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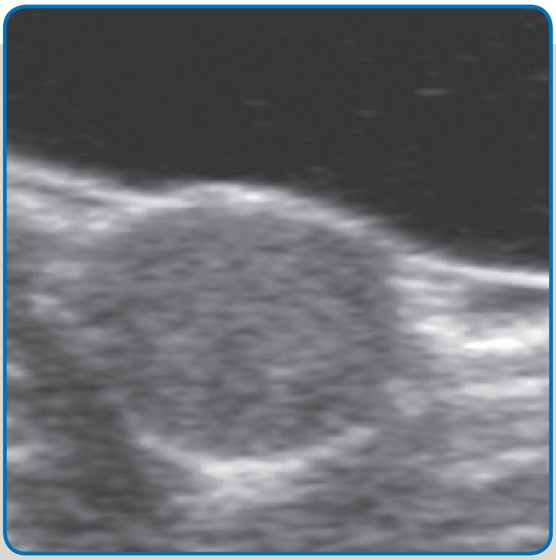
Minimalistic immunologically defined gene expression T helper cell 1 (MIDGE-Th1®) vectors coding for Interleukin 12 and -18 in combination with the transfection agent SAINT-18 have systemic antitumoral effects on equine melanomas



STIFTUNG TIERÄRZTLICHE HOCHSCHULE HANNOVER

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University of Veterinary Medicine Hannover

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to my family

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Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microliter
2D / 3D	Two / three dimensional
3LL-D122-tumor cells	(Lewis lung carcinoma metastatic variant 3LL-D122 cells)
^{51}Cr	Chromium
A2a	Adenosine receptor
Ab	Antibodies
ADCC	Antibody depended cytotoxicity
Adh-Trp2	Adenovirus vector coding for human tyrosinase Related protein
ALP1	amphiphysin like protein 1
Aminoacids	according to the recommendations for nomenclature for aminoacids of the International Union of Pure and Applied Chemistry and International Union of Biochemistry and Molecular Biology, Joint Commission on Biochemical Nomenclature (IUPAC-IUB, JCBN, 1983)
ANOVA	Analysis of variance
ASIP	Agouti signaling peptide
B16 Melanoma	Amelanotic mouse melanoma
BCG	Bacillus Calmette-Guérin
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin

cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CHO	Chinese hamster ovary
cm^2	Square centimetre
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CpG	Cytosine and guanine separated by a phosphate
CT	Computed tomography
CT 26	colon tumor 26 (murine)
CTL	Cytotoxic T lymphocytes
d	Days
DC	Dendritic cells
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DMSO	Dimethyl sulfoxide (CH3)2SO
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
EcoRI, Eco31-I	Endonuclease enzyme isolated from strains of E. coli, used as a restriction enzyme
EDC	Equine dermal cell
eGFP	Enhanced green fluorescent protein
EL4	Murine T-cell lymphoma
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
eq	equine

FACS	Fluorescence-activated cell sorting
Fas ligand / FasL	Homotrimeric type II transmembrane protein
Fc	Fragment crystallizable region (the tail region of an antibody)
FCS	Fetal Calf Serum
FITC	fluorescein isothiocyanate
FL1, FL2	Fluorescence 1, Fluorescence 2
FNA	Fine needle aspirates
FoxP3	forkhead box Protein 3
FSC	forward scatter
g	Gram
g	gravitational acceleration
G	Gauge
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp100	Glycoprotein 100
Gαs receptors	G protein-coupled receptors
h	Hours
H_2O	Water
H2R	Histamine receptor 2
HBsAg	Hepatitis B virus surface antigen
HEK 293	Human Embryonic Kidney
HER2/neu	
hgp100	Human glycoprotein 100
HIV	Human immunodeficiency virus
HMB45	Human Melanoma Black 45

HSVtk	Herpes simplex virus-thymidine kinase
hTERT	Human telomerase reverse transcriptase
htyr	Human tyrosinase
HVJ-AVE	Hemagglutinating virus of Japan-artificial viral envelope
Hz	Hertz
i.c.	Intracutaneous
i.d.	Intradermal
i.m.	Intramuscular
ICAM I	intercellular adhesion molecule I
ICS	Intracellular cytokine staining
IFNγ	Interferon-gamma
IL	Interleukin
ILRAP	IL 1beta receptor antagonist protein
IRAK	Interleukin-1 Receptor-associated Kinase
JAK-STAT	Janus Kinase - Signal Transducer and Activator of Transcription
kb	Kilo-base
kDa	Kilodalton
kg	Kilogram
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	Lipopolysaccharide
mel	Melanoma
mAb	Monoclonal Antibodies
MAGE	Melanoma AntiGEn

