1 Introduction

Equine melanoma is a neoplasm occurring frequently in aging grey horses. Typical predilection sites are the ventral aspect of the tail, genitalia, anal region, and parotid gland. Clinical problems are mainly caused by localisation of melanomas increasing in size.

So far, therapies like surgical excision, cryotherapy, radiation, intralesional cisplatin injection and systemic treatment with cimetidine have not been curative. Studies in mice, dogs, and humans showed that xenogenic DNA vaccination, with DNA coding for the melanoma differentiation antigens glycoprotein 100 (gp100) or tyrosinase (tyr), caused an antitumoral response by stimulating specific immunity against these proteins.

The administration of DNA coding for interleukin (IL) 12 and -18 was shown to have adjuvant effects in combination with vaccination, as well as synergistic antitumoral effects. Treatment of equine melanomas with IL12 or IL18 DNA has been partially efficient.

The purpose of this study was to evaluate safety, clinical efficacy and immunological effects of the treatment of equine melanomas with xenogenic human DNA coding for melanoma differentiation antigens gp100 and tyr in combination with equine IL12 and -18 DNA or the interleukin DNA alone.

2 Literature

2.1 Equine melanoma

2.1.1 Incidence and classification

Equine melanoma, a tumor of pigment producing cells, is a common skin tumor in the horse, with a prevalence of 3.8 % (SUNDBERG et al. 1977). Four different types of equine melanomas are described (VALENTINE 1995):

1) Melanocytic naevus, a solitary, good demarcated lesion, develops in younger horses of every coat colour. There is no predilection site described. Excision is curative for these lesions. The melanocytic naevi are localisated at the dermoepithelial site and contain large, moderate pleomorph and epitheloid to fusiform cells.

2) Malignant melanoma can occur in horses of each age and of every coat colour, in young horses and congenital (HAMILTON a. BYERLY 1974; COX et al. 1989). It might develop in different localisations of the body (KUNZE et al. 1986; HONNAS et al. 1990). Cells are exceedingly pleomorph, slightly pigmented and mitoses are frequently seen in histology (ROELS et al. 2000).

3) Dermal melanoma is localized in the dermis of adult grey horses. Localisation sites on the body are variable. Lesions are well deliminated and occur solitary or multiple. In general surgical therapy is curative. Cells are small, homogenous and round or fusiforme. The cytoplasma is intensively pigmented.

4) Dermal melanomatosis, the fourth type of equine melanoma, also develops exclusively in grey horses (VALENTINE 1995). With increasing age and depigmentation of the coat, the majority of this population is affected (VALENTINE 1995; SUTTON A. COLEMAN 1997). In a study concerning a Lippizaner horse population only 5.75 % of individuals older than 16 years were free of melanomas (SELTENHAMMER et al. 2003).

In the following, characteristics of dermal melanomatosis are described in detail. The term melanoma is used as a synonym for dermal melanomatosis.

2.1.2 Clinical signs and diagnosis

Typical predilection sites are the ventral aspect of the tail, the perineum, genitals and parotid region (RODRÍGUEZ et al. 1997; FLEURY et al. 2000; SELTENHAMMER et al. 2003). In addition, melanomas develop internally and can be diagnosed via endoscopy or necropsy (FLEURY et al. 2000; MACGILLIVRAY et al. 2002). Computed tomography (CT) (SASAKI et al. 2007) or magnetic resonance tomography (MRT) examination (GERLACH et al. 2007) are further diagnostic modalities to diagnose melanomas in the head region.

Clinical symptoms are usually related to lesion location, including interference with defecation, weight loss, colic, edema, dyspnoea, epistaxis (FLEURY et al. 2000; MACGILLIVRAY et al. 2002), paresis (TRAVER et al. 1977; KIRKER-HEAD et al. 1985; DE BLAAUW et al. 2003), or ataxia (MACGILLIVRAY et al. 2002; PATTERSON-KANE a. GINN 2003), dysuria and abnormalities involving the genitalia, including priapism (BLANCHARD et al. 1991). Localized sweating and cutaneous hyperthermia could be observed after sympathetic denervation due to a melanoma in the region of the cervicothoracic ganglion (MURRAY et al. 1997). Lesions, normally covered by intact epidermis, may ulcerate and become infected (FLEURY et al. 2000).

2.1.3 Histology and immunohistology

Histological evaluation has shown that melanoma development begins with accumulation of melanin around the follicles of permanent hairs (RODRÍGUEZ et al. 1997; SUTTON a. COLEMAN 1997) or dermal apocrine sweat glands (FLEURY et al. 2000). Histological benign and malignant forms of grey horse melanoma (dermal melanomatosis) have been differentiated (SELTENHAMMER et al. 2004).

The benign form of grey horse melanoma is characterized by intradermal and subcutaneous heavily pigmented areas with well defined boundaries. Cells in the melanomas are of heterogenic appearance. Epitheloid cells with big spheric nucleoli, spindle shaped cells, balloon cells and a few polyhedral cells were found (ROELS et al. 2000; SELTENHAMMER et al. 2004). Lesions are characterized by a small

amount of mitotic figures, frequent nuclear atypia (FLEURY et al. 2000; SELTENHAMMER et al. 2004) and no inflammation or junctional activity (RODRÍGUEZ et al. 1997). Benign melanomas contain melanomacrophages with melanin-loaded granules beneath apoptotic cells. Proliferating melanocytes and marked melanin synthesis are found at the margins of the lesions (SELTENHAMMER et al. 2004).

In the malignant grey horse melanoma, anisocytosis and anisocariosis are observed. Tumors are poorly demarcated from the surrounding tissue. Lesions have a heterogeneous appearance with irregular melanin distribution and increased vascularisation. Cells are mostly pleomorphic, epitheloid and balloon shaped and show high numbers of mitotic figures (SELTENHAMMER et al. 2004).

The lesions are located exclusively in the dermis and have no junctional activity. The epidermis is not involved (FLEURY et al. 2000; SELTENHAMMER et al. 2004).

Immunohistology has been performed for melanoma differentiation antigens glycoprotein 100 (gp100) and tyrosinase (tyr). Expression of gp100 was greater in malignant lesions. Tyrosinase expression was shown in malignant grey horse melanomas whereas in the benign form only low levels were detected (SELTENHAMMER et al. 2004). The calcium binding protein S100 (Stefansson et al. 1982; Kahn et al. 1983), which is expressed in melanocytes and melanoma cells, was detected in all kinds of melanomas (SUTTON A. COLEMAN 1997; ROELS et al. 2000; SELTENHAMMER et al. 2004). The proliferation markers Ki-67 (a nuclear antigen expressed during cell division) and PCNA (a co-factor of DNA polymerase δ) had a higher (SELTENHAMMER et al. 2004) or comparable (ROELS et al. 2000) expression in malignant melanomas compared to benign melanomas. CD44, a hyaluronic acid receptor which is important for cell migration, lymphocyte homing, adhesion during hematopoesis, and lymphocyte activation was detected in malignant in a higher amount than in benign lesions (SELTENHAMMER et al. 2004). Only metastatic melanomas stained positive for p53 (ROELS et al. 2000), a tumor suppressor protein which is able to arrest cell cycle (PERRY et al. 1993, FRAGKOS et al. 2011). It was concluded that, because of the immune histological staining and

histological features, equine melanoma is a true neoplasm (SELTENHAMMER et al. 2004).

2.1.4 Etiology

The exact etiology of equine melanoma is still unclear. Due to the frequently benign appearance of melanotic lesions not only a neoplastic tumorigenesis but also a non neoplastic etiology was discussed. Some authors suggest that equine melanoma is a storage disease caused by disturbed melanin metabolism or impaired melanin transport to the hair (RODRÍGUEZ et al. 1997; SUTTON A. COLEMAN 1997).

