I INTRODUCTION

1 Equine malignant melanoma

Equine melanoma or Equine Malignant Melanoma (EMM) as recently named by Moore *et al.* (MOORE 2012) is a common pigmented neoplasm, characterised by an uncontrolled growth of melanocytes. It is seen more frequenctly in grey- and white-coated horses and often in advanced stages presented as a multicentric malignancy.

The prevalence EMM in grey-horses increases with aging (VALENTINE 1995; JEGLUM 1999; SMITH et al. 2002) and independently of the gender, 80% of grey horses older than 15 years (VALENTINE 1995; JEGLUM 1999; SELTENHAMMER et al. 2003) develop EMM.

The pathological nature of EMM has been discussed for several years. Some authors suggested it was a benign pigment cell dysplasia, while others agree it is a true neoplasm. The concept of equine melanoma as a neoplasia with malignant potential became commonly accepted in the last years. Further, Scott affirmed that at least 66% of the melanocytic tumors in horses become malignant (SCOTT 1988).

Single or multiple to confluent tumors are commonly located in the deep dermis of the perineum, undersurface of the tail, anal, perianal and genital regions, perineum, and lip commissures (VALENTINE 1995; FLEURY et al. 2000). EMM affecting solid-coloured horses are usually solitary cutaneous masses, which can be “benign” or malignant with no obvious predilection sites (VALENTINE 1995).

1.1 Clinical presentation

Many melanomas present initially as single, small, raised nodules in the perineum, multiple nodules may be seen at once (FLEURY et al. 2000; SELTENHAMMER et al. 2003). In the initial stages in which there is little growth, lesions may exist for many years without causing clinical problems. This changes when lesions enlarge and coalesce. Therefore, the symptoms are generally related to the location and size of the tumor, including altered defecation, weight loss, colic, oedema, dyspnoea, epistaxis, paresis, or ataxia, dysuria and abnormalities involving the genitalia, including priapism. (JEGLUM 1999; FLEURY et al. 2000; MACGILLIVRAY et al. 2002). Lesions, normally covered by intact epidermis, may ulcerate and become infected (FLEURY et al. 2000).

Moore et al. suggested that since almost invariably, tumor nodules that are expanding are quite firm, this could be due the activation of fibroplasia by tumor cells or in response to tumor cell growth. Similarly the well-vascularised tumors may reflect enhanced angiogenesis as a result of tumor cell proliferation (MOORE 2012).

1.2 Current therapies for equine malignant melanoma

A variety of therapies have been developed and tested, nevertheless there is no widely accepted efficient treatment for EMM.

Excision of small, single melanomas is possible with apparent success (ROWE u. SULLINS 2004). Cure is not always achieved since new melanoma might develop in other locations and remaining melanoma might continue growing (PASCOE 1999). When the lesions are multiple and also invading internal organs, curative surgical treatment is not feasible (VALENTINE 1995). Surgical excision may be palliative if defecation or other physiological functions are disturbed (ROWE u. SULLINS 2004). Recent reports demonstrated that the intratumoral injection of cisplatin reduced the size of melanoma with an 81% success rate. Therapy was less effective in larger more advanced tumors and the development of new melanoma lesions continued outside the treated area (THEON et al. 2007). The antagonisation of histamine (which activates T-suppressor cells via H2R) using oral administration of cimetidin showed to have an antitumoral effect, reducing the number and size of the tumors (GOETZ et al. 1990). However, more recent studies found no significant effect on the number or size of the melanomas after cimetidine treatment (WARNICK 1995; LAUS 2010).

2 Immune-mediated melanoma therapy

Conventional therapy only palliates the disease and is not curative in the advanced stages. Therefore, a variety of approaches, which rely on the valuable properties of the immune system are being developed. Intensive research has been performed especially on human, mice (human melanoma model) and canine melanoma.

Different studies have shown the protective and or curative functions of the immune system, particularly against human colon carcinoma (DOHLSTEN et al. 1995; CORREALE et al. 2005; UYL-DE GROOT et al. 2005) and melanoma (SEIGLER et al. 1979; YEE et al. 2000; HODI et al. 2010).

Burnet reported, in the mid-nineties, that the immune system influences the development of neopastic diseases (BURNET 1967) and Kobayashi observed that

immunosuppressed human patients with reduced NK-cell cytotoxicity (Chediak-Higashi-syndrome) showed a significantly higher risk to develop malignant processes (N. KOBAYASHI 1985). Additionally, patients treated with immunosuppressive drugs are more likely to get non-viral induced neoplasia than untreated individuals (BIRKELAND et al. 1995; SHEIL et al. 1997).

Immunity against cancer involves several components. Interferon gamma (IFN-γ), produced by NK and T cells, with antiproliferative (KOMINSKY et al. 1998) and antiangiogenetic effects as well as an innate immune activation function (IKEDA et al. 2002) is one important factor. It directs the immune answer into a Th1 direction, activates the NK cell function, influences antigen-processing and –presentation, stimulates antiviral functions and apoptosis (BRUNDA et al. 1995). Mice with defective IFN-γ signal transmission developed more neoplasia compared to wild-type mice after aging or the treatment with chemical carcinogen 3`-methylcholanthrene (IKEHARA et al. 1984; SHANKARAN et al. 2001).