MART A	Melanoma differentiation antigens
MCA	3`-methylcholanthrene
MDCT	Modified discrete cosine transform
MEM	Minimal Essential Medium Eagle
mg	Milligram
MgCl2	Magnesium chloride
MHC	major histocompatibility complex
MHz	MegaHertz
MIDGE	Minimalistic Immunogenically Defined Gene Expression
min	Minutes
ml	Millilitre
mM	Millimolar
mm^3	Cubic millimetre
MRI	Magnetic resonance imaging
mu tyr	Murine tyrosinase
$Na_2\,^{51}CrO_4$	Natriumchromat
NaAcid	Natrium acid
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK cells	Natural killer cells
NLS	Nuclear localization signal or sequence
O_2	Oxygen
ODN	Oligodeoxyribonucleotide
OptiMEM®	Modification of Eagle's Minimum Essential Media

p	P-value / probability
p.o.	Per os
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
pCI neo	promoter Chimeric Intron neomycin (Mammalian Expression Vector)
PCR	Polymerase chain reaction
PET	Positron emission tomography
PMA	Phorbol 12 myristat 13-acetat
pMCV1.4 vector	Merkel Cell polyomavirus replication plasmid
PMELmut1re, -mut2re, -mut1li, -mut2li	Mutated primer for PCR of gp100
PNK	Polynucleotidkinase
polyA	Poly adenosinemonophosphate
QS-21	Purified plant extract derived from the *Quillaja saponaria* (Soap bark tree)
rGM-CSF	Recombinant Granulocyte-macrophage colony-stimulating factor
rpm	Rounds per minute
RT	Room temperature
RLU	Relative light units
SAINT-18	Synthetic, Amphiphilic, INTeractive (1-methyl-4-(cis-9-dioleyl) methyl-pyridinium-chloride)
SCID	Severe Combined Immunodeficiency

SCK	(murine Mammary carcinoma)
SSC	Side scatter
STAT	Signal Transducers and Activators of Transcription
STX17	Syntaxin membrane integrated Q-SNARE proteins participating in exocytosis
SV40	Simian virus 40
TaqPolymerase LC	Thermostable DNA polymerase named after the thermophilic bacterium Thermus aquaticus
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th1	T helper cell
TNF	Tumor necrosis factor
Tyr	Tyrosinase
U	Units
VCAM1	Vascular cell adhesion molecule 1
VRP	Venezuelan equine encephalitis virus Replicon Particle
y	Year

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.

3 Material und Methods

3.1 Patients

Twenty-eight probands were recruited by announcement. Informed consent was obtained from all animal owners. Inclusion criteria were presence of one or more melanomas and unaffected general condition. No systemic immunomodifying treatment and no local treatment of melanomas were administered during two weeks before immunisation.

Initially, a total of 28 patients participated in the study, with each group containing 9 (group IL12/18 and tyr) or 10 (gp100) individuals respectively (table 1).

Table 1: Patient demographics and group assignment

ID	Age (years)	Breed	Gender	color	Number of Melanoma	FNA	Group	Localisation
1	22	Warmblood	m	flea bitten	>7	no	IL12/18	vt, mu, o
2	18	Warmblood	g	flea bitten	1	yes	IL12/18	vt
3	18	unclassified	g	flea bitten	1	yes	gp100	pa
4	19	Arabian	s	flea bitten	>7	yes	tyr	pa, vt
5	6	Lusitano	s	cremello	1	no	gp100	e
6	12	Andalusian	m	dappled	>7	yes	gp100	pa, vt, mu, p, o
7	18	Andalusian	s	grey	>7	no	gp100	e, vt, ge
8	20	Icelandic-Horse	m	grey	>7	yes	IL12/18	pa, vt, ge
9	15	Berber	s	grey	>7	yes	gp100	pa, vt
10	12	Berber	m	grey	>7	yes	IL12/18	pa, vt, ge, p
11	15	Arabian	s	grey	>7	yes	tyr	pa, vt, mu
12	20	Carmague	s	grey	4	no	tyr	vt
13	11	Carmague	m	dappled	>7	no	IL12/18	vt, pa, ge, p
14	11	Shetland	g	dappled	3	no	IL12/18	pa, ge, vt
15	15	Shetland	m	grey	>7	no	tyr	vt
16	14	Andalusian	s	grey	>7	no	gp100	pa, vt
17	13	Warmblood	m	flea-bitten	2	yes	tyr	pa, vt
18	17	Andalusian	m	grey	>7	yes	IL12/18	pa, vt, ge, mu, o
19	16	Warmblood	m	grey	>7	yes	gp100	pa, vt, ge
20	15	Arabian	g	flea-bitten	>7	yes	gp100	p
21	22	Trakehner	g	grey	>7	yes	tyr	pa, vt
22	19	Arabian	g	grey	1	yes	tyr	vt
23	14	Warmblood	m	flea-bitten	2	no	gp100	pa, vt
24	14	Arabian	g	flea-bitten	1	yes	IL12/18	p
25	16	Warmblood	m	flea-bitten	>7	yes	IL12/18	pa, vt, ge
26	13	Arabian	m	grey	>7	yes	tyr	pa, vt, ge, mu, o
27	11	Hunter	g	grey	1	yes	tyr	vt
28	22	Icelandic-Horse	m	flea-bitten	>7	yes	gp100	pa, vt, ge