Equine melanoma develops associated with greying. Predilection sites of melanomas are located where greying starts (SUTTON A. COLEMAN 1997; SELTENHAMMER et al. 2003) and are also related to migration paths and sites of progenitor cells from the neural crest (SUTTON a. COLEMAN 1997). The mechanism of melanoma formation in coherence to greying is not clear and metabolism or transport alterations (RODRÍGUEZ et al. 1997; SUTTON A. COLEMAN 1997) as well as transformation (SELTENHAMMER et al. 2004) are discussed as possible options.

A polygenic genetic impact for this disease was found. The expression of the genes coding for melanocyte protein 17 precursor (Pmel17/gp100) and tyrosinase-related protein 1 (TYRP1/gp75) was different in the skin of coloured horses (normal), the skin of grey horses (low) and melanomas (high) (RIEDER et al. 2000). Recently, it was shown that grey horses share some genetic modifications which are not found in horses of different color. A 4.6 kb duplication in the intron 6 of the syntaxin (STX) 17 gene is correlated with the grey phenotype, and homozygote animals have been found to have a higher prevalence of melanomas. This was also shown for a homozygote recessive agouti signalling peptide (ASIP) genotype (ROSENGREN PIELBERG et al. 2008). The products of these two genes interact with the cell cycle regulator proteins rat sarcoma (ras) (Q. ZHANG et al. 2005) and nuclear receptor subfamily 4, group A, member 3 (NR4A3) and may therefore lead to proliferation or transformation (ROSENGREN PIELBERG et al. 2008).

2.1.5 Therapy

Several therapies for equine melanomas have been developed.

Excision of small, singular melanomas is possible (PILSWORTH a. KNOTTENBELT 2006). Curative surgical treatment of melanomatosis is not feasible because lesions are multiple and occur also in internal organs (VALENTINE 1995). Nevertheless, surgical treatment may be palliative if defecation or other physiological functions are disturbed. Primary closure of excision site is not always possible, and reconstruction of anatomical structures may be necessary (ROWE a. SULLINS 2004).

Though cryosurgery can be used after surgical debulking, the success rate is low (PILSWORTH a. KNOTTENBELT 2006).

Radiotherapy with cobalt caused no significant reduction in size (MONTES 1997).

Local injection of cisplatin was shown to lead to a reduction in size of treated melanomas. However, new melanomas at other sites of the body developed during the therapy (THEON et al. 2007).

Histamine is able to activate T-suppressor cells via histamine receptor 2 (H2R) (ROCKLIN a. HABEREK-DAVIDSON 1981), therefore antagonisation of this effect can lead to an enhanced immune response (OSBAND et al. 1981). Thus cimetidin, an antagonist of the H2R, has been shown to have an antitumoral effect in lung cancer in two human patients (ARMITAGE a. SIDNER 1979). GOETZ et al. (1990) found a reduction in size of equine melanomas and disappearance of lesions in horses treated with Cimetidine (2.5 mg/kg every 8h p.o.), whereas WARNICK et al. (1995) observed no significant effect of number or size reduction after cimetidine treatment.

For unspecific immunologic treatment Bacillus Calmette-Guérin (BCG) has been used, but therapy was not successful (KOBLUK 1995).

Because of the genetic influence of the aetiology of melanoma (RIEDER et al. 2000; PIELBERG et al. 2005; ROSENGREN PIELBERG et al. 2008), it has been propagated to reduce melanomatosis by selection in breeding (RIEDER et al. 2000).

2.2 Importance of immunity against neoplasia

Intervention of the immune system in neoplastic diseases was first proposed over one hundred years ago (EHRLICH 1909) and was further supported by BURNET et al. (1967). Clinical observations have suggested a protective or curative function of the immune system. Immunoreaction against colon carcinomas (GALON et al. 2006) or melanomas (CLARK et al. 1989; CLEMENTE et al. 1996) was associated with a better prognosis in humans. Spontaneous regression was documented for melanomas in human (NATHANSON 1976; FERRADINI et al. 1993; MENZIES a. MCCARTHY 1997); in these cases perivascular infiltration with lymphocytes was found (NATHANSON 1976; FERRADINI et al. 1993).

Primary immunosuppressed patients suffering from diseases manifested in reduced cytotoxicity of NK-cells like Chediak-Higashi-syndrome, have a 200-fold risk of developing malignant processes (N. KOBAYASHI 1985). Furthermore, transplant patients treated with immunosuppressive drugs show higher incidences for non-viral induced neoplasia than untreated individuals (PENN 1988; BIRKELAND et al. 1995; MA et al. 1996; SHEIL et al. 1997; DUNN et al. 2004a).

The importance of the immune system could be demonstrated further in mice with defective interferon-gamma (IFNγ) signal transmission. During ageing or after treatment with the chemical carcinogen 3`-methylcholanthrene (MCA) IFNγ signal transmission was impaired, and as a consequence mice developed more neoplasia compared to wild type mice (IKEHARA et al. 1984; SHANKARAN et al. 2001).

2.2.1 Properties of cancer immunity

Immunity against cancer is based on several components:

One crucial factor is the production of IFNγ (DUNN et al. 2004b; SWANN a. SMYTH 2007). IFNγ has antiproliferative (KOMINSKY et al. 1998) and antiangiogenetic effects as well as an innate immune activation function (IKEDA et al. 2002). It directs the immune answer into a Th1 direction and activates NK cell function. Several genes are regulated by IFNγ, influencing the antigen-processing and -presentation, antiviral functions, antiproliferative, apoptotic and other immunemodulatory effects

(as reviewed by SCHRODER et al. 2004). A deficiency of IFNγ leads to a higher incidence of chemically induced and spontaneous tumors:

As mentioned above, studies in mice lacking IFNγ-producing cells showed that the development of sarcoma after injection of MCA is faster than in wild type mice (SHANKARAN et al. 2001). IFNγ-insensitive fibrosarcoma cells can induce tumors in immunocompetent mice (DIGHE et al. 1994). The incidence of spontaneous tumors is higher in mice with deficient IFNγ signal transduction (signal transducer and activator of transcription 1 (STAT1)-deficient mice) (KAPLAN et al. 1998; SHANKARAN et al. 2001).

Perforin, another effector molecule of anti cancer immunity, is stored in granules in NK and CD8+ cells and is delivered by degranulation. Perforin is able to form a pore into, and therefore to lyse, target-cells (MASSON, TSCHOPP 1985). Perforin knockout mice were susceptible to develop MCA induced (STREET et al. 2001) and spontaneous (SMYTH et al. 2000) sarcoma.

Cytotoxic $CD8^+$ cells are able to lyse cells which present an epitope in combination with MHC-I complex. Tumor infiltrating lymphocytes improve the prognosis of patients with neoplastic disease (GALON et al. 2006). The role of T-cells was affirmed in immunodeficient mice: mice deficient for recombinase-activating-gene-2 (RAG-2) which is essential for the generation of mature B and T lymphocytes (SHANKARAN et al. 2001) and severe combined immunodeficiency (SCID) mice with interleukin-receptor-gene mutation and a lack of T and B lymphocytes (SWANN a. SMYTH 2007) showed a higher incidence for tumor development compared to wild-type mice.

NK-cells are another important factor for anticancer immunity. The role of NK-cells was, as pointed out above, first mentioned after detection of the higher incidence for neoplasia in Chediak Higashi Syndrome patients showing reduced cytotoxicity of NK-cells (N. KOBAYASHI 1985). NK-cells are responsible for tumor rejection and protection against metastases as shown in knockout mice (HALIOTIS et al. 1985; SMYTH 2008). NK-cells are most important for the elimination of cancer cells which express no MHC-I complexes (SMYTH et al. 2000).

Eventually, antibodies can play a role in cancer immunity, too (DI GAETANO et al. 2003; WEINER et al. 2010). Interaction of the Fc (Fragment, crystallisable) -segment of antibodies specific for tumor cell surface antigens, with the Fc-receptor on immune cells, leads to antibody dependent cytotoxicity (ADCC) (CARTRON et al. 2002; NIMMERJAHN a. RAVETCH 2006). Antigens of destroyed cells may afterwards be presented in MHC-II and also, in cross presentation, in MHC-I molecules (ALBERT et al. 1998; WEINER et al. 2010).

