^{+}$Tregs in the peripheral blood of canine cancer patients compared to healthy dogs. Although mean Treg percentages were significantly higher in dogs with cancer compared to the healthy dogs, considerable variation exists in cancer patients with a proportion of patients having Tregs percentages similar as those of healthy dogs. This might seem unexpected in a population of dogs with advanced disease, as it is accepted that a tumor has to induce immune tolerance for progression and

inducing Tregs is a major way to accomplish this. However, there are several possible explanations for this. Firstly, local proliferation or selective migration can cause elevated intratumoral Tregs concentration without elevated circulating Tregs.[229] Moreover, not only the number of Tregs, but also their functionality may be increased in cancer patients.[250] Lastly, tumors have a vast repertoire of escaping immunity outside Tregs, so it is possible that individual tumors accomplish the same goal in different ways, a feature characteristic for many tumor traits. Some examples include tumor antigen loss, downregulation of MHC molecules and resistance to cytotoxic pathways.[5] In contrast with previous studies that did not use the anti-CD25 antibody, we could not discover an association between the percentage of Tregs and the tumor type nor the presence of metastasis.[232,233] Limited patient numbers in the referenced studies as well as in the current study may explain this discrepancy.

Metronomic cyclophosphamide at 12.5 mg/m² decreased the percentage of these Tregs in cancer patients. This is in agreement with previous studies in human patients.[220] Although Burton *et al*. could not detect a significant difference in the percentage of $FoxP3^+CD4^+$ cells after treatment with 12.5 mg/m^2 in dogs with cancer, the authors stated that that was probably due to insufficient power, as absolute number of these cells were decreased and only five dogs were included.[156] As Tregs are considered the main obstacle for successful immune therapy, our metronomic cyclophosphamide regimen can thus be an adequate supportive treatment for immunotherapy in dogs.[151] Metronomic temozolomide at 6.6 mg/m² had no effect on the percentage of Tregs. This may be explained by the dosage, as only one dose based on extrapolation of an experiment on rats was evaluated.[222] In that experiment, depletion was already evident after one week. A recent study in human cancer patients also reports a decrease in percentage Tregs as soon as one week after treatment with low dose temozolomide.[221] However, in this study of Ridolfi *et al*. the dose (75 mg/m^2) was only 2.5 times lower than the conventional cytotoxic dose and only given for two weeks. Therefore, this study did not use a truly metronomic regimen. Consequently, grade II adverse effects occurred frequently and dosage had to be reduced in some patients. From preclinical studies for the approval of temozolomide for human use, it is evident that dogs are more sensitive for this drug, with human therapeutic doses of 200 mg/m^2 within the lethal range for dogs.[251] Nevertheless, since our dose was much lower than the one reported in the human study, a dose escalation study in dogs for metronomic temozolomide might identify a dose with selective toxicity to Tregs.

Baseline plasma VEGF levels were significantly higher in cancer patients than in healthy control dogs. Moreover, baseline plasma VEGF levels were also higher in cancer patients when metastases were present. This corresponds with previous findings in human and canine patients.[252-255] The TSP-1 concentration in dogs with cancer was markedly lower than in healthy dogs. This has also been reported in human cancer patients.[256] To our knowledge, this is the first time that plasma TSP-1 concentrations have been measured in canine cancer patients. The distinct difference observed in this study warrants further investigation of the use of TSP-1 as a tumor marker. It would be interesting to evaluate if TSP-1 measurements could aid in early detection of disease. As all patients in this study had advanced stages of disease, this could not be evaluated. An additional control group consisting of dogs with various other diseases could provide more information about the specificity of this potential marker. Despite the pathological levels of TSP-1 and VEGF at baseline, no difference after treatment with metronomic cyclophosphamide and/or temozolomide could be observed. This is in agreement with Burton *et al*., who also failed to detect an effect on tumor microvessel density after treatment with cyclophosphamide at 12.5 mg/m^2.[156] Immune modulating effects occur indeed at lower dosages of metronomic chemotherapy than anti-angiogenic effects.[219] The efficiency of metronomic chemotherapy is mainly dependent on anti-angiogenic activity.[240] The determination of metronomic dosage in veterinary, but also human medicine, has been empirical so far.[257] For cyclophosphamide, doses between 10 and 25 mg/m^2 for dogs are reported, with no evidence based rationale to select an optimal dose at this moment.[152-155,157] This can be partly explained by the vast number of patients required to conduct a comparative study based on clinical efficacy.[258] For different chemotherapeutic agents, it has been proven that their metronomic mode of action involves modulation of secretion of VEGF and TSP-1 and it has been demonstrated that the concentration of these factors in the circulation can be affected by metronomic chemotherapy in human patients.[238,241-244,246] Therefore, these factors can be valuable pharmacodynamic markers for dose optimization studies. This study demonstrates that circulating concentrations of these markers are different for dogs with cancer compared to healthy dogs, providing a window to show therapeutic modulation. Evaluation of these biomarkers in canine cancer patients treated with higher doses of metronomic chemotherapy would thus be highly interesting.

Cyclophosphamide currently is the drug of choice for metronomic treatment in veterinary as well as in human medicine.[257] Although low doses of cyclophosphamide are considered safe,

sterile hemorrhagic cystitis, a common side effect in patients that receive high doses of this drug, has also been observed in about 10% of canine patients that received metronomic cyclophosphamide for long periods.[152,153] Also in our study, one dog developed cyclophosphamide induced cystitis, with other causes ruled out by urinalysis, urine culture and ultrasound. Considering the relative high frequency of this side effect, alternatives for metronomic cyclophosphamide should be evaluated in veterinary oncology. Therefore, in this study, we studied for the first time metronomic temozolomide (6.6 mg/m^2 daily) in dogs with cancer. However, since no biological effect of temozolomide could be demonstrated, additional studies with higher doses are advocated.

Most of previous studies investigating Tregs in dogs used only CD4 and FoxP3 for Treg identification.[156,157,230-233,249] In four studies an anti-human CD25 antibody is used, which cross reacts with canine CD25 but with too low affinity to ensure sufficient sensitivity.[236,237,259,260] To our knowledge, this is the first study analyzing Tregs with the anti-canine CD25 antibody, developed by Abrams and coworkers, in dogs with cancer.[235] It has already been established that in healthy dogs a significant proportion of non-regulatory CD25 negative FoxP3$^+$CD4$^+$cells exists and thus that the proportion of FoxP3$^+$CD4$^+$cells is an overestimation of the proportion of true Tregs.[237] This is confirmed by our data in cancer patients. FoxP3$^+$CD4$^+$ T cells without suppressive capacity have been identified earlier, in humans as well as in dogs.[259,261] It has been reported that these CD25 negative cells constitute a peripheral inactive reservoir, capable of converting to fully functional CD25$^+$ Tregs. CD25 expression is thus an activation marker for Tregs, similar as for conventional CD4 cells.[262] To our knowledge, this is the first time that in addition to increased CD25$^+$FoxP3$^+$CD4$^+$, increased CD25$^-$FoxP3$^+$CD4$^+$ cells are reported in cancer patients. In other physiological and pathological conditions, also a parallel increase in CD25$^+$ and CD25$^-$ FoxP3$^+$CD4$^+$ is reported.[263,264]

6. Acknowledgements

We would like to thank prof. dr. Chris Vervaet of the Faculty of Pharmaceutical Sciences for help with the reformulation of the drugs used in this study. The financial support of Ghent University (BOF) is acknowledged with gratitude.

CHAPTER 3

Immunogenicity and safety of xenogeneic vascular endothelial growth factor receptor-2 DNA vaccination in mice and dogs

This chapter is based on Denies S., Cicchelero L., Polis I., Sanders N.N. Immunogenicity and safety of xenogeneic vascular endothelial growth factor receptor-2 DNA vaccination in mice and dogs. Under revision by Oncotarget.

1. Abstract

Vascular endothelial growth factor receptor 2 (VEGFR-2) is an attractive target in the treatment of cancer due to its crucial role in angiogenesis. In this study a DNA vaccine coding for human VEGFR-2 was evaluated in healthy mice and dogs as a xenogeneic vaccination approach. In mice, three doses (5, 15 and 25 µg) and vaccination schedules (prime, prime-boost, prime-boost-boost) were evaluated. The vaccine was administered by intradermal injection followed by electroporation under general anesthesia. Cellular immune responses were measured by intracellular staining of interferon-gamma (IFN-gamma) and a cytotoxicity assay. Serum antibodies were determined by ELISA. Safety was assessed by measuring regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) and by performing a wound healing assay. The VEFGR-2 DNA vaccine was subsequently evaluated in healthy dogs, which were vaccinated three times with 100 µg with a two week interval. The vaccine was administered by intradermal injection followed by electroporation under sedation and local anesthesia. Cellular immune responses in dogs were measured by intracellular IFN-gamma staining and antibodies by a flow cytometric assay. In mice, maximal cellular immune responses were already observed after two vaccinations with 5 µg. Humoral immune responses continued to increase with higher dose and number of vaccinations. No abnormities in the levels of Tregs, MDSCs or wound healing were observed. In dogs, the vaccine was also capable of eliciting a cellular and humoral immune response, but only after three vaccinations. No adverse effects were observed, but tolerability of the electroporation protocol in dogs was poor with sedation and local anesthesia. As the immunogenicity and safety of this xenogeneic VEGFR-2 DNA vaccine is demonstrated in dogs and mice, this will facilitate its evaluation in tumor bearing animals, ranging from preliminary rodent models to dogs with spontaneous tumors.

2. Introduction

It is well-known that the immune system is capable to recognize tumor cells and to establish a specific long-term antitumor response. Therefore, vaccination against tumor antigens holds great promises for the treatment of cancer.[5] However, due to complex interactions between tumor cells and the immune system and the problem of tolerance, the translation of the theoretical potential of tumor vaccines into an effective clinical response has been difficult.[265]

In healthy individuals, VEGFR-2 is expressed on virtually all endothelial cells as well as on certain other cell types including retinal progenitor cells, megakaryocytes and pancreatic duct cells. In cancer patients, VEGFR-2 is strongly overexpressed on endothelial cells.[266] VEGFR-2 can thus be classified as a ubiquitous tumor antigen. The potential of raising an effective immune response against these tumor antigens without causing autoimmunity can be explained by the level of overexpression in tumors, reaching the threshold for T cell recognition and thus breaking immune tolerance.[27] As it is difficult to raise a strong immune response against self-antigens, xenogeneic vaccination, which involves the use of an antigen of a different species, has been proven to result in more potent immune responses.[267]

VEGFR-2 as vaccine target has some important advantages. Tumor cells have grown under the selective pressure of the immune system. Evading immune control is one of the hallmarks of tumors, with down regulation of MHC-1 molecules and evasion of cytotoxic effects of immune cells as two common examples.[5] These immune evasive strategies acquired during tumor growth make tumor cells difficult targets for vaccine strategies. Moreover, because of their genetic instability, tumor antigens in tumor cells are prone to mutations, which leads to escape mutants that further decrease the efficacy of tumor vaccines.[5] In contrast, endothelial cells of tumoral blood vessels have not acquired immune evasive strategies and are genetically stable.[268] They are thus more vulnerable to vaccination strategies. Targeting tumor vasculature instead of tumor cells directly has proven its value, for instance by the success of bevacizumab.[266]

Although targeting VEGFR-2 is primarily an anti-angiogenic treatment, certain tumors also express VEGFR-2, serving as an autocrine growth factor for these cells.[269] In these tumor types VEGFR-2 vaccination can also induce a direct antitumor effect. Moreover, VEGFR-2 is expressed on Tregs and MDSCs.[270,271] Targeting these cells via VEGFR-2 vaccination can have a self-reinforcing effect, as these cells suppress anti-tumor immune responses.[5]

VEGF/VEGFR-2 targeting has been explored extensively with different treatment modalities, with some of them approved for clinical use.[268] In preclinical studies vaccination against VEGFR-2 has also been explored.[272] Vaccination has some important advantages. It is more specific than small molecule inhibitors and compared to monoclonal antibodies the polyclonal antibody response evoked by vaccination may have a higher antigen neutralizing

capacity. Both monoclonal antibodies and small molecule inhibitors have to be administered frequently, which makes vaccination a much more cost efficient and patient friendly therapy.[268] Different vaccine platforms exist. DNA vaccination has the advantage that the antigen is synthetized intracellularly, leading to a robust cellular immune response.[273] Moreover, DNA vaccines often make use of plasmids that contain immune stimulating unmethylated CpG motives.[274] Electroporation mediated delivery of DNA vaccines can further support the development of an immune response because the limited tissue damage caused by electroporation also serves as an adjuvant.[275] Delivery of DNA vaccines via electroporation has also the advantage that the vaccine manufacture is more economical, uniform and suitable for large scale production than when viral or chemical carriers are used for the delivery.[276,277]

In this study we evaluated in healthy mice and dogs the safety and immunogenicity of a xenogeneic VEGFR-2 DNA vaccine. The DNA vaccine was administered as a naked plasmid in combination with needle array electroporation.

3. Materials and Methods

Tumor cell lines and VEGFR-2 plasmid

The B16-F10 tumor cell line was generously provided by Johan Grooten (Department of Biomedical Molecular Biology, Ghent University, Belgium) and was stably transduced with luciferase as described earlier.[278] Briefly, retroviral vectors encoding luciferase were produced in HEK293T cells by calcium phosphate transfection. Virions were harvested after 48 and 72h, filtered and concentrated by ultracentrifugation. Transduction the B16-F10 cells was performed in the presence of 8 µg/ml polybrene and transfection efficiency was evaluated by bioluminescence. The canine hemangiosarcoma (HSA) cell line was generously provided by Douglas Thamm (College of Veterinary Medicine and Biomedical Sciences, Colorado State University, USA). These cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, 100 IU/ml penicillin and 1 mmol/ml L-glutamine (Invitrogen, Carlsbad, USA). The plasmid encoding human VEGFR-2 was purchased from Invivogen (Toulouse, France). A full description of the plasmid can be found on the website of the manufacturer.[279]Purification of the plasmids

was done with the EndoFree Giga kit, following the instructions of the manufacturer (Qiagen, Valencia, CA, USA).

DNA vaccine doses and vaccination schedules in mice

Healthy C57BL/6JRj mice of 8 weeks old were used for this optimization study (Janvier Breeding Center, Le Genest St. Isle, France). Three doses (5 µg, 15 µg and 25 µg) and three vaccination schedules (prime, prime-boost, prime-boost-boost) were evaluated with 4 mice for each condition (Fig. 1). A group of untreated mice served as control (n=4). The VEGFR-2 plasmid was injected intradermally in 20 µl calcium and magnesium free phosphate buffered saline (PBS; Invitrogen) followed by electroporation with the BTX Agilepulse device (Harvard apparatus, Holliston, USA) using 4 mm gap needle electrodes. The following electroporation protocol was used: 2 pulses of 450 V with a pulse duration of 0.05 ms and a 300 ms interval, followed by 8 pulses of 100 V with a pulse duration of 10 ms and a 300 ms interval. Mice were anesthetized with isoflurane (2%) during this procedure. The interval between the vaccinations was 2 weeks and mice were euthanized 2 weeks after the last vaccination. This experiment was approved by the ethical committee of the Faculty of Veterinary Medicine (Ghent University; EC2013/77) 2013_40

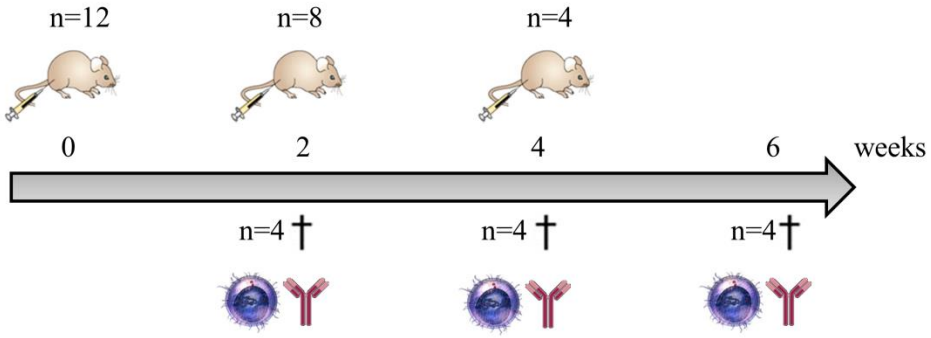

Figure 1: Vaccination schedule in mice. Mice were vaccinated with two weeks interval, two weeks after each vaccination four mice were sacrificed to measure cellular and humoral immune response. This vaccination schedule was used to evaluate three different doses of the VEGFR-2 DNA vaccine (5, 15 and 25 µg).

Assessing cellular immune response in vaccinated mice

Splenocytes were isolated two weeks after the last vaccination. To assess the cytotoxic response to cells expressing the target, splenocytes were co-incubated with bioluminescent B16-F10 melanoma cells that were *in vitro* electroporated with a plasmid encoding human VEGFR-2. Electroporation was performed in 2mm cuvettes with the BTX ECM 830 device (Harvard apparatus, Holliston, USA). Two pulses of 140 V with a pulse duration of 5 ms and

100 ms interval were used. As a control for specificity to the VEGFR-2 target, B16-F10 melanoma cells that were mock electroporated were also included. Absence of natural VEGFR-2 expression on the B16-F10 cells and induction of expression after electroporation were assessed by flow cytometry with a rabbit antibody that binds mouse VEGFR-2 and an Alexafluor-688 conjugated secondary goat anti-rabbit antibody (Abcam, Cambrige, UK). To measure the tumor killing capacity of lymphocytes from vaccinated and non-vaccinated mice we incubated $2x10^5$ splenocytes with $1x10^4$ B16-F10 cells. After 24 hours the bioluminescent signal, which is related to the number of living cells, was measured with an IVIS lumina II (Perkin-Elmer, Zaventem, Belgium).

Additionally, the IFN-gamma response was also measured. In more detail, $1x10^6$ splenocytes were stimulated overnight with 2 µg of murine VEGFR-2 protein (Bio-Connect, Te Huissen, The Netherlands). Subsequently, the protein transport inhibitor brefeldin A was added and after 4 hours the cells were incubated overnight in fixation buffer (ImTec Diagnostics, Antwerp, Belgium). The following day the cells were permeabilized and the intracellular staining of IFN-gamma was performed with an APC-conjugated anti-IFN-gamma antibody (Imtec Diagnostics). The cells were analyzed with an Accuri C6 flow cytometer (BD Biosciences, Erembodegem, Belgium) and events in the permeabilized lymphocyte gate were selected for analysis. Controls for specificity of the cytokine response were assessed by including non-stimulated splenocytes in the analysis.

Assessment humoral immune response in vaccinated mice

Blood was collected 2 weeks after the last vaccination of each vaccination schedule by cardiac puncture under terminal anesthesia. Blood was centrifuged (10 min, 3000 g, Eppendorf centrifuge) and serum was collected and stored at -80 °C until analysis. Microtiter plates were coated overnight with 1 µg murine VEGFR-2 protein in coating buffer and loaded with serial dilutions of serum in ELISA assay diluent for 2 hours (Imtec Diagnostics). After washing, a HRP-conjugated anti-mouse IgG antibody was added, followed by development with TMB substrate and measuring absorbance with a microplate reader at 450nm (ImTec Diagnostics). The titer was determined as the limiting dilution with a signal exceeding mean plus twice the standard deviation of the signal from control samples (non-vaccinated mice).

Assessment of Tregs and MDSCs levels

As VEGFR-2 expression is reported on Tregs and MDSCs, the effect of vaccination on normal levels of these cells was assessed as a measure of safety. Splenocytes were isolated when mice were sacrificed and the percentage of Tregs and MDSCs were measured by flow cytometry. Regulatory T cells were stained with a FITC-conjugated anti-CD25 antibody, an APC-conjugated CD4 antibody and a PE-conjugated anti-FoxP3 antibody after fixation and permeabilization with commercial buffers (Ebioscience, Vienna, Austria). MDSCs were stained with a FITC-conjugated anti-CD11b antibody and a PE-conjugated anti-Gr1 antibody (Ebioscience). The cells were analyzed with an Accuri C6 flow cytometer and events in the lymphocyte gate were selected for analysis. Color compensation was based on fluorescence minus one (FMO) controls.

Assessment of wound healing in vaccinated mice

To further assess safety of VEGFR-2 vaccination, a wound healing assay was performed. Two circular full-thickness dermal wounds were created between the shoulder blades with a 2 mm punch biopter one week after the last vaccination. These wounds were allowed to heal spontaneously and the days until macroscopic closure of the wound were determined, as described earlier.[280]

Vaccination of dogs

Six laboratory Beagle dogs were vaccinated three times with 100 µg of human VEGFR-2 plasmid dissolved in 100 µl magnesium and calcium free PBS. The vaccine was injected intradermally on the flank followed by electroporation with the BTX Agilepulse device. Following electroporation protocol was used: 2 pulses of 450 V with a pulse duration of 0.05 ms and a 0.2 ms interval, followed by 8 pulses of 100 V with a pulse duration of 10 ms and a 20 ms interval using 4 mm gap needle electrodes. Dogs were sedated by intravenous injection of 0.2mg/kg butorphanol (Dolorex, Intervet, Boxmeer, Nederland). Local anesthesia was obtained by subcutaneous injection of 0.5 ml of lidocaïne 2% (Xylocaïne, Recipharm Monts, Monts, France) around the vaccination site. This experiment was approved by the ethical committee of the Faculty of Veterinary Medicine (Ghent University; EC2013/40).

Assessment of cellular immune response in vaccinated dogs

Blood was collected before each vaccination and two weeks after each vaccination. Blood was collected in EDTA tubes and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation with Ficoll-Paque 1.007 g/ml (Invitrogen). The canine HSA cell line was used to assess a cytotoxic response against VEGFR-2 expressing cells. Expression of VEGFR-2 by these cells was confirmed with an anti-VEGFR-2 antibody and a secondary Alexa fluor 688-conjugated goat anti-rabbit antibody (both from Abcam). Subsequently, the canine HSA cells were *in vitro* transfected with eGFP via electroporation with the BTX device. A cytotoxicity assay was performed by incubating $2x10^5$ PBMC with $1x10^4$ eGFP transfected HSA cells and 24 hours later the number of eGFP expressing cells was analyzed by flow cytometry as a measure of PBMC mediated killing of VEGFR-2 positive cells. PBMC obtained before vaccination served as controls.

Additionally, the IFN-gamma response was measured after overnight incubation of $1x10^6$ PBMC with 2 µg human VEGFR-2 protein, as a recombinant canine VEGFR-2 protein is not available (Acro Biosystems, London, UK). Subsequently, brefeldin A was added followed after 4 hours by overnight incubation in fixation buffer (Ebioscience).The next day, PBMC were stained with a FITC-conjugated anti-IFN-gamma antibody after permeabilization with a commercial buffer (Life Technologies, Ghent, Belgium). The cells were analyzed with an Accuri C6 flow cytometer and events in the permeabilized lymphocyte gate were selected for analysis. Controls for specificity of the cytokine response were assessed by including non-stimulated PBMC in the analysis.

Assessment of the humoral immune response in vaccinated dogs

Plasma was collected two weeks after each vaccination. HSA tumor cells were washed and resuspended in 50 µl of diluted plasma (1/8 dilution in FACS buffer, Imtec Diagnostics). Cells were incubated for one hour at 37 °C and 5% CO_2. Subsequently the cells were washed and stained with APC-conjugated anti-dog IgG antibodies (Imtec Diagnostics) and the number of positive cells was analyzed via flow cytometry. Control for specificity of staining included HSA cells that were not incubated with plasma and stained with the secondary antibodies.

Assessment of tolerability and safety of the vaccination in dogs

As this is the first time that the Agilepulse was used for electroporation of dogs, an important outcome measurement was also the tolerability to the electroporation. Acute stress and pain signals during the electroporation as well as the aspect and sensitivity of the vaccination site the days after electroporation were monitored. A thorough physical examination including Doppler blood pressure monitoring was performed the week after each vaccination to assess safety of the vaccine.

Statistical analyses

Data were analyzed with the SPSS software version 19 (IBM, Brussels, Belgium). The effect of DNA vaccine dose and vaccination schedule on the cytotoxicity and IFN-gamma secretion of murine splenocytes were analyzed with a general linear model. The effect of DNA vaccine dose and vaccination schedule on antibody titers in serum of mice were analyzed with an exact linear by linear association test. The effect of vaccination on IFN-gamma secretion of canine PBMC was analyzed with a repeated measures ANOVA. The effect of vaccination on antibody bound HSA cells were analyzed with the non-parametric Friedman test. For post hoc tests, correction for multiple comparisons was performed with the Tukey method. Data are represented as mean ± standard deviation, unless otherwise specified.

4. Results

Effect of vaccine dose and vaccination schedule on the immune response in mice

Cellular immune response in mice

To study the influence of the VEGFR-2 DNA dose and vaccination schedule on the induction of tumor antigen specific cytotoxic T lymphocytes, we incubated splenocytes with tumor cells expressing human VEGFR-2 at an effector:target ratio of 20:1. Splenocytes from vaccinated mice demonstrated significant higher cytotoxicity than those from control mice ($p=0.015$) without a significant effect of the DNA vaccine dose (Fig. 2). When we tested the effect of the different vaccination schedules on the cytotoxic T-lymphocytes we found that one boost is superior to none ($p=0.002$). Overall, there was no significant difference between three and two vaccinations, although for 5 µg a trend towards better response after three

vaccinations is visible. After co-incubation with splenocytes from mice vaccinated at least two times there were on average 52 ± 14% surviving target cells, compared to 89 ± 13% when co-incubated with splenocytes from non-vaccinated mice.

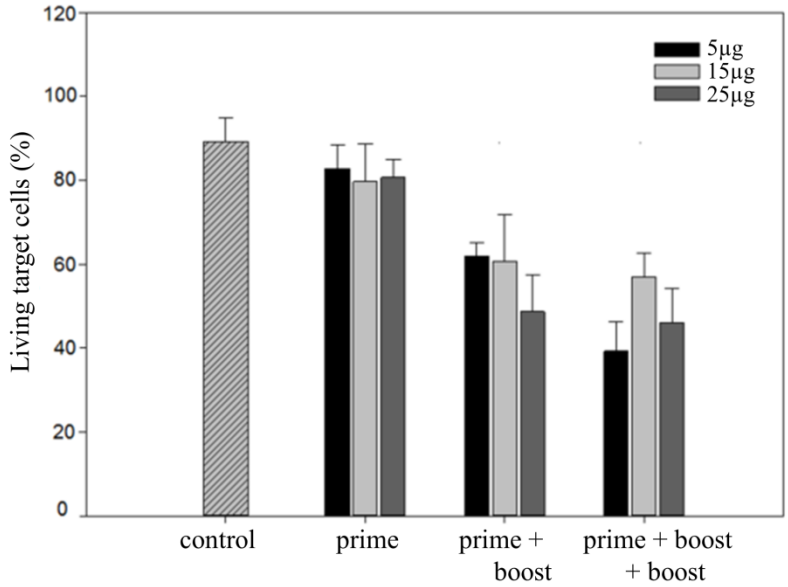

Fig. 2: Cytotoxic activity of splenocytes isolated from non-vaccinated and vaccinated mice that received one (prime), two (prime + boost), or three (prime + boost + boost) VEGFR-2 DNA vaccinations. Three different DNA vaccine doses (5, 15 or 25 µg) were tested. Two weeks after the last vaccination splenocytes were collected and their capacity to lyse VEGFR-2 expressing target cells was measured. Control indicates non-vaccinated mice. Bars represent mean, error bars standard error of the mean (n=4).

Analysis of IFN-gamma secretion led to the same conclusions (Fig. 3). After overnight incubation with a murine VEGFR-2 protein there were on average 14 ± 5% IFN-gamma positive lymphocytes in the splenocytes of mice that received at least two vaccinations, compared to 1 ± 1% in splenocytes from non-vaccinated mice.

Table 1: Effect of dose and schedule on antibody titers

	Prime	**Prime + boost**	**Prime + boost + boost**
5 µg	<4	<4	<4
15 µg	<4	4	100
25 µg	<4	400	800

All four mice within each group had the same antibody titer.

Humoral immune response in mice

The impact of the VEGFR-2 DNA dose and vaccination schedule on the induction of antibodies is summarized in table 1. Both DNA vaccine dose and vaccination schedule had a significant impact on antibody titer ($p<0.001$ and $p=0.003$ respectively). Antibody responses were undetectable in mice vaccinated with the lowest dose (5 µg) and for all doses after only one vaccination. The highest antibody titers were present in serum from mice vaccinated three times with 25 µg, reaching an antibody titer of 800 in this group.

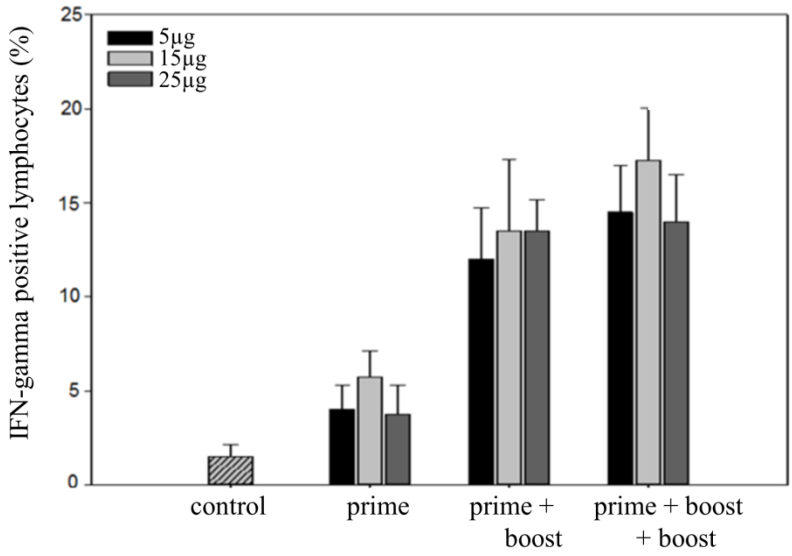

Fig. 3: Interferon-gamma response of splenocytes isolated from non-vaccinated and vaccinated mice that received one (prime), two (prime + boost), or three (prime + boost + boost) VEGFR-2 DNA vaccinations. Three different DNA vaccine doses (5, 15 or 25 µg) were tested. Two weeks after the last vaccination splenocytes were collected and stimulated with murine VEGFR-2 protein. Following stimulation the percentage of IFN-gamma positive splenocytes was measured by flow cytometry after intracellular cytokine staining. Control indicates non-vaccinated mice. Bars represent mean, error bars standard error of the mean (n=4).