The cytotoxic effects of T cells classify them as crucial for cancer immunity. Human patients with neoplastic disease with lymphocyte infiltration of their tumor have a better prognosis than the ones without this infiltration (GALON et al. 2006). Immunodeficient mice (unable to generate mature B and T lymphocytes) and mice with severe combined immunodeficiency showed higher tumor development than wild-type mice (SHANKARAN et al. 2001; SWANN u. SMYTH 2007)

NK-cells are also essential. Haliotis et al showed that in knockout mice NK-cells were responsible for tumor rejection (HALIOTIS et al. 1985). Furthermore, Smyth et al. demonstrated that NK-cells are responsible for the elimination of cancer cells not expressing the MHCI marker (SMYTH et al. 2000).

Also antibodies (Abs) play a role in cancer immunity. The activation of the complement pathway leads to the production of chemotactic molecules able to stimulate a cellular and humoral response (DI GAETANO et al. 2003) and the interaction of the Fc-end of antitumor specific Abs with the Fc-receptor of immune cells generates Ab-dependent cytotoxicity (CARTRON et al. 2002; NIMMERJAHN u. RAVETCH 2006)

Even though several immune mechanisms are responsible to prevent neoplastic changes, tumor cells have different techniques to escape from immune recognition. The non-recognition of tumors can occur when tumor antigens (Ags) are missing, the Ag expression during tumor growth is reduced or absent and when cells are unable

to present the Ags to the immune system (ALGARRA et al. 2000; MARINCOLA et al. 2000; SELIGER et al. 2000). T-cell recognition of tumor cells can also be reduced by decreased binding of the T-cell receptor to MHCI complexes (BUBENIK 2004). Tumor cells are able to secrete immunosuppressive mediators like NO (ZHANG u. XU 2001), transforming growth factor beta (BECK et al. 2001) and Interleukin 10 (KAWAMURA et al. 2002). Further, the immune system suppression is also possible after the induction or presence of regulatory T cells (CURIEL 2007).

Based on the immune system mechanisms associated with cancer, a variety of therapies that stimulate or inhibit specific immunological responses have been evaluated. Some of these approaches are the use of modified autologous tumor cells (LOTEM et al. 2009), specific peptides as tumor vaccines (LEWIS et al. 2000; GYORFFY et al. 2005), cytokines (ROSENBERG et al. 1994; NAGAI et al. 2000; KISHIDA et al. 2001; P. LEE et al. 2001) and vaccines (MORTON et al. 1992; SONDAK et al. 2002; BERGMAN et al. 2006; FINOCCHIARO u. GLIKIN 2008).

3 Interleukin-12 in cancer therapy

Different cytokines have been evaluated and applied in cancer therapy (e.g. IL-12, IL-2, IFN-γ, IFN-alfa, etc.)(ROSENBERG et al. 1994; FISHER et al. 2000; KIRKWOOD et al. 2000; P. LEE et al. 2001; IKEDA et al. 2002).

The ability of IL-12 to facilitate cell-mediated immune responses, including enhancement of NK cytotoxicity, generation of CTL, and macrophage activation, suggests that it could have a role in both the innate and adaptive resistance mechanisms against tumors (ANDREWS et al. 1993).

IL-12 is a potent inducer of cytokine production, particularly IFN-γ, in T and NK cells; a growth factor for pre-activated T and NK cells; and an enhancer of cytotoxic activity in both $CD8^+$ T and NK cells (KOBAYASHI et al. 1989). IL-12 is produced by phagocytes, lymphocytes and dendritic cells (DCs) (D'ANDREA et al. 1992). In addition to its role in the phagocytic cell activation mechanism early in the inflammatory response to infections, IL- 12 stimulates the generation of IL-2 and IFN-γ producing Th1 cells (MANETTI et al. 1993) and the optimal differentiation of cytotoxic T lymphocytes (GATELY et al. 1992). IL-12 leads to the differentiation and proliferation of activated CD8+ and CD4+ cells increasing the cytotoxic potential of CD8+ and NK cells (ROBERTSON u. RITZ 1996). IL-12 also up-regulates the expression of MHCI, MHCII and ICAM-I molecules, enhancing tumor recognition

(YUE et al. 1999). Additionally, IL-12 was shown to inhibit angiogenesis essential to stop tumor growth (YAO et al. 2000; L. HEINZERLING et al. 2002).