2.2.2 Tumor escape

Neoplasia can escape from immune recognition. Non-immunogenicity of a tumor may exist because the tumor never had immunogenic antigens, lost the antigens during growth or because cells cannot present antigens to the immune system (ALGARRA et al. 2000; MARINCOLA et al. 2000; SELIGER et al. 2000).

Recognition of tumor cells by T-cells can be reduced because of modulated peptide binding to MHC-I complexes or decreased binding of MHC-I-peptide complexes to T-cell receptors. This phenomenon, in turn, may lead to increased recognition of tumor cells by NK-cells (BUBENIK 2004).

Cytotoxicity can be reduced by binding of perforin at the tumor cell surface so that the molecule is unable to perform its function (LEHMANN et al. 2000).

Fas ligand (REAL et al. 2001) or TNFα-related apoptosis inducing ligand receptor (SHIN et al. 2001) can be down regulated or mutated.

Immunosuppressive mediators like nitric oxide (NO) (X. M. ZHANG a. XU 2001), Transforming Growth Factor beta (TGFβ) (BECK et al. 2001) and Interleukin-10 (IL10) (KAWAMURA et al. 2002) can be produced by tumor cells.

Immunosuppressive cytokines expressed by tumor cells are able to activate regulatory T-cells (CURIEL 2007). Regulatory T-cells suppress the immune system with TGFβ, IL35 and contact dependent mechanisms (COLLISON et al. 2007; CURIEL 2007) thereby reducing any antitumoral immune response.

Tumor cells can induce tolerance or anergy in immune cells (KOWALCZYK 2002).

The microenvironment of tumors may contain regulatory immune cells like myeloid-derived suppressor cells (GALLINA et al. 2006; SICA a. BRONTE 2007) and regulatory T-cells (WOLF et al. 2005; CURIEL 2007; NUMMER et al. 2007).

Endothelial adhesion molecules can be down regulated in newly formed vessels in tumors which reduce adhesion of immune cells in these regions (RYSCHICH et al. 2002).

Indolamin 2.3 dioxygenase, a catabolic enzyme in the tryptophan metabolism, can be produced by tumor cells. It reduces levels of tryptophan, and therefore T-cell proliferation is suppressed (UYTTENHOVE et al. 2003; MUNN a. MELLOR 2007).

2.2.3 Tumor antigens

Tumor immunity might develop against several groups of antigens.

Cancer/Testis-antigens are normally expressed in germ cells and neoplastic tissue. Because germ cells normally lack MHC-I/II complexes, antigens are not presented to T-cells. For that reason, germ cell antigens are unknown to the immune system so that autoimmunity might arise (SCANLAN et al. 2004; WEIDE et al. 2008).

Mutated antigens develop after point mutations (GILBOA 1999). They are highly immunogenic (LENNERZ et al. 2005) but difficult targets for immunotherapy because they are different in each patient (WEIDE et al. 2008). They might be used in form of tumor cell lysate vaccines (PARMIANI et al. 2007).

Another group are products of **over expressed genes** like human telomerase reverse transcriptase (hTERT) (VONDERHEIDE et al. 1999) which is expressed in 85% of solid human tumors like prostate carcinoma (SHAY a. BACCHETTI 1997) and melanoma (FULLEN et al. 2005). A therapeutic advantage is this antigen's limited capacity for escape from immunity because the protein is essential for proliferation of the tumor.

Differentiation antigens are specific for a tumor cell type and the corresponding healthy tissue (WEIDE et al. 2008). Examples for melanoma differentiation antigens are Melanoma Antigen Recognized by T-cells A (MART A), glycoprotein 100 (gp100) and tyrosinase (GRIFFIOEN et al. 2001). Differentiation antigens are normally

protected by self-tolerance (NISHIKAWA et al. 2005). Differentiation antigens are not ideal as targets for immunotherapy because autoimmunity might develop against the healthy tissue. Autoimmunity against melanocytes clinically manifested in vitiligo correlated with survival time (BOASBERG et al. 2006).

Important criteria for the selection of a target for immunotherapy are the immunogenicity of the antigen and the prerequisite that the antigen is, because of possible autoimmunity, not essential for the health and survival of an organism (WEIDE et al. 2008).

2.3 Immunologic Therapy against cancer

Based on the knowledge that the immune system participates in the combat of neoplasia (IKEHARA et al. 1984; CLARK et al. 1989; CLEMENTE et al. 1996; SHANKARAN et al. 2001; BOASBERG et al. 2006), several strategies have been evaluated to induce and intensify immunological responses (RIBAS et al. 2003).

2.3.1 Autologous tumor cells for immunological treatment of cancer

Autologous tumor cells can be utilized for therapy after *in vitro* modification (radiation) to minimize the risk of tumor dissemination. Cells possess many distinct antigens and are different for each individuum. Therapies using melanoma cells alone (LOTEM et al. 2009) or in combination with immune stimulatory adjuvants like BCG (BAARS et al. 2000; LOTEM et al. 2002) or BCG and recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (LEONG et al. 1999) lead to stimulation of CD4+ cells (LOTEM et al. 2009), prolonged survival (LOTEM et al. 2002) and delayed-type hypersensitivity (DTH) (BAARS et al. 2000) in men. A partial response in four out of eight patients and delayed type hypersensitivity (DTH) could be induced by tumor cell lysate pulsed Dendritic Cell (DC) vaccination (MAIER et al. 2003).

In equine sarcoids regression of tumors was observed after autologous implantation of sarcoid tissue which was previously freezed in liquid nitrogen (ESPY 2008) or after intradermal injection of polymerized sarcoids (HALLAMAA 2007).

For equine melanoma, the first description of this type of vaccine was made by MERTENS (1924) who injected autologous carbol-treated melanoma cells

subcutaneously into a melanoma bearing horse. No curative effect was observed in this case report.

More recently, autologous vaccines prepared from biopsies were used in combination with cimetidin in the therapy of equine melanomas in two horses. Outcome was not reported (MACGILLIVRAY et al. 2002).

A combination of excision and subsequent treatment of the incisional area and remaining melanomas with a combination of DNA lipoplexes coding for HSV*tk* gene, gangciclovir and subcutaneous injection of a vaccine obtained from excised melanomas combined with CHO cells transfected with hIL2 and GM-CSF has resulted in complete or partial regression of melanomas (FINOCCHIARO et al. 2009).

In conclusion, the preparation and antigen composition is varying in different whole tumor cell vaccines. Evaluation of immune answers is difficult, and comparison of different vaccines is not possible (SPEISER et al. 2003).

2.3.2 Peptides used as tumor vaccines

After identification of proteins and peptides specific for tumors it was attempted to obtain a more specific therapy by using these for vaccination (MANDELBOIM et al. 1995). Intradermal immunisation using tyrosinase peptide resulted in increased proliferation of specific cytotoxic T-lymphocytes (CTL) in human melanoma patients (LEWIS et al. 2000). CTL primed *in vitro* with tyrosinase peptide were able to induce a clinical regression in 2 of 10 stage 4 melanoma patients (MITCHELL et al. 2002).

Dendritic cells pulsed with antigenic peptides could induce a cytotoxic T-cell answer as well as clinical regression of metastases or partial response in human melanomas (THURNER et al. 1999; BANCHEREAU et al. 2001; LINETTE et al. 2005).

In a mouse model, treatment with peptide pulsed DC could protect mice against lethal challenge with tumor cells and resulted in regression of established neoplasia (MAYORDOMO et al. 1995).

DC were also used, transfected with xenogenic human DNA coding for gp100 in order to produce this protein, in malignant melanomas of dogs and could induce a specific stimulation of cytotoxic T-cells (GYORFFY et al. 2005).