Safety in mice

Vaccination with DNA encoding human VEGFR-2 had no effect on normal levels of Tregs (2.38 ± 0.49 in non-vaccinated mice versus 2.57 ± 0.51% in vaccinated mice) or MDSCs in the spleen of healthy mice (1.68 ± 0.35% in non-vaccinated mice versus 1.70 ± 0.32% in vaccinated mice). There was also no effect on the mean time until wound closure (5 ± 1 days for both vaccinated and non-vaccinated mice).

Immunogenicity in dogs

Cellular immune response in dogs

Before the start of the vaccination and two weeks after each vaccination PBMC were collected from the dogs and stimulated with human VEGFR-2. Subsequently, the percentage of IFN-gamma positive PMBC was measured by flow cytometry after intracellular staining. After the first and second vaccination we could not detect a significant IFN-gamma response compared to baseline (data not shown). However, after the third vaccination (i.e. second boost) the percentage of IFN-gamma positive lymphocytes were significantly increased with an average of 9 ± 0.05% of IFN-gamma positive lymphocytes compared to 4 ± 0.02% at baseline (p=0.04) (Fig. 4).

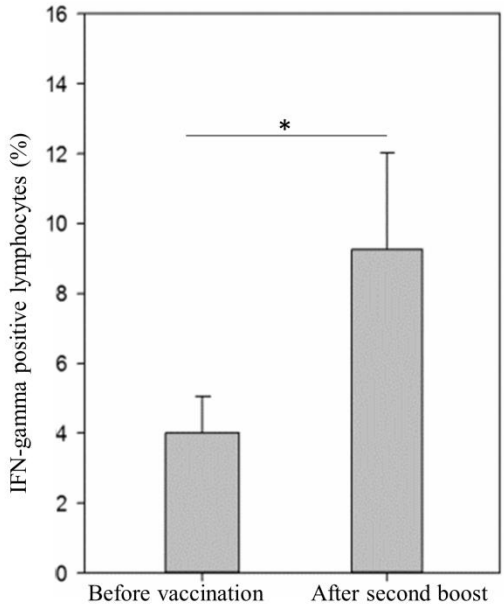

Fig. 4: Interferon-gamma response of lymphocytes isolated from dogs before vaccination and after a second boost with the VEGFR-2 DNA vaccine. Two weeks after the second boost lymphocytes were collected and stimulated with human VEGFR-2. Following stimulating the percentage of IFN-gamma positive lymphocytes was measured by flow cytometry after intracellular cytokine staining. Bars represent mean, error bars standard error of the mean. (*p<0.05)

We also tried to measure the cytotoxic T cell response after VEGFR-2 DNA vaccination of dogs by co-incubating PBMC with a canine HSA cell line. However, at baseline (i.e. before vaccination) there was already a considerable cytotoxic response of isolated PBMC towards the canine HSA cell line, with only 26 ± 9% HSA cells surviving the co-incubation. No increase in this cytotoxic response was observed after the last vaccination (data not shown).

This demonstrates that the allogeneic nature of the target cells already triggered a significant cytotoxic response from naïve PBMC, as previously reported for human PBMC.[281] It is hypothesized that this response is already at maximal capacity at baseline explaining no increase after vaccination.

Humoral Immune response in dogs

In vaccinated dogs antibody levels against VEGFR-2 had significantly increased (relative to baseline levels) after the third vaccination (p=0.03). When looking at individual dogs, four out of six dogs had a clear increase in antibodies after the second boost (Fig. 5).

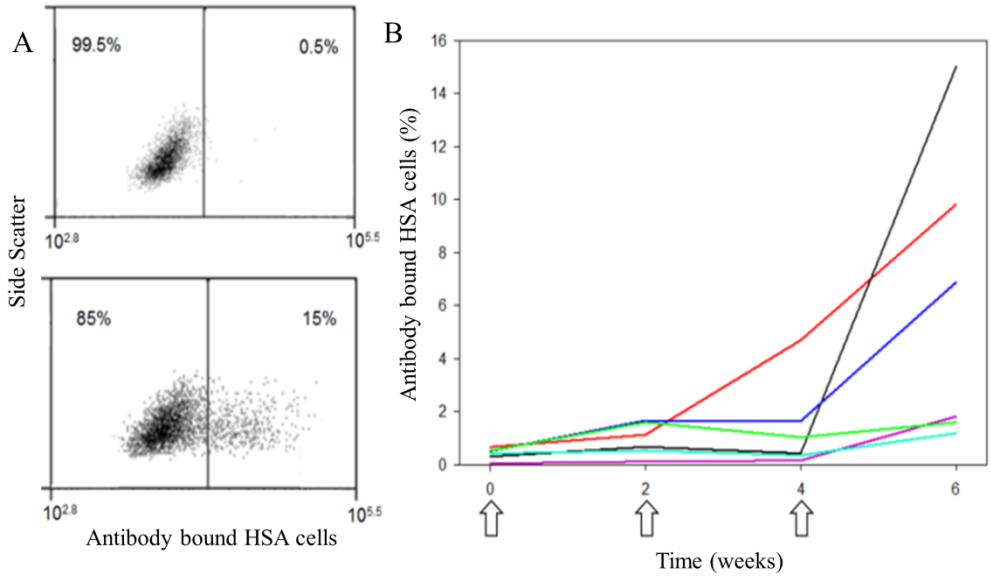

Fig. 5: (A) Flow cytometric plots illustrating the evaluation of antibody response by the amount of VEGFR-2 expressing target cells bound with antibodies present in serum via a secondary anti-dog IgG antibody (upper: at baseline, lower: after the last vaccination of the same dog).(B): time course of the antibody response for individual dogs, arrows indicate vaccination moments and each colored line represents an individual dog. HSA=hemangiosarcoma.

Tolerability in dogs

The delivery of the very short electric pulses caused in all dogs a startle response characterized by withdraw movements, vocalization and stress-induced anal sac excretion. This only occurred during electroporation, which lasted less than one second. Immediately after electroporation, dogs exhibited normal behavior and no reaction when manipulating the

electroporation site could be elicited. With the Agilepulse device, actual resistance of the tissue during electroporation is measured and saved. Hence it could be checked whether the electrodes remained in place during the withdraw movements. Based on these resistance data we could conclude that the withdraw movements did not affect any of the electroporations. This is most likely because the total duration of pulses is less than a second. Twenty-four hours after electroporation, the electroporation site was red and swollen, which evolved over two weeks to an epidermal crust. All physical parameters (heart rate, respiration rate, temperature, blood pressure) remained stable during the vaccination period.

5. Discussion

This study demonstrates that a human VEGFR-2 DNA vaccine, administered in combination with electroporation, is capable of inducing a cellular and humoral immune response in mice and dogs. The dose escalation study in mice demonstrated that it was easier to elicit a cellular immune response than a humoral immune response. This is in agreement with other studies with DNA vaccines and can be explained by the limited amounts of proteins released after DNA vaccination, which is insufficient for a robust antibody response.[282] A very small amount of antigen when presented within an antigen presenting cell, either directly by transfection or indirectly via cross-presentation, is sufficient to prime strong cytotoxic T cell responses.[282] In our mice study a cellular immune response was already observed after prime-boost vaccination with 5 µg pDNA, while higher DNA vaccine doses and at least two vaccinations were needed to elicit a humoral immune response. VEGFR-2 is a transmembrane protein, and thus accessible for both arms of the immune system.[266] Indeed, it has been reported that both a humoral and a cellular response against VEGFR-2 can convey anti-tumor effects.[283] Further evaluation of the efficacy of our VEGFR-2 DNA vaccine in tumor bearing mice should thus be done with at least 25 µg and three vaccinations. Vaccination against VEGFR-2 did not affect wound healing or normal levels of Tregs or MDSCs in healthy mice, two important factors that could warn for side-effects impeding clinical use. Both Tregs and MDSCs significantly upregulate their VEGFR-2 expression in response to tumor secreted cytokines.[270,271] It is thus possible that in tumor bearing hosts, VEGFR-2 vaccination has a differential effect on the percentage of Tregs and MDSCs compared to healthy individuals.

Dose extrapolation of DNA vaccines from mice to humans or other animals is difficult. A linear extrapolation based on body weight is often used to calculate the dose in other species. From such calculations it is frequently concluded that the doses are too high to be feasible in humans. However, this reasoning is not correct. Firstly, correct allometric translation of drug doses should be done based on body surface area, which results in drug doses 0.08 times less than when based on simple conversion based on body weight.[284] Secondly, allometric scaling of vaccine doses is not relevant, as this is based on pharmacokinetic characteristics that do not influence the interactions between antigen and immune cells after local injection.[285] Many other species specific factors do influence vaccine efficiency, that are too complex to allow for a scaling formula. Therefore, the DNA vaccine dose for dogs in this study (100 µg) was not based on a conversion from the results in mice, but on studies that tested xenogeneic anti-cancer DNA vaccines in dogs delivered with electroporation.[56,286] The xenogeneic VEGFR-2 DNA vaccine was successful in inducing a cellular and humoral immune response in dogs. A cellular immune response was evident by interferon-gamma secretion after incubation with human VEGFR-2 protein. The immune response against human VEGFR-2 required more boosts in dogs compared to mice, with a clear response only after three vaccinations. The higher degree of homology between human and canine VEGR-2 (93%) compared to murine VEGFR-2 (86%) may explain the higher immunogenicity of our vaccine in mice. However, it has been shown that one xenogeneic peptipe epitope is equally efficient in breaking tolerance as the full length xenogeneic protein, suggesting that the degree of heterology is not critical.[287] Additionally, also in other studies with xenogeneic DNA vaccines in dogs, immune responses are only evident after multiple boosts, suggesting this might be required in dogs independent of the vaccine composition [56,286].

The normal levels of Tregs and MDSCs, wound healing in healthy mice and physiological parameters (heart rate, respiration rate, temperature and blood pressure) in healthy dogs are not influenced by the vaccine. This indicates that no acute side effects occur after administration of our VEGFR-2 DNA vaccine. The tolerability of the electroporation on butorphanol sedated dogs in combination with local anesthesia is poor. As humans treated with the same protocol report good tolerability of electroporation and given the nature of reactions of the dogs, responses are believed to come from startling more than pain.[288,289] Based on the reaction of the dogs one may consider to use higher levels of sedation or even anesthesia during electroporation. Because of the very short duration of the entire procedure

and based on evaluation of experienced veterinarians supervising the experiment this is not ethically imposed, considering discomfort caused by additional manipulations and recovery associated with deep sedation or anesthesia. However, since the safety of the operators is also an aspect that has to be taken into account, higher levels of sedation are in our opinion advised. The inflammation and limited tissue damage observed on the vaccination site illustrates the adjuvant effect of electroporation and did not impact the dogs' welfare in any way.

Different DNA vaccines targeting VEGFR-2 have been evaluated in the past. This wide interest in VEGFR-2 vaccination demonstrates the potential of this approach. The majority of studies with VEGFR-2 DNA vaccines used a viral, bacterial or chemical vector to facilitate the delivery.[280,283,290-296] However, uniform large scale production is difficult with chemical and biological vectors, making clinical use outside a research context challenging.[276,277] Moreover, biological vectors also raise safety concerns.[40] Vaccination with naked plasmid is evaluated in three research papers.[297-299] Transfection efficiency of naked plasmid however is very low, making this approach far from optimal. However, when naked plasmid is delivered by the aid of a physical gene delivery method it is possible to combine the advantage of industrial suitable vaccine manufacturing with high transfection efficiency.[300] Moreover, physical gene delivery methods lack vector related toxicity issues. Electroporation mediated delivery of a VEGFR-2 DNA vaccine has only been evaluated once in a preclinical setting.[301] However, the vaccine in this study was developed to include only B cell epitopes, excluding cellular immune responses, whereas our vaccine was able to elicit both humoral and cellular immune responses.

As the immunogenicity and safety of this xenogeneic VEGFR-2 DNA vaccine is demonstrated in dogs and mice, the next step is the evaluation of its efficacy in animals with cancer. Spontaneously arising tumors in dogs can be a very valuable intermediate model, potentially limiting the now tremendous amount of negative phase I trials in human patients because of the low predictive power of rodent models.[302] Many tumors in dogs share biological, histological and clinical characteristics with their human counterparts and responses to treatment are very similar. Genetic research has revealed that tumors in humans and dogs undergo nearly identical genetic changes. An additional advantage of dogs is their body size, allowing surgical interventions, medical imaging and tissue/blood sampling much

like in human patient.[303] Several tumor vaccines have first been evaluated in canine patients, followed by a clinical trial in humans.[62,185,304,305] The evaluation of tumor vaccines in clinical trials with dogs can also lead to licensing of the vaccine for veterinary use. For example, the licensing of Oncept, a xenogeneic DNA vaccine encoding human tyrosinase for the treatment of melanoma in dogs has led to its widespread use in veterinary oncology.[306] Similar to humans VEGFR-2 overexpression is confirmed in numerous canine cancer types.[307-315] In conclusion, our data and the many reports on VEGFR-2 will facilitate the evaluation of our xenogeneic VEGFR-2 DNA vaccine in animals with cancer, ranging from preliminary rodent models to highly translational models of dogs with spontaneous tumors.

6. Acknowledgements

We would like to thank Bieke Weyn and Manuella Borloo for their assistance during the vaccinations of the laboratory dogs. We want to thank Prof. Douglas Thamm (College of Veterinary Medicine and Biomedical Sciences, Colorado State University, USA) for providing the canine HSA cell line. The financial support of Ghent University (BOF) is acknowledged with gratitude.

CHAPTER 4

Xenogeneic vascular endothelial growth factor-2 vaccination in tumor bearing mice

Sofie Denies, Laetitia Cicchelero, Niek N. Sanders

Chapter 4: Xenogeneic VEGFR-2 vaccination in tumor bearing mice.

1. Abstract

In this study a xenogeneic DNA vaccine encoding for human vascular endothelial growth factor receptor-2 (hVEGFR-2) was evaluated in two murine tumor models, the B16-F10 melanoma and the EO771 breast carcinoma model. The hVEGFR-2 DNA vaccine was administered by intradermal injection followed by electroporation. The immunogenicity and the biological efficacy of the vaccine was tested in (1) a prophylactic setting, (2) a therapeutic setting and (3) a therapeutic setting combined with surgical removal of the primary tumor. In the prophylactic and therapeutic setting, 14 vaccinated and 14 control mice were included per tumor model. Ten mice were followed for tumor growth and survival and 4 mice per group were sacrificed for biological read-outs. The systemic cellular immune response was measured by a bioluminescence based cytotoxicity assay with VEGFR-2 expressing target cells. Humoral immune responses were quantified by ELISA. *Ex vivo* bioluminescence imaging of organs was used to detect (micro)metastases. For the experiment where vaccination was combined with surgery, ten vaccinated and ten control mice were included per tumor model, and all ten were sacrificed three weeks after removal of the primary tumor for *ex vivo* bioluminescent quantification of (micro)metastases. A cellular and humoral immune response was present in prophylactically and therapeutically vaccinated mice, in both tumor models. Nevertheless, survival in prophylactically vaccinated mice was only moderately increased, and no beneficial effect on survival in therapeutically vaccinated mice could be demonstrated. Unexpectedly, the vaccine caused an increased quantity of early micrometastases in the liver. Lung metastases were not increased by the vaccine. These early liver micrometastes did however not grow into macroscopic metastases in either control or vaccinated mice when allowed to develop further after surgical removal of the primary tumor.

2. Introduction

It is well-known that the immune system is capable to recognize tumor cells and to establish a specific long term anti-tumor response.[5] Therefore, vaccination against tumor antigens holds great promises in the treatment for cancer. However, it has been difficult to translate the theoretical potential of tumor vaccines in clinical efficacy due to the capacity of tumor cells to create an immunosuppressive environment and to downregulate the expression of

tumor antigens.[265] Therefore, VEGFR-2 as vaccine target has some important advantages. Instead of the tumor cells, which are sculptured by the selective pressure of the immune system, VEGFR-2 vaccines target tumor associated endothelial cells, which have not acquired immune evasive strategies and are not prone to the development of escape mutants.[5,268] Additionally, it is known that certain tumor cells also express VEGFR-2. Therefore, the anti-angiogenic effect of VEGFR-2 based vaccines can be complemented by a direct anti-tumor action in these tumors.[269] Moreover, certain tumor associated regulatory T cells and myeloid derived suppressor cells express VEGFR-2, possibly enabling a self-enforcing mechanism of VEGFR-2 based vaccines, as depletion of these cells improves the efficacy of cancer vaccine.[5,270,271,316]

VEGF/VEGFR-2 targeting has been explored extensively with different treatment modalities, with some of them approved for clinical use.[268] In preclinical studies vaccination against VEGFR-2 has also been explored.[317] In these studies dendritic cells, DNA or proteins based VEGFR-2 vaccines have been tested. The advantage of DNA vaccines for tumor vaccination is that the antigens are synthetized intracellularly, leading to a robust cellular immune response.[273] The majority of studies with VEGFR-2 DNA vaccines used viral, bacterial or chemical carriers.[280,283,291-293,295,318-321] However, reproducible large scale production is difficult with chemical and biological carriers, making clinical use outside a research context challenging.[276,277] Moreover, biological carriers also raise safety concerns.[40] Vaccination with naked (i.e. unformulated) plasmid DNA (pDNA) encoding VEGFR-2 has been evaluated in a few publications.[297-299] However, the transfection efficiency of naked pDNA is very low, making this approach far from optimal. By using physical delivery methods, e.g. electroporation, it is possible to combine the manufacturing advantages of unformulated DNA vaccines with high transfection efficiency.[300] Moreover, physical delivery methods do also not have carrier related toxicity issues. Therefore, in this study, we evaluated for the first time the vaccination efficacy after electroporation of a naked pDNA vaccine encoding human VEGFR-2 (hVEGFR-2). Human VEGFR-2 is used as antigen, as xenogeneic vaccination can help to overcome immune tolerance.[267] Additionally, the development of metastases in different organs is monitored by bioluminescence imaging of excised organs, which allows the detection of micrometastases. This is necessary as it has been demonstrated, in a preclinical and clinical setting, that targeting VEGFR-2, by monoclonal antibodies as well as receptor inhibitors, can induce metastases.[322-324] In experiments with vaccination against

VEGFR-2, the effect of VEGFR-2 vaccination on metastases has been insufficiently examined.

3. Materials and Methods

Tumor cell lines, VEGFR-2 plasmid and mice

The B16-F10[325] and the EO771[326] tumor cell lines were generously provided by respectively Johan Grooten (Department of Biomedical Molecular Biology, Ghent University, Belgium) and Jo Van Ginderachter (Cellular and Molecular Immunology, VIB, Free University of Brussels). Both cell lines were transduced with luciferase as described earlier.[278] Briefly, retroviruses were produced in HEK293T cells by calcium phosphate transfection, harvested after 48 and 72h, filtered and concentrated by ultracentrifugation. Transduction was performed in the presence of 8 µg/ml polybrene and efficiency was evaluated by bioluminescence. Both cell lines were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, 100 IU/ml penicillin and 1 mmol/ml L-glutamine (Invitrogen, Carlsbad, CA, USA). For tumor inoculation, the cells were harvested by trypsinization and washed twice with calcium and magnesium free Dulbecco's phosphate buffered saline (PBS, Invitrogen). Human VEGFR-2 plasmid was purchased form Invivogen (Toulouse, France). A full description of the plasmid can be found on the website of the manufacturer.[279] Purification of the plasmids was done with the EndoFree Giga kit, following the instructions of the manufacturer (Qiagen, Valencia, CA, USA). For all experiments, C57BL/6JRj mice of 8 weeks were used (Janvier Breeding Center, Le Genest St. Isle, France).

Study design

The experiments were approved by the ethical committee of the Faculty of Veterinary Medicine (Ghent University; EC-DI 2014-45).

For prophylactic vaccination, 56 mice were randomly allocated into four groups of 14 mice. The first and third group were challenged with respectively B16-F10 and EO771 cells without prior vaccination (control groups, table 1). The second and fourth group were vaccinated and subsequently challenged with respectively B16-F10 or EO771 cells (Fig. 1

and table 1). The vaccination schedule consisted of three intradermal injections near the tail base of 25 µg human VEGFR-2 pDNA in calcium and magnesium free PBS followed by electroporation with the BTX Agilepulse device (Harvard apparatus, Holliston, MA, USA) using 4 mm gap needle electrodes. The following electroporation protocol was used: 2 pulses of 450 V with a pulse duration of 0.05 ms and a 300 ms interval, followed by 8 pulses of 100 V with a pulse duration of 10 ms and a 300 ms interval. The interval between the vaccinations was two weeks. One week after the final vaccination, the mice from group one and three were subcutaneously inoculated in the right flank with $2x10^5$ B16-F10 cells, while the mice from group two and four were inoculated with $5x10^5$ EO771 cells. Once weekly, tumor volume was measured with a digital caliper (formula: $1/2(\text{Length} \times \text{Width}^2)$). Additionally, the bioluminescence signal of the primary tumor was also measured 10 minutes after intraperitoneal injection of 100 µl of D-luciferin (15 mg/ml, Goldbio Technology, St. Louis, USA) with an IVIS lumina II (Perkin-Elmer, Zaventem, Belgium). Four mice of each group were randomly selected to be sacrificed three weeks after tumor inoculation, to isolate blood and immune cells from the spleen and to detect via *ex vivo* bioluminescence imaging (micro)metastases in the excised livers and lungs. The remaining mice (10 per group) were followed for tumor growth and survival and were euthanized when humane endpoints were reached. These humane endpoints included tumor size (> 1cm3), weight loss (>20%) or self-mutilation.

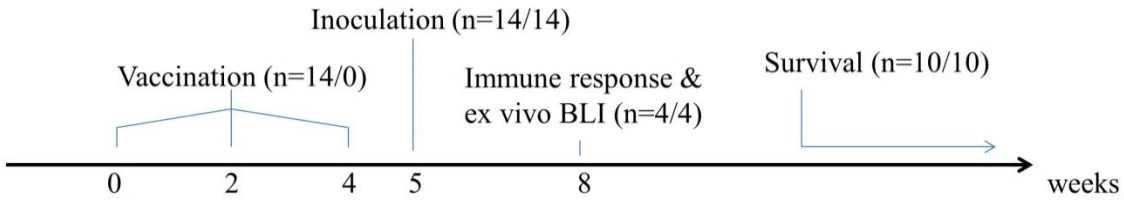

Fig. 1: Design of the prophylactic vaccination study (groups 1 to 4 in table 1). This experiment was performed in both the B16-F10 melanoma and EO771 breast carcinoma model. Sample size between parentheses represent mice numbers in vaccinated groups (left) and control group (right). BLI=bioluminescence imaging.

For therapeutic vaccination, 28 mice were inoculated in the flank with $2x10^5$ B16-F10 cells and another group of 28 mice with $5x10^5$ E0771 cells. As soon as tumors became palpable (circa 15 mm^3), mice were randomly allocated to serve as non-vaccinated control (n=14, group 5 and 7, table 1) or to receive the vaccination schedule (n = 14, group 6 and 8, table 1 and Fig. 2) described above. Due to rapid tumor growth, vaccination interval was adjusted to

one week. Tumor volume and bioluminescent signal of the primary tumor was measured weekly as described above. Four mice of each group were sacrificed one week after the last vaccination to measure immune responses and to screen for (micro)metastases in the livers and lungs via *ex vivo* bioluminescence imaging. However, because most mice in the control and vaccinated groups reached humane endpoints before this time, the mice that were sacrificed were not randomly selected as initially planned, but were instead the mice that were still alive at that time. Tumor growth and survival were analyzed for all 14 mice until the time of sacrifice, with mice sacrificed for the biological read-out censored.

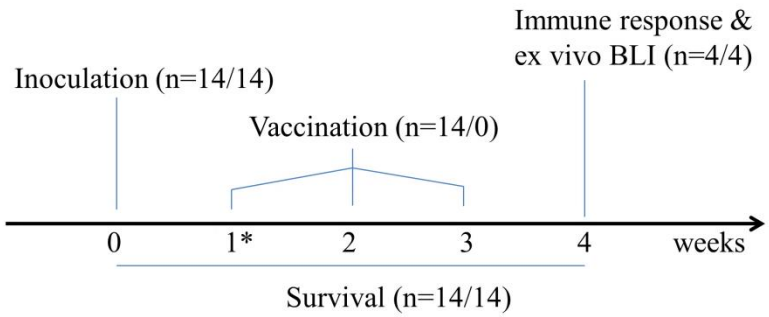

Fig. 2: Design of the therapeutic vaccination study (groups 5 to 8 in table 1). This experiment was performed in both the B16-F10 melanoma and EO771 breast carcinoma model. Sample size between parentheses represent mice numbers in vaccinated groups (left) and control group (right). BLI=bioluminescence imaging,*this time point varied individually per mouse depending on first appearance of a palpable tumor.

To study over a longer time period the effect of VEGFR-2 DNA vaccination on metastasis we surgically removed the tumor (Fig. 3). In more detail, 20 mice were inoculated with either B16-F10 or E0771 cells as described above. When tumors became palpable, mice were randomly allocated to serve as surgery-only control (n=10, group 9 and 11, table 1) or surgery-vaccination group (n=10, group 10 and 12, table 1). At this time point vaccination was started in the latter group and consisted out of three intradermal vaccinations with a time interval of one week (see above). Three weeks after inoculation, primary tumors (600-1000 mm^3) were surgically removed. Three weeks later, mice were sacrificed for the detection of macrometastases (visual inspection) and ex vivo bioluminescent imaging of organs (liver, spleen and lungs).

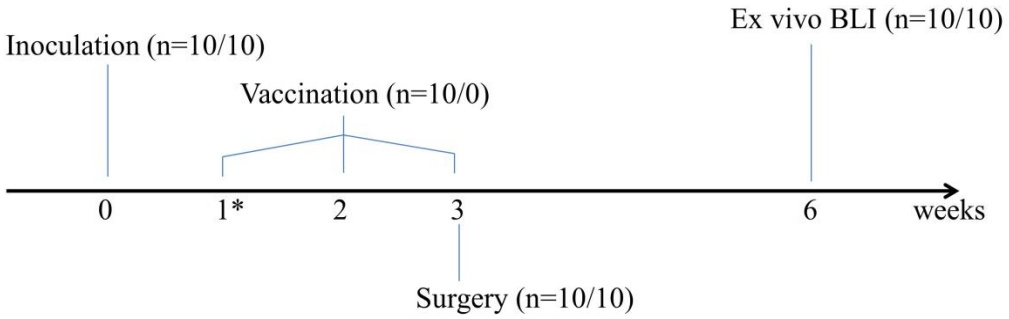

Fig. 3: Design of the surgery-vaccination study (groups 9 to 12 in table 1). This experiment was performed in both the B16-F10 melanoma and EO771 breast carcinoma model. Sample size between parentheses represent mice numbers in vaccinated groups (left) and control group (right). BLI=bioluminescence imaging,*this time point varied individually per moues depending on first appearance of a palpable tumor.

Follow-up of the immune response

In chapter 3 we have characterized the immune response after vaccination of healthy mice and dogs with hVEGFR-2 pDNA. In this study the most important assays were repeated. Splenocytes were isolated when mice were sacrificed. To assess cytotoxic response to cells expressing the target (i.e. VEGFR-2), splenocytes were coincubated with bioluminescent B16-F10 cells that had been *in vitro* electroporated with the VEGFR-2 pDNA (BTX ECM 830 electroporator, Harvard apparatus, Holliston, USA; 2 pulses of 140 V with a pulse duration of 5 ms and a 100 ms interval). As a control for specificity to the hVEGFR-2 target, B16-F10 melanoma cells that were mock electroporated were also included. Absence of natural VEGFR-2 expression on the bioluminescent B16-F10 cells and induction of expression after electroporation were assessed by flow cytometry with an anti-VEGFR-2 antibody combined with an Alexafluor-688 conjugated secondary goat anti-rabbit antibody (Abcam, Cambrige, UK). A cytotoxicity assay was performed by incubating $2x10^5$ splenocytes with $1x10^4$ B16-F10 cells. After 24 hours the bioluminescent signal, which is related to the number of living cells, was measured with an IVIS lumina II (Perkin-Elmer, Zaventem, Belgium).

Table 1: Overview of all experimental groups included in this study.

Group	Treatment	Inoculation	Follow-up
1	None	B16-F10	Immune response & ex vivo BLI (n=4) + survival (n=10)
2	hVEGFR-2 (P)		
3	None	EO771	
4	hVEGFR-2 (P)		
5	None	B16-F10	Immune response, ex vivo BLI & survival (n=14)
6	hVEGFR-2 (T)		
7	None	EO771	
8	hVEGFR-2 (T)		
9	Surgery	B16-F10	Ex vivo BLI (n=10)
10	Surgery+hVEGFR-2 (T)		
11	Surgery	EO771	
12	Surgery+hVEGFR-2 (T)		

hVEGFR-2: human vascular endothelial growth factor-2 vaccination; P=prophylactic, T=therapeutic; BLI=bioluminescence imaging

Blood was taken by cardiac puncture under terminal anesthesia when mice were sacrificed and after centrifugation serum was collected and stored at -80 °C until analysis. To assess humoral immune response, microtiter plates were coated with 1 µg murine VEGFR-2 protein, blocked with blocking buffer and loaded with serial dilutions of serum (Bio-Connect, Te Huissen, The Netherlands). After washing, a HRP-conjugated anti-mouse IgG antibody was added, followed by development with TMB substrate and measuring absorbance with a microplate reader at 450 nm (ImTec Diagnostics, Antwerp, Belgium). The titer was determined as the limited dilution with a signal exceeding mean plus two times the standard deviation of the signal from control samples (non-vaccinated mice).

Statistical analysis

Data were analyzed with the SPSS software version 19 (IBM, Brussels, Belgium). The effect of vaccination on the cytotoxicity and IFN-gamma secretion of murine splenocytes were analyzed with a student t-test. The effect of vaccination on antibody titers were analyzed with an exact linear by linear association test. For post hoc tests, correction for multiple comparisons was performed with the Tukey method. Survival between treatment groups were

compared with Kaplan-Meier analysis. Bioluminescence data was compared with a one-way ANOVA after log transformation.

4. Results

Immune response

There was a significant (p=0.001) cytotoxic T lymphocyte (CTLs) response in prophylactically vaccinated mice (Group 2 and 4) compared to control mice (Group 1 and 3). In more detail, 4 weeks after the last vaccination (i.e. three weeks after tumor inoculation) lymphocytes from the B16-F10 and EO771 challenged mice killed, on average, 44% more VEGFR-2 positive target cells than lymphocytes from non-vaccinated mice (Fig. 4A). Additionally, vaccinated mice also developed a significant humoral response, with antibody titers in the vaccinated groups ranging from 1:400 to 1:1600, whereas in the control groups no antibody response could be detected (p<0.001, table 2).