IL-12 unlike other cytokines, was shown to have a heterodimeric structure of 70 kD (p70), formed by two covalently linked glycosylated chains of approximately 40 kD (p40) and 35 kD (p35) (KOBAYASHI et al. 1989). Cotransfection with both cDNA in the same cells is required for secretion of the biologically active form of IL-12 (GUBLER et al. 1991). It was reported that in the mouse recombinant free p40 inhibits the biologic activity of the p70 heterodimer, suggesting that p40 may act as a physiologic antagonist of IL-12 (MATTNER et al. 1993). Further it was observed that the inhibitory activity of recombinant murine p40 resides primarily in p40 homodimers formed by recombinant protein and it is not clear yet whether natural p40 also has antagonistic activity; moreover little if any antagonistic activity was demonstrable with human recombinant p40 (TRINCHIERI 1994). The IL-12 receptors are located on activated T- and NK-cells (PRESKY et al. 1996) and the receptor production is induced by the activation of T-cell receptors, IFN-γ and IL-12 (ROGGE et al. 1997).

Reduction and remission of neoplasia after applying IL-12 was seen in mice (BRUNDA et al. 1993; LUCAS et al. 2002), humans (CEBON et al. 2003; L. HEINZERLING et al. 2005) and horses (L. M. HEINZERLING et al. 2001; MÜLLER et al. 2011a; MÜLLER et al. 2011b).

3.1 IL-12 in melanoma therapy

Research on human melanoma has been largely performed using the mice melanoma B16 model. Treatment with recombinant IL-12 or IL-12 gene therapy (IL-12 cDNA gene gun delivery) of mice bearing the B16 melanoma induced tumor regression; however the protein therapy resulted in a high level of mortality, weight loss, splenomegaly, fur ruffling and lethargy (RAKHMILEVICH et al. 1999).

Schultz et al. administered IL-12 as intramuscular plasmid DNA injection (encoding both IL-12 subunits) into mice bearing B16 melanoma reaching long-lasting antimetastatic effects. Additionally, they reported that the IL-12 DNA treatment is required during the early phases of metastasis formation, being the treatment ineffective when administered later (SCHULTZ et al. 1999).

Similarly to this approach, Kishida et al. injected plasmid vectors codeing the IL-12 and -18 genes intratumorally (B16 melanoma) followed by *in vivo* electroporation. The IL-12 gene transfection resulted in significant suppression of tumor growth, while the therapeutic effect was further improved by co-transfection with IL-12 and -18

genes. Repetitive co-transfection of the expression plasimds resulted in significant prolongation of survival of the animals (KISHIDA et al. 2001)

Lucas et al. confirmed these results after performing a similar study. B16 melanoma bearing mice were treated with plasmid DNA encoding IL-12 (intratumoral and intramuscular), followed by *in vivo* electroporation. The intratumoral treatment resulted in the cure of 47% of tumor-bearing mice, and 70% of them became resistant to the challenge with B16F10 cells. Interestingly the intramuscular treatment did not result in tumor regression. The intratumoral treatment also increased levels of IL-12 and IFN-γ within the tumors, the influx of lymphocytes into the tumors, and reduction in vascularity. Treatment was not effective in a nude mouse model, supporting the role of T cells in the regression of melanoma (LUCAS et al. 2002).

Recently, a significant anti-tumor activity was achieved after DCs genetically engineered by IL-12 plasmid DNA were injected intratumorally into mice carrying a B16 melanoma mass (YOSHIDA et al. 2010).

The intravenous injection of recombinant human IL-12 into twelve human patients with melanoma in a phase I dose escalation trial was performed. After treatment, one patient showed transient complete response and four had no disease progression. The common toxicities reported were fever/chills, fatigue, nausea, vomiting, and headache. Also routine laboratory changes like anemia, neutropenia, lymphopenia, hyperglycemia, thrombocytopenia and hypoalbuminemia were found. Dose-limiting toxicities were oral stomatitis and liver function test abnormalities. The maximum tolerated dose was associated with asymptomatic hepatic function test abnormalities and an on study death but was otherwise well tolerated by the rest of the patients (ATKINS et al. 1997).

Based on this previous study, the same research group conducted the phase II study. The maximum tolerated dose (500ng/kg) of recombinant human IL-12 was administered to 17 patients, resulting in severe unexpected toxicities, 12 patients were hospitalized and two patients died (LEONARD et al. 1997).

Bajetta et al. also administered human recombinant IL-12 (subcutaneously) to 10 patients with progressive metastatic melanoma. Toxicity during the treatment consisted mainly in flu-like syndrome and transient increase in transaminasemia and triglyceridemia. After the first cycle of treatment, regression of subcutaneous nodules (2 of 3 patients), superficial adenopathies (1 of 3 patients), and hepatic metastases (1 of 3 patients) were observed (BAJETTA et al. 1998).

To test the enhancing qualities of IL-12, addition of this cytokine to an immunization protocol was performed. Patients with high risk resected stage III and IV melanoma were immunized with two tumor antigens epitope peptides (gp100 and tyrosinase). Patients received the immunization with or without the addition of IL-12 (30ng/Kg i.v.). Local pain and granuloma formation, fever, and letargy were observed. A significant proportion of the patients mounted an antigen-specific immune response and IL-12 increased this response. The authors concluded that these results support further development of IL-12 as a vaccine adjuvant (P. LEE et al. 2001).