2.4 DNA vaccines

Vaccination using DNA holds some advantages compared to lysed tumor cells, proteins or peptides. First, DNA is inexpensive, can be produced in large quantities, storage as well as handling is uncomplicated (WEIDE et al. 2008). In addition, vaccination can be performed with DNA coding for several proteins or peptides (PILSWORTH a. KNOTTENBELT 2006), and MHC restriction, which limits the use of some peptides to individuals with compatible MHC haplotype, is not relevant. Humoral as well as cellular immunity can be induced (DUNHAM 2002; HAUPT et al. 2002).

In vivo transfection, a crucial event of DNA vaccination, was proven after intramuscular injection of DNA coding for luciferase in mouse (WOLFF et al. 1990).

Injected plasmids have to get into the cell were they remain episomally in the nucleus and are not integrated in the genome of the eukaryotic cell (WOLFF et al. 1990). Gene expression could be detected up to 19 months after injection which may lead to prolonged stimulation of the immune system (WOLFF et al. 1992). Proteins are processed to peptides, associated with MHC-I and presented on the cell surface (TOWNSEND a. TROWSDALE 1993).

If a cell expressing the target protein is lysed or the protein is secreted, the protein can be phagocyted by antigen-presenting cells and presented on cell surface in complex with MHC-II (HAUPT et al. 2002).

T-helper and effector cells can be induced systemically, and memory cells might be generated (SRINIVASAN a. WOLCHOK 2004).

In several species, DNA vaccines are protective against infectious agents (HO et al. 1998; LARSEN et al. 1998; R. WANG et al. 1998; EDGEWORTH et al. 2002; DHAMA et al. 2008). In horses, immunity against EHV-1 could be induced

experimentally using a DNA based vaccine (PAILLOT et al. 2006). Four veterinary vaccines are commercially available (KUTZLER a. WEINER 2008). An immune reaction against neoplasia could be induced using DNA vaccines in experimental settings (CHEN et al. 1998; SCHREURS et al. 1998; ZHOU et al. 1999; MENDIRATTA et al. 2001; HAUPT et al. 2002; WEIDE et al. 2008).

2.4.1 Overcoming self tolerance by xenovaccination

Because differentiation antigens are normally protected by self tolerance, immunogenicity of vaccines targeting these proteins is weak, and a crucial aspect of vaccination is to overcome self-tolerance mechanisms (HOUGHTON 1994; NAFTZGER et al. 1996).

In a mouse model the efficacy of vaccination with xenoprotein could be demonstrated (NAFTZGER et al. 1996). Also the use of DNA coding for xenogenic antigens provides an option to overcome self-tolerance. Administration of DNA coding for xenogenic melanocyte differentiation antigens was able to induce an immunological response against melanomas in several species (OVERWIJK et al. 1998; WEBER et al. 1998; ZHOU et al. 1999; GOLD et al. 2003; BERGMAN et al. 2006, YUAN et al. 2009). The immune system first recognizes the foreign protein and in a cross reaction, also the autologous protein is involved in the immunological answer (HAUPT et al. 2002; HOUGHTON a. GUEVARA-PATINO 2004; SRINIVASAN a. WOLCHOK 2004). Because of this mechanism, depigmentation, as a result of autoimmunity to melanocytes, is a possible side-effect (NAFTZGER et al. 1996; WEBER et al. 1998; BOWNE et al. 1999; BERGMAN et al. 2006).

2.4.2 DNA vaccination using minimalistic immunologically defined gene expression (MIDGE®) vectors

Plasmid DNA, normally used for gene therapy, contains backbone DNA which is required for the production. Unmethylated CpG motifs can exist in this plasmid backbone in large amounts, and may have an unspecific and unpredictable immune stimulatory effect (HEMMI et al. 2000; KLINMAN et al. 2009). Because of less backbone DNA in the minimalistic immunologically defined gene expression

(MIDGE®) vectors, the absolute dose of administered DNA can be reduced in comparison with normal plasmids. The administration of plasmids into an organism holds the risk that antibiotic resistance genes contained in plasmids can be expressed (GLENTING a. WESSELS 2005).

Using MIDGE®-vectors these problems can be avoided (LEUTENEGGER et al. 2000). These vectors contain no antibiotic resistance genes and only low numbers of CpG-motifs. They are made of linear double stranded DNA which is covalently closed with single-stranded hairpin loops at the ends (LEUTENEGGER et al. 2000). Consequently, MIDGE® vectors contain only the promotor, the coding sequence and a poly A-sequence.

2.5 Immunologic therapy against melanoma

Several melanoma antigens have been tested for their immunogenic effects, administered as protein, peptide or DNA (HOUGHTON 1994; LEWIS et al. 2000; RAMIREZ-MONTAGUT et al. 2003; SPEISER et al. 2003; SRINIVASAN a. WOLCHOK 2004).

The first molecularly identified melanoma differentiation antigen proven to be immunogenic was called MAGE (Melanoma Antigen) (VAN DER BRUGGEN et al. 1991). Progenitor cells recognizing MAGE can also be found in healthy individuals (LONCHAY et al. 2004). MAGE peptide vaccination resulted in regression of metastases without evidence of specific CTL response (MARCHAND et al. 1999). Targeting a different antigen, MART1, or also called **mel**anocyte **an**tigen A (Melan-A), an increase of specific T-cells could be observed after MART 1 peptide vaccination without clinical regression of tumors (CORMIER et al. 1997). These studies illustrate the potential paradox between clinical and immunological outcome.

Vaccination against gp100 caused clinical and immunological reactions in several studies (HUANG et al. 1998; ROSENBERG et al. 1998; SCHREURS et al. 1998; ZHOU et al. 1999; HAWKINS et al. 2000; SMITH et al. 2003). Furthermore, tyrosinase, an important enzyme of the melanin synthesis pathway, was also found to be an appropriate target protein for immunologic therapeutic strategies (LEWIS et al. 2000; MITCHELL et al. 2002; SCHAED et al. 2002; WOLCHOK a. CHAPMAN

2002; GOLDBERG et al. 2005; BERGMAN et al. 2006; WOLCHOK et al. 2007). These latter two proteins were used as targets for immune therapy in this study and are described in the following.

2.5.1 The use of gp100 in immunotherapy

Human gp100 (hgp 100) is a glycoprotein built of two disulfide-linked subunits (BERSON et al. 2001). It is localized in membranes of premelanosomes and premelanogenetic cytosolic vesicles and is involved in the conversion of 5.6-dihydroxyindole-2-carboxylic acid (DHICA) to melanin. It is a component of a complex of membrane bound oxidoreductases (LEE et al. 1996). This protein has been shown to be immunogenic, and it is an important target for specific immunotherapy (ZHAI et al. 1996). Several epitopes of gp100 were identified as recognized by specific cytotoxic T-cells (BAKKER et al. 1994; KAWAKAMI et al. 1994; KAWAKAMI et al. 1995) as well as T helper cells (HALDER et al. 1997).

Peptides cleaved from gp100 were able to increase the number of cytotoxic T-cells identified by tetramer assay in 28/29 human melanoma patients when combined with incomplete Freund's adjuvant (IFA) (SMITH et al. 2003). Regression was shown in 42% and a cellular response in 91% of human melanoma patients after vaccination with the peptide containing the aminoacids 209-217 of gp100 ($gp100_{209–217}$) and IL2 (ROSENBERG et al. 1998).

DNA coding for gp100 has been used in several studies. Mice vaccinated with DNA coding for human gp100 (hgp100) generated IgG and CTL specific for hgp100 and a protection against hgp100 expressing B16 cells. Tumor infiltrating T-lymphocytes showed cross reactivity to murine gp100. Splenocytes of vaccinated mice, transferred to non vaccinated mice, could convey immunity, indicating a cellular based immune reaction (SCHREURS et al. 1998).

Immunisation with plasmid DNA encoding hgp100 incorporated in hemagglutinating virus of Japan (HVJ-AVE) liposomes resulted in humoral and cellular immunity as well as longer survival of vaccinated mice (ZHOU et al. 1999).