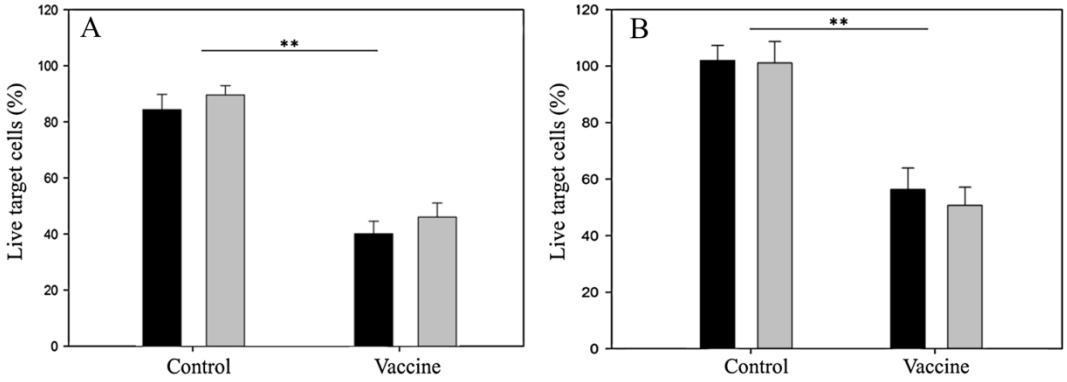

Fig. 4: Splenocytes of prophylactically (4A) or therapeutically (4B) vaccinated (groups 2, 4 and 6, 8 from table 1) and control mice (groups 1, 3 and 5, 7 from table 1) were coincubated with VEGFR-2 expressing target cells. After 24h, a significant cytotoxic response of splenocytes from vaccinated mice is evident. (black: B16-F10 bearing mice, grey: EO771 bearing mice) (**p<0.01)

A similar systemic immune response was present in the therapeutically vaccinated mice. One week after the last vaccination (i.e. four weeks after tumor inoculation) splenocytes from the B16-F10 and EO771 bearing mice (Groups 6 and 8) killed, on average, 40 % more VEGFR-2 positive targets than lymphocytes from non-vaccinated mice (Groups 5 and 7, Fig. 4B). Antibody responses in the therapeutically vaccinated mice were also higher than in control mice (p<0.001, table 2). No difference in immune response between tumor models or between vaccination schedules (prophylactic/therapeutic) were evident.

Table 2: Number of mice in each group per antibody titer

Treatment group	Antibody titer		
	400	800	1600
Group 2	1	3	-
Group 4	-	2	2
Group 6	3	1	-
Group 8	1	1	2

Groups: see table 1. Non-vaccinated mice are not shown in the table as all control mice had no detectable antibody levels.

Effect of prophylactic vaccination on tumor growth and survival

The VEGFR-2 DNA vaccine was able to slow down the growth of B16-F10 and EO771 tumors when mice were vaccinated before they were inoculated with the tumor cells (Fig. 5 and 6). The bioluminescence signal of the tumor followed a similar trend as the tumor volume (data not shown). This decrease in tumor growth led to a modest increase in survival of the vaccinated mice (Fig. 5 and 6). In detail, vaccination was able to significantly increase median survival of B16-F10 challenged mice from 25 days to 30 days, and of EO771 challenged mice from 38 days to 48 days (Fig. 2, p=0.004 and p=0.002 respectively). However, prophylactic vaccination was not protective as all vaccinated mice developed a tumor.

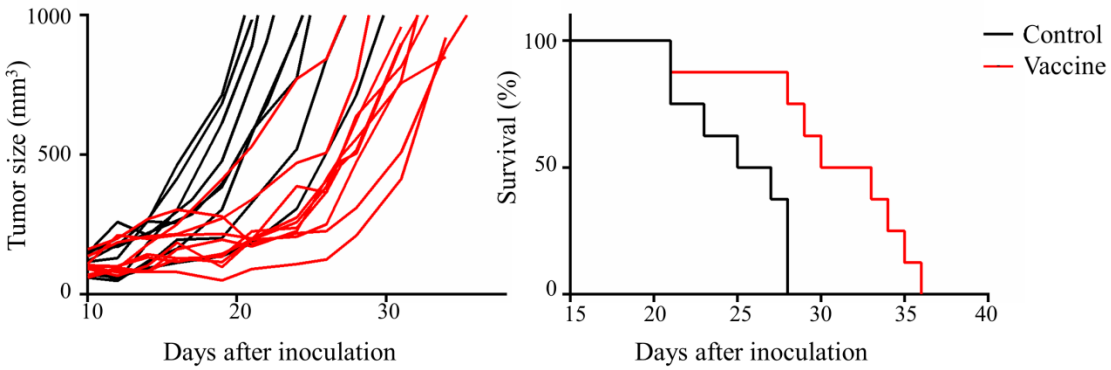

Fig. 5: Individual tumor growth curves (left) and survival curves (right) for prophylactically vaccinated (group 2) and control mice (group 1) inoculated with B16-F10.

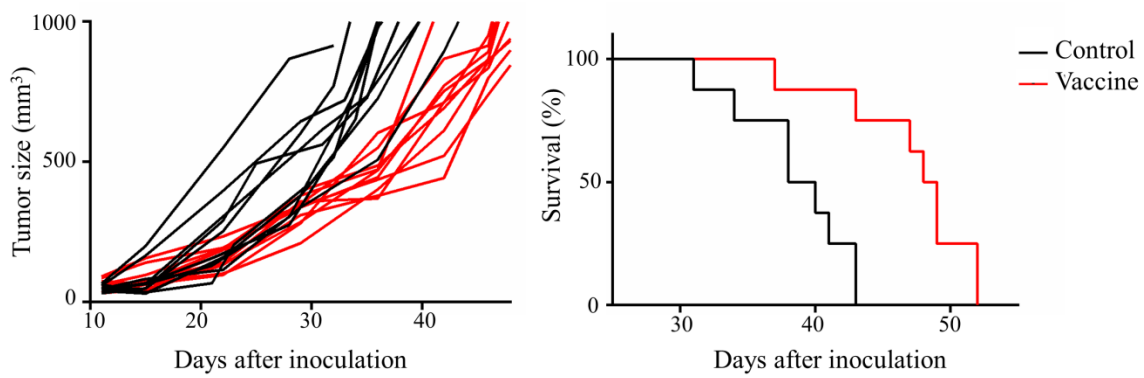

Fig. 6: Individual tumor growth curves (left) and survival curves (right) for prophylactically vaccinated (group 4) and control mice (group 3) inoculated with EO771.

Effect of therapeutic vaccination on tumor growth and survival

The VEGFR-2 DNA vaccine was subsequently tested in mice that already had a tumor. The volumes of the B16-F10 and EO771 tumors did not significantly decrease after vaccination with the VEGFR-2 DNA vaccine and survival was not affected by vaccination (Fig. 7 and 8). Only a proportion of mice survived long enough to receive the complete rounds of vaccinations.

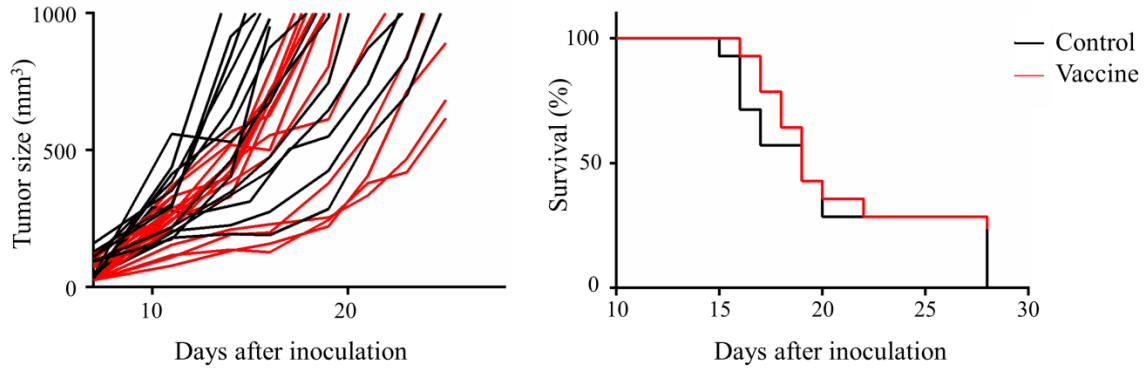

Fig. 7: Individual tumor growth curves (left) and survival curves (right) for therapeutically vaccinated (group 6) and control mice (group 5) inoculated with B16-F10.

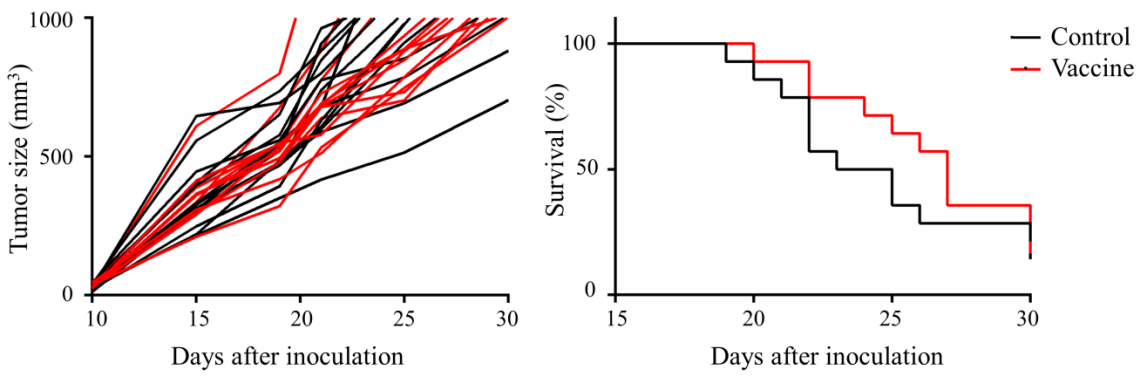

Fig. 8: Individual tumor growth curves (left) and survival curves (right) for therapeutically vaccinated (group 8) and control mice (group 7) inoculated with EO771.

At day 27, i.e. 6 days after the last vaccination, clear differences between the bioluminescence patterns of the tumors of vaccinated and non-vaccinated were noticed (Fig. 9). Indeed, the ratio of the tumor bioluminescence to the tumor volume was significantly lower for vaccinated then control mice, suggesting less viable tumor mass in the vaccinated mice ($p=0.01$, Fig. 10). It is important to note that these measurements are not from randomly selected mice per group, but only from mice in each group that survived up until this time point (4 control mice and 5 vaccinated mice for B16-F10 and 4 control mice and 8 vaccinated mice for EO771). At earlier time points there was no difference in bioluminescence signal between vaccinated and non-vaccinated mice (data not shown).

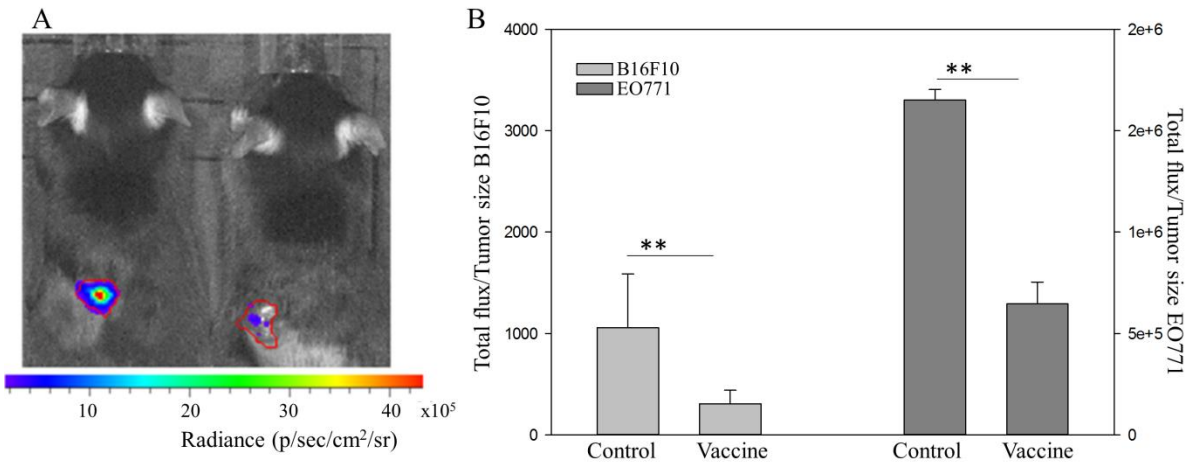

Fig. 9: Bioluminescence signals of the tumors of the therapeutically vaccinated mice. (A) Representative *in vivo* bioluminescence image of a control (left) and vaccinated (right) B16-F10 tumor mouse 4 weeks after tumor inoculation (i.e. 1week after the last vaccination) (B) Bar chart of the ratio of the tumor bioluminescent to the tumor volume one week after the third therapeutic vaccination. (**$p<0.01$)

Metastases

Development of metastases in B16-F10 tumor mice

Prophylactic and therapeutic vaccination had the same effect on the development of metastases. Four weeks after the last prophylactic vaccination (i.e. 3 weeks after tumor inoculation) only microscopic metastases were detected in the liver of vaccinated and control mice (Fig. 10). The metastases were visible as multiple bioluminescent spots. Remarkably, the total bioluminescence signal of these spots was significant higher in the vaccinated than in the non-vaccinated mice (Fig. 10). Similarly, mice with established tumors before vaccination (i.e. therapeutic vaccination) had one week after the last vaccination (i.e. 4 weeks after tumor inoculation) only microscopic metastases in the liver. Interestingly, the bioluminescence signal of the metastases in these therapeutically vaccinated mice was again significantly higher than the bioluminescence signal of the metastases of the control mice (Fig. 10). No metastases in the lungs could be detected.

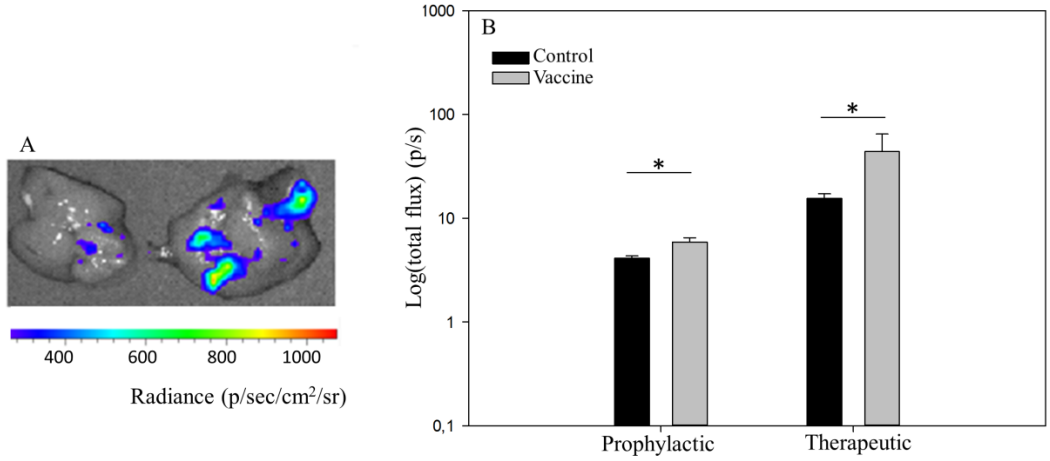

Fig. 10: B16-F10 liver metastases in control, prophylactic and therapeutic vaccinated mice. (A) Representative bioluminescence image of the liver of a control (left) and vaccinated mouse (right). (B) Bar chart showing the bioluminescent signal in livers of control mice and prophylactic and therapeutic vaccinated mice. (*$p<0.05$)

Development of metastases in EO771 tumor mice

Four weeks after the last prophylactic vaccination (i.e. three weeks after tumor inoculation) only micrometastases were detected in the liver and lung of prophylactic vaccinated and control mice. The metastases were visible as bioluminescent spots. Again, the average total bioluminescence signal of these spots was significantly higher in the vaccinated than in the non-vaccinated mice (Fig. 11). Likewise, therapeutic vaccinated mice had, one week after the

last vaccination (i.e. 4 weeks after tumor inoculation), only microscopic metastases in the liver and lung and also in this tumor model the bioluminescence signal of these metastases in the liver of vaccinated mice was significantly higher than the bioluminescence signal of the liver metastases of the control mice (Fig. 11). On the other hand, the bioluminescence signal of the lung metastases in the vaccinated mice was not significantly different from the bioluminescence signal of the lung metastases in the control mice (Fig 11).

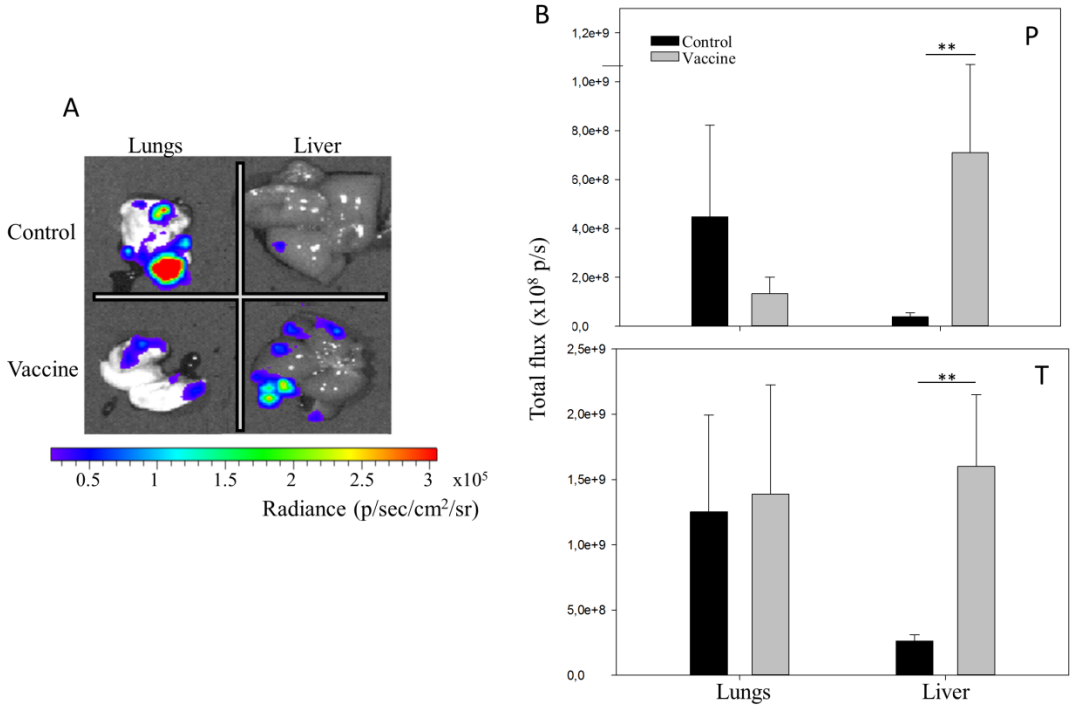

Fig. 11: EO771 lung and liver metastases in control, prophylactic and therapeutic vaccinated mice. (A) Representative bioluminescence image of the lung and liver of a control (upper row) and a vaccinated mouse (lower row). (B) Bar chart showing the bioluminescent signal in the lungs and livers of control mice and prophylactically (upper) and therapeutically (lower) vaccinated mice. (**p<0.01)

Long term effect of vaccination on metastases

When metastases were allowed to develop further for three weeks after surgical removal of the primary tumor (6 weeks after inoculation), we found in both tumor models small macroscopic metastatic nodules in the lungs of therapeutic vaccinated mice as well as control mice (Fig. 12).

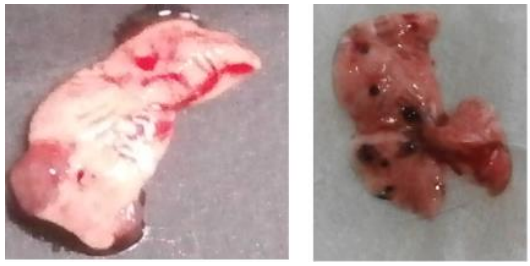

Fig. 12: Representative images demonstrating the size of macroscopic tumor nodules in the lungs from animals three weeks after surgical removal of the primary tumor (left: EO771/right: B16-F10). There was no difference between control and vaccinated animals.

On visual inspection as well as by bioluminescence measurements there was no difference between control mice and vaccinated mice (data not shown). In the liver the metastases remained microscopic in both tumor models. In control mice, the total bioluminescence signal of these liver metastases (6 weeks after inoculation) was increased in comparison with earlier time point (3 and 4 weeks after inoculation). In vaccinated mice, there was no further increase in bioluminescent signal of the liver micrometastases compared to earlier time points. As a consequence the bioluminescence signals of the metastases in the livers and lungs were, three weeks after removal of the primary tumor, not significant different between vaccinated and control mice. This was true for both tumor models.

5. Discussion

In this study, VEGFR-2 vaccination was evaluated in three different settings in two tumor models (B16-F10 and EO771). The three settings we tested were: (1) prophylactic vaccination, (2) therapeutic vaccination and (3) therapeutic vaccination in combination with surgery. In a prophylactic setting, survival of vaccinated mice was modestly increased. In a therapeutic setting, only a small proportion of mice survived long enough to receive the complete rounds of vaccinations, as both B16-F10 and EO771 are very aggressive tumor models. The time necessary to develop an anti-tumor immune response and associated clinical benefit is also an issue in clinical immunotherapy.[265] Although tumor size was comparable between control and vaccinated animals after therapeutic vaccination, the ratio of bioluminescent signal per tumor volume was significantly different. This confirms that classical volumetric tumor measurements are not ideal to detect a treatment effect of anti-VEGF/VEGFR-2 agents.[327] Our fining corresponds with other reports of loss of viable tumor

mass without significant alterations in tumor size measurements after treatment with anti-VEGF/VEGFR agents.[327] Although it is difficult to compare between studies, as often tumor type and vaccination scheme are different, our effect on primary tumor growth and survival seems less than that for other VEGFR-2 vaccine formulations. This coincides with a more limited humoral immune response in our study compared with others, whereas cellular immune responses are comparable. This link between humoral immune response and clinical efficacy for VEGFR-2 vaccination is confirmed by the study of Xu and others.[298] In this study mice were also vaccinated with naked plasmid, but without electroporation, and they observed a low humoral response associated with only a limited effect on tumor growth. After conjugation with the C3d molecular adjuvant, humoral responses were markedly increased and this corresponded with a much more pronounced effect on tumor growth. Limited humoral immune responses after DNA vaccination are due to the fact that only very small amounts of proteins are secreted.[282] Improving humoral immunity of our vaccine, without losing the advantage of a safe and easy to manufacture formulation, could be attempted by increasing DNA dose, combining it with a protein vaccine or incorporating molecular adjuvants.[298,328,329]

DNA vaccination with human VEGFR-2 had a clear effect on metastasis. At an early time point (i.e. 3-4 weeks after tumor inoculation) liver micrometastases were significant higher in both tumor models after prophylactic and therapeutic vaccination. In contrast, the micrometastases in the lungs of EO771 mice were not significantly different between prophylactic/therapeutic vaccinated and non-vaccinated mice. Based on these observations it seems that the seeding of circulating tumor cells (CTCs) in the liver is easier in vaccinated mice than in control mice. Probably VEGFR-2 vaccination caused hypoxia in the primary tumor, which is in agreement with a lower bioluminescence signal of the primary tumor in the vaccinated mice. Hence, CTCs of VEGFR-2 vaccinated mice are probably more adapted to a hypoxic environment than the CTCs of non-vaccinated mice and this allows them to proliferate sooner after dissemination in the liver, which is known to be a relative hypoxic organ.[330] On the other hand, the lungs have high basal levels of oxygen perfusion. In such an oxygen rich environment CTCs of vaccinated mice which are adapted to a hypoxic milieu, do not have an advantage over CTCs of non-vaccinated for colonization.[330] This may explain the differential effect of VEGFR2 vaccination on lung and liver metastases. It has been demonstrated in the past that hypoxia in the primary tumor caused by anti-angiogenic

treatment can influence multiple crucial steps in the metastatic process.[327,331] Additionally, an association between hypoxic markers in the primary tumor and liver metastases has been demonstrated earlier.[332] We report, to our knowledge, for the first time that VEGFR-2 vaccination promotes early liver metastases. In the past, the effect of VEGFR-2 vaccination on spontaneous arising metastases has been investigated in three studies. However, only metastases in the lungs and not the livers were examined in these studies.[280,292,333,334] The other studies that investigated the effect of VEGFR-2 vaccination on metastasis used an experimental metastasis model that consisted of the injection of tumor cells in the tail vein.[291,294,295,318,320,335,336] With such metastatic models it is not possible to evaluate the effect of treatments that modulate the metastatic capacity of CTCs originating from a primary tumor.

When metastases were allowed to develop further after surgical removal of the primary tumor, both EO711 and B16-F10 tumor bearing mice developed macroscopic lung metastases, while the liver metastases stayed microscopic. Interestingly, the micrometastases in the liver of control animals increased to the same level as those in vaccinated mice, whose metastases had not increased compared to earlier time points. This indicates that for B16-F10 and EO771 cells the liver is not an adequate metastatic site for the development of macroscopic metastases, which is in agreement with previous findings.[337,338] The higher metastatic capacity of tumor cells from VEGFR-2 vaccinated mice thus only applies for initial colonization of the liver, but not for macroscopic tumor growth. Indeed, forming micrometastases is a result of the capacity to survive in the foreign environment of the distant organ, whereas forming macroscopic metastases is another process depending on many other factors.[339] So, on the long term no overall increase in liver metastases between VEGFR-2 vaccinated and control mice is observed. Furthermore, no difference in the development of lung metastases between control and vaccinated animals was observed, in contrast to previous studies that found decreased lung metastases in VEGFR-2 vaccinated mice.[280,333,334] Next to different tumor types used in the other studies compared to ours, another possible explanation is the time of read-out. Metastatic tumor nodules in our experiment were considerably smaller than those reported in other studies. It is possible that the effect of VEGFR-2 vaccination on metastases becomes more pronounced with increasing metastatic tumor size, as the dependence on neovascularization will increase accordingly.

6. Conclusion

In summary, the VEGFR-2 DNA vaccine resulted in a limited effect on primary tumor growth, which may be enhanced by adjusting the vaccination schedule to induce a more potent humoral immune response. Interestingly, for the first time, we have shown VEGFR-2 vaccination to affect metastatic behavior, in two different tumor models. This consisted of accelerated occurrence of liver micrometastases. As for both tumor models the liver is not an adequate site for the development of macrometastases, the clinical consequences of this phenomenon cannot be evaluated. Therefore, it would be highly interesting to investigate this in tumor models with the liver as preferential site of metastasis. Moreover, further research regarding the mechanisms behind this process could identify anti-metastatic complementary treatments, which may significantly improve efficacy of VEGFR-2 vaccination as well as other anti-angiogenic treatment.

CHAPTER 5

In vitro exploration of a myeloid derived suppressor cell line as vehicle for cancer gene therapy

Sofie Denies, Sean McCafferty, Cynthia Ghekiere, Laetitia Cicchelero, Niek N. Sanders

This chapter contains preliminary data

Chapter 5: In vitro exploration of a MDSCs cell line as vehicle for cancer gene therapy

1. Abstract

Recent research has demonstrated that cell-mediated gene therapy can be an interesting method to obtain local intratumoral expression of therapeutic proteins. In cell-mediated cancer gene therapy, cells that naturally home to tumor tissue are *in vitro* transfected with the protein of interest followed by systemic infusion of the cells. Myeloid derived suppressor cells (MDSCs) home strongly to tumor tissue. In this work we explored the possibility to use MDSCs, derived from a murine cell line, as a cellular vehicle to transport anti-cancer agents like interleukin-12 (IL-12) to tumors. A drawback of using MDSCs as cellular vehicles for tumor targeted delivery is that they cause immunosuppression. Therefore, IL-12, a very potent anti-tumor cytokine, was used because it can reprogram the immunosuppressive MDSCs into immune stimulating immune cells. We demonstrated *in vitro* that the cell line derived MDSCs strongly migrate towards tumor cells. The transfectability of these cells with electroporation and lipofectamine 2000 was evaluated with reporter plasmids. DNA electroporation was not an adequate method as it caused massive cell death. This was not due to electroporation-induced cell damage, but due to the intracellular presence of plasmids. In contrast, cell death was not significant after transfection with lipofectamine 2000. However, transfection efficiency remained modest (40%) and transgene expression was almost completely lost after 72h. Nevertheless, we demonstrated, with an IL-12 encoding plasmid, that this relatively low percentage of transfected MDSCs could produce biologically relevant IL-12 amounts. Additionally, the produced IL-12 caused an upregulation of CD80, which is a confirmation that IL-12 decreases the immune suppressive capacities of MDSCs. However, in a preliminary *in vitro* cytotoxicity assay we found that IL-12 transfection reduced, but not completely eliminated the immunosuppressive capacity of the MDSCs. In conclusion, our data show that the MDSC cell line can be transfected with lipofectamine 2000. Additionally, the MDSC cell line has the tumor migratory capacity to be used as a cellular vehicle for cancer gene therapy. However, the immunosuppressive effects of these cells will be an important challenge for their use. In that context, an efficient transfection protocol, leading to a higher percentage of IL-12 transfected MDSCs and a higher concentration of IL-12 is critical.

2. Introduction

Myeloid derived suppressor cells (MDSCs) are immature myeloid cells that are strongly immunosuppressive and pro-angiogenic. They are induced by tumor derived cytokines and accumulate into the tumor micro-environment to protect tumor cells from immune destruction and promote angiogenesis. As these are crucial mechanisms for tumor progression, MDSCs have been identified as important targets in the treatment against cancer.[340] However, their ability to migrate to tumor tissue can also be exploited by using these cells as vehicles to deliver anti-tumor agents specifically to tumor cells.[341] This includes, but is not limited to, cell-mediated gene therapy.[342] Gene therapy has gained much attention as a treatment modality against cancer. Amongst other applications, cytokine therapy by gene transfer is highly interesting, considering the short half-life and potential toxicity of most cytokines impeding systemic use. Gene therapy can provide a constant source of local cytokine production within the tumor, eliminating the need for frequent administration of high amounts of protein to obtain similar effects.[343] However, many gene therapy approaches rely on local applications, either by practical constraints due to the method of gene transfer (physical methods) or by the lack of specificity after systemic injection (chemical or viral methods).[344]

Mesenchymal stem cells (MSCs) are currently the most common cell type that is explored for intratumoral delivery of drugs.[345] However, MSCs also have tumor-stimulating properties. In addition, the large size of MSCs poses problems with passing the capillary bed in the lungs after systemic administration.[346] MDSCs are much smaller and as immune cells more fit to efficiently migrate through the body. Tumor infiltrative T cells or genetically modified T cells are other cell types used for tumor-specific delivery.[347] They have the advantage of being anti-tumoral in contrast to MDSCs and MSCs. However, as part of their immune-evasive strategies, tumors do not secrete cytokines to attract T cells and T cells that do extravasate will have to deal with a microenvironment that is very hostile for T cells. MDSCs are strongly attracted by tumor cells and the tumor microenvironment supports their survival and expansion. Indeed, the excellent tumor specific accumulation of transferred MDSCs compared to other immune cells has been recently demonstrated.[341] A recent study with freshly isolated syngeneic MDSCs as carriers for an oncolytic virus proves that the overall outcome of MDSCs-mediated therapy can be tumoricidal.[341] Additionally, it has been

demonstrated that MDSCs can be reprogrammed in in immune stimulating immune cells by interleukin-12 (IL-12).[126,132] Therefore, it is possible that for the delivery of IL-12, the pro-tumor characteristics of MDSCs are of less importance, if they lose this trait under influence of the IL-12 they produce.