Cebon et al. applied melanoma peptide antigens (Melan-A) combined with the administration of a low dose of recombinant IL-12 to stage III and IV melanoma patients. This low dose approach was introduced by the authors to avoid the toxicity results of higher doses of recombinant IL-12. The therapy was well tolerated, being the only adverse event influenza-like symptoms. Clinical responses included a complete response, a partial response and mixed responses in pulmonary, pleural and nodal disease. Clinical and immunological activity (Melan-A lysing capable CD4+ and CD8+ lymphocyte infiltration) were observed after the administration of peptides either with or without low dose recombinant human IL-12 (CEBON et al. 2003).

Heinzerling et al. applied DNA encoding human IL-12 into melanomas of human patients based on the efficacy obtained previously in preclinical melanoma studies with mice and grey horses (L. M. HEINZERLING et al. 2001; L. HEINZERLING et al. 2002). Low, medium and high doses of plasmid DNA encoding human IL-12 were injected into lesions of nine patients with stage IV malignant melanoma. Therapy was well tolerated and three of nine patients experienced a clinical response (two with stable disease, one complete remission). Biopsies of the responders showed local reduction of angiogenesis, lymphocyte infiltration and some increase in IL-12, IP-10, and IFN-γ. Serum levels revealed fluctuations (L. HEINZERLING et al. 2005).

Similarly, Mahvi et al. injected intratumorally DNA coding for human IL-12 to melanoma patients reporting a significant size reduction of the primary tumors (MAHVI et al. 2007). Further, Daud et al. applied intratumoral IL-12 DNA electroporation in humans with metastatic melanoma resulting in complete regression (two of 19 patients) and stable disease or partial regression (eight of 19 patients)(DAUD et al. 2008).

In dogs the search for a curative therapy against canine malignant melanoma (CMM) is still ongoing. CMM is also a spontaneous, aggressive, and metastatic neoplasm.

Therefore, similar approaches to the ones used in humans have been developed and applied in dogs. Bergman et al. used an intramuscular xenogeneic DNA vaccination with genes encoding human tyrosinase (potent stimulator of cytotoxic T-cell responses) in dogs with stage II-IV CMM. Vaccination was well tolerated, one dog showed complete clinical response in multiple lung metastases for 329 days, four dogs had long-term survivals (421, 588+, 501 and 496 days), and four others were euthanized because of progression of the primary tumor. The authors concluded that this was a safe and a potential good therapeutic modality against advanced CMM (BERGMAN et al. 2003). A similar study performed by the same group supported these results. Intramuscular xenogeneic DNA vaccination with genes encoding human and or murine tyrosinase was applied to stage II-IV CMM dogs. Vaccination was also well tolerated, prolonged significantly the median survival time of all treated dogs and induced a 2- to 5-fold increase in circulating antibodies to human tyrosinase (BERGMAN et al. 2006).

Recently a vaccine to trat canine stage II-III oral melanoma got an USDA (United States Department of Agriculture) approval (February 2010; ONCEPT™ Canine Melanoma Vaccine; Merial Company, Duluth, GA, USA). There are still ongoing studies and little is known about the therapy success. Nevertheless, Bergman et al. results and the introduction of this vaccine are promising approaches in the search for CMM cure. The use of the vaccine prolongs survival time, but only in not severe stages of the disease remission has been observed. The vaccination certainly induces an efficient immune system stimulation, but possibly the solely use of tyrosinase is not enough. The addition of IL-12 to the treatment protocol, as a vaccine adjuvant, could benefitial. Adding the IL-12 immune system stimulatory functions to the proven tyrosinase effects a more efficient respond with possible higher remission rates could be reached.

3.2 Adjuvant effects of IL-12

IL-12 has been used in combination with other approaches to enhance cell mediated immunity (IWASAKI et al. 1997; KIM et al. 1999). Recently, IL-12 is being applied as a novel vaccine adjuvant (GHERARDI et al. 2001; PORTIELJE et al. 2003). In tumor vaccination, the co-administration of genes encoding for IL-12 and various tumor antigens clearly enhanced the anti-tumor effects, with inhibition of tumor growth and eradication of established tumors (TAN et al. 1999; AMICI et al. 2000; KIM et al. 2001). An important and promising vaccination strategy consisted of the addition of

IL-12 to DC-based vaccines (ZITVOGEL et al. 1996; FALLARINO et al. 1999; MELERO et al. 1999; KOIDO et al. 2000; YOSHIDA et al. 2010), resulting in the augmented anti-tumor effect of the vaccine.

3.3 DCs and the immune-mediated therapy

DCs are known as potent antigen-presenting cells able to induce effective immune responses. Their characteristics make them suitable for different therapeutic approaches that aim to stimulate or suppress immune responses (JONULEIT et al. 2001). In humans, DCs are particularly used in cancer therapy (RIBAS et al. 2002; SCHULER et al. 2003); immune-mediated therapy (FIGDOR et al. 2004) and prevention and or treatment of autoimmune diseases (LO u. CLARE-SALZLER 2006).