Legend:

m: mare
g: gelding
s: stallion
FNA: fine needle aspirate

vt: ventral tail
p: parotid region
ge: genitals
pa: perianal

e: eyelid
mu: muscle
o: other regions

One patient, horse number 5, group gp100, was excluded on day 78 because of surgical excision of the single melanoma, localized at the eyelid. Since the melanoma continuously increased in size a surgical intervention was indicated.

Age of included horses reached from 6 to 22 years (15.6 ± 3.89 years). Six horses had one, two horses two, one horse three, one horse four and 18 horses had more than seven melanomas. Horses were of different breeds (6 warmbloods, 6 Arabian horses, 4 Andalusian horse, 2 Icelandic horses, 2 Berber, 2 Carmague horses, 2 Shetland ponies, one Trakehner, one hunter and one unclassified horse). There were 13 mares, 8 geldings and 6 stallions.The degree of greying was incomplete in 13 horses with a flea bitten pattern in 10 horses and a dappled pattern in 3 horses. 14 horses showed a grey coat color. Melanomas were localized at the ventral site of the tail (24), perianal (18), at the genitals (10), in the muscles (5), at the parotic gland (5) at the eyelid (1) ond in other regions (4) (table 1).

Pre-trial evaluation included a physical examination, complete blood count (packed cell volume, erythrocytes, haemoglobin, mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), total nucleated cell count, platelet count, eosinophils, basophils, monocytes, mature neutrophils, band neutrophils) and blood biochemistry profile (total protein, Mg^{2+}, Na^{+}, Cl^{-}, Ca^{2+}, Mg^{2+}, phosphate, glucose, lactate, urea, enzymatic activity of gamma-glutamyltransferase and lactatdehydrogenase). Age, breed, gender, degree of greying and number of melanomas were documented. Diagnosis was made on the basis of signalement, physical examination, location and appearance of the tumor. To confirm the diagnosis, fine needle aspirates (FNA) of melanomas were examined by board certified pathologists in 20 of 28 horses. Aspirates were collected using a 22 gauge needle and a ten millilitre syringe. Cytologic diagnosis for each sample was equine melanoma (table 1).

For the evaluation of unspecific side effects and for *in vivo* transfection tests with luciferase DNA, nine study horses in unaffected general condition, properties of the Clinic for Horses of the University of Veterinary Medicine Hannover, Foundation, were used (see 3.6 and 3.8). If a horse was used for more than one purpose, a recovery time of at least two weeks was left between experiments.

The mean age of the horses was 13.2 ± 5.76 years. There were 2 geldings and 7 mares and the horses were coloured grey (n=3), brown (n=5) and chestnut (n=1) (table 2).

Table 2: Horses used for unspecific side effect evaluation

Pferd_Number	Group	gender	age	colour	luciferase assay
1	SAINT-18/Luc	mare	5	brown	yes
2	SAINT-18/Luc	gelding	17	brown	yes
3	SAINT-18/Luc	mare	5	brown	
4	SAINT-18/Luc	mare	13	grey	yes
5	SAINT-18/Luc	mare	14	grey	
1	SAINT-18	mare	5	brown	
6	SAINT-18	mare	22	grey	
7	SAINT-18	gelding	16	chestnut	
2	IL12/18	gelding	17	brown	
8	IL12/18	mare	14	brown	
9	IL12/18	mare	17	brown	

Probands were treated in the Clinic for Horses of the University of Veterinary Medicine Hannover, Foundation, from November 2009 to July 2010 in agreement with the ethical guidelines of the law of animal welfare (Tierschutzgesetz) approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), approval no. 08/1522.

3.2 Groups

The 28 study horses were assigned to the treatment groups (IL12/18, gp100 and tyr) by a stratified biphasic model. Therefore, horses were scaled into two subgroups based on age (years: y; < 15 y or ≥ 15 y) and two subgroups based on number of melanomas (melanoma/s: mel; < 7 mel or ≥ 7 mel) to avoid a bias caused by imbalance of these criteria in the treatment groups. Three treatment groups (all groups received: SAINT-18 (0.75 mmol/ 1mg MIDGE-Th1® DNA), eqIL12-MIDGE-Th1® and MIDGE-Th1® eqILRAP-IL18, group IL12/18: no additional DNA, group tyrosinase (tyr): 500 µg htyr MIDGE-Th1®, group glycoprotein (gp) 100: 500 µg hgp100 MIDGE-Th1®) were assigned by random selection out of the four aforementioned subgroups.