IL-12 is also a very potent anti-tumor cytokine that stimulates T cells and natural killer (NK) cells to attack tumor cells. However, IL-12 results in major toxicity when delivered systemically.[133] Therefore, intratumoral administration of viral or non-viral vectors that encode IL-12 has been used in many pre-clinical and clinical studies.[180] However, a disadvantage of intratumoral IL-12 gene transfer is that it is difficult to apply to internal tumors and metastases whose exact location is not always known. Therefore, specific targeting of IL-12 to these hard-to-reach tumors after systemic administration would be a breakthrough.

In the past a murine MDSC cell line has been established.[348] Using a cell line instead of freshly isolated MDSCs greatly facilitates preclinical evaluation as it requires fewer animals and generates more reproducible data. In this study, we investigated if this previously described MDSC cell line can be transfected with reporter plasmids by physical and chemical methods. These methods have the advantage over viral gene transfer to be more versatile, thus allowing for quick evaluation of different therapeutic molecules. Transfection with IL-12 was performed to quantify IL-12 production capacity of this approach and to investigate the effect of IL-12 transfection on the immunosuppressive capacity of the MDSCs. Lastly, we investigated by an *in vitro* assay if this MDSC cell line has retained its capacity to migrate in response to tumor secreted cytokines.

3. Materials and Methods

Cell lines and plasmids

The MDSC cell line was generously provided by Vincenzo Bronte (Department of Oncology and Surgical Sciences, Padova, Italy). These cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, 100 IU/ml penicillin and 1 mmol/ml L-glutamine (Invitrogen, Carlsbad, USA). pGL4 luciferase

plasmid (Promega, Massachussets, USA) and eGFP-C1 plasmid (generously provided by K. Remaut, laboratory for general biochemistry and physical pharmacy, Ghent University, Belgium) were used as reporter plasmids. For IL-12 transfections, murine pORF-mIL-12 plasmid was used (Invivogen, Toulouse, France). All plasmids were purified with Qiagen Endofree Giga purification kit following instructions of the manufacturer (Qiagen, Valencia, USA).

Electroporation

In vitro electroporation was performed with the ECM830 device (BTX Harvardt apparatus, Holliston, Massachusetts, USA). After initial optimization (range 100-500 V), the optimal electroporation protocol consisted of 2 pulses of 140 V and 5 ms length with an interval of 100 ms. One million cells were transfected in 2 mm cuvettes with 5 µg pGL4 plasmid in 80 µl calcium and magnesium free phosphate buffered saline (PBS, Invitrogen). Also cells that underwent electroporation without plasmid were included. After electroporation, cells were transferred to a 96 well plate and bioluminescent signal was measured with an optical imaging system after 4h, 24h, 48h and 72h (IVIS Lumina II, Perkin-Elmer, Zaventem, Belgium). Viability of the cells was evaluated 24h after electroporation with AlamarBlue (Invitrogen) and by microscopic evaluation. Electroporation and biological read-outs were performed at least in duplicate per experiment and repeated once in an independent experiment.

Lipofection

Lipofection was performed with lipofectamine 2000 (LF2000, Invitrogen) with optimization following the instructions of the manufacturer. Following ratios of volume (µl) LF2000 to weight (µg) of plasmid DNA (pDNA) were evaluated: 1:1, 1:1.25, 1:1.7, 1:2.5. Cells were seeded 24h before lipofection in a 96 well plate at two densities, $5x10^3$ and $1x10^4$ cells in 100 µl. Lipofection was done with 100 ng pGL4 plasmid per well in serum containing medium without antibiotics. All transfections performed to find the optimal protocol were performed in duplicate per experiment and repeated once in an independent experiment. Viability was evaluated by flow-cytometric determination of cell numbers within healthy FSC-SSC profile that were negative for 7-AAD (Ebioscience, Vienna, Austria). Additionally, 24h after transfection cell viability was also qualitatively evaluated by microscopic evaluation. Flow cytometry was done with an Accuri C6 flow cytometer (BD, Biosciences, Erembodegem,

Belgium). With the most optimal transfection condition (LF2000:pDNA ratio 1:1.25, and seeding density of $5x10^3$ MDSCs/96 well) we studied the evolution of the luciferase expression by measuring the bioluminescence 24h, 48h and 72h after transfection. The number of transfected MDSCs was determined by flow cytometry after transfection with an eGFP plasmid. All transfections were performed in triplicate.

IL-12 transfection and effect on costimulatory molecules

MDSCs were transfected with IL-12 plasmid using the optimal protocol identified for LF2000. After 24h, medium was collected and stored at -20 °C. The concentration of IL-12 in the medium was determined with an ELISA following the instructions of the manufacturer (ImTec Diagnostics, Belgium, Antwerp). To study the effect of IL-12 plasmid transfection on the expression of costimulatory molecules by MDSCs we stained the MDSCs 24h after transfection with the pGL4 plasmid (control) or the IL-12 plasmid with FITC-conjugated anti-CD40, APC-conjugated anti-CD80 and PE-conjugated anti-CD86 antibodies (Ebioscience). The cells were subsequently analyzed with an Accuri C6 flow cytometer. Color compensation was set based on fluorescence minus one controls. Transfections and read-outs were performed in triplicates and positive results confirmed by an independent experiment with four replicates.

Effect of IL-12 transfection on the immunosuppressive capacity of MDSCs

To study the effect of IL-12 transfected MDSCs on the tumor cell killing capacity of lymphocytes we added non-transfected MDSCs and IL-12 transfected MDSCs to co-cultures of luciferase positive B16-F10 cells [278] and freshly isolated splenocytes from healthy 12 week old C57BL/6 mice (Janvier Breeding Center, Le Genest St. Isle, France). Co-cultures of B16-F10 cells with freshly isolated splenocytes and B16-F10 alone served as additional controls. The ability of splenocytes to interfere with B16-F10 growth was measured by comparing the bioluminescent signal from the co-incubation conditions to the bioluminescent signal from B16-F10 alone. Splenocytes were co-cultered with tumor cells in a 20:1 ratio and MDSCs were added in a 1:10 ratio with tumor cells. After 24h, the bioluminescent signal was measured with an optical imaging system (IVIS Lumina II). Four replicates were included, each with splenocytes from separate mice.

In vitro migration assay

To investigate if the cell line derived MDSCs have retained their ability to migrate in response to tumoral cytokines, an *in vitro* migration assay was performed with 24 well 8µm transwells (VWR, Leuven, Belgium). B16-F10 cells were stained with CFSE (ImTec Diagnostics) and $1x10^4$ cells seeded in the bottom compartment of the transwells. In control wells, serum containing medium without any cells was added in the bottom compartment. After 24h, $2x10^5$ MDSCs in serum free medium were added on the upper compartment. After 4h, nonadherent and adherent cells in the lower compartment were collected by centrifuging the medium and by trypsinization following instructions of the manufacturer (VWR). CFSE negative cells representing the migrated MDSCs were counted by flow cytometry. Specific migration towards tumor cells was calculated by the ratio of migrated MDSCs in compartments with tumor cells to migrated MDSCs in compartments with medium. The assay was performed in triplicate.

Statistical analysis

Data were analyzed with the SPSS software version 19 (IBM, Brussels, Belgium). Results of the AlamarBlue assay and the immune assay were analyzed with Kruskal-Wallis test. Flow cytometric data and results of the migration assay were analyzed with a student t-test.

4. Results

Electroporation mediated transfection of MDSCs

Electroporation of MDSCs with a luciferase plasmid resulted in a clear bioluminescent signal after 4h, demonstrating efficient uptake of the plasmid. However, after 24h, a tremendous decrease in signal was observed, leading to loss of signal after 72h (Fig. 1). This is explained by significant ($p=0.02$) loss of viability of transfected cells 24h after electroporation (Fig. 2). The viability, assessed by AlamarBlue, of MDSCs electroporated with plasmid was only 54% compared with untreated cells and on microscopic evaluation electroporated cells showed signs of apoptosis. This cell death was not caused by electroporation-induced cell damage, as such a massive cell death was not observed in cells electroporated without plasmid. Indeed, MDSCs that were electroporated without plasmid had on average 90% viability compared to untreated cells and did not show abnormalities on microscopic evaluation (Fig. 2).

Lipofectamine mediated transfection of MDSCs

As electroporation is not an adequate method for transfecting MDSCs, transfection with LF2000 was evaluated. Optimization of the transfection protocol of LF2000 was done with a luciferase plasmid and by measuring the bioluminescent signal 24h after transfection. The best results were obtained at LF2000:pDNA ratio of 1:1.25 (volume:weight ratio; (µl:µg)) and a seeding density of $5x10^3$ MDSCs per 96 well (Fig. 3). After 24h the viability of the transfected MDSCs was, compared to untreated MDSCs, on average 85% (95% CI 63-94%).

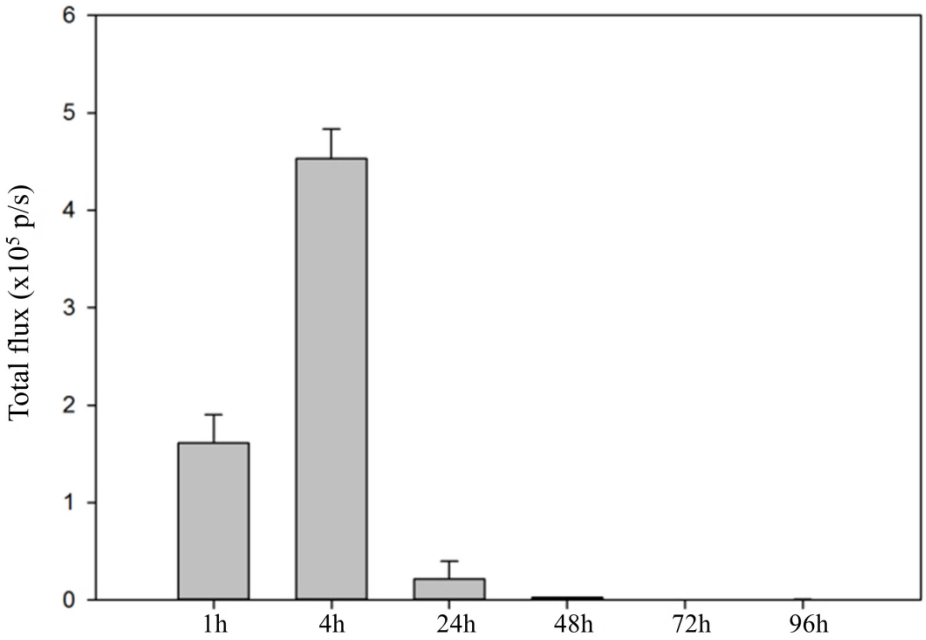

Fig. 1: Transfection of MDSCs with electroporation. MDSCs were electroporated with 100 ng pGL4 plasmid and the bioluminescent signal was followed as a function of time. (n=2 and repeated once in an independent experiment)

The bioluminescence signal of a fixed number of cells decreases after 48h to less than half of its signal 24h after transfection. However, the total signal after 48h, bioluminescence signal per cell multiplied by the total amount of cells after 48h, is not significantly different from the total signal after 24h. This indicates that the drop in bioluminescence signal per cell is mainly due to a dilution of the luciferase, and hence also of the plasmids, during cell division. Consequently, after 48h degradation of plasmids is not responsible for the drop of the bioluminescence signal in each cell. However, between 48h and 72h, the bioluminescence signal per cell decreases faster than that the cells increase in number and hence the total

bioluminescence signal also decreases significantly compared to 24h and 48h after transfection. Plasmid degradation or transgene silencing becomes thus much more important after 48h (Fig. 4). Cells were also transfected with eGFP to quantify the proportion of positive cells 24h after transfection. On average, 43 ± 3% of the cells were positive. Further kinetic studies with the eGFP plasmid were not performed, as the long half-life of eGFP (24h) complicates interpretation.

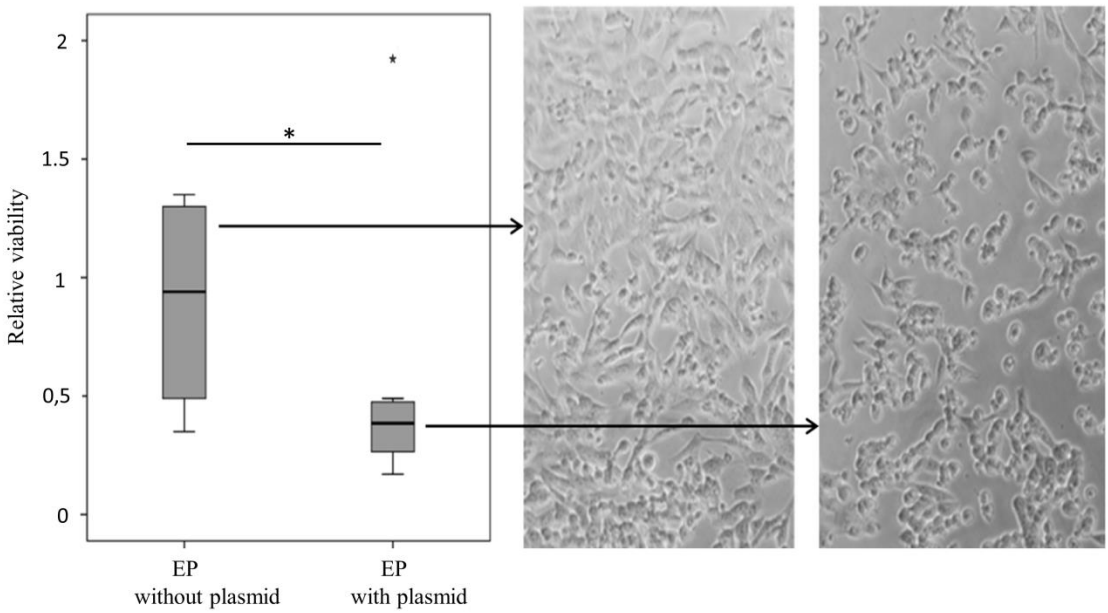

Fig. 2: Relative viability of MDSCs 24h after electroporation with and without pGL4 plasmid compared to untreated cells measured by AlamarBlue (left) and by microscopic evaluation (right). EP = electroporation (n = 4 and repeated in an independent experiment, * $p < 0.05$)

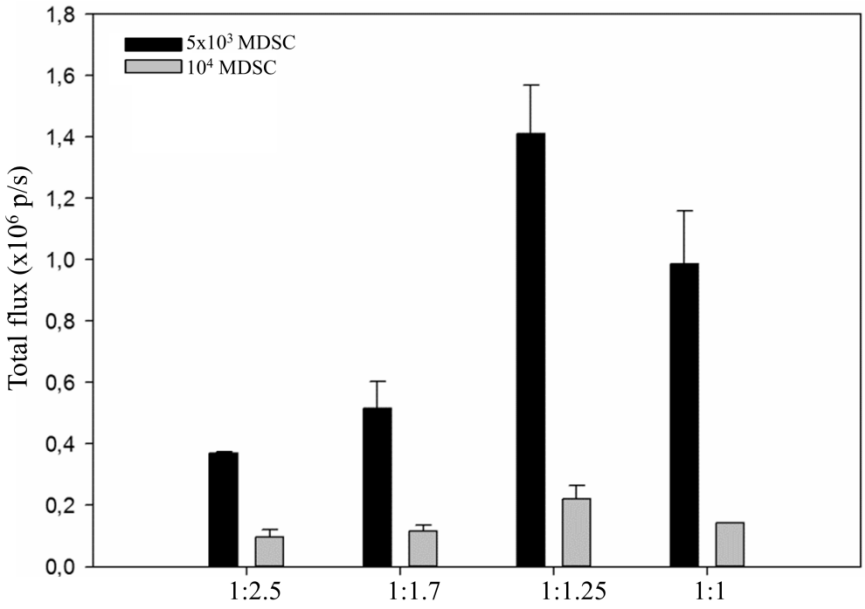

Fig. 3: Bioluminescent signal 24h after lipofectamine 2000 mediated transfection of MDSCs with a luciferase encoding plasmid. Different lipofectamine:pDNA ratios (x-axis) and two cell densities were evaluated. (n=2 and repeated in an independent experiment)

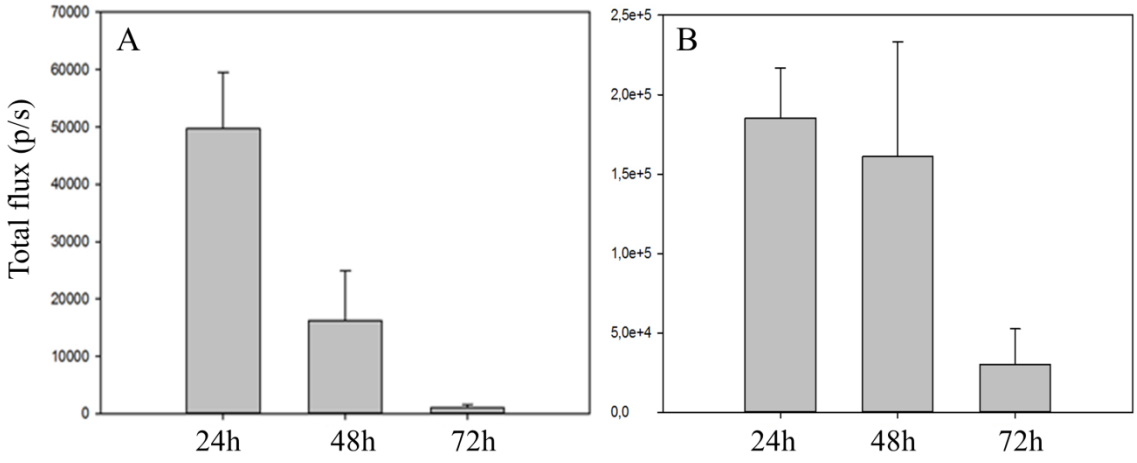

Fig. 4: Bioluminescent signal of MDSCs after lipofectamine 2000 mediated transfection of MDSCs with a luciferase encoding plasmid. (A) Bioluminescence signal per $5x10^3$ cells 24h, 48h and 72h after transfection. (B) Total bioluminescence signal 24h, 48h and 72h after transfection. (n=3 and repeated in an independent experiment)

Lipofectamine 2000 mediated transfection of MDSCs with a IL-12 encoding plasmid

To have an idea about the IL-12 production capacity of IL-12 transfected MDSCs we determined the amount of IL-12 produced after transfection of the MDSCs with an IL-12

encoding plasmid. Twenty-four hours after transfection 18 ± 3 ng of IL-12 per one million MDSCs was produced in the medium. MDSCs that were not transfected or transfected with a luciferase encoding plasmid did not secrete detectable amounts of IL-12.

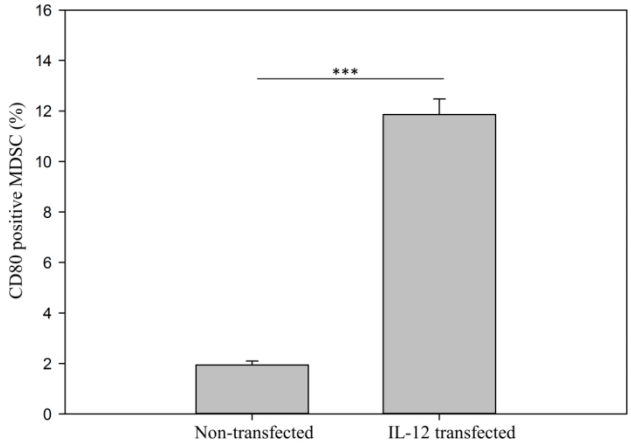

Fig. 5: CD80 expression on non-transfected MDSCs and on MDSCs transfected with an IL-12 encoding plasmid. CD80 expression was measured 24h after transfection. Transfections were performed with lipofectamine 2000. (n=4 and repeated in an independent experiment, ***p<0.001)

Twenty-four hours after transfection we also determined the expression of the activation markers CD40, CD80 and CD86 on the surface of the MDSCs. Lipofection of the MDSCs with a plasmid encoding luciferase (control) or IL-12 did not significantly change the expression of CD40 and CD86. However, the expression of CD80 was significantly upregulated after lipofection of the IL-12 encoding plasmid (p<0.001, Fig. 5). This was not observed after lipofection of a luciferase encoding plasmid.

Effect of IL-12 transfected MDSCs on the tumor killing capacity of lymphocytes

The effect of IL-12 transfected MDSCs on the tumor killing capacity of lymphocytes was studied by coincubating either non-transfected or IL-12 transfected MDSCs with freshly isolated splenocytes and bioluminescent B16-F10 tumor cells. In the absence of MDSCs splenocytes reduce the median growth of B16-F10 tumor cells with 40% (p=0.04) compared to B16-F10 tumor cells without splenocytes (Fig. 6). In the presence of non-transfected MDSCs splenocytes were no longer able to reduce the growth of the B16-F10 cells with a median growth inhibition of -4% (Fig. 6). Interestingly, the splenocytes almost completely regained their ability to suppress tumor cell growth when IL-12 transfected MDSCs are

added to the splenocytes and B16-F10 cells which resulted in a median growth inhibition of 23% (p=0.07, Fig. 6).

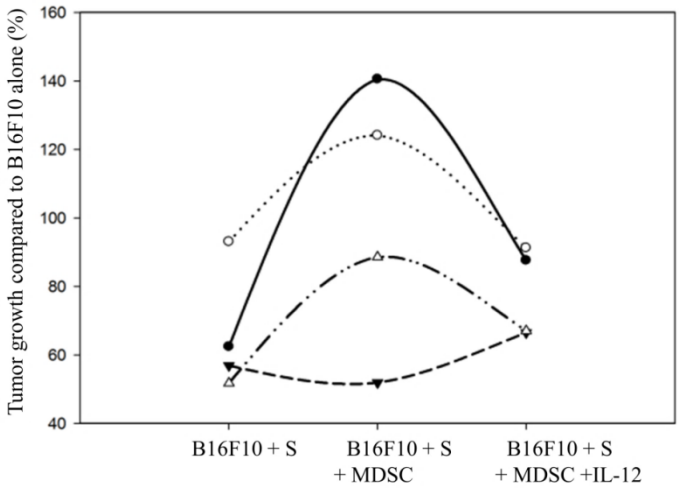

Fig. 6: Relative growth of B16-F10 tumor cells in the presence of splenocytes from 4 different mice (separate lines) with and without the presence of non-transfected and IL-12 transfected MDSCs. S=splenocytes.

In vitro migration

An *in vitro* migration assay (Fig. 8) was performed to determine whether the cell line derived MDSCs still have the capacity to migrate in response to tumoral cytokines. This assay demonstrated that MDSCs migrate on average three times more to the bottom compartment when B16-F10 cells are present in the bottom compartment instead of culture medium (Fig. 8, p=0.03).

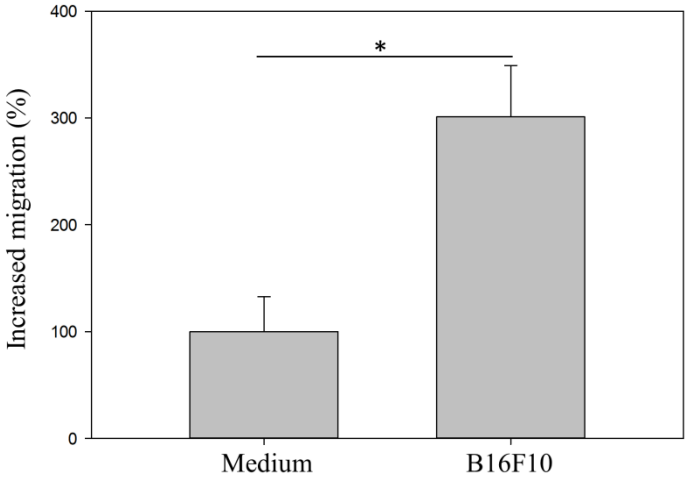

Fig. 8: Migration of MDSCs in a transwell system with only medium or B16-F10 cells with medium in the bottom compartment. The migration of the MDSCs when only medium was present in the bottom compartment was set as 100%. (n=3, *p<0.05)

5. Discussion

A good cellular vehicle for cancer gene therapy should fulfill two important criteria. A first prerequisite is that the cells should efficiently migrate towards tumor cells. A second requirement is that the cells can be efficiently transfected without major cytotoxic effects. In this study we determined whether a MDSC cell line meets these requirements. Using an *in vitro* migration assay we demonstrated that these MDSCs migrate under the influence of tumor secreted factors, which confirms the potential of these cells as vehicles for tumor targeted delivery. Nevertheless, *in vivo* migration studies are necessary to confirm this finding. Subsequently, the possibilities to transfect this MDSC cell line with a luciferase reporter plasmid was studied. Electroporation was found not to be an adequate transfection method for these MDSCs due to the induction of cell death. Cell death was only observed when cells where electroporated with a plasmid, and not after mock electroporation (i.e. without plasmid). It can thus be concluded that the cell death is not due to the physical stress of electroporation, but due to the intracellular delivery of the plasmid. Most likely apoptosis or pyroptosis was induced by immune recognition of the foreign plasmid by intracellular pattern recognition receptors (PRRs) like e.g. DNA-dependent activator of interferon regulatory factors.[349] Recognition of pDNA by intracellular PRRs has been described and well characterized in other myeloid cell lines.[350,351] In contrast, transfection with LF2000 did not result in significant death of the MDSCs. This discrepancy with electroporation has also been observed by others and could be explained by the massive cytoplasmic influx of naked plasmid during electroporation and hence massive activation of the PRRs.[351] Nevertheless, the immune related effects after plasmid electroporation are not necessarily coupled with transfection efficiency, so it is possible that by further optimizing the protocol, especially the pulse length, an efficient electroporation protocol can be found.[352]

Lipofectamine 2000 mediated transfection of the MDSCs with an eGFP plasmid resulted in 43% eGFP positive cells. Transgene expression per cell decreased significantly after 48 hours but the total expression by the total population of transfected cells remained similar, suggesting initially a dilution by cell division. After 72 hours also the total expression decreased significantly, suggesting plasmid degradation or transgene silencing. Increased rate of plasmid degradation over time can be explained by the initial very high amounts of plasmid DNA per cell, vastly exceeding the capacity of endonucleases present in each

individual cell. As the plasmid becomes more diluted, more endonucleases per plasmid copy will become available, and degradation will occur more rapidly. Also transgene silencing could explain the loss of signal after 72 hours. Indeed, transgene silencing can occur immediately after transfection but also after a certain period of adequate expression.[353]

To be effective as a cellular vehicle for tumor targeted delivery the pDNA transfected MDSCs in this work should migrate within 48h after transfection towards the tumor. Additionally, the temporarily expression of the therapeutic molecule, like e.g. IL-12, has to be sufficient for inducing a biological effect. Preliminary *in vivo* data demonstrated that the cell line derived MDSCs indeed migrate to tumor tissue within 48h (data not shown).Twenty-four hours after transfection the MDSCs had produced 18 ng of IL-12. In the literature it has been demonstrated that IL-12 transfected cells, which produce similar amounts of IL-12, can convey anti-tumor effects in mice and humans.[354,355] The IL-12 production capacity of MDSCs after transfection with LF2000 should thus be sufficient. However, there is no data on how long this production capacity should be maintained to result in anti-tumor effects.

The MDSC cell line reacted itself on the IL-12 it produces by upregulating CD80. Depending on the other receptors present on immune effector cells, CD80 can either act as a costimulatory (CD28 receptor) or inhibitory (CTLA-4 receptor) signal.[356] The functional effect of this upregulation can thus not be predicted. It is however known that MDSCs in a tumor microenvironment also upregulate CD80 expression upon IL-12 treatment and this is associated with a decrease in their immunosuppressive function.[126,132] This is encouraging, because using IL-12 as a therapeutic protein has thus the potential to decrease the immunosuppressiveness of MDSCs. Nevertheless, in an *in vitro* killing assay we found that IL-12 transfection reduced, but not completely eliminated the immunosuppressive capacity of the MDSCs. Better anti-tumor effects could be potentially obtained by introducing a selection marker in the IL-12 plasmid, as only 43% of the MDSCs were currently transfected. Only selecting IL-12 transfected MDSCs will result in the same IL-12 concentration with less MDSCs, potentially tipping the balance more in the direction of anti-tumor effects of IL-12 than towards the pro-tumor effects of MDSCs.

GENERAL DISCUSSION

1. Overview

An in depth discussion and relation to the literature of the specific results is included in the different chapters. Here, a more general discussion of the treatment modalities used in this thesis is presented.

Gene therapy

A large part of this thesis is relying on gene therapy to convey therapeutic effects. For IL-12 gene therapy, the rationale is a constant local source of IL-12 production, necessary because of its systemic toxicity and short half-life. For genetic tumor vaccination, the expression of the antigen by the cells after gene therapy mimics best the actual situation of target cells expressing the antigen.

Despite these advantages, one might question the clinical potential of gene therapy as until now only one approach based on gene therapy (unrelated to cancer) is approved for clinical use by the European Medicines Agency (EMA), and none by the FDA.[357] The entire field of gene therapy has been severely hindered by the occurrence of serious adverse effects in the first clinical trials. In 1999, a patient died after a massive immune response against the viral vector, which prompted the FDA to re-evaluate the regulation of gene therapy trials.[358] Later, a second major setback happened, when the initial success of total cure of an otherwise fatal immunodeficiency disorder was overshadowed by the development of a leukaemia-like disorder in two patients as a result of insertional mutagenesis.[359]

However, these serious adverse effects are vector-related and are not a risk for plasmid based gene therapy, the method selected in this thesis.[360] In the clinical trial database of the Journal of Gene Medicine (Wiley Database), 2210 clinical trials with gene therapy are listed, with 64% of them in oncology and 79 already in phase III trials. Most common gene types under consideration are antigens (20%) and cytokines (16%).[361] These data clearly indicate the potential of gene therapy in the treatment of cancer in general, and more specifically for vaccination and cytokine therapy as well as its suitability to proceed into clinical evaluation.