3.3.1 DCs in melanoma therapy

In humans the first DC-vaccination studies used DCs directly isolated *ex vivo* from blood, having the disadvantage of low yield of DCs (HSU et al. 1996; SCHULER et al. 2003). However, effective and clinically applicable methods based on magnetic cell sorting techniques are now available, making it possible to compare the immunogenic properties of distinct DC subsets, such as plasmacytoid DCs, to the various myeloid DC subsets that have been used so far (SCHULER et al. 2003). DC vaccination of melanoma patients appears to be safe and to produce encouraging clinical responses. A single deep dermal injection of matured Mo-DCs pulsed with foreign peptides (keyhole limpet hemocyanin (KLH), tetanus toxoid (TT), flu matrix peptide) rapidly induced the proliferation of KLH-specific Th1 cells and expanded the flu-specific $CD8^+$ CTL population (DHODAPKAR et al. 2000). Similarly, Mo-DCs loaded with KLH and tumor peptides or tumor cell lysates were intranodally delivered inducing antigen-specific immunity during DC vaccination (NESTLE et al. 1998). Responses were evident in 5 out of 16 evaluated patients (two complete responses, three partial responses) with regression of metastases in various organs and most of the clinical responses were durable over several years (NESTLE et al. 1998). Banchereau and colleagues pulsed $CD34^+$-derived DCs simultaneously with KLH and four HLA-A2.1 restricted melanoma antigens (melanA, tyrosinase, gp100 and Mage-3), vaccinated subcutaneously 18 stage IV melanoma patients and found that regression of tumor metastasis was observed in 7 out of 17 evaluated patients (BANCHEREAU et al. 2001). The overall immunity to melanoma antigens after

vaccination was clearly associated with the clinical outcome (BANCHEREAU et al. 2001).

In horses, DCs are currently also being generated and used for the treatment of some skin tumours or DC-based immunotherapy (VECCHIONE et al. 2002; LOEWENSTEIN u. MUELLER 2009; STEINBACH et al. 2009).

A preliminary study treated horses with sarcoids intradermally using a DC-based vaccine (DCs were activated with autologous tumor lysates), the treatment was well tolerated but no correlation was found between the used therapy and the clinical outcome (STEINBACH et al. 2009).

Dietze et al. generated high quantities of equine DCs to develop a DC-based immunotherapy against equine summer eczema. Generated DCs were activated with fluorescence-labelled culicoides protein and the subsequent mRNA expression of IL-12 was analysed. After stimulation increased IL-12 mRNA expression by DCs was detected, suggesting that these loaded DCs could be able to induce Th1 polarisation, enabling a deviation from pathogenic Th2 response in patients with summer eczema (DIETZE 2009).

Monocyte-derived DCs can be generated *in vitro* from peripheral blood CD14+ monocytes after adding specific growth factors to the culture medium (KIERTSCHER u. ROTH 1996; ROMANI et al. 1996). Different protocols were reported to allow DC generation in horses and modification of these protocols resulted in increased numbers of generated DCs (HAMMOND et al. 1999; MAUEL et al. 2006; DIETZE et al. 2008). Unfortunately, the achieved efficiency of this process in horses is still poor, resulting in unsatisfying yields of generated DCs.

4 Equine malignant melanoma immune-mediated therapy

Research results for human melanoma, particularly the IL-12 mediated approaches, contributed to develop immune-mediated studies in horses. Heinzerling et al. suggested that the spontaneous developed metastatic melanoma in horses is similar to the development of human melanoma, and therefore this specie would be a more optimal animal model than the mice model (induced melanoma) to study new immunotherapy protocols (L. M. HEINZERLING et al. 2001). In their first study, plasmid DNA coding for IL-12 applied intratumorally into established metastases induced significant regression in all twelve treated lesions (seven horses), complete

disappearance was observed in one lesion and the treatment was well tolerated (L. M. HEINZERLING et al. 2001).