In particular, mice vaccinated with hgp100 DNA showed 50 % less lung metastases after injection of B16 melanoma cells than unvaccinated mice. Thirty (90) day tumor-free survival time was 68 % (47 %) for vaccinated and 4% for non vaccinated animals. In the cited study three immunisations resulted in significantly higher tumor-free survival than a single treatment ($p < 0.05$) or no treatment ($p < 0.001$). Immunity was based on cellular mechanisms. Autoimmunity was observed in form of depigmentation in mice treated with hgp100 DNA (HAWKINS et al. 2000).

Furthermore, after vaccination of melanoma patients with human and mouse gp100 plasmids, there was only one responder showing specific IFNγ producing cells. With specific MHC-I/peptide tetramers in 5 out of 18 patients specific T-cells could be detected (YUAN et al. 2009).

To evaluate the similarity of the aminoacid sequences of human and equine gp100 was compared using the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome, 17.11.2010) The comparison of the amino acid sequences of equine and human gp100 showed a homology of 83%.

2.5.2 The use of tyrosinase in immunotherapy

Tyrosinase is a copper containing enzyme (monophenol monooxygenase) catalyzing the rate limiting step of the reaction of L-Tyrosin, L-Dopa (Levo-Dihydroxyphenylalanin) and O_2 to L-Dopa, Dopachinon and H_2O (HEARING a. TSUKAMOTO 1991; YAMAGUCHI a. HEARING 2009). It is a transmembrane protein of the melanosomes. Tyrosinase is expressed in every melanin producing cell (HEARING a. TSUKAMOTO 1991). For that reason it has been used for vaccination against melanomas (V. G. BRICHARD et al. 1996).

Although even in healthy individuals anti tyrosinase antibodies were detected, antibody titres in patients with malignant melanomas and patients with vitiligo exceeded those (MERIMSKY et al. 1996).

Tyrosinase is recognized by antibodies (BERGMAN et al. 2003; BERGMAN et al. 2006), $CD4^+$ (TOPALIAN et al. 1994) and $CD8^+$ cells (V. BRICHARD et al. 1993). In melanoma patients, CTLs, recognizing peptides of tyrosinase, could be detected (WOLFEL et al. 1994; P. P. LEE et al. 1999).

Immunotherapy targeting tyrosinase was performed with peptides and DNA:

In patients suffering from malignant melanoma, an increase of cytotoxic T-lymphocytes recognizing a specific peptide complexed with HLA-A*0201 was detected by ELISPOT analysis after intradermal vaccination with tyrosinase protein and the immune adjuvant QS-21 (a purified plant extract derived from the Soap bark tree (*Quillaja saponaria*)) (LEWIS et al. 2000).

Another study using vaccination with tyr peptides showed that GM-CSF or QS-21 were appropriate adjuvants for the induction of specific T-cells (SCHAED et al. 2002).

CTL primed *in vitro* against a tyrosinase peptide were able to induce clinical regression in 2 of 10 melanoma patients (MITCHELL et al. 2002).

Tumor-free survival, IFNγ production by CD8 positive cells, and antibody production were compared after vaccination using plasmid DNA and propagation incompetent virus-like replicon particle vaccines (VRP) in mice. An antitumor response and immune reaction was shown for xenogenic plasmid DNA vaccination and for syngenic VRP vaccination (GOLDBERG et al. 2005).

Dogs suffering from canine malignant melanoma were treated with DNA coding for human tyrosinase, murine tyrosinase, murine gp75 (mu-gp75, tyrosinase related protein), human granulocyte macrophage colony stimulating factor (hGM-CSF) and a combination of murine tyrosinase and hGM-CSF. Combination of hGM-CSF and murine tyrosinase resulted in longer survival (>402d (median not reached)) compared to human tyrosinase (389d), mu-gp75 (153d), human GM-CSF (148d) or murine tyrosinase alone (224d) (BERGMAN et al. 2006).

Antibodies against human and canine tyrosinase were detected in plasma of 3 of 9 dogs vaccinated with 100 (1 dog) resp. 1500 (2 dogs) µg human tyrosinase DNA. Dogs with detectable antibodies had a longer survival time (LIAO et al. 2006).

In humans, murine tyrosinase and human tyrosinase were used in a crossover designed trial in doses of 100, 500 and 1500µg. Patients received three xenogenic and three syngenic vaccinations of one dose level every three weeks. Using tetramer assay and intracellular IFNγ staining, specific T-cells could be demonstrated between 3 weeks and 3 months after the last injection. No differences due to dose, assigned schedule or T-cell response were found. No specific tyrosinase antibodies were detected (WOLCHOK et al. 2007).

To evaluate the similarity of the aminoacid sequences of human and equine gp100 and tyrosinase were compared using the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome, 17.11.2010). The comparison of the amino acid sequences of equine and human gp100 and tyrosinase showed a homology of 84%.

2.6 Cytokines

Cytokines like IL12 and IL 18 have been used to potentiate immune reactions (DISIS et al. 2003). These cytokines trigger an augmentation of cellular immunity and a reduction of angiogenesis in tumors (IWASAKI et al. 1997; OKADA et al. 1997; CHOW et al. 1998; COUGHLIN et al. 1998; J. J. KIM et al. 1998; J. J. KIM et al. 1999a; J. J. KIM et al. 1999b; SIN et al. 1999; GHERARDI et al. 2000).

Applied systemically as recombinant protein, IL12 causes several side effects like fever, chills, fatigue, headache, nausea, vomiting (ORANGE et al. 1995; ATKINS et al. 1997; BAJETTA et al. 1998; MOTZER et al. 1998; PORTIELJE et al. 1999) and even death (COHEN 1995; LEONARD et al. 1997).

In contrast, cytokine DNA is able to potentiate an immune reaction but shows less toxicity than recombinant protein due to a constant plasma level, rather than high, toxic, and short-lived plasma levels of recombinant proteins (SCHULTZ et al. 1999; COLOMBO a. TRINCHIERI 2002)

2.6.1 Interleukin 12

Interleukin 12 (IL12) is a heterodimeric protein consisting of a constitutively produced 35kDa (IL12α) chain and a stimuli-related expressed 40kDa (IL12β) chain (M. KOBAYASHI et al. 1989; D'ANDREA et al. 1992). It is produced by phagocytes, lymphocytes and dendritic cells (D'ANDREA et al. 1992; MACATONIA et al. 1995). Its expression can be induced by different pathogens (MA and TRINCHIERI 2001). Interleukin10 (D'ANDREA et al. 1993; ASTE-AMEZAGA et al. 1998) TGFβ (ADACHI et al. 1998) and TNFα (ALEXOPOULOU et al. 2001) decrease the production. Also the binding of ligands to different receptors (Guanine Nucleotide-Binding Protein-Coupled$_{\alpha s}$ ($G_{\alpha s}PC$)-, α_2 adrenerg-, adenosine$_{2a}$ (A_{2a})-, H2- and vasoactive intestinal peptide- (VIP) receptor) is able to decrease IL12 production (BRAUN a. KELSALL 2001).

The IL12 receptor consists of two chains transducing the signal by JAK-STAT (Januskinase-signal transducer and activator of transcription) mechanism. Receptors are located on activated T- and NK-cells (PRESKY et al. 1996). Receptor production is induced by activation of IFNγ, IL12 and the TCR itself (ROGGE et al. 1997; SZABO et al. 1997).

IL12 leads to improved tumor recognition by up regulation of MHC-I and II molecules as well as intercellular adhesion molecule I (ICAM-I) on melanoma cells (YUE et al. 1999).

In addition IL12 was shown to have an antiangiogenetic effect as an important factor for tumor regression (VOEST et al. 1995; COUGHLIN et al. 1998; YAO et al. 2000; GEE et al. 2001; L. HEINZERLING et al. 2002).