Alternatives for gene therapy do exist, both for sustained local production of IL-12 and cancer vaccination approaches.[362-364] However, gene therapy often compares advantageous

compared to the alternatives because of low production cost and high stability of plasmid DNA.[360]

Vaccination against self-antigens

In this thesis we vaccinated against self-antigens. The principle of vaccination against self-antigens raises two important questions; can it be effective and if so can it be safe. Autoreactive T cells are a normal component of the peripheral T cell repertoire. In contrary to early believes, T cells are to a certain extent cross-reactive and deletion of all autoreactive T cells would compromise the recognition of non-self-antigens. Instead, deletion is based on avidity, the concentration of antigen necessary for the induction of T cell activation and effector functions. T cells with high avidity (low threshold) for self-antigens are deleted, but cells with low avidity for self-antigens are allowed in the periphery. They will not be activated by physiological levels of self-antigen expression but can have at the same time a high avidity for non-self-antigens, thereby contributing to immunity against pathogens.[365] In conclusion, the presence of autoreactive T cells proves that vaccination against self-antigens is theoretically possible and the control of avidity provides a window of doing so without causing overt autoimmunity.

The targets for vaccination against self-antigens are thus low avidity T cells. These cells are very difficult to activate with the native antigen, even at high antigen concentration.[366] However, the discovery of altered peptide ligands was a major breakthrough in the manipulation of these low avidity T cells. Epitopes that only differ from the native self-epitopes by one or a few amino acids influence the stability of MHC-peptide-TCR binding and can lead to efficient fully functional activation of low avidity T cells. Once activated, these low avidity T cells can exert their cytolytic functions against target cells expressing high levels of the native antigen.[366,367] During activation, avidity of T cells is increased, requiring less antigen for exerting effector functions.[368] The extent of this avidity increase depends on the structure of the altered peptide ligand and this can exceed the safety threshold for autoimmunity. However, peripheral tolerance mechanisms are in place to delete these high avidity T cells after activation.[369] Similarly, the increase of avidity in memory T cells is controlled in the case of self-antigens by peripheral tolerance mechanisms.[370,371] Therefore, with altered peptide ligands, it is possible to activate low avidity T cells that will only recognize cells overexpressing the antigen above physiological levels, providing a clear

therapeutic window for vaccination against self-antigens. One way to provide altered peptide ligands is to use xenogeneic vaccination, the strategy used in this thesis.[287]

This mode of action has a clear implication for therapeutic use: only patients that have a strong overexpression of the antigen can benefit from such vaccination strategy. For VEGFR-2, this has been demonstrated in a wide variety of tumor types.[372] Ideally, patient selection occurs on an individual basis, for instance by non-invasive imaging of VEGFR-2 expression by PET.[373] For VEGFR-2, the safety margin between normal and tumoral expression appears to be quite wide, as even the adoptive transfer of CAR T cells recognizing VEGFR-2, having higher avidity than naturally evoked T cells, seems to be safe in rodents and is currently tested in humans (NCT01218867).[374]

Despite the theoretical foundation of a safety margin as discussed above, one has to keep in mind that vaccination can have a life-long effect that is difficult to control afterwards. Moreover, the safety of vaccines against self-antigens relies on intact tolerance mechanisms that are yet incompletely understood.[365] For now, only one tumor vaccine directed to self-antigen is approved for clinical use. The bottleneck in the approval of tumor vaccines is currently mainly limited efficacy, and not toxicity.[375] Time will tell if, when tumor vaccines become more efficacious, the risk for autoimmunity will become an important limitation in the regulatory approval of such vaccines. Progress in induction of antigen-specific tolerance might diminish this risk in the near future, providing a safety mechanism when unforeseen autoimmune reactions occur.[376]

An alternative to obtain tumor reactive CD8 cells is the adoptive transfer of (genetically modified) T cells (TIL therapy, TCR modified T cells and CAR T cells).[377] Very impressive results have been published, but it is also associated with severe toxicity, either by the lymphodepletion and high dose IL-2 therapy necessary for TIL-therapy or on-target toxicity because of the unnatural affinity of genetically modified T cells, which resulted in death of patients in three trials.[377] Moreover, adoptive transfer is technically challenging, whereas vaccination is a simple procedure.

Anti-angiogenesis

The VEGFR-2 vaccine evaluated in this thesis relies on an anti-angiogenic mechanism of action. The rationale for anti-angiogenic treatment has been postulated in the early 1970's and is still sound: tumors need blood supply to grow beyond 2mm, hence targeting tumoral blood vessels will result in anti-tumor activity. Although different anti-angiogenic drugs have been approved for clinical use (e.g. bevacizumab (an anti-VEGF monoclonal antibody) and sunitinib (a small molecule inhibitor)), results are rather disappointing. Survival benefit is measured in the range of months, with durable responses very rare.[378] The reason is the occurrence of resistance, because of many redundant pathways in the regulation of angiogenesis.[379]

However, in contrast to traditional anti-angiogenic drugs that inhibit a pathway, VEGFR-2 vaccination will ideally result in destruction of the endothelial cells. It is conceivable that this will have profound implications for the occurrence of resistance. In our models however, vaccination against VEGFR-2 did not have a significant impact on tumor growth. A possible explanation is the known limited efficacy of tumor vaccines as monotherapy, due to the local immunosuppressive tumor microenvironment. If the vaccine can be rendered more efficacious, a direct comparison with traditional anti-angiogenic drugs regarding the development of resistance would be highly interesting, possibly revealing a unique advantage of active immunization as anti-angiogenic treatment.

Another limitation of anti-angiogenic treatment is that total eradication of tumor cells is not possible. In the best case, if anti-angiogenic treatment is completely effective, small dormant tumor islands, capable of surviving without blood vessel supply, will persist and regrow when anti-angiogenic treatment is discontinued.[380] Again, active immunization may offer a unique advantage, as memory immune cells can convey life-long control forcing tumor cells to stay dormant.

Importantly, influencing angiogenesis can have an effect on invasion and metastasis pathways, which we also observed with our vaccine.[327] As HIF-1a is a major mediator of the response of tumor cells to hypoxia, combining anti-angiogenic treatment with an HIF-1a inhibitor (e.g. topotecan) may disrupt this association.[381] In this context, an interesting tool in the development of anti-angiogenic therapeutic interventions is mathematical modelling by computational biology.[382] With the vast knowledge of signalling pathways implicated in

angiogenesis and interactions of these pathways with other biological processes, such effects can hopefully in the future be predicted early on instead of being dependent on observations in preclinical experiments.

Apart from VEGFR-2 vaccination, also IL-12 and metronomic cyclophosphamide have anti-angiogenic activity. Combining these treatments could also work synergistically in that context, on top of their synergistic immunological effects. This is especially interesting, considering that the key to improving anti-angiogenic treatment is thought to be combination therapy acting on multiple pathways thereby limiting escape strategies.[379]

In conclusion, although anti-angiogenesis in general or our VEGFR-2 vaccine has not yet been able to meet its potential, we still believe firmly in the concept of anti-angiogenic treatment and especially by active immunization. However, combination of our vaccine with agents that tackle the immunosuppressive tumor environment, increase the immunogenicity of the vaccine and abolish hypoxia mediated deleterious effects is deemed necessary for successful anti-tumor activity.

Immune modulation by selective depletion of immune subsets

In this thesis, regulatory T cells were targeted by metronomic cyclophosphamide. Selective toxicity of chemotherapeutica for certain cell types might seem unexpected but several mechanisms have been elucidated. The selectivity of low dose cyclophosphamide for Tregs is explained by reduced detoxification of cyclophosphamide, because Tregs contain low ATP levels.[147] Another example includes selective toxicity of 5-fluorouracil (5-FU), a thymidylate synthase inhibitor, for MDSCs. MDSCs are much more sensitive to 5-FU because they expression much lower levels of thymidylate synthase than other cells.[383] As both MDSC and regulatory T cells are major regulators of tumor induced immune suppression, strategies to revert immune suppression should at least address both cell types. However, many immune subsets can have an immune-regulatory phenotype, including NK cells, dendritic cells, B cells and macrophages.[384-387] Moreover, also non-immune stromal cell suppress immune responses in cancer.[388]

Therefore, it might be more meaningful to interfere more downstream on T cells rather than targeting a specific immunosuppressive cell type. Immune checkpoint inhibitors use this strategy by blocking inhibitory pathways on T cells and have shown impressive efficacy.[389] However, more downstream interference also will have a more profound impact,

circumventing many natural control mechanisms. Indeed, autoimmune manifestations after immune checkpoint therapy are common.[390] Especially with combination therapies, its safety will have to be carefully determined.

Immunotherapy

Last but not least, although the recent successes of immunotherapy are considered a major breakthrough in cancer treatment, one might wonder if this is not premature, considering the low number of responding patients. However, for the first time in decades of intensive research, immunotherapy gives hope that a cure of cancer may be possible after all. Although significant progress has been made with chemotherapy and targeted therapies, durable response also after cessation of treatment remains very rare.[391,392] The occurrence of enduring responses and even complete responses in cancers that were incurable in the past makes immuno-oncology an important emerging field, deserving the attention it has been receiving in recent years.[392]

Only a low number of patients respond to cancer immunotherapy. So the question remains how to improve the proportion of responding patients. An important factor could be the combination of different immunotherapeutic agents, the foundation of this thesis. For instance, the success of immune checkpoint inhibitors is related to the mutation rate of tumors, representing immunogenic tumors.[393] It is indeed logical that immune checkpoint inhibitors only have an effect if activated anti-tumoral T cells are naturally present. For inherently non-immunogenic tumors, this can be achieved by combining with a vaccination approach. Similarly, with vaccination you might elicit activated T cells, but if this is not combined with a strategy to revert immune suppression, these activated T cells will be rendered useless in the tumor microenvironment. Therefore, combining immunotherapeutic agents may be strongly synergistic and be the key to increasing the number of responding patients.

Another important improvement would be the discovery of predictive biomarkers.[394] The discrepancy between the high proportion of patients that do not respond and the low proportion of patients that have a long lasting response clearly indicate subpopulations. If we can identify the patients with the potential to respond to certain immunotherapeutic treatments, a patient tailored treatment selection could be applied resulting in an overall higher number of responding patients. Additionally, biomarkers could also be used for

follow-up during treatment.[395] Clinical response to immunotherapy can take a long time to develop, if surrogate biomarkers are developed that can evaluate sooner if the treatment is working, non-responding patients can be identified earlier and switched to another treatment.

Also combination with non-immunotherapeutic treatments could significantly impact the response rate to immunotherapy. Firstly, non-immunotherapeutic treatments can work synergistically with immunotherapy. For instance, different chemotherapeutics and radiation induce immunogenic cell death and surgical debulking removes the gross immunosuppressive environment.[396-398] Secondly, traditional treatments can just provide the time for immunotherapy to work. As said before, clinical responses to immunotherapy can take months to develop, and some patients with advanced disease do not have that time. On the other hand, optimal combinations and timing will have to be carefully determined. Chemotherapy and radiation therapy can result in immune depletion, and also surgery is associated with an immunosuppressive state.[399-401]

In conclusion, immunotherapy makes the dream of curing cancer within reasonable reach, which justifies the excitement it currently receives. However, the low number of responding patients remains a sore point. Many potential strategies to increase this number can be hypothesized which will take considerable time to validate and optimize, but hopefully finally result in what has long seemed like a lost battle, a cure against cancer.

2. Limitations and reflections

In **chapter 1** not all stand-alone therapies and possible combinations are evaluated, but instead the different groups were selected from a practical point of view. This was done to comply with the 3 R principles. No treatment groups without IL-12 were included, as there was ample prior knowledge that this would be the most potent component of the combination treatment. Two key questions needed addressing. First, has metronomic cyclophosphamide value as Treg depleting agent on top of IL-12? Although IL-12 has no direct effect on Tregs, it is possible that they deplete Tregs indirectly, for instance via their effect on MDSCs.[126] Therefore, groups with IL-12 stand-alone therapy and the combination of IL-12 and metronomic cyclophosphamide were included. It was concluded that only metronomic cyclophosphamide could deplete Tregs. Because of the known beneficial effects of Tregs

depletion for immunotherapy, and the low cost, easy administration and good tolerability, this information was enough to decide to include it in the combination therapy.[402] Tumor vaccination is much more expensive and less convenient. Therefore, a direct comparison of IL-12 and metronomic chemotherapy and the full combination was deemed necessary, to provide a proof of concept that vaccination indeed could have and added value in this combination therapy, which was indeed the case. An important limitation is that the results were only obtained in one tumor model. Because of the restrictions of rodent tumor models, we plan to directly confirm the potential of this combination treatment in dogs, instead of evaluating it in more rodent tumor models.

More information could potentially have been obtained from the clinical study in **chapter 2**. For metronomic temozolomide, the selection of only one dose was from a scientific point of view clearly not optimal, especially when one knows that no information is available in the literature about the optimal metronomic dose of temozolomide. In the same context, the evaluation of the angiogenic markers could have been much more informative if more doses of both chemotherapeutic drugs were included, allowing for a pharmacodynamic dose-response to be evaluated. Both shortcomings in this chapter were a result of the limited number of canine cancer patients that were eligible for the study. This study was conducted at the university veterinary clinic (Ghent University) and metronomic chemotherapy was proposed next to other treatments to owners. Owners that visit the university veterinary clinic want the best treatment for their pets and are less inclined to choose for this palliative treatment. Accrual of patients in first-line veterinary practices may lead to higher patient numbers for metronomic chemotherapy, where convenience and costs are important considerations next to the chance and degree of efficacy. Another limitation of this study is the absence of a clinical follow-up. It was not deemed feasible to have enough patient numbers to include a control group. Moreover, also as a single-arm trial, the high heterogeneity of patients precludes meaningful conclusions of efficacy. Therefore, conclusions were restricted to the repeated measurements of biological parameters.

The evaluation of the VEGFR-2 vaccine in **chapter 3 and 4** and the MDSC cell line as vehicle for gene therapy in **chapter 5** is only the beginning. More research is needed to clarify the mechanism by which VEGFR-2 vaccination promotes early liver micrometastases and its relevance in tumor models where the liver is an important site of metastases has to be determined. Moreover, despite the occurrence of an immune response, anti-tumor effects of

the vaccine are ranging from modest in a prophylactic setting to absent in a therapeutic setting. Therefore, at this moment, the positive result in chapter 3 regarding immunogenicity of the vaccine are very conditional on better efficacy of the vaccine by for instance combination treatment and better understanding of the pro-metastatic effects. For the MDSC cell line, further optimization of transfection and demonstration of tumor-specific migration *in vivo* are some of the following steps to be undertaken before therapeutic evaluation can be started.

3. Perspectives

The question is not if immunotherapy will become a mainstay in cancer treatment, but when. What is unique and very promising for immunotherapy compared to other treatments is not the proportion of patients that benefit from it, but the extent to which certain patients benefit. Patients with advanced disease, where all other treatment options fail, have experienced complete responses on immunotherapy.[392] Most success has been achieved with ipilimumab. For example when melanoma patients are treated with ipilimumab around 20% survive up to 3 years. Interestingly, after this time point survival curves form a plateau at least up to 10 years, meaning that when achieving this milestone chance of dying of melanoma are minimal.[403] Although long term survival does not automatically mean that patients are completely free from tumor, investigators have already introduced the concept of curative potential of immunotherapy.[404] Although the extent to which patients might benefit is spectacular, the reality is that most patients do not. It is believed that the answer to increase responding patients lies in combinatory immunotherapy.

The work in this thesis has set the first steps towards the evaluation of such a combinatory treatment in dogs. This is continued in the Laboratory of Gene therapy with clinical trials in dogs with intratumoral IL-12 gene therapy and the combination of intratumoral IL-12 gene therapy and metronomic cyclophosphamide ongoing. The latter combination will be complemented with a vaccination approach when this is available. Also the research on MDSCs as cellular vehicles for tumor targeted delivery of IL-12 will be continued.

SUMMARY

The use of the immune system as a therapy against cancer is an option explored since the late 19th century. However, so far results have been disappointing. This can be explained by the vast complexity of interactions between tumor and immune cells, of which many details still have not been elucidated. Indeed, earlier believes that the immune system fails to recognize the tumor as a malignant process and should thus be taught to do so have now been overthrown. Paradoxically, it is the actions of the immune system that select those mutated cells with strategies to evade attacks of the same immune system. These defensive strategies will often abuse those mechanisms used by the body to prevent autoimmunity and excessive immune responses.

As these mechanisms work via different pathways, it can be understood that immunotherapy should likely address several of these pathways to be effective. Therefore, a combination immunotherapy based on three mechanisms is evaluated in this thesis. Firstly, tumor vaccination is used to address the deficient antigen presentation in tumors, which promotes tolerance rather than active immune responses. Secondly, interleukin-12 (IL-12) gene therapy is used to convert the immunosuppressive microenvironment, elicited by the tumor, toward immune stimulatory. Indeed IL-12 stimulation has the capacity to stimulate T cells, make NK cells cytotoxic and turn the normally immunosuppressive myeloid derived suppressor cells (MDSCs) into antigen presenting cells. As the final part of this combination therapy, metronomic chemotherapy will be included to diminish the effect of regulatory T cells (Tregs), also an important population of immunosuppressive cells.

The final goal of this thesis is the evaluation of this combination therapy in dogs, both from a veterinary perspective and to maximize the potential of this species as a highly translational research model.

Chapter 1 describes the application this therapy in a mouse model of melanoma. Mice were treated with IL-12 gene therapy alone, or with addition of metronomic cyclophosphamide (CPX), or with genetic human tyrosinase (hTyr) vaccination and metronomic CPX. These different groups were compared for both immunological effects and survival. The IL-12 gene therapy group showed a considerable clinical effect, with necrosis of the tumor already after one day. This resulted in complete disappearance of the tumor in 60% of the mice. Because of the rapidness of the onset of necrosis we investigated possible directly cytotoxic effects.

However, no direct cytotoxicity of IL-12 could be elicited *in vitro*. To confirm the involvement of the immune system we investigated *in vitro* if NK or CD8 cells were able to respond this rapidly to IL-12. Indeed, NK cells, but not CD8, showed heightened cytotoxicity towards tumor cells 24 hours after IL-12 exposure. The addition of metronomic CPX induced a reduction in the number of Tregs, not observed with IL-12 as monotherapy. This addition also resulted in longer survival in mice whose tumors never completely regressed, although the number of mice with such complete regression did not increase. The final addition of tumor vaccination did increase the number of mice with complete regression. Most of these extra mice had tumors that fully regressed only at later time-points, a phenomenon not observed without the vaccination addition. Furthermore, only in this group a specific cellular immune response was found after a tumor rechallenge in cured mice. A humoral immune response however, was present in all three groups after tumor rechallenge.

Chapter 2 describes a clinical study in dogs, in which the potential of metronomic cyclophosphamide (12.5 mg/m^2) and temozolomide (6.6 mg/m^2), separately or combined, to decrease Tregs in cancer patients was investigated. Additionally, vascular growth factor (VEGF) and thrombospondin-1 (TSP-1), two important angiogenetic markers, were quantified to assess the anti-angiogenetic potency of this treatment. Regulatory T cells decreased significantly when dogs were treated with cyclophosphamide, but not for temozolomide. Since temozolomide was used for the first time with this purpose in dogs, the dosage was highly experimental. Therefore, it is very likely that this finding is a result of an inappropriate dose rather than unsuitability of this drug. Metronomic cyclophosphamide however, can be used with the current dosage for this purpose in dogs. Both treatments did not affect the angiogenetic biomarkers VEGF or TSP-1, but again both treatments should be used at different doses before inferring on the value of these markers in dogs.

Chapters 3 and 4 describe the evaluation of a universal tumor vaccine, which would allow a broad application of this combination therapy in veterinary oncological patients. A target holding this promise is vascular endothelial growth factor receptor-2 (VEGFR-2), a receptor considerably expressed in tumor blood vessels. Therefore, the immunogenicity of a human VEGFR-2 plasmid was evaluated in healthy mice for different doses and vaccination schedules. This confirmed earlier reports that induction of a humoral immune response required higher doses and more boosts than the cellular immune response. The safety of

vaccination against VGEFR-2 was tested in two ways. On the one hand, wound healing, which is physiologically supported by VGEFR-2, was assessed and found to be normal. On the other hand two cell populations expressing VEGFR-2, i.e. Tregs and MDSCs, were followed and found to remain unharmed by the vaccination. Consequently, the same vaccination was evaluated in healthy dogs. Also in this species we were able to elicit both a cellular and humoral immune response, albeit only after the third vaccination. This experiment also made clear that light sedation and local anesthesia are not sufficient. However, no adverse effects were observed.

Finally, in chapter 4 the vaccine was evaluated in a mouse model of breast cancer and melanoma in both a preventive and curative design. Although increased survival was only observed with preventive use, curative vaccination decreased bioluminescence compared to equally large control tumors probably indicating central necrosis. An unexpected finding was that both vaccination strategies increased the presence of micrometastases in the liver, even though no such effect was found in the lungs. Mice had to be euthanized before these metastases could become macroscopic. Therefore, to evaluate the progression of these metastases, an additional group was included were the primary tumor was surgically removed. Although metastases in the lung progressed, liver metastases remained microscopic. Additionally, liver metastases of control animals increased to the same level as those of vaccinated animals. The relevance of these early vaccine-induced liver metastases can thus not be sufficiently assessed with these data. Possible explanations based on literature can be the adaptation of the primary tumor to vaccine-induced hypoxia, not only stimulating metastasis formation, but also selecting cells that thrive under hypoxia. Indeed, relative hypoxia is known to be present in the liver, but naturally not in the lungs. The ultimate consequences of this metastasis induction should be evaluated with tumor models, contrary to the current ones, for which the liver is a natural metastatic site.

Chapter 5 describes the first steps toward broadening the application of IL-12 therapy to tumors and metastases that are not accessible for electroporation. This poses a challenge since systemic administration of IL-12 is impeded by its toxic side-effects. A possible solution to this challenge is to use cells with the capacity to migrate to tumor tissue, that have been loaded with therapeutic agents. For IL-12, cells could be transfected *in vitro* with a plasmid encoding IL-12, be systemically infused after which they will migrate to the tumor

and express the plasmid locally. MDSCs migrate strongly towards tumor tissue and hence could be used for this approach. However they also have pro-tumoral effects. Therefore, the loaded therapeutic agent should have an effect great enough to (over)compensate for these effects. An advantage of IL-12 in this light is its ability to diminish the pro-tumoral characteristics of these MDSCs, apart from its own anti-tumor effects. In this chapter we used a murine MDSC cell line, and evaluate if this cell line has retained the ability to migrate towards tumor cells and if this cell line could be transfected with physical (electroporation) or chemical (lipofectamine 2000) methods. These transfection methods have the advantage to allow rapid evaluation of candidate therapeutic agents. However, electroporation induced extensive cell death, but only when MDSCs were transfected with plasmid. Previous research has indeed shown that large quantities intracellular plasmid after electroporation can induce apoptosis via immunological reaction of cells to the plasmid. No significant cell death was however observed with the chemical transfection vector that was used, i.e. lipofectamine 2000. Using this method we observed a transfection efficiency of only 43% and a strong decrease of expression after 72 hours. However, during this time MDSCs could produce therapeutically relevant concentrations of IL-12. Moreover, MDSCs responded to their own IL-12 production by upregulation of CD80, which is associated with decreased immunosuppressive function. This was indeed confirmed in a preliminary *in vitro* experiment, but the final effect remained pro-tumoral. The *in vitro* migration assay confirmed that these cells strongly migrate towards tumor secreted factors. In conclusion, this promising method could be exploited if more efficient transfection would be achieved. Indeed, the majority of MDSCs are currently not transfected and thus do not contribute to therapeutic effect, leaving only their pro-tumor characteristics.

SAMENVATTING

Kanker behandelen door het eigen immuunsysteem in te schakelen wordt reeds sinds 1890 verkend, maar heeft lang enkel tot teleurstellende resultaten geleid. Dit is te verklaren door de enorme complexiteit van de interacties tussen tumorcellen en immuuncellen, die lang onvoldoende gekend was. Waar men vroeger dacht dat het immuunsysteem de tumor niet herkent als een indringer, en de sleutel tot succesvolle behandeling dus zou zijn om het immuunsysteem hiervan bewust te maken, weet men nu dat dit allesbehalve waar is. Een tumor is niet onzichtbaar voor het immuunsysteem, maar heeft in de loop van zijn ontwikkeling allerlei strategieën ontwikkeld om zich te beschermen tegen een aanval van het immuunsysteem. Het misbruikt hierbij mechanismen die fysiologisch aanwezig zijn in het lichaam om het immuunsysteem te controleren, om auto-immuniteit en een overdadige immuunrespons te voorkomen.

Daar de tumor een effectieve immuunrespons op verschillende niveaus verhindert, moet immuuntherapie ook op verschillende niveaus ingrijpen om deze situatie weer om te keren. In deze thesis werd een combinatietherapie geëvalueerd die aan deze voorwaarde voldoet. Tumor vaccinatie werd geïncludeerd omdat antigen presentatie in de tumor inefficiënt gebeurt, wat aanleiding geeft tot tolerantie in plaats van activatie. Interleukine-12 (IL-12) gentherapie helpt de lokale micro-omgeving van de tumor te converteren van immuunsuppressief naar immuunstimulerend. IL-12 kan het effect van vaccinatie versterken door T cellen lokaal te stimuleren en ook complementeren door daarnaast NK cellen aan te zetten tot cytotoxiciteit. Verder werden myeloid derived suppressor cells (MDSCs), immunosuppressieve cellen gerekruteerd door de tumor, onder invloed van IL-12 omgezet naar niet-immunosuppressieve antigen-presenterende cellen. Ten laatste werd metronomische chemotherapie aan de combinatiebehandeling toegevoegd om zo ook het effect van regulatorische T cellen (Tregs), de tweede belangrijke populatie van immunosuppressieve cellen, te kunnen verminderenen. Het uiteindelijke doel is de evaluatie van deze combinatiebehandeling in honden, zowel voor de hond als doeldier als voor model voor de mens.

In hoofdstuk één werd deze combinatie getest in een melanoma muismodel. Muizen met een tumor werden behandeld met IL-12 gentherapie, IL-12 gentherapie en metronomische cyclofosfamide of IL-12 gentherapie, metronomische cyclofosfamide en genetisch vaccinatie met human tyrosinase. De immunologische effecten in de verschillende groepen werd nagegaan, en overleving vergeleken. IL-12 gentherapie had op zichzelf een zeer sterk

klinisch effect. Eén dag na de behandeling trad er al een duidelijke necrose van de tumor op. Bij ongeveer 60% van de muizen verdween de tumor uiteindelijk ook volledig. Dit zeer snelle effect zou kunnen duiden op een rechtstreeks effect van IL-12 op de tumorcellen, zonder tussenkomst van het immuunsysteem. Dit werd nagegaan in een *in vitro* test, maar een direct cytotoxisch effect van IL-12 op de tumorcellen was afwezig. In een andere *in vitro* test werd nagegaan of NK cellen of CD8 cellen zo snel kunnen reageren op IL-12 stimulatie en dit dus een mogelijke verklaring is voor het snelle *in vivo* effect. Daaruit bleek dat NK cellen, maar niet CD8 cellen binnen 24 uur verhoogde cytotoxiciteit ten opzichte van de tumorcellen vertoonden onder invloed van IL-12. IL-12 behandeling op zichzelf had geen effect op Tregs, wat wel gezien werd in de combinatiegroep IL-12 gentherapie + metronomische cyclofosfamide. Toevoeging van metronomische cyclofosfamide resulteerde niet in een verhoogd aantal muizen waarbij de tumor volledig verdwijnt, maar wel in een langere overleving van de muizen waarbij de tumor niet volledig verdween. Als het vaccin werd toegevoegd aan de behandeling, leidde dit tot een verhoogd aantal muizen waarbij de tumor volledig verdween. Dit laatste werd gekarakteriseerd door bijkomende muizen met een regresserende tumor op een later tijdspunt dan zonder het vaccin. Alleen in de gevaccineerde groep kon een specifieke cellulaire immuunrespons aangetoond worden na een rechallenge in de genezen muizen. In de groepen die geen vaccin kregen toegediend, was er op dat moment enkel een humorale immuunrespons aanwezig.

In een eerste klinische studie werd nagegaan of metronomische cyclofosfamide (aan een dosis van 12.5 mg/m^2) ook bij honden met kanker de Tregs kan doen dalen. Een tweede chemotherapeuticum met een effect op Tregs bij muizen en mensen, temozolomide (aan 6.6 mg/m^2) werd eveneens geëvalueerd, net zoals de combinatie van beide geneesmiddelen. Naast het effect op Tregs in het bloed, werden ook twee belangrijke angiogenetische merkers (vascular endothelial growth factor en thrombospondine-1) geëvalueerd. Het is immers gekend dat metronomische chemotherapie ook een anti-angiogenetische werking heeft. Metronomische cyclofosfamide resulteerde in een significante daling van Tregs. Metronomische temozolomide had geen effect op de hoeveelheid Tregs, waarschijnlijk door een te lage dosis. Beide chemotherapeutica hadden geen effect op de angiogenetische merkers. Er kan geconcludeerd worden dat metronomische cyclofosfamide aan de geëvalueerde dosis bij honden kan gebruikt worden als Treg depleterende behandeling in immunotherapie. Voor metronomische temozolomide is meer onderzoek vereist met hogere dosissen. Aangezien beide metronomische chemotherapeutica geen effect hadden op de

angiogenetische merkers, maar ook maar één dosis geëvalueerd werd, kunnen er geen conclusies getrokken worden over de farmacodynamische waarde van deze merkers bij de hond.