Each treatment group included 9 horses (gp100 included initially ten, but after exclusion of horse number 5 nine horses). The age distribution and number of melanomas in the groups was homogenous (table 3).

During the treatment and evaluation period the treatment groups were blinded for the treating and examining persons, the supervisors as well as for all other persons involved in handling of the horse by a color code.

Table 3: Distribution of the age and number of melanomas in the subgroups

melanomas (number), age (y) / group	<7mel, <15y	<7mel, >15y	>7mel, <15y	>7mel, >15y
IL12/18	2	1	2	4
gp100	1	1	2	5
tyr	2	2	1	4

The horses for the evaluation of unspecific side effects were assigned to the groups by random selection.

3.3 Gene synthesis

DNA coding for human glycoprotein 100 (hgp100), courtesy of Dr. Robbins und Dr. Rosenberg, National Cancer Institute, was obtained in form of the plasmid pcDNA3-gp100. To produce the MIDGE® vectors coding for gp100, plasmid was digested by the restriction endonuclease Eco31-I. Eco31-I restriction enzyme sites existing in the gene were eliminated by PCR-based, site directed mismatched primer mutagenesis before. Two primers, PMELmut1re and PMEL17mut2li (Microsynth, Balgach, Switzerland; table 4), contained the mutated recognition site and additionally a recognition site for Eco31-I outside the coding sequence. The other primers (PMEL17li and PMEL17 re) contained no mutation.

Table 4: Primer used for site directed mismatched primer mutagenesis

PCR1	PMEL17li	5'-ATT ATA GAA TTC ATG GAT CTG CTA AAA AGA TG-3'
	PMEL17mut1re	5'-AAA CAA GGT CTC TCT TCT GGG ACC AAG AGC-3'
PCR2	PMEL17mut2li	5'-AAC AAC GGT CTC TGG GTG GAA ACC ACA GCT AG-3'
	PMEL17re	5'-TTA TAT GAG CTC AGA CCT GCT GCC CAC-3'

By using Eco31-I for digestion of the PCR products, an enzyme that slices outside its recognition site, designated DNA sequences were obtained. The generated gene sequences contained the beginning and the end sequence of the gene.

PCR was performed using

- 5 µl $MgCl_2$- buffer (MBI Fermentas, Vilnius, Lithuania)
- 3 µl dNTPs
- 2.8 µl $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania)
- 1.5 µl PMEL17 li-Primer (10 µM) and 1.5 µl PMEL17 mut1 re-Primer (10µM)

or 1.5 µl PMEL17 mut2li-Primer (10 µM) and 1.5 µl PMEL17 re-Primer (10µM)

- 1 µl pcDNA3 gp100 solution
- 2 µl TaqPolymerase LC (MBI Fermentas, Vilnius, Lithuania)
- 33.2 µl H2O

in a thermo cycler (T Personal 20, Biometra Göttingen, Germany). Thermo cycling parameters employed were 30 s at 94 °C, 30 s at 37 °C and 60 s at 72 °C for 30 cycles. PCR products were isolated by agarose gel electrophoresis (30 min; 120 V, Gene ReadIR 4200, LiCOR Biosciences, 1 % agarose) and digested with Eco31-I (MBI Fermentas, Vilnius, Lithuania). Therefore 50 µl DNA solution, 4 µl ECO31-I (10 u/µl) and 6 µl G-Puffer (Green-buffer containing 10 mM Tris-HCl (pH 7.5 at 37 °C), 10 mM $MgCl_2$,50 mM NaCl, 0.1 mg/ml BSA; MBI Fermentas, Vilnius, Lithuania) were incubated at 37 °C over night. After gel electrophoresis, DNA was harvested and purification was performed (NucleoSpin® 8 Extract II, Macherey-Nagel GmbH&Co.KG, Düren, Germany). Therefore, the agarose gel containing DNA of interest was cut out and diluted for 30 minutes in the NT-Puffer, a binding buffer (containing chaotropic salt, guanidine thiocyanate), at 37 °C. Solution was filled on a filter column and washed with 600 µl NT3 wash buffer. Fifty µl NE elution buffer was pipetted on the filter and after a minute, the solution was centrifuged in a 2 ml collection tube (1 min, 13000 *g*). Products were called PCR1 (primer used: PMEL17li and PMEL17mut1re) and PCR2 (primer used: PMEL17mut2li and PMEL17re).