Based on these positive results, a larger study using 26 grey horses was performed to confirm the ability of IL-12 and -18 to inhibit tumor growth after intratumoral DNA injection (PATRIZIA STÄHLI 2005). Animals were treated intratumorally with plasmid DNA encoding equine IL-12 or -18. Significant tumor size regression after IL-18 treatment was observed, whereas the IL-12 group showed a regression tendency (calliper measurement). No significant change in size or vascularisation was reported after ultrasound measurement. Peritumoral infiltration of inflammation cells was seen in six of 15 horses treated with IL-12 or -18 (PATRIZIA STÄHLI 2005). A similar study was recently published by Müller et al.. Grey horses with metastatic melanoma were used for a double-blind placebo-controlled study (MÜLLER et al. 2011a). Empty plasmid DNA, plasmid DNA encoding IL-12 or -18 was applied intratumorally. Treatment response was assessed measuring tumor size (calliper and ultrasound measurement) and histological analysis of tumor biopsies. The treatment was safe and well tolerated by all animals. Significant tumor regression was reported in both treated groups, while in the control group (empty plasmid DNA treatment) slight tumor growth was detected. Peritumoral infiltration of inflammatory cells was seen in seven of ten treated tumors (IL-12 or -18 treatments). Finally, authors concluded that the obtained results indicate that intratumoral treatment with IL-12 and -18 encoding plasmid DNA has antitumor effects in horses and holds promise for the treatment of patients with metastatic melanoma (MÜLLER et al. 2011a). A follow up study was performed by the same group to assess the *in vivo* gene transfer in the IL-12 treatment of grey horse melanoma (MÜLLER et al. 2011b). The in-vivo induction of IFN-γ expression in grey horses with metastatic melanoma after the intratumoral injection of plasmid DNA coding for equine IL-12 was evaluated. After the intratumorally injection of plasmid DNA coding for IL-12 in seven horses, peripheral blood and tumor biopsies from the injection site were taken. Quantitative real-time PCR results showed fast elimination kinetics of plasmid DNA in peripheral blood and increased IFN-γ expression after IL-12 plasmid injection in the taken biopsies (MÜLLER et al. 2011b). These last results from Müller et al. confirmed in horses the biological activity of the injected IL-12 plasmid DNA after intratumoral injection (MÜLLER et al. 2011b).

The analysis of the obtained results (cell infiltration, change in tumor growth, etc.) after the IL-12 immune-mediated melanoma transfection approaches, it is essential to determine the biological activity of the transfected DNA. Ideally the IL-12 concentrations in serum or biopsies after treatment should be measured. Unfortunately a specific equine enzyme linked assay for IL-12 and or IFN-gamma quantification is so far not available. Therefore, previous studies determined indirectly the presence of IL-12 in samples performing *in vitro* stimulation of PBMCs or analysing biopsies from the treated tumors using RT-PCR for IFN-gamma detection (PATRIZIA STÄHLI 2005; MÜLLER et al. 2011a).

5 Recombinant IL-12 toxicity and gene therapy

The application of recombinant IL-12 into patients caused several side effects like fever, chills, fatigue, headache, nausea and vomiting (ATKINS et al. 1997; BAJETTA et al. 1998; PORTIELJE et al. 1999) and the unexpected death of two patients (LEONARD et al. 1997). Researchers investigated the death cause and concluded that the toxic reactions that killed the patients were caused by the administration of recombinant IL-12, a highly toxic drug when given in multiple high doses (COHEN 1995). Negative effects could be avoided if multiple doses are preceded by a single dose followed by a rest period. This initial dose apparently imprints a memory on the immune system and avoids detrimental overreactions (COHEN 1995).

The side effects caused by using recombinant IL-12 stimulated further studies to use low dose recombinant IL-12 or gene therapy, applying DNA encoding IL-12. Cytokine DNA is able to potentiate the same immune reaction but shows less toxicity than recombinant proteins due to the body's own protein production with a constant plasma level, rather than high, toxic and short-lived plasma levels of recombinant proteins (SCHULTZ et al. 1999; COLOMBO u. TRINCHIERI 2002).

6 Gene therapeutic approaches

Gene therapy is currently being applied to treat different diseases. Several trials employing gene therapy protocols were used in melanoma patients (PORTIELJE et al. 2003; L. HEINZERLING et al. 2005; MAHVI et al. 2007). Successful gene therapy requires the development of an efficient method to introduce therapeutic genes into target cells *in vivo*. The vector needs to be selective and efficient, delivering exogeneous DNA to target cells with minimal toxicity. The used vectors can be separated into two categories: viral and non-viral vectors.

6.1 Viral vectors

To enter the cells they use the natural ability of viruses (retrovirus, adenovirus, adeno-associated viruses) and express their own proteins, allowing a high transfection rate with rapid transcription of the foreign material inserted in the viral genome. However, their use is limited by various factors. Safety issues were raised following death of a patient during a clinical trial using viral vectors (SOMIA u. VERMA 2000). Only small sequences of DNA can be inserted in the virus genome and large-scale production is difficult to achieve. Further, viruses induce severe immune and inflammatory responses in patients. Lastly, also insertional mutagenesis and oncogenic effects can occur (K. Y. LEE et al. 1998).

6.2 Non-viral vectors

The limitations of viral vectors, particularly regarding safety, have led to the evaluation and development of alternative vectors based on non-viral systems. Non-viral vectors are less toxic, less immunogenic (lack of specific immune response), and easier to prepare (large-scale production) than viral vectors (NISHIKAWA u. HUANG 2001). Nevertheless, they have also some disadvantages including lower efficiency compared with viral vectors and short duration of gene expression (LI u. HUANG 2000). The physicochemical properties of the DNA-vector complex affect its passage through capillaries, extravasation, capture by the mononuclear phagocytes and uptake by target cells. Therefore, successful gene therapy will be achieved by developing a well designed efficient non-viral vector that overcomes these delivery barriers (NISHIKAWA u. HUANG 2001).