IL12 directs the immune system towards a Th1 response (HSIEH et al. 1993; MANETTI et al. 1993; TRINCHIERI 2003) by differentiation of T helper cells to Th1 cells (ROBERTSON a. RITZ 1996). Via IL12, Th1 cells are stimulated to produce cytokines (IFNγ, TNFα, IL2, IL3, IL8, IL10) (TRINCHIERI 1993). For this reason it is supposed to be a good adjuvant for cancer vaccines in which a Th1 answer is aimed for (AFONSO et al. 1994).

IL12 leads to differentiation (ROMAGNANI 1992; SCOTT 1993) and proliferation (ROBERTSON a. RITZ 1996) of activated $CD8^+$ and $CD4^+$ cells. Moreover, the cytotoxic potential of $CD8^+$ cells and NK cells is increased by IL12 stimulation (ROBERTSON a. RITZ 1996). NK cells are important targets for the stimulation by IL12 as shown in knockout mice experiments (RAKHMILEVICH et al. 2004). In aforementioned CTL and NK cells, transcription of effector molecules like granzyme B and perforin (CESANO et al. 1993; SALCEDO et al. 1993; ASTE-AMEZAGA et al. 1994), and adhesion molecules (ROBERTSON et al. 1992; RABINOWICH et al. 1993) are increased by IL12.

Most importantly, IL 12 stimulates T and NK cells to produce IFNγ (TRINCHIERI 2003). As anti IFNγ mAk inhibited the IL12 effects, IFNγ can be regarded as essential for the effect of IL12 (NASTALA et al. 1994; ZOU et al. 1995). It in turn induces a further increase in production of IL12 and therefore a positive feedback (STERN et al. 1990; D'ANDREA et al. 1992; PERUSSIA et al. 1992; COLOMBO a. TRINCHIERI 2002).

2.6.1.1 Antitumoral effects of IL12

Reduction and regression of neoplasia after IL12 therapy could be shown in mouse models (BRUNDA et al. 1993; NASTALA et al. 1994; BRUNDA a. GATELY 1995; MU et al. 1995; ZOU et al. 1995). Antitumoral effects were attained in a model of transplanted (FALLARINO et al. 1996) or induced tumors (NOGUCHI et al. 1996).

Subcutaneously administered recombinant IL12 reduced metastases in human melanoma (BAJETTA et al. 1998).

Administration of DNA coding for IL12 lead to a reduction of metastases, to prolonged survival, and to reduction and regression of tumors in several mouse tumor models (RAKHMILEVICH et al. 1996; MENDIRATTA et al. 1999; WANG et al. 1999). Its effects compared favourable to gene therapy coding for other cytokines (IL-2, IL-4, IL-6, IFN-*γ*, TNF-*α*, GM-CSF) (RAKHMILEVICH et al. 1997).

In melanoma mouse models, DNA coding for IL12 had an antimetastatic effect (SCHULTZ et al. 1999; SCHULTZ et al. 2000). Intratumoral electroporation could

reduce growth (LUCAS a. HELLER 2003) and induce a regression (LUCAS et al. 2002) of injected melanoma cells.

In spontaneous melanoma in humans, IL12 DNA injected intratumorally could induce a size reduction of primary tumors (MAHVI et al. 2007). IL12 DNA electroporated in human metastatic melanoma lesions induced complete regression even in distant tumors in 2 of 19 patients and stable disease or partial regression in 8 patients (DAUD et al. 2008).

In equine melanomas, human DNA injected into melanomas was effective in reducing the size and in one tumor complete regression of the injected melanomas (HEINZERLING et al. 2001). Using equine DNA, a significant size reduction was achieved (MÜLLER et al. 2011).

2.6.2 Interleukin 18

IL18 is an 18 kDa glycoprotein (DINARELLO 1999), an important factor for IFNγ production and has been called IFNγ-inducing factor for this reason (KOHNO et al. 1997). IL18 alone induces little IFNγ production, but potentiates other stimuli like IL12, LPS and other bacterial products (DINARELLO et al. 1998; FANTUZZI a. DINARELLO 1999; NETEA et al. 2000).

It is synthesized in macrophages, dendritic cells, ceratinocytes, osteoblasts, adrenal cortical cells, intestinal epithelia, microglia and synovial fibroblasts (CONTI et al. 1997; MATSUI et al. 1997; STOLL et al. 1997; UDAGAWA et al. 1997; STOLL et al. 1998; GRACIE et al. 1999; PIZARRO et al. 1999; PRINZ a. HANISCH 1999). Stimuli for production are LPS, exotoxins of gram-positive bacteria, and diverse microbial products (DINARELLO 1999). Regulation is achieved by two promotors which can be stimulated by LPS (TONE et al. 1997; Y. M. KIM et al. 1999c). NFκB is also involved in the regulation of IL18 synthesis (GRACIE et al. 2003).

Receptors for IL18 are localized on macrophages, neutrophilic granulocytes, NK cells, endothelial and smooth muscle cells (HYODO et al. 1999; LEUNG et al. 2001; GERDES et al. 2002).

Further, signal transduction is realized by activation of myeloid differentiation factor 88 and IL1R associated kinase (IRAK), a mechanism shared with other IL1 cytokines (WESCHE et al. 1997; ADACHI et al. 1998; KANAKARAJ et al. 1999).

IL18 enhances a T and NK cell maturation, cytotoxicity and cytokine production. Expression of apoptosis stimulating fragment ligand (FasL) on NK cells, and therefore their cytotoxicity, is enhanced (DAO et al. 1996; TSUTSUI et al. 1996). Expression of IL4, IL6, IL10, IL13, IFNγ and TNFα can be induced by IL18 (HOSHINO et al. 1999; HOSHINO et al. 2000; NETEA et al. 2000; YOSHIMOTO et al. 2000).

Depending on the milieu, a Th1 or Th2 answer can be favoured (XU et al. 2000). In neutrophils, activation and synthesis of reactive O_2 metabolites and cytokines can be provoked by IL18 (LEUNG et al. 2001).

Expression of intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) and E-Selectin is upregulated in endothelial cells and synovial fibroblasts (MOREL et al. 2001).

2.6.2.1 Antitumoral effects of IL18

The antitumoral effects of IL18 were shown in mice models. After intratumoral injection of IL18 DNA, numbers of effector T and NK cells increased in the spleen and in peripheral blood; moreover, $CD4^+$ cells showed enhanced IFNγ production (CHANG et al. 2007). The antitumoral effect was shown to be depended on IFNγ production as it can be neutralized by administration of IFNγ antibodies. Melanomas, build up from melanoma cells transfected with IL18 gene and injected in mice, showed retarded growth, reduced vascularisation and enhanced necrosis. Antiangiogenesis in tumors induced by IL18 depended on up regulation of antiangiogenic chemokines, interferon inducible protein 10 and monokine induced by IFNγ, and the down regulation of angiogenin (NAGAI et al. 2002). In a comparison of effects of intratumorally injected equine IL18 and IL12 DNA in equine melanomas both therapeutics caused a significant size reduction of spontaneously developing melanomas in horses (MÜLLER et al. 2011).

IL18 fulfills its function after secretion. Ligation of the IL-1beta receptor antagonist protein (ILRAP)-signal sequence to the original gene (IL 18) proved to lead to an augmented secretion of the cytokine (O'DONOVAN et al. 2004).

2.6.3 Combination of IL12 and IL18

IL12 and IL18 were shown to have synergistic antitumoral effects (COUGHLIN et al. 1998; HARA et al. 2000; KISHIDA et al. 2001).

The effect of the combination of IL12 and IL18 was shown to be dependent on NK cells and their IFNγ production (SUBLESKI et al. 2006).

Synergy arises from IL12 activated STAT4 which increases the IL18 induced AP1 binding to the IFNγ promoter (BARBULESCU et al. 1998; NAKAHIRA et al. 2002). IL12 and IL18 also act together via non receptor tyrosine protein kinase (tyk2) signalling (SHIMODA et al. 2002). IL12 and IL18 synergistically induce indolaminedioxigenase (I2.3DO) via lymphocytes secreting IFNγ. The I2.3DO degrades tryptophan which subsequently results in apoptosis of osteosarcoma cells (LIEBAU et al. 2002a). Furthermore, IL12 causes a higher expression rate of the IL18 receptor (AHN et al. 1997).