To produce the internal part of the gene, pcDNA3gp100 was transformed using heat shock transformation into XL1-Blue® chemo-competent 4 (CC4) *E. coli* (XL-1 Blue by Agilent Technologies Sales & Services GmbH & Co.KG Life Sciences & Chemical Analysis, Waldbronn, Germany; CC4 by Mologen AG, Berlin, Germany). In detail, 1 µl pcDNA3gp100 was incubated on ice with 100 µl *E.coli* for 20 minutes. Then the suspension was incubated for 45 seconds in a 42 °C water bath and was subsequently passed on ice. The suspension was diluted in 1 ml Super Optimal Catabolite Repression (SOC) medium (Super Optimal Broth (SOB) medium + 20 % Glucose (both Carl Roth GmbH&Co. KG, Karlsruhe, Germany)) and incubated for 90 minutes at 220 rpm in an incubation shaker. Bacteria were plated on LB (lysogeny broth) selection agar (Carl Roth GmbH&Co. KG, Karlsruhe, Germany) containing 50 µg/ml ampicillin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

Bacteria were incubated for 16 hours at 37 °C. DNA was isolated using NucleoSpin® 8 Extract II (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Therefore bacteria were separated from medium by centrifugation (2 min/13000 *g*), diluted in 250 µg re-suspension buffer and then lysed by slewing after addition of 250 µl lysis buffer. After five minutes, 300 µl neutralisation buffer were added. Solution was centrifuged for 10 minutes at 13000 *g*. Solution was filtered through a filter spin column and washed with 600 µl wash buffer. DNA was eluted using 50 µl elution buffer (NE-buffer). 50 µl NE-buffer was pipetted on the filter and after a minute, the solution was centrifuged in a 2 ml collection tube (1 min, 13000 *g*).

DNA isolated from these bacteria was digested with Eco31-I using 2 µl of DNA solution, 1 µl Eco31-I, 22 µl G-buffer, 15 µl H_20 at 37 °C for 1 h.

The approximately 840 bp long part was isolated by agarose gel electrophoresis (30 min; 120 V) and DNA purification was performed as described above. DNA sequence was called middle part (MP).

PCR products PCR1&2 and MP were ligated (PCR1 85 µl, MP1 85 µl, PCR2 85 µl, 30 µl G-Puffer, 5 µl Eco31-I, 3 µl Ligase (MBI Fermentas, Vilnius, Lithuania), 20 µl ATP (MBI Fermentas, Vilnius, Lithuania)) over night at 37 °C.

The ligation product was digested using 40 µl DNA solution, 12 µl tango buffer (MBI Fermentas, Vilnius, Lithuania), 2 µl EcoR-I, 2 µl SacI (MBI Fermentas, Vilnius, Lithuania) and 4 µl H_2O at 37 °C over night). Thereafter, it was cloned into pMCV1.4 using 0,5 µl pMCV1.4, 1 µl ligase buffer (MBI Fermentas, Vilnius, Lithuania), 1 µl ATP, 1 µl T4Ligase (MBI Fermentas, Vilnius, Lithuania), 6,5 µl ligation product, 1 h at room temperature and transformed into XL1 *E.coli* (as previously described) in order to amplify the plasmid. The insertion of the gene in the pMCV1.4 vector was necessary for the production of MIDGE-Th1® vectors.

Equine IL18 (eqIL18), courtesy of L. Nicholson, University of Glasgow, codes for mature IL18. In order to produce a more effective secreted form of IL18, the IL18 sequence was modified by ligating the oligonucleotides ILRAP fw1, -fw2, -rv1 and rv-

2 (Microsynth, Balgach, Switzerland, table 5) coding for the IL1beta receptor antagonist protein (ILRAP) signal sequence to the original gene (eqILRAP-IL18).

Table 5: Oligonucleotids used for ILRAP synthesis

ilrap-1	-fwd	CAT GGA AAT CTG CAG AGG CCT CCG CAG TCA CCT AAT CAC
	-rv	TTA GGT GAC TGC GGA GGC CTC TGC AGA TTT CCA TGG TAC
ilrap-2	-fwd	TCT CCT CCT CTT CCT GTT CCA TTC AGA GAC GAT CTG CCT GC
	-rv	GCA GAT CGT CTC TGA ATG GAA CAG GAA GAG GAG GAG AGT GA

Dephosphorylated oligonucleotides were diluted to 100 µM using tri-distilled water. Phosphorylation was performed by incubation of 23 µl oligonucleotide ILRAP rv-1 and fw2 with 3 µl polynucleotidkinase (PNK) A buffer (MBI Fermentas, Vilnius, Lithuania), 1 µl PNK and 3 µl ATP at 37 °C for one hour. Enzyme was inactivated subsequently at 75 °C for 20 minutes. Phosphorylated oligonucleotides were annealed with dephosphorylated oligonucleotides (ILRAP rv1 with ILRAP fw1; ILRAP fw2 with ILRAP rv2). Therefore, 23 µl dephosporylated oligonucleotides were added, incubated for three minutes at 75 °C, and cooled down. Intended for ligation, 39 µl of the annealed oligonucleotides were incubated for one hour with 10 µl ligase buffer, 10 µl ATP, and 2 µl T4 ligase at room temperature.