6.2.1 Non-viral transfection methods

6.2.1.1 Naked DNA injection

The simplest approach for DNA delivery is the injection of naked DNA. The first report was made by Wolf et al were gene expression after intramuscular injection of naked plasmid DNA was achieved (WOLFF et al. 1990). Intratumoral injection of naked DNA can also induce transgene expression at a level that it is enough to elicit therapeutic effects (NOMURA et al. 1999). However, when injected intramuscularly the uptake of plasmid DNA by muscle cells is relatively inefficient and limited to cells adjacent to the track of injection (WOLFF et al. 1991). For systemic administration plasmid DNA should be protected from degradation before reaching target cells

(NISHIKAWA u. HUANG 2001). In mice, after systemic administration of plasmid DNA, rapid degradation by nucleases and clearance by the mononuclear phagocyte system with a disappearance half-life of less than 5 minutes was reported (KAWABATA et al. 1995). Hence, administered systemically plasmid DNA requires a delivery system, such as cationic liposomes, that protects it from *in vivo* degradation (NISHIKAWA u. HUANG 2001).

6.2.1.2 Gene gun

Shooting naked plasmid DNA into target cells or tissues can be carried out by using a gene gun which uses gold nanoparticles coated with DNA (NISHIKAWA u. HUANG 2001). Transfection efficiency varies among tissues (10-20% skin, 1-5% muscle) (YANG et al. 1990) and gene expression can last up to 14-60 days (NISHIKAWA u. HUANG 2001). However, the major limitation is the shallow penetration into the tissue (in mice the depth did not exceed 0.5 mm) (ZELENIN et al. 1997).

6.2.1.3 Electroporation

Intense short electric pulses are applied reversibly to permeabilize the cell membrane allowing extracellular molecules to enter the cell. It has been applied to introduce plasmid DNA into tissues such as skin, liver, melanoma, and muscle (NISHIKAWA u. HUANG 2001). Naked plasmid DNA is injected into the tissue and electric pulses are applied with needle- or calliper-type electrodes. This pulses generally increase gene expression up to 1000-fold compared with injection of naked plasmid DNA without electroporation (NISHIKAWA u. HUANG 2001). Nevertheless, electroporation parameters and outcome greatly differ depending on the target tissue (NISHIKAWA u. HUANG 2001).

6.2.1.4 Cationic lipid (liposome)-DNA complex (lipoplex)

The addition of cationic lipids to plasmid DNA decreases its negative charge and facilitates its interaction with cell membranes. Cationic lipid-DNA complex has been used successfully to deliver plasmid DNA to lung, brain, tumors and skin by local administration or after intravenous injection (NISHIKAWA u. HUANG 2001). Complexes formed between the cationic lipid and DNA are rapidly cleared from the bloodstream and have been found to be widely distributed in the body (LIU u. HUANG 2002). The transfection efficiency of liposome-DNA complexes *in vivo* has been shown to be relatively low, especially when compared to viral vectors

(AUDOUY et al. 2002). One explanation for the relatively poor transfection efficiency is that they are susceptible to disruption by serum proteins (LIU u. HUANG 2002). Various proteins are known to bind to liposomes *in vitro* and *in vivo*, which may lead to membrane destabilization (MANSOURI et al. 2004). Additionally, it is also well documented that *in vitro* liposome-DNA complexes are directly cytotoxic.

6.2.1.5 Cationic polymer-DNA complex (polyplex)

High molecular weight cationic polymers are more effective in condensing DNA than cationic liposomes and can enhance cellular uptake of plasmid DNA by nonspecific adsorptive endocytosis (NISHIKAWA u. HUANG 2001). Transfection efficiency largely depends on its molecular weight and iso-form (FISCHER et al. 1999). In addition to natural polymers, synthetic ones have been developed to increase transfection efficiency, decreasing toxicity. Maheshwari et al. reported that Intratumoral injection of a biodegradable synthetic polymer increased significantly gene expression (MAHESHWARI et al. 2000). Cationic polymer-DNA complexes have also some disadvantages such as toxicity, lack of biodegradability, low field of gene transfection, biocompatibility and in particular, low transfection efficiency (OUPICKY et al. 2002).

7 Transfection reagents

Several reagents have been developed to overcome the limitations of transfection. The majority of the available transfection reagents introduce foreign DNA into cells by lipofection. The reagents generally contain lipid subunits that form liposomes in an aqueous environment entrapping the DNA, the generated DNA-containing liposomes can then fuse with the plasma membrane of cells and the DNA is able to enter the cell for replication. Transfection reagents commonly applied and also used in this study were Lipofectamine® (Invitrogen Life Technologies, Darmstadt, Germany), FuGENE® HD (Promega, Mannheim, Germany), X-tremeGENE (Roche applied sciences, Mannheim, Germany) and Mirus TransIT® (Mirus, MoBiTec GmbH, Göttingen, Germany).