Combination of IL12 plasmid transfected into osteosarcoma cells with recombinant IL18 induced increased IFNγ production in mononuclear cells (LIEBAU et al. 2002b).

Also in animal models such as mice with B16 melanomas, injection of DNA coding for IL12 and IL18, and subsequent electroporation resulted in prolonged survival and reduction of melanoma size, and was more effective than IL12 DNA alone (KISHIDA et al. 2001). This was also shown in CT26 murine tumors. $CD8^+$ cells and IFNγ were detected in higher levels in IL12 and 18 DNA treated tumors. By comparison of IL12 DNA treatment and the combination of IL12 and IL18 DNA, tumor growth was inhibited more using the combination compared to treatment with IL12 DNA alone. Only the combination of IL12 and IL18 suppressed collateral tumor growth (TAMURA et al. 2003).

Combination of IL12 and IL18 caused an augmentation of cellular immunity and a reduction of angiogenesis (COUGHLIN et al. 1998; KISHIDA et al. 2001). IL12 and

IL18, transfected in SCK mouse mamma carcinoma cells, lead to reduced angiogenesis in tumors grown out of these cells. But also the growth of distant tumors and tumors build of cells injected 3 days before immunotherapy was reduced (COUGHLIN et al. 1998).

The co-administration of IL12 and IL18 was shown to reduce the toxicity of IL12 because of enhanced IL10 synthesis. The antitumor activity was not affected (RODRIGUEZ-GALAN et al. 2009).

2.6.4 Adjuvant effects of cytokines

The cytokines IL12 and 18 were used as adjuvant in immunotherapy targeting specific antigens. Combined with DNA vaccines against leishmaniosis or Feline Leukaemia Virus (FeLV) an increased cellular immune response was induced, which was shown to be protective against challenge infection (HANLON et al. 2001; TAPIA et al. 2003). For vaccination against Feline Immunodeficacy Virus (FIV) a combination of FIV gene and IL12 was successfully used (BORETTI et al. 2000).

Also in tumor vaccination, an adjuvant effect was shown. The co-administration of IL12 and human gp100 DNA was shown to have a significant enhanced efficacy against tumor growth. The mice used in this B16 melanoma model, were shown to have a prolonged survival in comparison to vaccination with xenogenic DNA alone (ELZAOUK et al. 2006).

2.7 Determination of tumor regression

To define the clinical response to treatment, special criteria were introduced (WHO 1979; MILLER et al. 1981). These were later modified (THERASSE et al. 2000). The Response Evaluation Criteria in Solid Tumors (RECIST) criteria (THERASSE et al. 2000) use only one dimension (the greatest diameter) and define complete response as a disappearance of all measured lesions, partial response as decrease of the sum of maximal diameter of up to 10 target lesions ≥30% compared to baseline. Progressive disease is defined as an increase of the sum of the greatest diameter in

all target lesions to ≥20% compared to smallest sum of greatest diameters during the study. Results between progressive and partial response are defined as stable disease.

2.8 Detection of specific effector cells

Peptides of tumor antigens in complex with MHC-I molecules can be recognized by specific $CD8^+$ cells (CTL) with their T-cell receptor (ROMERO et al. 1998b). The specific immunoreactions induced by recognition of epitope/MHC-I complexes can be measured.

2.8.1 *In vivo* detection of specific CTL

Specific T-cell reactions can be measured *in vivo* based on a delayed type hypersensitivity reaction. After injection of antigen, skin biopsies can be examined by immunohistology to specify inflammatory cells (JAEGER et al. 1996; K. TAMURA et al. 2008).

2.8.2 *In vitro* CTL assays

In vitro assays can measure specific lysis of target-cells by CTL and cytokine or effector molecule production after specific stimulation. Phenotypes of CTL can be analysed by analysis of TCR specificity or genotype.

2.8.2.1 ^{51}Cr release-assay

The ^{51}Cr-release assay is based on the lysis of target-cells presenting the required epitope complexed with MHC-I by specific cytotoxic T-cells. Target-cells are incubated with $Na_2{}^{51}CrO_4$, which they take up. Vital cells do not release ^{51}Cr spontaneously. After incubation with antigen-specific effector T-cells, target-cells are lysed and ^{51}Cr can be measured in the supernatant (BRUNNER et al. 1968).

The test recognizes one specific T-cell in 1000 peripheral blood mononuclear cells (PBMC). With a frequency of 10-20% of $CD8^+$ cells in PBMCs one of 100-200 $CD8^+$ have to be specific to be detected (ROMERO et al. 1998a). To increase the number of specific cytotoxic T-cells and to detect lower frequencies, cells were co-cultured *in vitro* for expansion of specific T-cell clones with stimulator cells (presenting the

epitope in MHC-I and expressing co-stimulatory molecules) and cytokines (IL2) (MARCHAND et al. 1995; RIVOLTINI et al. 1995; JAEGER et al. 1996; SALGALLER et al. 1996; CORMIER et al. 1997; ROMERO et al. 1998a; SPEISER et al. 2003). As stimulator cells, tumor cells (SPEISER et al. 2003) or cells with appropriate MHC-I haplotype presenting the epitope and co-stimulatory molecules, for example peptide-pulsed PBMC can be used (ROMERO et al. 1998a).

Crucial for the assay is not only specificity of the T-cells but also their functionality because only cells able to lyse the target-cells are detected. The test has been used as a standard method, is highly reproducible and easy to perform. Because of the low frequencies of specific cytotoxic T-cells seen in neoplastic disease *in vitro* expansion is necessary. Cells, which undergo apoptosis or do not proliferate in reaction to the stimulus, are not counted; thus resulting in a systematic bias. Cells, which are not functional in the *in vitro* model, are also not recognized (ROMERO et al. 1998b). This assay has been used to detect cytotoxic T-cells after vaccination or infection in horses (ALLEN et al. 1995; HAMMOND et al. 1998; CASTILLO-OLIVARES et al. 2003; KYDD et al. 2006; PAILLOT et al. 2006; TAGMYER et al. 2007).

To detect tumor-antigen-specific CTL in mice and human this assay has also been used frequently (PARKHURST et al. 1996; SALGALLER et al. 1996; ZHOU et al. 1999; MENDIRATTA et al. 2001; GOLDBERG et al. 2005).

2.8.2.2 ELISPOT (Enzyme linked immunospot)

This assay detects cells producing cytokines after specific *in vitro* stimulation. Different molecules can be detected with varying results, for example INFγ, TNFα (human) (HERR et al. 1996), granzyme B (equine) (PIUKO 2007), perforin or IL2, IL5 and IL10 (human) (ROMAGNANI 1995). Similarly, for different stimulator methods results varied (PASS et al. 1998). However, the use of $CD8^+$ specific molecules like granzyme B can specify the information content of this assay (PIUKO 2007).

Most frequently, detection of IFNγ is used. This cytokine is secreted not only by $CD8^+$ cells but also by $CD4^+$, $CD5^+$, NK- and B-cells (BREATHNACH et al. 2005). Previous

selection of $CD8^+$ cells can specify the information content of this assay (ROMERO et al. 1998a).

In short, the essay works as follows: PBMCs or $CD8^+$ cells are seeded in microtiter-plates covered with antibodies directed against the cytokine or effector molecule of interest and stimulatory cells are added.

Effector cells recognizing the stimulating antigen produce cytokines or effector molecules, which bind to the specific antibodies. Soluble anti-effector molecule antibodies detect bound molecules after removal of cells. A secondary anti species antibody, coupled with an enzyme (alcaline phosphatase or horseradish peroxidase), binds to bound primary antibodies. Antibody binding can be detected after adding a substrate for this enzyme (ROMERO et al. 1998a).