The eqILRAP-IL18 gene was digested with EcoRI and SacI and cloned into the pMCV1.4 vector as described above, which was used to produce the MIDGE-Th1® vectors.

The vectors coding for equine Interleukin (eqIL) 12, provided by Intervet (Intervet International, Boxmeer, The Netherlands) and for tyrosinase (MOLOGEN AG, Berlin, Germany) already existed in form of pMCV1.4 vectors at the MOLOGEN AG (Berlin, Germany) with no restriction enzyme sites for Eco31-I.

3.3.1 Synthesis of MIDGE-Th1® vectors

The construction and synthesis of MIDGE® vectors was done in analogy to the method described by SCHIRMBECK et al. (2001) and was performed by MOLOGEN AG (Berlin, Germany). The expression cassettes, containing the CMV immediate-early enhancer/promoter, a chimeric intron, the gene sequence (hgp100, htyr, eqIL12 or ILRAP-eqIL18) and the SV40 late protein polyA site were inserted into the plasmid pMCV1.4. A digestion with the restriction-endonuclease Eco31-I (MBI Fermentas, Vilnius, Lithuania) was performed to set the expression cassette free. Afterwards it was ligated to a hairpin oligodeoxyribonucleotide (ODN) on each free end. One of them was, in analogy to SCHIRMBECK et al. (2001), linked to a peptide nuclear localization sequence (NLS, a peptide with the amino acid sequence PKKKRKVEDPYC). Unbound DNA fragments were digested with T7 DNA polymerase (MBI Fermentas, Vilnius, Lithuania) followed by purification.

3.4 Preparation of SAINT-18/DNA complexes

The transfection reagent Synthetic, Amphiphilic, INTeractive® 18 (SAINT-18, 1-methyl-4-(cis-9-dioleyl) methyl-pyridinium-chloride, Synvolux Therapeutics, Groningen, The Netherlands) dissolved in water in the adequate concentration for the absolute amount of DNA was vortexed (neoLab-Reagenzglasmixer, Vortex neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg, Germany) for one minute. SAINT-18 (0,5 ml) was mixed manually with the DNA dissolved in concentrated buffer (0,5 ml; PBS-Dulbecco w/o Ca^{2+} and Mg^{2+}, Biochrom AG, Berlin, Germany) and incubated for approximately ten minutes prior to injection. The concentration of molecules in the PBS in the final mixture was: NaCl: 137 mmol/l, KCl: 2.7 mmol/l, Na_2HPO_4 x 2 H_2O: 10 mmol/l and KH_2PO_4: 2.0 mmol/l. MIDGE-Th1®/SAINT-18 complexes were formed at a ratio of 1 mg DNA to 0.75 µmol SAINT-18 and the final volume was 1 ml for each DNA/SAINT-18 mixture. For injection of IL12/18-MIDGE-Th1® or SAINT-18 alone the solutions were prepared as described above with aqua ad injectabila (0,5 ml, for MIDGE-TH1®-vectors) or double concentrated PBS (0,5 ml, for SAINT-18), respectively.

3.5 Control of *in vivo* transfection by luciferase assay

To prove expression of the MIDGE-Th1® vector in combination with SAINT-18 transfection agent a MIDGE-Th1® vector coding for the reporter gene luciferase was injected intradermally and intramuscularly in 3 horses (900 µg MIDGE-TH1®-Luciferase and 0.675 µmol SAINT-18 in a total volume of 1 ml).

MIDGE-Th1® and SAINT-18 complexes were prepared as described above. Complexes were injected intradermally (1 ml distributed to 3 injection sites; BD Microlance 3 Needle, 27 G x 0,75 inch, Beckton Dickinson, Heidelberg, Germany; Syringe, Injekt, 1 ml, Braun, Melsungen, Germany) in the skin of the neck in three horses and intramuscularly in the *M. sacrococcygeus ventralis lateralis* and *M. levator nasolabialis* (1 ml distributed to 2 injection sites; BD Microlance 3 Needle, 22 G x 1.5 inch, Beckton Dickinson, Heidelberg, Germany) in one of the three horses. Areas of skin and muscle of one horse in a distance of 5 cm adjacent to the injection site where no manipulation had taken place, served as negative controls. Skin biopsies were harvested after 24 hours in two horses, and after 2 weeks in one horse. Muscle biopsies five days after injection (Biopsy Punch 0,8cm, Stiefel, GlaxoSmithKline GmbH & Co. KG, Munich, Germany). For skin biopsies in the two horses in which the biopsies were harvested after 24 hours, horses were sedated with butorphanol (10-25 µg/kg Alvegesic®, cp Pharma, Burgdorf, Germany) and detomidine (10-15 µg/kg, Domosedan® Pfizer, Berlin, Germany) and local anaesthesia was performed using 1ml of 2% lidocaine (Vétoquinol AG, Ravensburg, Germany). The skin biopsies harvested after two weeks and muscle biopsies were performed under general anaesthesia (directly before euthanasia due to other reasons) in one horse.