8 Transfection efficiency

The effectiveness of the protocols applying plasmid DNA, in some cases, has not been well achieved, mainly because of the inefficiency of the gene transfer vectors used (MANSOURI et al. 2004).

Most of the results presented above after applying DNA encoding for IL-12 in melanoma therapy report tumor regression or tumor growth stop and in rare cases complete remission. Several limitations could explain this poor treatment respond. Immune system suppression or escape mechanisms of tumor cells are reasonable causes, but also reduced transfection efficiency with low protein expression.

8.1 Transfection efficiency enhancers

Various methods and modifications are currently evaluated to increase efficiency and reduce toxicity.

8.1.1 Nanoparticles (NPs)

The valuable characteristics of NPs make them suitable to act as plasmid DNA carriers and transfection enhancers. Transfection protocols using NPs have been evaluated in recent studies (BERTRAM 2006; P. GHOSH et al. 2008a; S. PETERSEN et al. 2009).

8.1.2 Gold Nanoparticles (AuNPs)

AuNPs are a good complement for the existing transfection protocols. AuNPs have a big surface, adjustable stability, low toxicity and can protect DNA from enzymatic disassembling (P. S. GHOSH et al. 2008b). AuNPs are in the focus of intense research due to their chemical stability, electro-density and -affinity to biomolecules such as DNA, when charged (ROSI et al. 2006). This inherent charge given to these AuNPs, make them interesting for DNA-binding and cell transfection.

8.1.3 Magnetic Nanoparticles

Magnetic NPs loaded with the nucleic acid of interest have been used to increase transfection efficiency by applying magnetic force to the DNA-NP complexes. These magnetic DNA-NP complexes are drawn towards the outer cell membrane via magnetic force and are subsequently taken up by the cell via endocytosis (BERTRAM 2006).

9 Equine IL-12 mammalian expression vectors

The heterodimeric nature of IL-12 presents challenges to the expression of the protein *in vitro* and *in vivo*. Both IL-12 subunits are translated separately and then processed by the cell to a joint complex. IL-12 expression strategies developed to overcome these limitations and achieve equimolar expression of each subunit include

the separation of the cDNAs (p35 and p40) by an internal ribosome entry site (IRES) (ZITVOGEL et al. 1994) or physical linking through a flexible peptide coding region (flexi) (ANDERSON et al. 1997). Equine IL-12 p35 and p40 subunits were cloned and sequenced by Nicolson et al. (NICOLSON et al. 1999) and the production of biologically active equine IL-12 in different expression systems was reported by McMonagle et al. (MCMONAGLE et al. 2001). The production of equine IL-12 after applying different expression vectors was measured and the “flexi” constructs were associated with superior expression of bioactive IL-12 than the IRES IL-12 constructs (MCMONAGLE et al. 2001). The lack of incorporated markers in these constructs (like GFP) makes it difficult to assess transfection efficiency. Therefore, to analyse accurately and if needed improve transfection efficiency one of the aims of these study was to built additional IL-12 DNA expression constructs using a pIRES-hrGFPII expression vector for the simultaneous but separate expression of a green fluorescent protein (hrGFP) and the protein of interest (equine IL-12) allowing to assess easily transfection efficiencies by GFP detection using fluorescence microscopy or flow cytometry.

10 Protein Expression verification/quantification after IL-12 treatment

The evaluation and further improvement of IL-12 mediated equine melanoma therapy requires an assay that directly or indirectly measures the actual concentration of equine IL-12 in serum or plasma of treated patients. Müller et al. assessed indirectly *in vitro* the IL-12 expression of transfected cells with equine IL-12 encoding vectors. The ability of the supernatants of transfected cells to induce IFN-gamma (IFN-γ) expression in equine PBMCs -by measuring the IFN-γ expression via RT-PCR- was determined (MÜLLER et al. 2011a), reporting an increased IFN-γ production in PBMCs stimulated with transfection supernatants. Furthermore, the IFN-γ mRNA expression was increased in biopsies of equine melanomas treated intratumorally with equine IL-12 coding vectors (MÜLLER et al. 2011b). A direct quantitative analysis of the acting cytokines (IL-12 and IFN-γ) could not be performed due to missing options for an accurate estimation of the present protein concentration.

So far, no specific functional enzyme linked assays for equine IL-12 or IFN-γ have been reported. Therefore, IL-12 expression after cell transfection with equine IL-12 encoding vectors was herein primarily assessed using immunofluorescence and

Western Blotting. Afterwards, different antibodies against human and bovine IL-12 and, bovine IFN-γ were evaluated for cross reactivity with equine IL-12 and IFN-γ to establish a bead-based Luminex cytokine assay. These established and evaluated bead-based assays were subsequently used to quantify separately and simultaneously equine IL-12 and IFN-γ concentrations in supernatants from stimulated PBMCs, lysates/supernatants of transfected cells with equine IL-12 encoding vectors and in several equine serum dilutions.