Om de combinatiebehandeling ruimer toepasbaar te maken dan enkel melanoma zou een universeel kankervaccin nodig zijn. Daarom werd in hoofdstukken 3 en 4 een universeel tumorvaccin geëvalueerd. Dit bestaat uit een plasmide coderend voor humaan vascular endothelial growth factor-2 (VEGFR-2), toegediend in combinatie met elektroporatie. VEGFR-2 wordt in belangrijke mate tot expressie gebracht in tumorale bloedvaten. In gezonde muizen werd de immunogeniciteit van human VEGFR-2 DNA vaccin bij verschillende dosissen en vaccinatieschema's bestudeerd. Hierbij werd duidelijk dat de ontwikkeling van een humorale immuunrespons een hogere dosis en meer boosts nodig had dan de ontwikkeling van een cellulaire immuunrespons. Dit is een gekend fenomeen bij genetische vaccinatie. Aangezien VEGFR-2 de wondheling ondersteunt werd door middel van een wondhelingassay de veiligheid van het vaccin nagegaan. Geen vertraagde wondheling werd waargenomen. Ook bleven de percentages van Tregs en MDSCs, die eveneens VEGFR-2 tot expressie kunnen brengen, onveranderd bij gevaccineerde muizen. Vervolgens werd het vaccin geëvalueerd in gezonde honden. Hierbij werd duidelijk dat het voorziene protocol van lichte sedatie en lokale anesthesie niet aan te raden is voor elektroporatie bij honden. Heftige schrikreacties werden uitgelokt, die weliswaar van zeer korte duur waren. Een cellulaire en humorale immuunrespons kon worden opgewekt, zij het enkel aantoonbaar na de derde vaccinatie. Het vaccin werd vervolgens geëvalueerd in muizen met kanker, in een borstkankermodel en melanomamodel. Effect op tumorgrootte en overleving was matig wanneer profylactisch werd gevaccineerd en afwezig wanneer therapeutisch werd gevaccineerd. Bij de therapeutische vaccinatie werd wel een verschil in bioluminescentiesignaal van de tumor opgemerkt, suggestief voor het optreden van necrose. Onverwacht was er in beide tumormodellen, zowel bij profylactische als therapeutische vaccinatie, een sterkere aanwezigheid van micrometastasen in de lever. De micrometastasen in de longen waren niet verschillend tussen gevaccineerde en niet-gevaccineerde muizen. Om dit effect op levermetastasen verder te kunnen opvolgen, werd vaccinatie herhaald in combinatie met chirurgische verwijdering van de primaire tumor. Hoewel de longmetastases verder waren ontwikkeld, bleven de metastases in de lever microscopisch en kwamen tot op hetzelfde niveau in niet-gevaccineerde en gevaccineerde muizen. Beide tumormodellen geven inderdaad geen aanleiding tot macrometastasen in de lever. De relevantie van het

optreden van vervroegde micrometastasen in de lever kan dus niet voldoende beoordeeld worden. Meer onderzoek is nodig om het mechanisme van dit effect te onderzoeken, wat op basis van de literatuur zou kunnen verklaard worden door adaptatie van de cellen in de primaire tumor aan hypoxie, wat ook in zekere mate aanwezig is in de lever. Het uiteindelijk effect zou eveneens moeten beoordeeld worden in een model waar de lever een belangrijk orgaan voor metastases vormt.

Aangezien IL-12 gentherapie zoals toegepast in hoofdstuk 1 enkel toepasbaar is voor tumoren die uitwendig bereikbaar zijn voor elektroporatie, werd ook op dit vlak de eerste stappen gezet naar een bredere toepasbaarheid. Aangezien IL-12 toxisch is bij hoge systemische concentraties, is de uitdaging om bij systemische toediening toch enkel intratumoraal hoge concentraties te krijgen. Een mogelijke oplossing is het gebruik van cellen die na systemische toediening migreren naar tumorweefsel, die zo gebruikt kunnen worden als transportmiddel voor geneesmiddelen. In het geval van IL-12 kunnen deze cellen *in vitro* getransfecteerd worden met plasmide coderend voor IL-12, systemische toegediend worden waarna zij naar de tumor zullen migreren en daar het plasmide tot expressie brengen. MDSCs migreren zeer sterk naar tumorweefsel en kunnen dus voor dit doeleinde gebruikt worden. Een nadeel van MDSCs is echter dat ze tumorgroei sterk stimuleren, het antitumor effect van het therapeuticum moet dit dus sterk kunnen compenseren. Een voordeel van het gebruik van deze cellen voor de toediening van IL-12 is dat IL-12 in staat is om de protumor eigenschappen van MDSCs te verminderen. Er is een muriene MDSCs cellijn ontwikkeld. Het gebruik van deze cellijn in plaats van vers geïsoleerde MDSCs vergemakkelijkt sterk het preklinische onderzoek, zowel qua tijd, reproduceerbaarheid en benodigde dieren. In het laatste hoofdstuk van deze thesis werd *in vitro* nagegaan of deze cellijn de eigenschap heeft behouden om te migreren naar tumorweefsel. Dit bleek het geval te zijn, waaruit werd besloten dat verder onderzoek met deze cellijn voor het beoogde doeleinde interessant is. Een vereiste hiervoor is dat de cellen *in vitro* getransfecteerd kunnen worden met een therapeutisch plasmide. Niet-virale transfectie is hiervoor interessant daar met deze techniek een snelle initiële evaluatie van kandidaat therapeutica kan gebeuren. Elektroporatie werd geëvalueerd maar bleek niet geschikt daar er een sterke celdood optrad wanneer MDSCs met plasmiden werden geëlektroporeerd. Voor gelijkaardige cellijnen is reeds aangetoond dat de massale hoeveelheden intracellulair plasmide na elektroporatie apoptose induceert via een immunologische reactie van de cellen. Daarom werd overgestapt naar een chemische vector voor transfectie, namelijk lipofectamine 2000. Hierbij werd geen significante celdood

waargenomen. De transfectie-efficiënte bedroeg echter slechts 43% en de expressie van het transgen was na 72 uur fors gedaald. Wanneer de cellen op deze manier werden getransfecteerd met IL-12, konden therapeutisch relevante hoeveelheden geproduceerd worden. De MDSCs zelf reageerden ook op deze IL-12 productie met een opregulatie van CD80. Een opregulatie van CD80 op MDSCs o.i.v. IL-12 is in de literatuur beschreven en is geassocieerd met een vermindering van hun immunosuppressieve functies. Echter, een preliminaire *in vitro* proef suggereerde dat de immunosuppressieve functies inderdaad verminderd waren in aanwezigheid van IL-12, maar dat dit effect niet sterk genoeg was om de protumor eigenschappen van MDSCs te compenseren. Een efficiëntere transfectiemethode, waarbij er eenzelfde concentratie van IL-12 kan geproduceerd worden door minder MDSCs, zou dit evenwicht kunnen meer kunnen laten omslaan naar een therapeutisch effect.

REFERENCES

References

1. Coley WB. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Clinical orthopaedics and related research.* Jan 1991(262):3-11.
2. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. *Science.* Dec 20 2013;342(6165):1432-1433.
3. Flemming A. Deal watch: Pfizer and GSK join race for T cell cancer therapies. *Nature reviews. Drug discovery.* Aug 2014;13(8):568-569.
4. Sheridan C. Industry pursues co-stimulatory receptor immunomodulators to treat cancer. *Nat Biotechnol.* Mar 2013;31(3):181-183.
5. Swann JB, Smyth MJ. Review series Immune surveillance of tumors. *The Journal of Clinical Investigation.* 2007;117(5):1137-1146.
6. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* Mar 4 2011;144(5):646-674.
7. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer.* Apr 2012;12(4):265-277.
8. Hubo M, Trinschek B, Kryczanowsky F, Tuettenberg A, Steinbrink K, Jonuleit H. Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. *Frontiers in immunology.* 2013;4:82.
9. Wallet MA, Sen P, Tisch R. Immunoregulation of dendritic cells. *Clinical medicine & research.* Aug 2005;3(3):166-175.
10. Munz C, Steinman RM, Fujii S. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med.* Jul 18 2005;202(2):203-207.
11. Ikeda H, Old LJ, Schreiber RD. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine & growth factor reviews.* Apr 2002;13(2):95-109.
12. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer.* Apr 2002;2(4):277-288.
13. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol.* Oct 2002;2(10):735-747.
14. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol.* Apr 2012;12(4):239-252.
15. Marcus A, Gowen BG, Thompson TW, et al. Recognition of tumors by the innate immune system and natural killer cells. *Advances in immunology.* 2014;122:91-128.
16. Igney FH, Krammer PH. Immune escape of tumors: apoptosis resistance and tumor counterattack. *J Leukoc Biol.* Jun 2002;71(6):907-920.
17. O'Connell J, Bennett MW, O'Sullivan GC, Collins JK, Shanahan F. Fas counter-attack--the best form of tumor defense? *Nat Med.* Mar 1999;5(3):267-268.
18. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med.* Aug 2002;8(8):793-800.
19. Platten M, Wick W, Van den Eynde BJ. Tryptophan catabolism in cancer: beyond IDO and tryptophan depletion. *Cancer Res.* Nov 1 2012;72(21):5435-5440.
20. Burkholder B, Huang RY, Burgess R, et al. Tumor-induced perturbations of cytokines and immune cell networks. *Biochimica et biophysica acta.* Apr 2014;1845(2):182-201.
21. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol.* Apr 2012;12(4):269-281.

22. Poschke I, Mougiakakos D, Kiessling R. Camouflage and sabotage: tumor escape from the immune system. *Cancer immunology, immunotherapy : CII.* 2011;60(8):1161-1171.
23. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends in Molecular Medicine.* 2007;13(3):108-116.
24. Wesolowski R, Markowitz J, Carson WE. Myeloid derived suppressor cells – a new therapeutic target in the treatment of cancer. *Journal for ImmunoTherapy of Cancer.* 2013;1(1):10-10.
25. Ochsenbein AF. Principles of tumor immunosurveillance and implications for immunotherapy. *Cancer Gene Ther.* Dec 2002;9(12):1043-1055.
26. van Mierlo GJ, Boonman ZF, Dumortier HM, et al. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. *Journal of immunology.* Dec 1 2004;173(11):6753-6759.
27. Buonaguro L, Petrizzo A, Tornesello ML, Buonaguro FM. Translating tumor antigens into cancer vaccines. *Clin Vaccine Immunol.* Jan 2011;18(1):23-34.
28. Caballero OL, Chen YT. Cancer/testis (CT) antigens: Potential targets for immunotherapy. *Cancer Science.* 2009;100:2014-2021.
29. Weide B, Garbe C, Rammensee HG, Pascolo S. Plasmid DNA- and messenger RNA-based anti-cancer vaccination. *Immunology Letters.* 2008;115:33-42.
30. Liao JB. Viruses and human cancer. *The Yale journal of biology and medicine.* Dec 2006;79(3-4):115-122.
31. Kim TJ, Jin HT, Hur SY, et al. Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. *Nature communications.* 2014;5:5317.
32. Poljak M. Prophylactic human papillomavirus vaccination and primary prevention of cervical cancer: issues and challenges. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases.* Oct 2012;18 Suppl 5:64-69.
33. Srinivasan R, Wolchok JD. Tumor antigens for cancer immunotherapy: therapeutic potential of xenogeneic DNA vaccines. *Journal of translational medicine.* 2004;2:12-12.
34. Chiang CLL, Benencia F, Coukos G. Whole tumor antigen vaccines. *Seminars in Immunology.* 2010;22(3):132-143.
35. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature.* 12/22/2011 2011;480(7378):480-489.
36. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. *Clin Cancer Res.* Jun 1 2011;17(11):3520-3526.
37. Gallo P, Dharmapuri S, Nuzzo M, et al. Xenogeneic immunization in mice using HER2 DNA delivered by an adenoviral vector. *Int J Cancer.* Jan 1 2005;113(1):67-77.
38. Sahin U, Kariko K, Tureci O. mRNA-based therapeutics--developing a new class of drugs. *Nature reviews. Drug discovery.* Oct 2014;13(10):759-780.
39. Ferraro B, Morrow MP, Hutnick NA, Shin TH, Lucke CE, Weiner DB. Clinical applications of DNA vaccines: current progress. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* Aug 1 2011;53(3):296-302.
40. Baban CK, Cronin M, O'Hanlon D, O'Sullivan GC, Tangney M. Bacteria as vectors for gene therapy of cancer. *Bioeng Bugs.* Nov-Dec 2010;1(6):385-394.

41. Perez Sa, Von Hofe E, Kallinteris NL, et al. A new era in anticancer peptide vaccines. *Cancer.* 2010;116:2071-2080.
42. Draper SJ, Heeney JL. Viruses as vaccine vectors for infectious diseases and cancer. *Nature reviews. Microbiology.* 2010;8(jANuArY):62-73.
43. Khazaie K, Bonertz A, Beckhove P. Current developments with peptide-based human tumor vaccines. *Current opinion in oncology.* 2009;21:524-530.
44. Goubier A, Fuhrmann L, Forest L, et al. Superiority of needle-free transdermal plasmid delivery for the induction of antigen-specific IFNgamma T cell responses in the dog. *Vaccine.* 4/24/2008 2008;26(18):2186-2190.
45. Peruzzi D, Mesiti G, Ciliberto G, La MN, Aurisicchio L. Telomerase and HER-2/neu as targets of genetic cancer vaccines in dogs. *Vaccine.* 2/3/2010 2010;28(5):1201-1208.
46. Tamura K, Arai H, Ueno E, et al. Comparison of dendritic cell-mediated immune responses among canine malignant cells. *J.Vet.Med.Sci.* 9/2007 2007;69(9):925-930.
47. Tamura K, Yamada M, Isotani M, et al. Induction of dendritic cell-mediated immune responses against canine malignant melanoma cells. *Vet.J.* 1/2008 2008;175(1):126-129.
48. Bird RC, Deinnocentes P, Lenz S, Thacker EE, Curiel DT, Smith BF. An allogeneic hybrid-cell fusion vaccine against canine mammary cancer. *Vet.Immunol.Immunopathol.* 6/15/2008 2008;123(3-4):289-304.
49. Bird RC, Deinnocentes P, Church Bird AE, van Ginkel FW, Lindquist J, Smith BF. An autologous dendritic cell canine mammary tumor hybrid-cell fusion vaccine. *Cancer Immunol.Immunother.* 1/2011 2011;60(1):87-97.
50. Milner RJ, Salute M, Crawford C, Abbot JR, Farese J. The immune response to disialoganglioside GD3 vaccination in normal dogs: a melanoma surface antigen vaccine. *Vet.Immunol.Immunopathol.* 12/15/2006 2006;114(3-4):273-284.
51. Hogge GS, Burkholder JK, Culp J, et al. Preclinical development of human granulocyte-macrophage colony-stimulating factor-transfected melanoma cell vaccine using established canine cell lines and normal dogs. *Cancer Gene Ther.* 1/1999 1999;6(1):26-36.
52. Thacker EE, Nakayama M, Smith BF, et al. A genetically engineered adenovirus vector targeted to CD40 mediates transduction of canine dendritic cells and promotes antigen-specific immune responses in vivo. *Vaccine.* 11/23/2009 2009;27(50):7116-7124.
53. Smith BF, Baker HJ, Curiel DT, Jiang W, Conry RM. Humoral and cellular immune responses of dogs immunized with a nucleic acid vaccine encoding human carcinoembryonic antigen. *Gene Ther.* 7/1998 1998;5(7):865-868.
54. Yu WY, Chuang TF, Guichard C, et al. Chicken HSP70 DNA vaccine inhibits tumor growth in a canine cancer model. *Vaccine.* 4/18/2011 2011;29(18):3489-3500.
55. Pai CC, Kuo TF, Mao SJ, Chuang TF, Lin CS, Chu RM. Immunopathogenic behaviors of canine transmissible venereal tumor in dogs following an immunotherapy using dendritic/tumor cell hybrid. *Vet.Immunol.Immunopathol.* 2/15/2011 2011;139(2-4):187-199.
56. Peruzzi D, Mesiti G, Ciliberto G, La Monica N, Aurisicchio L. Telomerase and HER-2/neu as targets of genetic cancer vaccines in dogs. *Vaccine.* Feb 3 2010;28(5):1201-1208.
57. Murchison EP. Clonally transmissible cancers in dogs and Tasmanian devils. *Oncogene.* 12/2008 2008;27 Suppl 2:S19-S30.

58. Hurk vDL-vd, Babiuk SL, Babiuk LA. Strategies for improved formulation and delivery of DNA vaccines to veterinary target species. *Immunol.Rev.* 6/2004 2004;199:113-125.
59. Tang H, Foster NR, Grothey A, Ansell SM, Goldberg RM, Sargent DJ. Comparison of error rates in single-arm versus randomized phase II cancer clinical trials. *J.Clin.Oncol.* 4/10/2010 2010;28(11):1936-1941.
60. Sharma MR, Stadler WM, Ratain MJ. Randomized phase II trials: a long-term investment with promising returns. *J.Natl.Cancer Inst.* 7/20/2011 2011;103(14):1093-1100.
61. Farley J, Rose PG. Trial design for evaluation of novel targeted therapies. *Gynecol.Oncol.* 2/2010 2010;116(2):173-176.
62. Grosenbaugh DA, Leard AT, Bergman PJ, et al. Safety and efficacy of a xenogeneic DNA vaccine encoding for human tyrosinase as adjunctive treatment for oral malignant melanoma in dogs following surgical excision of the primary tumor. *Am J Vet Res.* Dec 2011;72(12):1631-1638.
63. Manley CA, Leibman NF, Wolchok JD, et al. Xenogeneic murine tyrosinase DNA vaccine for malignant melanoma of the digit of dogs. *J.Vet.Intern.Med.* 1/2011 2011;25(1):94-99.
64. Finocchiaro LM, Glikin GC. Cytokine-enhanced vaccine and suicide gene therapy as surgery adjuvant treatments for spontaneous canine melanoma: 9 years of follow-up. *Cancer Gene Ther.* Dec 2012;19(12):852-861.
65. Benjamini E, Theilen GH, Torten M, Fong S, Crow S, Henness AM. Tumor vaccines for immunotherapy of canine lymphosarcoma. *Ann.N.Y.Acad.Sci.* 1976 1976;277(00):305-312.
66. Peruzzi D, Gavazza A, Mesiti G, et al. A vaccine targeting telomerase enhances survival of dogs affected by B-cell lymphoma. *Mol.Ther.* 8/2010 2010;18(8):1559-1567.
67. U'Ren LW, Biller BJ, Elmslie RE, Thamm DH, Dow SW. Evaluation of a novel tumor vaccine in dogs with hemangiosarcoma. *J.Vet.Intern.Med.* 1/2007 2007;21(1):113-120.
68. Bergman PJ, Camps-Palau MA, McKnight JA, et al. Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. *Vaccine.* 5/22/2006 2006;24(21):4582-4585.
69. Bergman PJ, McKnight J, Novosad A, et al. Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. *Clin.Cancer Res.* 4/2003 2003;9(4):1284-1290.
70. Grosenbaugh DA, Leard AT, Bergman PJ, et al. Safety and efficacy of a xenogeneic DNA vaccine encoding for human tyrosinase as adjunctive treatment for oral malignant melanoma in dogs following surgical excision of the primary tumor. *Am.J.Vet.Res.* 12/2011 2011;72(12):1631-1638.
71. Liao JC, Gregor P, Wolchok JD, et al. Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. *Cancer Immun.* 2006 2006;6:8.
72. Michaeli Y, Denkberg G, Sinik K, et al. Expression hierarchy of T cell epitopes from melanoma differentiation antigens: unexpected high level presentation of tyrosinase-HLA-A2 Complexes revealed by peptide-specific, MHC-restricted, TCR-like antibodies. *J.Immunol.* 5/15/2009 2009;182(10):6328-6341.
73. MacEwen EG, Kurzman ID, Vail DM, et al. Adjuvant therapy for melanoma in dogs: results of randomized clinical trials using surgery, liposome-encapsulated muramyl

tripeptide, and granulocyte macrophage colony-stimulating factor. *Clin.Cancer Res.* 12/1999 1999;5(12):4249-4258.
74. Finocchiaro LM, Glikin GC. Cytokine-enhanced vaccine and suicide gene therapy as surgery adjuvant treatments for spontaneous canine melanoma. *Gene Ther.* 2/2008 2008;15(4):267-276.
75. Argyle DJ, Nasir L. Telomerase: a potential diagnostic and therapeutic tool in canine oncology. *Vet.Pathol.* 1/2003 2003;40(1):1-7.
76. Kow K, Thamm DH, Terry J, et al. Impact of telomerase status on canine osteosarcoma patients. *J.Vet.Intern.Med.* 11/2008 2008;22(6):1366-1372.
77. Long S, Argyle DJ, Nixon C, et al. Telomerase reverse transcriptase (TERT) expression and proliferation in canine brain tumours. *Neuropathol.Appl.Neurobiol.* 12/2006 2006;32(6):662-673.
78. Mandrioli L, Panarese S, Cesari A, Mandara MT, Marcato PS, Bettini G. Immunohistochemical expression of h-telomerase reverse transcriptase in canine and feline meningiomas. *J.Vet.Sci.* 6/2007 2007;8(2):111-115.
79. Jeglum KA, Young KM, Barnsley K, Whereat A. Chemotherapy versus chemotherapy with intralymphatic tumor cell vaccine in canine lymphoma. *Cancer.* 5/15/1988 1988;61(10):2042-2050.
80. Turek MM, Thamm DH, Mitzey A, et al. Human granulocyte – macrophage colony-stimulating factor DNA cationic-lipid complexed autologous tumour cell vaccination in the treatment of canine B-cell multicentric lymphoma. 2007:219-232.
81. Henson MS, Curtsinger JM, Larson VS, et al. Immunotherapy with autologous tumour antigen-coated microbeads (large multivalent immunogen), IL-2 and GM-CSF in dogs with spontaneous B-cell lymphoma. *Vet.Comp Oncol.* 6/2011 2011;9(2):95-105.
82. Sorenmo KU, Krick E, Coughlin CM, et al. CD40-activated B cell cancer vaccine improves second clinical remission and survival in privately owned dogs with non-Hodgkin's lymphoma. *PloS one.* 2011;6(8):e24167-e24167.
83. Jeglum KA, Young KM, Barnsley K, Whereat A, McGrath D, Hutson C. Intralymphatic autochthonous tumor cell vaccine in canine lymphoma. *J.Biol.Response Mod.* 4/1986 1986;5(2):168-175.
84. Turek MM, Thamm DH, Mitzey A, et al. Human granulocyte-macrophage colony-stimulating factor DNA cationic-lipid complexed autologous tumour cell vaccination in the treatment of canine B-cell multicentric lymphoma. *Vet.Comp Oncol.* 12/2007 2007;5(4):219-231.
85. Sorenmo KU, Krick E, Coughlin CM, et al. CD40-activated B cell cancer vaccine improves second clinical remission and survival in privately owned dogs with non-Hodgkin's lymphoma. *PLoS.One.* 2011 2011;6(8):e24167.
86. Mason NJ, Coughlin CM, Overley B, et al. RNA-loaded CD40-activated B cells stimulate antigen-specific T-cell responses in dogs with spontaneous lymphoma. *Gene Ther.* 7/2008 2008;15(13):955-965.
87. Shanbhogue AK, Karnad AB, Prasad SR. Tumor response evaluation in oncology: current update. *J.Comput.Assist.Tomogr.* 7/2010 2010;34(4):479-484.
88. Mackiewicz J, Mackiewicz A. Design of clinical trials for therapeutic cancer vaccines development. *Eur.J.Pharmacol.* 12/25/2009 2009;625(1-3):84-89.
89. Sasada T, Komatsu N, Suekane S, Yamada A, Noguchi M, Itoh K. Overcoming the hurdles of randomised clinical trials of therapeutic cancer vaccines. *Eur.J.Cancer.* 6/2010 2010;46(9):1514-1519.
90. Lesterhuis WJ, Haanen JB, Punt CJ. Cancer immunotherapy--revisited. *Nat.Rev.Drug Discov.* 8/2011 2011;10(8):591-600.

91. Hogge GS, Burkholder JK, Culp J, et al. Development of human granulocyte-macrophage colony-stimulating factor-transfected tumor cell vaccines for the treatment of spontaneous canine cancer. *Hum.Gene Ther.* 9/1/1998 1998;9(13):1851-1861.
92. Chauvet AE, Hogge GS, Sandin JA, Lipsitz D. Vertebrectomy, bone allograft fusion, and antitumor vaccination for the treatment of vertebral fibrosarcoma in a dog. *Vet.Surg.* 11/1999 1999;28(6):480-488.
93. Gyorffy S, Rodriguez-Lecompte JC, Woods JP, et al. Bone marrow-derived dendritic cell vaccination of dogs with naturally occurring melanoma by using human gp100 antigen. *J.Vet.Intern.Med.* 1/2005 2005;19(1):56-63.
94. Mito K, Sugiura K, Ueda K, et al. IFN{gamma} markedly cooperates with intratumoral dendritic cell vaccine in dog tumor models. *Cancer research.* 2010;70(18):7093-7101.
95. Alexander AN, Huelsmeyer MK, Mitzey A, et al. Development of an allogeneic whole-cell tumor vaccine expressing xenogeneic gp100 and its implementation in a phase II clinical trial in canine patients with malignant melanoma. *Cancer Immunol.Immunother.* 4/2006 2006;55(4):433-442.
96. Kamstock D, Elmslie R, Thamm D, Dow S. Evaluation of a xenogeneic VEGF vaccine in dogs with soft tissue sarcoma. *Cancer Immunology, Immunotherapy.* 2007;56:1299-1309.
97. Lawman M, Eidizadeh S, Selmon C, et al. Anti-tumor response induced by autologous cancer vaccine in canine lymphoma. 2008;6:827-840.
98. Finocchiaro LM, Villaverde MS, Gil-Cardeza ML, Riveros MD, Glikin GC. Cytokine-enhanced vaccine and interferon-beta plus suicide gene as combined therapy for spontaneous canine sarcomas. *Res.Vet.Sci.* 10/2011 2011;91(2):230-234.
99. Gabai V, Venanzi FM, Bagashova E, et al. Pilot study of p62 DNA vaccine in dogs with mammary tumors. *Oncotarget.* Dec 30 2014;5(24):12803-12810.
100. Mito K, Sugiura K, Ueda K, et al. IFN{gamma} markedly cooperates with intratumoral dendritic cell vaccine in dog tumor models. *Cancer Res.* 9/15/2010 2010;70(18):7093-7101.
101. Kamstock D, Elmslie R, Thamm D, Dow S. Evaluation of a xenogeneic VEGF vaccine in dogs with soft tissue sarcoma. *Cancer Immunol.Immunother.* 8/2007 2007;56(8):1299-1309.
102. Company page ImmuneFx. http://www.morphogenesis-inc.com/rd/technologies/immunefx/. Accessed 10/12, 2015.
103. Lawman M, Eidizadeh S, Selmon C, et al. Anti-tumor response induced by autologous cancer vaccine in canine lymphoma. *Cancer Therapy.* 2008 2008;6:827-840.
104. Pluhar GE, Grogan PT, Seiler C, et al. Anti-tumor immune response correlates with neurological symptoms in a dog with spontaneous astrocytoma treated by gene and vaccine therapy. *Vaccine.* 4/26/2010 2010;28(19):3371-3378.
105. Rossi A, Maione P, Schettino C, et al. Non-small-cell lung carcinoma vaccines in clinical trials. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):887-897.
106. Leffers N, Daemen T, Boezen HM, Melief KJ, Nijman HW. Vaccine-based clinical trials in ovarian cancer. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):775-784.
107. Mittendorf EA, Alatrash G, Xiao H, Clifton GT, Murray JL, Peoples GE. Breast cancer vaccines: ongoing National Cancer Institute-registered clinical trials. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):755-774.

108. Madan RA, Aragon-Ching JB, Gulley JL, Dahut WL. From clinical trials to clinical practice: therapeutic cancer vaccines for the treatment of prostate cancer. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):743-753.
109. Rezvani K, de LH. Vaccination strategies in lymphomas and leukaemias: recent progress. *Drugs.* 9/10/2011 2011;71(13):1659-1674.
110. Speetjens FM, Zeestraten EC, Kuppen PJ, Melief CJ, van der Burg SH. Colorectal cancer vaccines in clinical trials. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):899-921.
111. Klein O, Schmidt C, Knights A, Davis ID, Chen W, Cebon J. Melanoma vaccines: developments over the past 10 years. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):853-873.
112. Brookman-May S, Burger M, Wieland WF, Rossler W, May M, Denzinger S. Vaccination therapy in renal cell carcinoma: current position and future options in metastatic and localized disease. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):837-852.
113. Plate J. Clinical trials of vaccines for immunotherapy in pancreatic cancer. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):825-836.
114. Bridle BW. Neuroendocrine cancer vaccines in clinical trials. *Expert.Rev.Vaccines.* 6/2011 2011;10(6):811-823.
115. Ebben JD, Rocque BG, Kuo JS. Tumour vaccine approaches for CNS malignancies: progress to date. *Drugs.* 2009 2009;69(3):241-249.
116. Di LG, Ferro M, Buonerba C. Sipuleucel-T (Provenge(R)) for castration-resistant prostate cancer. *BJU.Int.* 12/16/2011 2011.
117. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat.Rev.Genet.* 10/2008 2008;9(10):776-788.
118. Janetzki S, Britten CM, Kalos M, et al. "MIATA"-minimal information about T cell assays. *Immunity.* 10/16/2009 2009;31(4):527-528.
119. Bergman PJ, Wolchok JD. Of mice and men (and dogs): development of a xenogeneic DNA vaccine for canine oral malignant melanoma. *Cancer Therapy.* 2008 2008;6:817-826.
120. Wolchok JD, Yuan J, Houghton AN, et al. Safety and immunogenicity of tyrosinase DNA vaccines in patients with melanoma. *Mol.Ther.* 11/2007 2007;15(11):2044-2050.
121. Vacchelli E, Galluzzi L, Eggermont A, et al. Trial Watch: Immunostimulatory cytokines. *Oncoimmunology.* Jul 1 2012;1(4):493-506.
122. Tarrant JM. Blood cytokines as biomarkers of in vivo toxicity in preclinical safety assessment: considerations for their use. *Toxicological sciences : an official journal of the Society of Toxicology.* Sep 2010;117(1):4-16.
123. Vazquez-Lombardi R, Roome B, Christ D. Molecular Engineering of Therapeutic Cytokines. *Antibodies.* 2013;2(3):426.
124. Lee S, Margolin K. Cytokines in cancer immunotherapy. *Cancers.* 2011;3(4):3856-3893.
125. Grunhagen DJ, de Wilt JH, Verhoef C, van Geel AN, Eggermont AM. TNF-based isolated limb perfusion in unresectable extremity desmoid tumours. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology.* Oct 2005;31(8):912-916.
126. Steding CE, Wu ST, Zhang Y, Jeng MH, Elzey BD, Kao C. The role of interleukin-12 on modulating myeloid-derived suppressor cells, increasing overall survival and reducing metastasis. *Immunology.* Jun 2011;133(2):221-238.
127. Lasek W, Zagozdzon R, Jakobisiak M. Interleukin 12: still a promising candidate for tumor immunotherapy? *Cancer Immunol Immunother.* May 2014;63(5):419-435.