Samples were directly placed in liquid nitrogen and stored at -80 °C until further processing for the luciferase assay (Luciferase Assay System, Promega Corporation, Madison, Wisconsin, USA) was performed.

Horses used for these experiments were owned by the Clinic for Horses, University of Veterinary Medicine Hannover, Foundation.

3.5.1 Luciferase assay

Before performing the luciferase assay, 20 mg of each biopsy were placed in a 2.0 ml tube. Five hundred µl Cell Culture Lysis Reagent® (Promega Corporation, Madison, Wisconsin, USA) and a Tungsten Carbide Bead (3 mm diameter) (Quiagen GmbH, Hilden, Germany) were added. Vials were placed in the TissueLyser II (Quiagen GmbH, Hilden, Germany) and were agitated at 30 Hz for 15 minutes. After centrifugation, (3 min at 10000 *g*) supernatants were transferred into another tube and were stored at -80 °C until assay was performed.

For the assay, supernatant and Luciferase Assay Reagent® (Promega Corporation, Madison, Wisconsin, USA) were equilibrated to room temperature. For each assay 100 µl of the Luciferase Assay Reagent were placed into a tube. Twenty µl supernatant were added and mixed by pipetting. Relative light units (RLUs) were measured for ten seconds by GloMax® 20/20 Luminometer (Promega Corporation, Madison, Wisconsin, USA). Measurements were done in duplicates. Transfected biopsies were compared concerning the RLUs to the controls. The RLUs measured indicated qualitatively the expression of luciferase.

3.6 Injection of MIDGE-Th1® coding for differentiation antigens (hgp100 and htyr) and interleukins (eqIL12 and eqILRAP-IL18)

Prior to each injection, on every day of hospitalisation and on day 120 a complete physical examination was performed. A complete blood count (packed cell volume, erythrocytes, haemoglobin, mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), total nucleated cell count, platelet count, eosinophils, basophils, monocytes, mature neutrophils, band neutrophils) was evaluated prior to ech injection and on day three after injection as well as on day 120.

The injections of MIDGE-Th1® vectors to groups IL12/18, gp100 and tyr were performed on days 1, 22 and 78 of the study. Patients were hospitalized from the day prior to injection until the fourth day after injection. The horses of the three groups were each treated on the three time points (table 6).

Table 6: Time points of injection and treatment substances used in the different groups

Group / Day	Group IL12/18	Group gp100	Group tyr
Day 1	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 0.3 µmol SAINT18	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 500µg hugp100 MIDGE-Th1® 0.675 µmol SAINT18	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 500µg hutyr MIDGE-Th1® 0.675 µmol SAINT18
Day 22	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 0.3 µmol SAINT18	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 500µg hugp100 MIDGE-Th1® 0.675 µmol SAINT18	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 500µg hutyr MIDGE-Th1® 0.675 µmol SAINT18
Day 78	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 0.3 µmol SAINT18	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 500µg hugp100 MIDGE-Th1® 0.675 µmol SAINT18	200µg eqIL12 MIDGE-Th1® 200µg eqILRAPIL18 MIDGE-Th1® 500µg hutyr MIDGE-Th1® 0.675 µmol SAINT18

Total volume of the DNA/SAINT-18 complexes was 1 ml. All horses received SAINT-18 (0.75µmol / 1mg MIDGE-Th1® DNA), 200 µg of MIDGE-Th1® vector coding for eqIL12 and 200 µg of MIDGE-Th1® eqILRAP-IL18. The control group (IL12/18), was treated with eqIL12 and eqILRAP-IL18 DNA only. Group gp100 was additionally treated with 500 µg MIDGE-Th1® vector coding for hgp100, group tyr with htyr

MIDGE-Th1®. 500 µl of the complexes were injected intradermally (i.d.; BD Microlance 3 needle, 27 G x 0,75 inch, Beckton Dickinson, Heidelberg, Germany; Syringe, Injekt, 1 ml, Braun, Melsungen, Germany) peritumoral to one selected well-measureable and easily-injectable melanoma (“locally treated melanoma”) and 500 µl intramuscularly (i.m.; BD Microlance 3 needle, 22G x 1.5 inch, Beckton Dickinson, Heidelberg, Germany) into the *Musculus semimembranosus* or the *Musculus serratus ventralis cervicis* depending on which site was closer to the localisation of the locally treated tumor.