This test correlates well with the ^{51}Cr-release-assay (CZERKINSKY et al. 1988; SCHEIBENBOGEN et al. 2000), yet it has an up to 100-fold higher sensitivity than the ^{51}Cr-release-assay (ROMERO et al. 1998a). One specific CTL in $5x10^4$ -10^6 PBMC can be detected (GAJEWSKI 2000; LETSCH et al. 2003; BOON et al. 2006). The ELISPOT assay is therefore the most sensitive *in vitro* assay for the detection of specific T-cells (LETSCH et al. 2003).

The result of the assay is not dependent on lysis of target-cells.

Although for ELISPOT assay, no *in vitro* expansion of CTLs is required, *in vitro* expansion was shown to increase sensitivity of this assay further (PASS et al. 1998).

For melanoma specific CTL this test has already been used successfully in human (HERR et al. 1996).

A commercial ELISPOT assay detecting IFNγ exists for horses (Equine IFN-gamma ELISpot Kit, R&D Systems, Minneapolis, MN, USA).

2.8.2.3 Intracellular cytokine staining (ICS)

The principle of ICS is comparable to ELISPOT. Cells are specifically stimulated *in vitro* to produce cytokines. Secretion of these cytokines is blocked using substances like brefeldin A which causes destruction of the golgi-apparatus. Cytokines accumulate in the cells (DONALDSON et al. 1992; KLAUSNER et al. 1992; VOGEL

et al. 1993). Subsequently, cells are fixed and permeabilized by a detergent. Afterwards, intracellular molecules can be stained using fluorescent-labelled monoclonal antibodies directed against the cytokine of interest. TNFα and IFNγ are recommended cytokines because low numbers of epitopes are needed for stimulation of their synthesis (FONTENEAU et al. 1997). An advantage of this detection method is the synchronic use of additional surface markers to characterize cytokine producing cell populations (JUNG et al. 1993; PRUSSIN a. METCALFE 1995). As well as IFNγ produced by $CD8^+$ cells also IFNγ produced by CD4-Th1 and other cells can be detected in flow cytometry (TOPALIAN et al. 1994; BARBULESCU et al. 1998; HINES et al. 2003; BREATHNACH et al. 2006).

This assay was used in the research of equine infectious diseases (PAILLOT et al. 2005; PAILLOT et al. 2006).

The method was modified to reach higher sensitivity. PBMCs or CD8+ cells were co-cultivated with stimulator cells to increase the frequency of specific IFNγ producing cells (PAILLOT et al. 2007).

In a study of Sun et al. (2003) the sensitivity of ICS has been shown to be comparable to that of an ELISPOT assay. Other reports point out that the sensitivity for ICS is limited to 40 specific T-cells in 10^6 PBMC and thereby less than that of an ELISPOT (LETSCH 2003).

The ICS seems in contrast to be more sensitive to detect T-cells secreting low amounts of IFNγ. That might be an advantage in the detection of tumor reactive T-cells (LETSCH 2003).

For the detection of specific cytotoxic T-cells against tyrosinase after xenogenic DNA vaccination against human tyrosinase in horses, recently an assay was validated. A tyrosinase peptide pool was used to stimulate the PBMCs. The production of IFNγ was measured by quantitative real-time-polymerase chain reaction (qRT-PCR) (LEMBCKE et al. 2012).

2.8.2.4 Tetramer-Assay

A direct method to detect specific T-cells is the staining with soluble MHC-I molecules complexed with epitope which bind to specific TCRs (ALTMAN et al. 1996; ROMERO et al. 1998b).

Low binding affinity can be circumvented by insertion of a gene segment coding for a biotinylation peptide at the C-terminus of MHC-I molecules. This leads to multimerisation and higher binding affinities. A fluorescent-marked avidin molecule renders detection of marked cells possible (ALTMAN et al. 1996; MEALEY et al. 2005). According to SUN (2003), the tetramer assay had a 5-fold higher sensitivity than ICS and ELISPOT. Also naive cells can be detected after preselection using tetramers and subsequent IFNγ staining (PITTET et al. 2001). No functionality (cytokine secretion or lytic potential) is needed to detect specific T-cells (P. P. LEE et al. 1999).

This assay has already been used in studies of equine disease (MEALEY et al. 2005) and neoplasia in other species (P. P. LEE et al. 1999; SPEISER et al. 2002; WOLCHOK et al. 2007; YUAN et al. 2009).

2.8.2.5 CTL detection in neoplasia

As mentioned above, all assays for the detection of cytotoxic T-cells like cytotoxicity assays (PARKHURST et al. 1996; SALGALLER et al. 1996; ZHOU et al. 1999; MENDIRATTA et al. 2001; GOLDBERG et al. 2005), cytokine production measurements (PARKHURST et al. 1996; SALGALLER et al. 1996; PARKHURST et al. 2004; GOLDBERG et al. 2005), and the tetramer assay (DUTOIT et al. 2002; WOLCHOK et al. 2007; YUAN et al. 2009) can be used in cancer research. In neoplasia, detection of CTLs is more difficult because of lower frequencies of CTL. Consequently, assays have to reach a high sensitivity.

GAJEWSKI (2000) mentioned that an augmentation of immunity resulting from vaccination is more important than the development of a high sensitive assay. In some studies there seems to be a discrepancy between clinical outcome and measured immunological response (CORMIER et al. 1997; NESTLE et al. 1998; ROSENBERG et al. 1998; GAJEWSKI 2000).

2.9 Antibody detection

As mentioned above, humoral immune mechanisms are also important in the antitumoral immune response. For detection of antibodies, the specific antigen is needed. Enzyme-linked immunosorbent assay (ELISA) is a frequently used method to detect antibodies in serum (ENGVALL a. PERLMANN 1971).

In cancer vaccination studies, many target antigens are used, and ELISAs have not always been established for these antigens.

To detect specific serum antibodies it is possible to use cancer cells expressing the protein of interest (WEBER et al. 1998) or transfected cells (SURMAN et al. 1998). Cells can be lysed and antigens purified or whole tumor cell lysate may be used as a source of antigen for ELISA (SURMAN et al. 1998). For the detection of tyrosinase antibodies, tumor (melanoma) cell lysate can be incubated with antibody containing sera and A-sepharose beads. The antigen binds to tyrosinase antibodies in the sera, if present and antigen-antibody-sepharose complexes are formed. Because of the high specific weight of the beads, complexes can be harvested by centrifugation. After L-DOPA substitution to the purified beads color changes can be recognized if antityrosinase antibodies were present in the serum (GOLDBERG et al. 2005). As an alternative tyrosinase source cell free (ZHOU et al. 1999), mushroom (MERIMSKY et al. 1996), or bacterial (GELBART et al. 2004) protein might be used. Recently, for the detection of specific tyrosinase antibodies after htyr-DNA vaccination in horses, an ELISA using the human tyrosinase protein was successfully validated. A tyrosinase peptide pool was used to stimulate the PBMCs. The production of IFNγ was measured by quantitative real-time-polymerase chain reaction (qRT-PCR) (LEMBCKE et al. 2012).

Flow cytometry is a further method to detect antibodies using cells expressing the antigen of interest as a substrate. Along the lines of ELISA technique, tumor cells or transfected cells can be used. Antibodies are detected after incubation of cells with serum and a secondary antibody against the tumor antigen (LIAO et al. 2006). For detection of intracellular antigens cells have to be permeabilized.

2.10 Luciferase assay

Luciferase from fireflies (*Lampyridae*) is a monomeric 61 kDa oxidative enzyme which catalyses the reaction of luciferin to oxiluciferin using ATP•Mg^{2+} as a cosubstrate and during the reaction a photon is emitted (THORNE et al. 2010). The firefly luciferase gene was started to be used as a reporter gene and initially cloned in 1985 (DE WET et al. 1985). The gene was optimized for expression and activity in mammalian cells (WOOD 1998).

The detection sensitivity of the luciferase assay is very high as it is able to detect up to 10^{-20} molecules (WOOD 1991). No specific immunological action of this protein was reported until now.