128. Cools N, Ponsaerts P, Van Tendeloo VF, Berneman ZN. Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. *J Leukoc Biol.* Dec 2007;82(6):1365-1374.
129. Curtsinger JM, Lins DC, Mescher MF. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J Exp Med.* May 5 2003;197(9):1141-1151.
130. Valenzuela J, Schmidt C, Mescher M. The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. *Journal of immunology.* Dec 15 2002;169(12):6842-6849.
131. Romee R, Leong JW, Fehniger TA. Utilizing cytokines to function-enable human NK cells for the immunotherapy of cancer. *Scientifica.* 2014;2014:205796.
132. Thaci B, Ahmed AU, Ulasov IV, et al. Depletion of myeloid-derived suppressor cells during interleukin-12 immunogene therapy does not confer a survival advantage in experimental malignant glioma. *Cancer Gene Ther.* Jan 2014;21(1):38-44.
133. Del Vecchio M, Bajetta E, Canova S, et al. Interleukin-12: biological properties and clinical application. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2007;13(16):4677-4685.
134. Dow SW, Elmslie RE, Willson AP, Roche L, Gorman C, Potter Ta. In vivo tumor transfection with superantigen plus cytokine genes induces tumor regression and prolongs survival in dogs with malignant melanoma. *Journal of Clinical Investigation.* 1998;101:2406-2414.
135. Dow S, Elmslie R, Kurzman I, MacEwen G, Pericle F, Liggitt D. Phase I study of liposome-DNA complexes encoding the interleukin-2 gene in dogs with osteosarcoma lung metastases. *Human gene therapy.* 2005;16(August):937-946.
136. Pavlin D, Cemazar M, Cör A, Sersa G, Pogacnik A, Tozon N. Electrogene therapy with interleukin-12 in canine mast cell tumors. *Radiology and Oncology.* 2010;45(1):31-39.
137. Cutrera J, King G, Jones P, et al. Safety and Efficacy of Tumor-targeted Interleukin 12 Gene Therapy in Treated and Non-treated, Metastatic Lesions. *Current gene therapy.* 2014;15(1):44-54.
138. Cutrera J, Torrero M, Shiomitsu K, Mauldin N, Li S. Intratumoral bleomycin and IL-12 electrochemogenetherapy for treating head and neck tumors in dogs. *Methods in molecular biology.* 2008;423:319-325.
139. Reed SD, Fulmer A, Buckholz J, et al. Bleomycin/interleukin-12 electrochemogenetherapy for treating naturally occurring spontaneous neoplasms in dogs. *Cancer Gene Ther.* Aug 2010;17(8):571-578.
140. Jourdier TM, Moste C, Bonnet MC, et al. Local immunotherapy of spontaneous feline fibrosarcomas using recombinant poxviruses expressing interleukin 2 (IL2). *Gene Ther.* Dec 2003;10(26):2126-2132.
141. Hampel V, Schwarz B, Kempf C, et al. Adjuvant immunotherapy of feline fibrosarcoma with recombinant feline interferon-omega. *J Vet Intern Med.* Nov-Dec 2007;21(6):1340-1346.
142. Jahnke A, Hirschberger J, Fischer C, et al. Intra-tumoral gene delivery of feIL-2, feIFN-gamma and feGM-CSF using magnetofection as a neoadjuvant treatment option for feline fibrosarcomas: a phase-I study. *Journal of veterinary medicine. A, Physiology, pathology, clinical medicine.* Dec 2007;54(10):599-606.
143. Siddiqui F, Li C-Y, Larue SM, et al. A phase I trial of hyperthermia-induced interleukin-12 gene therapy in spontaneously arising feline soft tissue sarcomas. *Molecular cancer therapeutics.* 2007;6(1):380-389.

144. Heinzerling LM, Feige K, Rieder S, et al. Tumor regression induced by intratumoral injection of DNA coding for human interleukin 12 into melanoma metastases in gray horses. *Journal of Molecular Medicine.* 2000;78:692-702.
145. Spoormakers TJ, Klein WR, Jacobs JJ, Van Den Ingh TS, Koten JW, Den Otter W. Comparison of the efficacy of local treatment of equine sarcoids with IL-2 or cisplatin/IL-2. *Cancer Immunol Immunother.* Mar 2003;52(3):179-184.
146. Hanahan D, Bergers G, Bergsland E. Less is, more, regularly: Metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. *Journal of Clinical Investigation.* 2000;105(8):1045-1047.
147. Zhao J, Cao Y, Lei Z, Yang Z, Zhang B, Huang B. Selective depletion of CD4+CD25+Foxp3+ regulatory T cells by low-dose cyclophosphamide is explained by reduced intracellular ATP levels. *Cancer research.* 2010;70(12):4850-4858.
148. Kerbel RS. Improving conventional or low dose metronomic chemotherapy with targeted antiangiogenic drugs. *Cancer research and treatment : official journal of Korean Cancer Association.* Dec 2007;39(4):150-159.
149. Andre N, Carre M, Pasquier E. Metronomics: towards personalized chemotherapy? *Nature reviews. Clinical oncology.* Jul 2014;11(7):413-431.
150. Hao YB, Yi SY, Ruan J, Zhao L, Nan KJ. New insights into metronomic chemotherapy-induced immunoregulation. *Cancer Lett.* Nov 28 2014;354(2):220-226.
151. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nature reviews. Immunology.* 2006;6(April):295-307.
152. Lana S, U'Ren L, Plaza S, et al. Continuous low-dose oral chemotherapy for adjuvant therapy of splenic hemangiosarcoma in dogs. *J Vet Intern Med.* Jul-Aug 2007;21(4):764-769.
153. Elmslie RE, Glawe P, Dow SW. Metronomic therapy with cyclophosphamide and piroxicam effectively delays tumor recurrence in dogs with incompletely resected soft tissue sarcomas. *J Vet Intern Med.* Nov-Dec 2008;22(6):1373-1379.
154. Marchetti V, Giorgi M, Fioravanti A, et al. First-line metronomic chemotherapy in a metastatic model of spontaneous canine tumours : a pilot study. 2012:1725-1730.
155. Spugnini EP, Buglioni S, Carocci F, et al. High dose lansoprazole combined with metronomic chemotherapy: a phase I/II study in companion animals with spontaneously occurring tumors. *J Transl Med.* 2014;12:225.
156. Burton JH, Mitchell L, Thamm DH, Dow SW, Biller BJ. Low-dose cyclophosphamide selectively decreases regulatory T cells and inhibits angiogenesis in dogs with soft tissue sarcoma. *Journal of Veterinary Internal Medicine.* 2011;25:920-926.
157. Mitchell L, Thamm DH, Biller BJ. Clinical and Immunomodulatory Effects of Toceranib Combined with Low-Dose Cyclophosphamide in Dogs with Cancer. *Journal of Veterinary Internal Medicine.* 2012;26:355-362.
158. Tripp CD, Fidel J, Anderson CL, et al. Tolerability of metronomic administration of lomustine in dogs with cancer. *J Vet Intern Med.* Mar-Apr 2011;25(2):278-284.
159. Leach TN, Childress MO, Greene SN, et al. Prospective trial of metronomic chlorambucil chemotherapy in dogs with naturally occurring cancer. *Veterinary and Comparative Oncology.* 2012;10:102-112.
160. Schrempp DR, Childress MO, Stewart JC, et al. Metronomic administration of chlorambucil for treatment of dogs with urinary bladder transitional cell carcinoma. *Journal of the American Veterinary Medical Association.* 2013;242(11):1534-1538.
161. Adams VJ, Evans KM, Sampson J, Wood JL. Methods and mortality results of a health survey of purebred dogs in the UK. *The Journal of small animal practice.* Oct 2010;51(10):512-524.

162. Pinho SS, Carvalho S, Cabral J, Reis CA, Gartner F. Canine tumors: a spontaneous animal model of human carcinogenesis. *Transl.Res.* 3/2012 2012;159(3):165-172.
163. Pang LY, Argyle DJ. Using naturally occurring tumours in dogs and cats to study telomerase and cancer stem cell biology. *Biochim.Biophys.Acta.* 4/2009 2009;1792(4):380-391.
164. Vail DM, MacEwen EG. Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer Invest.* 2000 2000;18(8):781-792.
165. Rankin KS, Starkey M, Lunec J, Gerrand CH, Murphy S, Biswas S. Of dogs and men: comparative biology as a tool for the discovery of novel biomarkers and drug development targets in osteosarcoma. *Pediatr.Blood Cancer.* 3/2012 2012;58(3):327-333.
166. Khanna C, London C, Vail D, Mazcko C, Hirschfeld S. Guiding the optimal translation of new cancer treatments from canine to human cancer patients. *Clin.Cancer Res.* 9/15/2009 2009;15(18):5671-5677.
167. Hangalapura BN, Timares L, Oosterhoff D, Scheper RJ, Curiel DT, de Gruijl TD. CD40-targeted adenoviral cancer vaccines: the long and winding road to the clinic. *J.Gene Med.* 1/7/2012 2012.
168. MacEwen EG, Kurzman ID, Rosenthal RC, et al. Therapy for osteosarcoma in dogs with intravenous injection of liposome-encapsulated muramyl tripeptide. *J.Natl.Cancer Inst.* 6/21/1989 1989;81(12):935-938.
169. Meyers PA, Schwartz CL, Krailo MD, et al. Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival--a report from the Children's Oncology Group. *J.Clin.Oncol.* 2/1/2008 2008;26(4):633-638.
170. Jain KK. Personalized cancer vaccines. *Expert.Opin.Biol.Ther.* 12/2010 2010;10(12):1637-1647.
171. Belardelli F, Ferrantini M. Cytokines as a link between innate and adaptive antitumor immunity. *Trends Immunol.* 4/2002 2002;23(4):201-208.
172. Poschke I, Mougiakakos D, Kiessling R. Camouflage and sabotage: tumor escape from the immune system. *Cancer Immunol.Immunother.* 8/2011 2011;60(8):1161-1171.
173. Trinchieri G. Recognition of major histocompatibility complex class I antigens by natural killer cells. *J.Exp.Med.* 8/1/1994 1994;180(2):417-421.
174. Zamai L, Ponti C, Mirandola P, et al. NK cells and cancer. *J.Immunol.* 4/1/2007 2007;178(7):4011-4016.
175. Asselin-Paturel C, Lassau N, Guinebretiere JM, et al. Transfer of the murine interleukin-12 gene in vivo by a Semliki Forest virus vector induces B16 tumor regression through inhibition of tumor blood vessel formation monitored by Doppler ultrasonography. *Gene Ther.* 4/1999 1999;6(4):606-615.
176. Steding CE, Wu ST, Zhang Y, Jeng MH, Elzey BD, Kao C. The role of interleukin-12 on modulating myeloid-derived suppressor cells, increasing overall survival and reducing metastasis. *Immunology.* 6/2011 2011;133(2):221-238.
177. Del VM, Bajetta E, Canova S, et al. Interleukin-12: biological properties and clinical application. *Clin.Cancer Res.* 8/15/2007 2007;13(16):4677-4685.
178. Lucas ML, Heller R. IL-12 gene therapy using an electrically mediated nonviral approach reduces metastatic growth of melanoma. *DNA Cell Biol.* 12/2003 2003;22(12):755-763.
179. Cemazar M, Jarm T, Sersa G. Cancer electrogene therapy with interleukin-12. *Curr.Gene Ther.* 8/2010 2010;10(4):300-311.
180. Cemazar M, Jarm T, Sersa G. Cancer electrogene therapy with interleukin-12. *Current Gene Therapy.* 2010;10(4):300-311.

181. Morris LF, Ribas A. Therapeutic cancer vaccines. *Surg.Oncol.Clin.N.Am.* 10/2007 2007;16(4):819-831, ix.
182. Stevenson FK, Ottensmeier CH, Rice J. DNA vaccines against cancer come of age. *Curr.Opin.Immunol.* 4/2010 2010;22(2):264-270.
183. Weide B, Garbe C, Rammensee HG, Pascolo S. Plasmid DNA- and messenger RNA-based anti-cancer vaccination. *Immunol.Lett.* 1/15/2008 2008;115(1):33-42.
184. Yuan J, Ku G, Adamow M, et al. Immunologic responses to xenogeneic tyrosinase DNA vaccine administered by electroporation in patients with malignant melanoma. *Journal for ImmunoTherapy of Cancer.* 2013 2013;1(1):20.
185. Yuan J, Ku GY, Adamow M, et al. Immunologic responses to xenogeneic tyrosinase DNA vaccine administered by electroporation in patients with malignant melanoma. *J Immunother Cancer.* 2013;1:20.
186. Wesolowski R, Markowitz J, Carson W. Myeloid derived suppressor cells - a new therapeutic target in the treatment of cancer. *Journal for ImmunoTherapy of Cancer.* 2013 2013;1(1):10.
187. Pere H, Tanchot C, Bayry J, et al. Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer. *Oncoimmunology.* 5/1/2012 2012;1(3):326-333.
188. Zhao J, Cao Y, Lei Z, Yang Z, Zhang B, Huang B. Selective depletion of CD4+CD25+Foxp3+ regulatory T cells by low-dose cyclophosphamide is explained by reduced intracellular ATP levels. *Cancer Res.* 6/15/2010 2010;70(12):4850-4858.
189. Van IK, Bethuyne J, Cool S, et al. A nanobody targeting the F-actin capping protein CapG restrains breast cancer metastasis. *Breast Cancer Res.* 12/13/2013 2013;15(6):R116.
190. Description IL-12 plasmid Invivogen. http://www.invivogen.com/PDF/pORF-mIL12(p35p40)_TDS.pdf?PHPSESSID=a92aede6098965f6a98c1df1edcd09a5, 2015.
191. Bergman PJ, McKnight J, Novosad A, et al. Long-Term Survival of Dogs with Advanced Malignant Melanoma after DNA Vaccination with Xenogeneic Human Tyrosinase : A Phase I Trial Advances in Brief Long-Term Survival of Dogs with Advanced Malignant Melanoma after DNA Vaccination with Xenogeneic Human. 2003:1284-1290.
192. Emmenegger U, Shaked Y, Man S, et al. Pharmacodynamic and pharmacokinetic study of chronic low-dose metronomic cyclophosphamide therapy in mice. *Mol.Cancer Ther.* 8/2007 2007;6(8):2280-2289.
193. Hung CF, Tsai YC, He L, Wu TC. Control of mesothelin-expressing ovarian cancer using adoptive transfer of mesothelin peptide-specific CD8+ T cells. *Gene Ther.* 6/2007 2007;14(12):921-929.
194. Othus M, Barlogie B, Leblanc ML, Crowley JJ. Cure models as a useful statistical tool for analyzing survival. *Clin.Cancer Res.* 7/15/2012 2012;18(14):3731-3736.
195. Divino CM, Chen SH, Yang W, Thung S, Brower ST, Woo SL. Anti-tumor immunity induced by interleukin-12 gene therapy in a metastatic model of breast cancer is mediated by natural killer cells. *Breast Cancer Res.Treat.* 3/2000 2000;60(2):129-134.
196. Komita H, Homma S, Saotome H, Zeniya M, Ohno T, Toda G. Interferon-gamma produced by interleukin-12-activated tumor infiltrating CD8+T cells directly induces apoptosis of mouse hepatocellular carcinoma. *J.Hepatol.* 11/2006 2006;45(5):662-672.
197. Hamano Y, Sugimoto H, Soubasakos MA, et al. Thrombospondin-1 associated with tumor microenvironment contributes to low-dose cyclophosphamide-mediated endothelial cell apoptosis and tumor growth suppression. *Cancer Res.* 3/1/2004 2004;64(5):1570-1574.

198. Becker JC, Houben R, Schrama D, Voigt H, Ugurel S, Reisfeld RA. Mouse models for melanoma: a personal perspective. *Exp.Dermatol.* 2/2010 2010;19(2):157-164.
199. Lucas ML, Heller L, Coppola D, Heller R. IL-12 plasmid delivery by in vivo electroporation for the successful treatment of established subcutaneous B16.F10 melanoma. *Mol.Ther.* 6/2002 2002;5(6):668-675.
200. Kim H, Sin JI. Electroporation driven delivery of both an IL-12 expressing plasmid and cisplatin synergizes to inhibit B16 melanoma tumor growth through an NK cell mediated tumor killing mechanism. *Hum.Vaccin.Immunother.* 11/1/2012 2012;8(11):1714-1721.
201. Airoldi I, Di CE, Cocco C, et al. Endogenous IL-12 triggers an antiangiogenic program in melanoma cells. *Proc.Natl.Acad.Sci.U.S.A.* 3/6/2007 2007;104(10):3996-4001.
202. Malvicini M, Rizzo M, Alaniz L, et al. A novel synergistic combination of cyclophosphamide and gene transfer of interleukin-12 eradicates colorectal carcinoma in mice. *Clin.Cancer Res.* 12/1/2009 2009;15(23):7256-7265.
203. Arulanandam BP, Mittler JN, Lee WT, O'Toole M, Metzger DW. Neonatal administration of IL-12 enhances the protective efficacy of antiviral vaccines. *J.Immunol.* 4/1/2000 2000;164(7):3698-3704.
204. Whiteside TL, Ferrone S. For breast cancer prognosis, immunoglobulin kappa chain surfaces to the top. *Clin.Cancer Res.* 5/1/2012 2012;18(9):2417-2419.
205. Schmidt M, Hellwig B, Hammad S, et al. A comprehensive analysis of human gene expression profiles identifies stromal immunoglobulin kappa C as a compatible prognostic marker in human solid tumors. *Clin.Cancer Res.* 5/1/2012 2012;18(9):2695-2703.
206. Mine T, Sato Y, Noguchi M, et al. Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing, peptide-specific cellular responses. *Clin.Cancer Res.* 2/1/2004 2004;10(3):929-937.
207. Gilbert AE, Karagiannis P, Dodev T, et al. Monitoring the systemic human memory B cell compartment of melanoma patients for anti-tumor IgG antibodies. *PLoS.One.* 2011 2011;6(4):e19330.
208. Pearce EL, Shen H. Generation of CD8 T cell memory is regulated by IL-12. *J.Immunol.* 8/15/2007 2007;179(4):2074-2081.
209. Wilson DC, Grotenbreg GM, Liu K, et al. Differential regulation of effector- and central-memory responses to Toxoplasma gondii Infection by IL-12 revealed by tracking of Tgd057-specific CD8+ T cells. *PLoS.Pathog.* 3/2010 2010;6(3):e1000815.
210. Kohlhapp FJ, Zloza A, O'Sullivan JA, et al. CD8(+) T cells sabotage their own memory potential through IFN-gamma-dependent modification of the IL-12/IL-15 receptor alpha axis on dendritic cells. *J.Immunol.* 4/15/2012 2012;188(8):3639-3647.
211. Becker JC, Varki N, Brocker EB, Reisfeld RA. Lymphocyte-mediated alopecia in C57BL/6 mice following successful immunotherapy for melanoma. *J.Invest Dermatol.* 10/1996 1996;107(4):627-632.
212. Bronte V, Apolloni E, Ronca R, et al. Genetic vaccination with "self" tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. *Cancer Res.* 1/15/2000 2000;60(2):253-258.
213. O'Leary JG, Goodarzi M, Drayton DL, von Andrian UH. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat.Immunol.* 5/2006 2006;7(5):507-516.

214. Sun JC, Madera S, Bezman NA, Beilke JN, Kaplan MH, Lanier LL. Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J.Exp.Med.* 5/7/2012 2012;209(5):947-954.
215. Zhang T, Scott JM, Hwang I, Kim S. Cutting edge: antibody-dependent memory-like NK cells distinguished by FcRgamma deficiency. *J.Immunol.* 2/15/2013 2013;190(4):1402-1406.
216. Miklavcic D, Sersa G, Brecelj E, et al. Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors. *Medical & biological engineering & computing.* Dec 2012;50(12):1213-1225.
217. Chinnasamy D, Yu Z, Kerkar SP, et al. Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. *Clin.Cancer Res.* 3/15/2012 2012;18(6):1672-1683.
218. Cutrera J, Dibra D, Xia X, Hasan A, Reed S, Li S. Discovery of a linear peptide for improving tumor targeting of gene products and treatment of distal tumors by IL-12 gene therapy. *Mol.Ther.* 8/2011 2011;19(8):1468-1477.
219. Landreneau JP, Shurin MR, Agassandian MV, Keskinov Aa, Ma Y, Shurin GV. Immunological Mechanisms of Low and Ultra-Low Dose Cancer Chemotherapy. *Cancer Microenvironment.* 2013:1-8.
220. Ghiringhelli F, Menard C, Puig PE, et al. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer immunology, immunotherapy : CII.* 2007;56(5):641-648.
221. Ridolfi L, Petrini M, Granato AM, et al. Low-dose temozolomide before dendritic-cell vaccination reduces (specifically) CD4+CD25++Foxp3+ regulatory T-cells in advanced melanoma patients. *Journal of translational medicine.* 2013;11(1):135-135.
222. Banissi C, Ghiringhelli F, Chen L, Carpentier AF. Treg depletion with a low-dose metronomic temozolomide regimen in a rat glioma model. *Cancer immunology, immunotherapy : CII.* 2009;58(10):1627-1634.
223. Kim T-G, Kim C-H, Park J-S, et al. Immunological factors relating to the antitumor effect of temozolomide chemoimmunotherapy in a murine glioma model. *Clinical and vaccine immunology : CVI.* 2010;17(1):143-153.
224. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature medicine.* 2004;10(9):942-949.
225. Cui Z, Le UM, Qiu F, Shaker DS. Learning from viruses: the necrotic bodies of tumor cells with intracellular synthetic dsRNA induced strong anti-tumor immune responses. *Pharmaceutical research.* 2007;24(9):1645-1652.
226. Liotta F, Gacci M, Frosali F, et al. Frequency of regulatory T cells in peripheral blood and in tumour-infiltrating lymphocytes correlates with poor prognosis in renal cell carcinoma. *BJU International.* 2011;107:1500-1506.
227. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res.* Feb 2003;9(2):606-612.
228. Ge Y, Domschke C, Stoiber N, et al. Metronomic cyclophosphamide treatment in metastasized breast cancer patients: Immunological effects and clinical outcome. *Cancer Immunology, Immunotherapy.* 2012;61:353-362.
229. Tominaga M, Horiuchi Y, Ichikawa M, et al. Flow cytometric analysis of peripheral blood and tumor-infiltrating regulatory T cells in dogs with oral malignant melanoma. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc.* May 2010;22(3):438-441.

230. Biller BJ, Elmslie RE, Burnett RC, Avery AC, Dow SW. Use of FoxP3 expression to identify regulatory T cells in healthy dogs and dogs with cancer. *Vet Immunol Immunopathol.* Mar 15 2007;116(1-2):69-78.
231. Biller BJ, Guth A, Burton JH, Dow SW. Decreased ratio of CD8+ T cells to regulatory T cells associated with decreased survival in dogs with osteosarcoma. *J Vet Intern Med.* Sep-Oct 2010;24(5):1118-1123.
232. Horiuchi Y, Tominaga M, Ichikawa M, et al. Increase of regulatory T cells in the peripheral blood of dogs with metastatic tumors. *Microbiology and immunology.* Aug 2009;53(8):468-474.
233. O'Neill K, Guth A, Biller B, Elmslie R, Dow S. Changes in regulatory T cells in dogs with cancer and associations with tumor type. *J Vet Intern Med.* Jul-Aug 2009;23(4):875-881.
234. Daniele N, Scerpa MC, Landi F, et al. T(reg) cells: collection, processing, storage and clinical use. *Pathology, research and practice.* 2011;207(4):209-215.
235. Abrams VK, Hwang B, Lesnikova M, et al. Veterinary Immunology and Immunopathology A novel monoclonal antibody specific for canine CD25 (P4A10): Selection and evaluation of canine Tregs. *Veterinary Immunology and Immunopathology.* 2010;135(3-4):257-265.
236. Pinheiro D, Singh Y, Grant CR, et al. Phenotypic and functional characterization of a CD4+ CD25high FOXP3high regulatory T-cell population in the dog. *Immunology.* 2011;132:111-122.
237. Knueppel A, Lange S, Sekora A, Altmann S, Freund M, Junghanss C. Phenotypic and functional characterization of freshly isolated and expanded canine regulatory T cells. *Experimental animals / Japanese Association for Laboratory Animal Science.* 2011;60(5):471-479.
238. Bocci G, Francia G, Man S, Lawler J, Kerbel RS. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. *Proceedings of the National Academy of Sciences of the United States of America.* 2003;100(22):12917-12922.
239. Hamano Y. Thrombospondin-1 Associated with Tumor Microenvironment Contributes to Low-Dose Cyclophosphamide-Mediated Endothelial Cell Apoptosis and Tumor Growth Suppression. *Cancer Research.* 2004;64(617):1570-1574.
240. Kerbel RS, Kamen Ba. The anti-angiogenic basis of metronomic chemotherapy. *Nature reviews. Cancer.* 2004;4(June):423-436.
241. Allegrini G, Falcone a, Fioravanti a, et al. A pharmacokinetic and pharmacodynamic study on metronomic irinotecan in metastatic colorectal cancer patients. *British journal of cancer.* 2008;98:1312-1319.
242. Lansiaux A, Salingue S, Dewitte A, Clisant S, Penel N. Circulating thrombospondin 1 level as a surrogate marker in patients receiving cyclophosphamide-based metronomic chemotherapy. *Investigational New Drugs.* 2012;30(may 2006):403-404.
243. Shi H, Jiang J, Ji J, et al. Anti-angiogenesis participates in antitumor effects of metronomic capecitabine on colon cancer. *Cancer Letters.* 2014;349:128-135.
244. Kieran MW, Turner CD, Rubin JB, et al. A feasibility trial of antiangiogenic (metronomic) chemotherapy in pediatric patients with recurrent or progressive cancer. *Journal of pediatric hematology/oncology.* Nov 2005;27(11):573-581.
245. Gupta K, Gupta P, Wild R, Ramakrishnan S, Hebbel RP. Binding and displacement of vascular endothelial growth factor (VEGF) by thrombospondin: effect on human microvascular endothelial cell proliferation and angiogenesis. *Angiogenesis.* 1999;3:147-158.

246. El-Arab LRE, Swellam M, El Mahdy MM. Metronomic chemotherapy in metastatic breast cancer: Impact on VEGF. *Journal of the Egyptian National Cancer Institute.* 2012;24(1):15-22.
247. Denies S, Sanders NN. Recent progress in canine tumor vaccination: potential applications for human tumor vaccines. *Expert Rev Vaccines.* Nov 2012;11(11):1375-1386.
248. Dervisis NG, Dominguez PA, Sarbu L, et al. Efficacy of temozolomide or dacarbazine in combination with an anthracycline for rescue chemotherapy in dogs with lymphoma. *J Am Vet Med Assoc.* Aug 15 2007;231(4):563-569.
249. Horiuchi Y, Tominaga M, Ichikawa M, et al. Relationship between regulatory and type 1 T cells in dogs with oral malignant melanoma. 2010(November 2009):152-159.
250. Drennan S, Stafford ND, Greenman J, Green VL. Increased frequency and suppressive activity of CD127low/- regulatory T cells in the peripheral circulation of patients with head and neck squamous cell carcinoma are associated with advanced stage and nodal involvement. *Immunology.* 2013;140:335-343.
251. EMEA. EMEA scientific discussion for the approval of Temodal. . 2004; http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000229/WC500035617.pdf. Accessed 2015/06/27, 2015.
252. Kato Y, Asano K, Mogi T, et al. Clinical significance of circulating vascular endothelial growth factor in dogs with mammary gland tumors. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science.* 2007;69:77-80.
253. Clifford CA, Hughes D, Beal MW, et al. Plasma vascular endothelial growth factor concentrations in healthy dogs and dogs with hemangiosarcoma. *J Vet Intern Med.* Mar-Apr 2001;15(2):131-135.
254. Karayiannakis AJ, Syrigos KN, Polychronidis A, et al. Circulating VEGF levels in the serum of gastric cancer patients: correlation with pathological variables, patient survival, and tumor surgery. *Annals of surgery.* 2002;236(1):37-42.
255. Li L, Wang L, Zhang W, et al. Correlation of serum VEGF levels with clinical stage, therapy efficacy, tumor metastasis and patient survival in ovarian cancer. *Anticancer Research.* 2004;24:1973-1979.
256. Fleitas T, Martínez-Sales V, Vila V, et al. VEGF and TSP1 levels correlate with prognosis in advanced non-small cell lung cancer. *Clinical and Translational Oncology.* 2013;15:897-902.
257. Lien K, Georgsdottir S, Sivanathan L, Chan K, Emmenegger U. Low-dose metronomic chemotherapy: A systematic literature analysis. *European Journal of Cancer.* 2013;49(16):3387-3395.
258. Schatzkin A, Gail M. The promise and peril of surrogate end points in cancer research. *Nature reviews. Cancer.* 2002;2(January):19-27.
259. Rissetto K. Canine T-regulatory cells in aging and cancer: quantifying a unique T cell subset. 2010:25-25.
260. Mizuno T, Suzuki R, Umeki S, Okuda M. Crossreactivity of antibodies to canine CD25 and Foxp3 and identification of canine CD4+CD25 +Foxp3+ cells in canine peripheral blood. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science.* 2009.
261. Miyara M, Yoshioka Y, Kitoh A, et al. Functional Delineation and Differentiation Dynamics of Human CD4+ T Cells Expressing the FoxP3 Transcription Factor. *Immunity.* 2009;30:899-911.

262. Zelenay S, Lopes-Carvalho T, Caramalho I, Moraes-Fontes MF, Rebelo M, Demengeot J. Foxp3+ CD25- CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion. *Proceedings of the National Academy of Sciences of the United States of America.* 2005;102(11):4091-4096.
263. Yan B, Liu Y. The Nature of Increased Circulating CD4CD25Foxp3 T Cells in Patients with Systemic Lupus Erythematosus: A Novel Hypothesis. *The open rheumatology journal.* 2009;3:22-24.
264. Dimova T, Nagaeva O, Stenqvist AC, et al. Maternal foxp3 expressing CD4 +CD25 + and CD4 +CD25 - regulatory T-cell populations are enriched in human early normal pregnancy decidua: A phenotypic study of paired decidual and peripheral blood samples. *American Journal of Reproductive Immunology.* 2011;66:44-56.
265. Lesterhuis WJ, Haanen JB, Punt CJ. Cancer immunotherapy--revisited. *Nature reviews. Drug discovery.* Aug 2011;10(8):591-600.
266. Youssoufian H, Hicklin DJ, Rowinsky EK. Review: monoclonal antibodies to the vascular endothelial growth factor receptor-2 in cancer therapy. *Clin Cancer Res.* Sep 15 2007;13:5544-5548.
267. Strioga MM, Darinskas A, Pasukoniene V, Mlynska A, Ostapenko V, Schijns V. Xenogeneic therapeutic cancer vaccines as breakers of immune tolerance for clinical application: to use or not to use? *Vaccine.* Jul 7 2014;32(32):4015-4024.
268. Wentink MQ, Huijbers EJ, de Gruijl TD, Verheul HM, Olsson AK, Griffioen AW. Vaccination approach to anti-angiogenic treatment of cancer. *Biochimica et biophysica acta.* Apr 2015;1855(2):155-171.
269. Goel HL, Mercurio AM. VEGF targets the tumour cell. *Nat Rev Cancer.* Dec 2013;13(12):871-882.
270. Yang L, DeBusk LM, Fukuda K, et al. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell.* Oct 2004;6(4):409-421.
271. Suzuki H, Onishi H, Wada J, et al. VEGFR2 is selectively expressed by FOXP3high CD4+ Treg. *Eur J Immunol.* Jan 2010;40(1):197-203.
272. Matejuk A, Leng Q, Chou ST, Mixson AJ. Vaccines targeting the neovasculature of tumors. *Vasc Cell.* 2011;3(1):7.
273. Morris LF, Ribas A. Therapeutic cancer vaccines. *Surgical oncology clinics of North America.* Oct 2007;16(4):819-831
274. Scheule RK. The role of CpG motifs in immunostimulation and gene therapy. *Advanced drug delivery reviews.* Nov 15 2000;44(2-3):119-134.
275. Stevenson FK, Ottensmeier CH, Rice J. DNA vaccines against cancer come of age. *Curr Opin Immunol.* Apr 2010;22(2):264-270.
276. Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther.* Dec 2002;9(24):1647-1652.
277. Xu L, Anchordoquy T. Drug delivery trends in clinical trials and translational medicine: challenges and opportunities in the delivery of nucleic acid-based therapeutics. *Journal of pharmaceutical sciences.* Jan 2011;100(1):38-52.
278. Denies S, Cicchelero L, Van Audenhove I, Sanders NN. Combination of interleukin-12 gene therapy, metronomic cyclophosphamide and DNA cancer vaccination directs all arms of the immune system towards tumor eradication. *J Control Release.* Aug 10 2014;187:175-182.
279. Description VEGFR-2 plasmid Invivogen. http://www.invivogen.com/PDF/pUNO1_hFLK1_mb_14A24v35.pdf, 2015.

280. Niethammer AG, Xiang R, Becker JC, et al. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat Med.* Dec 2002;8(12):1369-1375.
281. Anderson RJ, McBride CM, Hersh EM. Lymphocyte blastogenic responses to cultured allogeneic tumor cells in vitro. *Cancer Res.* May 1972;32(5):988-992.
282. Otten GR, Doe B, Schaefer M, et al. Relative potency of cellular and humoral immune responses induced by DNA vaccination. *Intervirology.* 2000;43(4-6):227-232.
283. Wang YS, Wang GQ, Wen YJ, et al. Immunity against tumor angiogenesis induced by a fusion vaccine with murine beta-defensin 2 and mFlk-1. *Clin Cancer Res.* Nov 15 2007;13:6779-6787.
284. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* Mar 2008;22(3):659-661.
285. Davis HL. Novel vaccines and adjuvant systems: the utility of animal models for predicting immunogenicity in humans. *Human vaccines.* May-Jun 2008;4(3):246-250.
286. Goubier A, Fuhrmann L, Forest L, et al. Superiority of needle-free transdermal plasmid delivery for the induction of antigen-specific IFNgamma T cell responses in the dog. *Vaccine.* Apr 24 2008;26(18):2186-2190.
287. Gold JS, Ferrone CR, Guevara-Patino JA, et al. A single heteroclitic epitope determines cancer immunity after xenogeneic DNA immunization against a tumor differentiation antigen. *Journal of immunology.* May 15 2003;170(10):5188-5194.
288. Tjelle TE, Rabussay D, Ottensmeier C, Mathiesen I, Kjeken R. Taking electroporation-based delivery of DNA vaccination into humans: a generic clinical protocol. *Methods in molecular biology.* 2008;423:497-507.
289. El-Kamary SS, Billington M, Deitz S, et al. Safety and tolerability of the Easy Vax clinical epidermal electroporation system in healthy adults. *Mol Ther.* Jan 2012;20(1):214-220.
290. Jellbauer S, Panthel K, Hetrodt JH, Russmann H. CD8 T-cell induction against vascular endothelial growth factor receptor 2 by Salmonella for vaccination purposes against a murine melanoma. *PLoS One.* 2012;7(4):
291. Seavey MM, Maciag PC, Al-Rawi N, Sewell D, Paterson Y. An anti-vascular endothelial growth factor receptor 2/fetal liver kinase-1 Listeria monocytogenes anti-angiogenesis cancer vaccine for the treatment of primary and metastatic Her-2/neu+ breast tumors in a mouse model. *Journal of immunology.* May 1 2009;182(9):5537-5546.
292. Xie K, Bai RZ, Wu Y, Liu Q, Liu K, Wei YQ. Anti-tumor effects of a human VEGFR-2-based DNA vaccine in mouse models. *Genet Vaccines Ther.* 2009;7:10.
293. Yan J, Jia R, Song H, et al. A promising new approach of VEGFR2-based DNA vaccine for tumor immunotherapy. *Immunol Lett.* Sep 22 2009;126(1-2):60-66.
294. Zhang J, Wang Y, Wu Y, et al. Mannan-modified adenovirus encoding VEGFR-2 as a vaccine to induce anti-tumor immunity. *J Cancer Res Clin Oncol.* May 2014;140(5):701-712.
295. Zhou H, Luo Y, Mizutani M, Mizutani N, Reisfeld RA, Xiang R. T cell-mediated suppression of angiogenesis results in tumor protective immunity. *Blood.* Sep 15 2005;106(6):2026-2032.
296. Schmitz-Winnenthal FH, Hohmann N, Niethammer AG, et al. Anti-angiogenic activity of VXM01, an oral T-cell vaccine against VEGF receptor 2, in patients with advanced pancreatic cancer: A randomized, placebo-controlled, phase 1 trial. *Oncoimmunology.* Apr 2015;4(4):

.
297. Liang PH, Zhang KQ, Xu GL, et al. Construction of a DNA vaccine encoding Flk-1 extracellular domain and C3d fusion gene and investigation of its suppressing effect on tumor growth. *Cancer Immunol Immunother.* Jan 2010;59(1):93-101.
298. Xu GL, Zhang KQ, Guo B, et al. Induction of protective and therapeutic antitumor immunity by a DNA vaccine with C3d as a molecular adjuvant. *Vaccine.* Oct 18 2010;28(44):7221-7227.
299. McKinney KA, Al-Rawi N, Maciag PC, Banyard DA, Sewell DA. Effect of a novel DNA vaccine on angiogenesis and tumor growth in vivo. *Arch Otolaryngol Head Neck Surg.* Sep 2010;136(9):859-864.
300. Wells DJ. Gene therapy progress and prospects: electroporation and other physical methods. *Gene Ther.* Sep 2004;11(18):1363-1369.
301. Kyutoku M, Nakagami H, Koriyama H, et al. Development of novel DNA vaccine for VEGF in murine cancer model. *Scientific reports.* 2013;3:3380.
302. Pinho SS, Carvalho S, Cabral J, Reis CA, Gartner F. Canine tumors: a spontaneous animal model of human carcinogenesis. *Translational research : the journal of laboratory and clinical medicine.* Mar 2012;159(3):165-172.
303. Vail DM, MacEwen EG. Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer investigation.* 2000;18(8):781-792.
304. Thacker EE, Nakayama M, Smith BF, et al. A genetically engineered adenovirus vector targeted to CD40 mediates transduction of canine dendritic cells and promotes antigen-specific immune responses in vivo. *Vaccine.* Nov 23 2009;27(50):7116-7124.
305. Hangalapura BN, Timares L, Oosterhoff D, Scheper RJ, Curiel DT, de Gruijl TD. CD40-targeted adenoviral cancer vaccines: the long and winding road to the clinic. *J Gene Med.* Jun 2012;14(6):416-427.
306. Bergman PJ, Wolchok JD. Of Mice and Men (and Dogs): development of a xenogeneic DNA vaccine for canine oral malignant melanoma Review Article. 2011;6:817-826.
307. Al-Dissi AN, Haines DM, Singh B, Kidney BA. Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor associated with tumor cell proliferation in canine cutaneous squamous cell carcinomas and trichoepitheliomas. *Vet Pathol.* Nov 2007;44(6):823-830.
308. Al-Dissi AN, Haines DM, Singh B, Kidney BA. Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor in canine cutaneous fibrosarcomas. *J Comp Pathol.* Nov 2009;141(4):229-236.
309. Al-Dissi AN, Haines DM, Singh B, Kidney BA. Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 in canine simple mammary gland adenocarcinomas. *Can Vet J.* Oct 2010;51(10):1109-1114.
310. Rawlings NG, Simko E, Bebchuk T, Caldwell SJ, Singh B. Localization of integrin alpha(v)beta3 and vascular endothelial growth factor receptor-2 (KDR/Flk-1) in cutaneous and oral melanomas of dog. *Histol Histopathol.* Jul 2003;18(3):819-826.
311. Rebuzzi L, Willmann M, Sonneck K, et al. Detection of vascular endothelial growth factor (VEGF) and VEGF receptors Flt-1 and KDR in canine mastocytoma cells. *Vet Immunol Immunopathol.* Feb 15 2007;115(3-4):320-333.
312. Restucci B, Borzacchiello G, Maiolino P, Martano M, Paciello O, Papparella S. Expression of vascular endothelial growth factor receptor Flk-1 in canine mammary tumours. *J Comp Pathol.* Feb-Apr 2004;130(2-3):99-104.
313. Urie BK, Russell DS, Kisseberth WC, London CA. Evaluation of expression and function of vascular endothelial growth factor receptor 2, platelet derived growth

factor receptors-alpha and -beta, KIT, and RET in canine apocrine gland anal sac adenocarcinoma and thyroid carcinoma. *BMC Vet Res.* 2012;8:67.
314. Wolfesberger B, Guija de Arespacohaga A, Willmann M, et al. Expression of vascular endothelial growth factor and its receptors in canine lymphoma. *J Comp Pathol.* Jul 2007;137(1):30-40.
315. Yonemaru K, Sakai H, Murakami M, Yanai T, Masegi T. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and their receptors (flt-1, flk-1, and flg-1) in canine vascular tumors. *Vet Pathol.* Nov 2006;43(6):971-980.
316. Terme M, Pernot S, Marcheteau E, et al. VEGFA-VEGFR pathway blockade inhibits tumor-induced regulatory T-cell proliferation in colorectal cancer. *Cancer Res.* Jan 15 2013;73(2):539-549.
317. Matejuk A, Leng Q, Chou S-T, Mixson AJ. Vaccines targeting the neovasculature of tumors. *Vascular cell.* 2011;3(1):7-7.
318. Dong J, Yang J, Ming QC, et al. A comparative study of gene vaccines encoding different extracellular domains of the vascular endothelial growth factor receptor 2 in the mouse model of colon adenocarcinoma CT-26. *Cancer Biology and Therapy.* 2008;7(February 2015):502-509.
319. Zhang J, Wang Y, Wu Y, et al. Mannan-modified adenovirus encoding VEGFR-2 as a vaccine to induce anti-tumor immunity. *Journal of Cancer Research and Clinical Oncology.* 2014;140:701-712.
320. Jellbauer S, Panthel K, Hetrodt JH, Rüssmann H. CD8 T-Cell Induction against Vascular Endothelial Growth Factor Receptor 2 by Salmonella for Vaccination Purposes against a Murine Melanoma. *PLoS ONE.* 2012;7(4):e34214-e34214.
321. Lu XL, Jiang XB, Liu RE, Zhang SM. The enhanced anti-angiogenic and antitumor effects of combining flk1-based DNA vaccine and IP-10. *Vaccine.* 2008;26:5352-5357.
322. Pàez-Ribes M, Allen E, Hudock J, et al. Antiangiogenic Therapy Elicits Malignant Progression of Tumors to Increased Local Invasion and Distant Metastasis. *Cancer Cell.* 2009;15:220-231.
323. Plimack ER, Tannir N, Lin E, Bekele BN, Jonasch E. Patterns of disease progression in metastatic renal cell carcinoma patients treated with antivascular agents and interferon: impact of therapy on recurrence patterns and outcome measures. *Cancer.* May 1 2009;115(9):1859-1866.
324. Zuniga RM, Torcuator R, Jain R, et al. Efficacy, safety and patterns of response and recurrence in patients with recurrent high-grade gliomas treated with bevacizumab plus irinotecan. *J Neurooncol.* Feb 2009;91(3):329-336.
325. Overwijk WW, Restifo NP. B16 as a mouse model for human melanoma. *Current protocols in immunology / edited by John E. Coligan ... [et al.].* May 2001;Chapter 20:Unit 20 21.
326. Johnstone CN, Smith YE, Cao Y, et al. Functional and molecular characterisation of EO771.LMB tumours, a new C57BL/6-mouse-derived model of spontaneously metastatic mammary cancer. *Disease models & mechanisms.* Mar 2015;8(3):237-251.
327. Moserle L, Casanovas O. Anti-angiogenesis and metastasis: a tumour and stromal cell alliance. *Journal of internal medicine.* Feb 2013;273(2):128-137.
328. Deck RR, DeWitt CM, Donnelly JJ, Liu MA, Ulmer JB. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine.* Jan 1997;15(1):71-78.
329. Li J, Valentin A, Kulkarni V, et al. HIV/SIV DNA vaccine combined with protein in a co-immunization protocol elicits highest humoral responses to envelope in mice and macaques. *Vaccine.* Aug 12 2013;31(36):3747-3755.

330. Sullivan WJ, Christofk HR. The Metabolic Milieu of Metastases. *Cell.* 2015;160(3):363-364.
331. Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: Master regulators of metastasis. *Clinical Cancer Research.* 2010;16(2):5928-5935.
332. Uemura M, Yamamoto H, Takemasa I, et al. Hypoxia-inducible adrenomedullin in colorectal cancer. *Anticancer Research.* 2011;31:507-514.
333. Li Y, Wang M-N, Li H, et al. Active immunization against the vascular endothelial growth factor receptor flk1 inhibits tumor angiogenesis and metastasis. *The Journal of experimental medicine.* 2002;195(12):1575-1584.
334. Xie K, Bai R-Z, Wu Y, Liu Q, Liu K, Wei Y-Q. Anti-tumor effects of a human VEGFR-2-based DNA vaccine in mouse models. *Genetic vaccines and therapy.* 2009;7:10-10.
335. Liu JY, Wei YQ, Yang L, et al. Immunotherapy of tumors with vaccine based on quail homologous vascular endothelial growth factor receptor-2. *Blood.* 2003;102(5):1815-1823.
336. Zuo SG, Chen Y, Wu ZP, et al. Orally administered DNA vaccine delivery by attenuated Salmonella typhimurium targeting fetal liver kinase 1 inhibits murine Lewis lung carcinoma growth and metastasis. *Biological & pharmaceutical bulletin.* 2010;33(2):174-182.
337. Ewens A, Mihich E, Ehrke MJ. Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma. *Anticancer Res.* Nov-Dec 2005;25(6B):3905-3915.
338. Winkelmann CT, Figueroa SD, Rold TL, Volkert WA, Hoffman TJ. Microimaging characterization of a B16-F10 melanoma metastasis mouse model. *Molecular imaging.* Apr-Jun 2006;5(2):105-114.
339. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell.* Oct 14 2011;147(2):275-292.
340. Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. *Nat Rev Cancer.* Oct 2013;13(10):739-752.
341. Eisenstein S, Coakley Ba, Briley-Saebo K, et al. Myeloid-derived suppressor cells as a vehicle for tumor-specific oncolytic viral therapy. *Cancer Research.* 2013;73:5003-5015.
342. Basel MT, Shrestha TB, Bossmann SH, Troyer DL. Cells as delivery vehicles for cancer therapeutics. *Therapeutic delivery.* May 2014;5(5):555-567.
343. Podhajcer OL, Lopez MV, Mazzolini G. Cytokine gene transfer for cancer therapy. *Cytokine & growth factor reviews.* Feb-Apr 2007;18(1-2):183-194.
344. Scanlon KJ. Cancer gene therapy: challenges and opportunities. *Anticancer Res.* Mar-Apr 2004;24(2A):501-504.
345. Hu YL, Fu YH, Tabata Y, Gao JQ. Mesenchymal stem cells: a promising targeted-delivery vehicle in cancer gene therapy. *J Control Release.* Oct 15 2010;147(2):154-162.
346. Ankrum J, Karp JM. Mesenchymal stem cell therapy: Two steps forward, one step back. *Trends Mol Med.* May 2010;16(5):203-209.
347. Harrington K, Alvarez-Vallina L, Crittenden M, et al. Cells as vehicles for cancer gene therapy: the missing link between targeted vectors and systemic delivery? *Hum Gene Ther.* Jul 20 2002;13(11):1263-1280.
348. Apolloni E, Bronte V, Mazzoni A, et al. Immortalized myeloid suppressor cells trigger apoptosis in antigen-activated T lymphocytes. *Journal of immunology.* Dec 15 2000;165(12):6723-6730.

349. Sagulenko V, Thygesen SJ, Sester DP, et al. AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell death and differentiation.* Sep 2013;20(9):1149-1160.
350. Stacey KJ, Ross IL, Hume DA. Electroporation and DNA-dependent cell death in murine macrophages. *Immunology and cell biology.* Apr 1993;71 (Pt 2):75-85.
351. Shimokawa T, Okumura K, Ra C. DNA induces apoptosis in electroporated human promonocytic cell line U937. *Biochemical and biophysical research communications.* Apr 2 2000;270(1):94-99.
352. Heller L, Todorovic V, Cemazar M. Electrotransfer of single-stranded or double-stranded DNA induces complete regression of palpable B16.F10 mouse melanomas. *Cancer Gene Ther.* Dec 2013;20(12):695-700.
353. Tolmachov OE, Subkhankulova T, Tolmachova T. Silencing of transgene expression: A gene therapy perspective
Gene Therapy – Tools and Potential Applications. InTech; 2013:49-68.
354. Wei LZ, Xu Y, Nelles EM, et al. Localized interleukin-12 delivery for immunotherapy of solid tumours. *J Cell Mol Med.* Nov 2013;17(11):1465-1474.
355. Zhang L, Morgan Ra, Beane JD, et al. Tumor Infiltrating Lymphocytes Genetically Engineered with an Inducible Gene Encoding Interleukin-12 for the Immunotherapy of Metastatic Melanoma. *Clinical Cancer Research.* 2015.
356. Manzotti CN, Liu MK, Burke F, Dussably L, Zheng Y, Sansom DM. Integration of CD28 and CTLA-4 function results in differential responses of T cells to CD80 and CD86. *Eur J Immunol.* Jun 2006;36(6):1413-1422.
357. Yla-Herttuala S. Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union. *Mol Ther.* Oct 2012;20(10):1831-1832.
358. Sibbald B. Death but one unintended consequence of gene-therapy trial. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne.* May 29 2001;164(11):1612.
359. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet.* May 2003;4(5):346-358.
360. Prather KJ, Sagar S, Murphy J, Chartrain M. Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification. *Enzyme and Microbial Technology.* 2003;33(7):865-883.
361. Journal of Gene Medicine Clinical Trial Database. http://www.abedia.com/wiley/. Accessed 2015/12/06.
362. Dermime S, Armstrong A, Hawkins RE, Stern PL. Cancer vaccines and immunotherapy. *British medical bulletin.* 2002;62:149-162.
363. Christian DA, Hunter CA. Particle-mediated delivery of cytokines for immunotherapy. *Immunotherapy.* Apr 2012;4(4):425-441.
364. Mitragotri S, Burke PA, Langer R. Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. *Nature reviews. Drug discovery.* Sep 2014;13(9):655-672.
365. Anderton SM, Wraith DC. Selection and fine-tuning of the autoimmune T-cell repertoire. *Nat Rev Immunol.* Jul 2002;2(7):487-498.
366. de Visser KE, Cordaro TA, Kessels HW, Tirion FH, Schumacher TN, Kruisbeek AM. Low-avidity self-specific T cells display a pronounced expansion defect that can be overcome by altered peptide ligands. *Journal of immunology.* Oct 1 2001;167(7):3818-3828.
367. Slansky JE, Rattis FM, Boyd LF, et al. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. *Immunity.* Oct 2000;13(4):529-538.

368. Fahmy TM, Bieler JG, Edidin M, Schneck JP. Increased TCR avidity after T cell activation: a mechanism for sensing low-density antigen. *Immunity.* Feb 2001;14(2):135-143.
369. McMahan RH, McWilliams JA, Jordan KR, Dow SW, Wilson DB, Slansky JE. Relating TCR-peptide-MHC affinity to immunogenicity for the design of tumor vaccines. *J Clin Invest.* Sep 2006;116(9):2543-2551.
370. Kreuwel HT, Aung S, Silao C, Sherman LA. Memory CD8(+) T cells undergo peripheral tolerance. *Immunity.* Jul 2002;17(1):73-81.
371. Turner MJ, Jellison ER, Lingenheld EG, Puddington L, Lefrancois L. Avidity maturation of memory CD8 T cells is limited by self-antigen expression. *J Exp Med.* Aug 4 2008;205(8):1859-1868.
372. Kampen KR. The mechanisms that regulate the localization and overexpression of VEGF receptor-2 are promising therapeutic targets in cancer biology. *Anti-cancer drugs.* Apr 2012;23(4):347-354.
373. Cai W, Chen K, Mohamedali KA, et al. PET of vascular endothelial growth factor receptor expression. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine.* Dec 2006;47(12):2048-2056.
374. Chinnasamy D, Yu Z, Theoret MR, et al. Gene therapy using genetically modified lymphocytes targeting VEGFR-2 inhibits the growth of vascularized syngenic tumors in mice. *J Clin Invest.* Nov 2010;120(11):3953-3968.
375. Kissick HT, Sanda MG. The role of active vaccination in cancer immunotherapy: lessons from clinical trials. *Curr Opin Immunol.* Aug 2015;35:15-22.
376. Van Brussel I, Lee WP, Rombouts M, et al. Tolerogenic dendritic cell vaccines to treat autoimmune diseases: can the unattainable dream turn into reality? *Autoimmunity reviews.* Feb 2014;13(2):138-150.
377. Kershaw MH, Westwood JA, Slaney CY, Darcy PK. Clinical application of genetically modified T cells in cancer therapy. *Clinical & translational immunology.* May 2014;3(5):e16.
378. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer.* Aug 2008;8(8):592-603.
379. Clarke JM, Hurwitz HI. Understanding and targeting resistance to anti-angiogenic therapies. *Journal of gastrointestinal oncology.* Sep 2013;4(3):253-263.
380. Kerbel RS. Tumor angiogenesis: past, present and the near future. *Carcinogenesis.* Mar 2000;21(3):505-515.
381. Hartwich J, Orr WS, Ng CY, Spence Y, Morton C, Davidoff AM. HIF-1alpha activation mediates resistance to anti-angiogenic therapy in neuroblastoma xenografts. *Journal of pediatric surgery.* Jan 2013;48(1):39-46.
382. Finley SD, Chu LH, Popel AS. Computational systems biology approaches to anti-angiogenic cancer therapeutics. *Drug Discov Today.* Feb 2015;20(2):187-197.
383. Ghiringhelli F, Apetoh L. Enhancing the anticancer effects of 5-fluorouracil: current challenges and future perspectives. *Biomedical journal.* Mar-Apr 2015;38(2):111-116.
384. Deniz G, Erten G, Kucuksezer UC, et al. Regulatory NK cells suppress antigen-specific T cell responses. *Journal of immunology.* Jan 15 2008;180(2):850-857.
385. Schmidt SV, Nino-Castro AC, Schultze JL. Regulatory dendritic cells: there is more than just immune activation. *Frontiers in immunology.* 2012;3:274.
386. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell.* Mar 18 2013;23(3):277-286.
387. He Y, Qian H, Liu Y, Duan L, Li Y, Shi G. The roles of regulatory B cells in cancer. *Journal of immunology research.* 2014;2014:215471.

388. Kraman M, Bambrough PJ, Arnold JN, et al. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science.* Nov 5 2010;330(6005):827-830.
389. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science.* Apr 3 2015;348(6230):56-61.
390. Kong YC, Flynn JC. Opportunistic Autoimmune Disorders Potentiated by Immune-Checkpoint Inhibitors Anti-CTLA-4 and Anti-PD-1. *Frontiers in immunology.* 2014;5:206.
391. Hanahan D. Rethinking the war on cancer. *Lancet.* Feb 8 2014;383(9916):558-563.
392. McDermott D, Lebbe C, Hodi FS, et al. Durable benefit and the potential for long-term survival with immunotherapy in advanced melanoma. *Cancer treatment reviews.* Oct 2014;40(9):1056-1064.
393. Platten M, Offringa R. Cancer immunotherapy: exploiting neoepitopes. *Cell research.* Aug 2015;25(8):887-888.
394. Mahoney KM, Atkins MB. Prognostic and predictive markers for the new immunotherapies. *Oncology.* Nov 2014;28 Suppl 3:39-48.
395. Hoos A. Evolution of end points for cancer immunotherapy trials. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO.* Sep 2012;23 Suppl 8:viii47-52.
396. Vanneman M, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer.* Apr 2012;12(4):237-251.
397. Golden EB, Apetoh L. Radiotherapy and immunogenic cell death. *Seminars in radiation oncology.* Jan 2015;25(1):11-17.
398. Pol J, Vacchelli E, Aranda F, et al. Trial Watch: Immunogenic cell death inducers for anticancer chemotherapy. *Oncoimmunology.* Apr 2015;4(4):e1008866.
399. Formenti SC, Demaria S. Radiation therapy to convert the tumor into an in situ vaccine. *International journal of radiation oncology, biology, physics.* Nov 15 2012;84(4):879-880.
400. Hogan BV, Peter MB, Shenoy HG, Horgan K, Hughes TA. Surgery induced immunosuppression. *The surgeon : journal of the Royal Colleges of Surgeons of Edinburgh and Ireland.* Feb 2011;9(1):38-43.
401. Drake CG. Combination immunotherapy approaches. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO.* Sep 2012;23 Suppl 8:viii41-46.
402. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol.* Apr 2006;6(4):295-307.
403. Schadendorf D, Hodi FS, Robert C, et al. Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol.* Jun 10 2015;33(17):1889-1894.
404. Rosenberg SA. Raising the bar: the curative potential of human cancer immunotherapy. *Science translational medicine.* Mar 28 2012;4(127):127ps128.

CURRICULUM VITAE

Curriculum vitae

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Professional experience

2015 - ...	**Associate research scientist Iteos Therapeutics**
2013-2015	**Member Ethical Committee**
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2011-2015	**PhD candidate at the Laboratory of Gene therapy, Faculty of Veterinary Medicine (Ghent University)**

Education

2014 – ...	**Master of Statistical Data Analysis (part time)**
2011	**Laboratory Animal Science (FELASA C)**
	With great distinction
2008 – 2011	**Master of Science in Veterinary Medicine, University of Ghent, Belgium**
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2005 – 2008	**Bachelor of Science in Veterinary Medicine, University of Antwerp, Belgium**
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Obtained funding & rewards

2014	**Best storm session presentation awarded of OncoPoint 2014**
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2011	**Personal PhD grant awarded by Special Research Fund (BOF) of Ghent University**

BIBLIOGRAPHY

Bibliography

Publications in Journals

- **Denies S**, Cicchelero L, Van Audenhove I, Sanders NN. (2014). Combination of interleukin-12 gene therapy, metronomic cyclophosphamide and DNA cancer vaccination directs all arms of the immune system towards tumor eradication. J Control Release 10;187:175-82. Impact factor: 7.261 / Ranking: Q1

- **Denies S.**, Sanders N.N. (2012). Recent progress in canine tumor vaccination: potential applications for human tumor vaccines. Expert reviews on vaccines, 11, 1375-86. Impact factor: 4.219 / Ranking: Q1

- Cicchelero L., **Denies S.**, Devriendt B., De Rooster H., Sanders N.N (2015). Can dendritic cells improve whole cancer cell vaccines based on immunogenically killed cancer cells? OncoImmunology. Impact factor: 6.266 / Ranking: Q1

- **Denies S.**, Cicchelero L., De Rooster H., Daminet S., Polis I., Van de Maele I., Sanders N.N. Evaluation of regulatory T cells and angiogeneic markers in canine cancer patients compared to a healthy control group and during metronomic chemotherapy. Veterinary and comparative oncology (accepted for publication). Impact factor: 2.733 / Ranking: Q1

- **S. Denies**, L. Cicchelero, I. Polis, I., N.N. Sanders. Immunogenicity and safety of xenogeneic VEGFR-2 DNA vaccination in mice and dogs. Oncotarget (under revision). Impact factor: 6.359 / Ranking: Q1

Conference Contributions

- **Denies S**., Cicchelero L., Sanders NN. (2015). Immunological and angiogenic markers during metronomic chemotherapy in spontaneous arising cancers in dogs. Cancer Immunotherapy 13th annual meeting, Mainz (Germany). Poster presentation.
- **Denies S,** Ciccelero L., de Rooster H., Daminet S., Polis I., Van de Maele I., Sanders N.N.(2015). Immunological and angiogenic markers during metronomic chemotherapy. ESVONC annual congress. Krakow (Poland). Oral presentation.

- **Denies S**.,Cicchelero L., Sanders NN. (2014). Evaluation of a combinatorial cancer immunotherapy approach. Cancer Immunotherapy 12th annual meeting, Mainz (Germany). Poster presentation.
- **Denies S.,** Cicchelero L., Sanders NN. (2014). Interleukine-12, metronomic cyclophosphamide and tyrosinase vaccine. 2nd OncoPoint Meeting, Ghent (Belgium). Oral presentation.
- Breyne K., **Denies S.**, Demon D., Sanders N.N., Meyer E. (2014). Optimalization and characterization of a novel intraductal mouse mammary tumor model. 2nd OncoPoint Meeting, Ghent (Belgium). Oral presentation.
- Gommeren K., de Laat B., **Denies S.**, Merveille A-C., Gomart S., Fergus A., Peeters D. (2012). Repeatability and reproducibility of transcranial doppler ultrasonography in healthy beagle dogs. 22nd ECVIM-CA Congress, Maastricht (Netherlands). Oral presentation.
- de Laat B., Gommeren K., **Denies S.**, Merveille A-C., Gomart S., Fergus A., Peeters D. (2012). Influence of sedatives, anticonvulsants and a negative chronotrope on transcranial doppler ultrasonography. 22nd ECVIM-CA Congress, Maastricht (Netherlands). Oral presentation.

Book chapters

Denies S., de Laat B.W.G.A, de Brouwer C.(2012). Training Critical Appraisal of a Topic, an essential manual in the age of Evidence Based Medicine.Chapter 2: Systemic literature research; from clinical question to evidence (special edition for veterinary medicine). Mediview BV, Maastricht (Netherlands)

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