3.7 Evaluation of unspecific side effects of treatment with MIDGE-Th1® vectors and SAINT18 alone or in combination

Five horses, received MIDGE-Th1® luciferase DNA (900 µg, dissolved in PBS to a total volume of 1 ml) and SAINT-18 (0.675 µmol dissolved in PBS to a total volume of 1 ml, group SAINT-18/luc) injected singularly intradermally and intramuscularly, prepared as described above, with the objective of evaluating systemic and local effects of the combination of MIDGE-Th1® DNA and transfection agent.

Additionally, eqIL12 and eqILRAP-IL18 MIDGE-Th1® vectors (group -/IL12/18 and SAINT-18/- were injected solitary in the previously described amounts i.d. and i.m. in another three (-/IL12/18) resp. four (SAINT-18/-) horses (table 7).

Table 7: Substances administered for the evaluation of unspecific side effects

group SAINT-18/Luc (DNA and transfection agent	**group SAINT-18/- (Transfection agent only)**	**group -/IL12/18 (DNA only)**
MIDGE-Th1® Luciferase+ SAINT-18	SAINT-18	MIDGE-Th1® IL12 and 18

Therefore clinical examination was performed prior to injection and every 3 hours after injection for 24 hours. Local effects were evaluated daily by adspection of the injection sites for at least one week. Swelling, reddening, exsudation and ulceration were documented (present or absent) as well as depigmentation in relation to the medicament distribution at the injection site (present or absent; if present: mild: depigmentation has a smaller area than injection site, moderate: depigmentation has the same area as the injection site, severe: depigmentation has a greater area as the injection site).

3.8 Clinical evaluation for determination of response to treatment

3.8.1 Clinical evaluation

A complete physical examination was performed at each day of hospitalisation to evaluate local as well as systemic reactions after injection. For the evaluation of local effects swelling, reddening, exsudation and ulceration were documented (present or absent) as well as depigmentation in relation to the medicament distribution at the injection site (present or absent; if present: mild: depigmentation has a smaller area than injection site, moderate: depigmentation has the same area as the injection site, severe: depigmentation has a greater area as the injection site).

3.8.2 Hematology and blood biochemistry

Hematology and blood biochemistry were performed on the days of injection (day 1), on the third day after injection (day 4), and on day 120, respectively. For hematology packed cell volume, erythrocytes, hemoglobin, mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), total nucleated cell count, platelet count, eosinophils, basophils, monocytes, mature neutrophils and band neutrophils were measured. Blood biochemistry profile included measurement of total protein, Mg^{2+}, Na^{+}, Cl^{-}, Ca^{2+}, Mg^{2+}, phosphate glucose, lactate, urea, as well as enzymatic activity of gamma-glutamyltransferase and lactatdehydrogenase.

3.8.3 Measurement of melanomas

The locally treated melanoma and a maximum of eight additional non locally treated melanomas were measured in each horse. However, when the total number of melanomas in one exceeded nine, nine representative tumors which were accessible by calliper and ultrasound measurements were chosen. In horses no. 6, 7, 8, 10, 13, 14, 18, 20, and 26 with multiple melanomas less than nine tumors were measureable due to poorly defined boundaries.

3.8.4 Calliper measurements

In each horse, melanomas were measured by calliper (Conmetall, Celle, Germany) immediately before injection on days one, 22, 78, and on day 120. The locally treated melanoma was additionally measured on days 4, 25 and 81. Length (longest diameter) and width (perpendicular diameter in relation to length) were documented in triplet-measurements to improve accuracy.

3.8.5 Ultrasonographic measurements

Ultrasonographic images were obtained at the greatest diameter of the melanomas synchronized to calliper measurements in both sagittal and transversal plain using an 8-12 MHz linear transducer on a LogiQ P5 ultrasound machine (General Electrics, Connecticut, USA). For better contact to the melanomas a stand-off distance (SONOKIT soft, 100 x 100 x 20 mm, Sonogel Vertriebs GmbH, Bad Camberg, Germany) and ultrasound contact gel were used. Images were obtained in triplets and by two examiners to improve accuracy. Length and depth were both measured in the image of the sagittal plane. Width was was measured in the image of the transversal plane. The measurements were obtained using the instrument-inherent software or after transfer in an imaging software (easyIMAGE, VetZ, Isernhagen, Germany). The appearance of the melanomas was characterized concerning texture and structure by echogenicity.

3.8.6 Calculation of tumor volume

For calliper measurements the tumor volume was calculated by the formula

$$\text{volume} = \text{length} \times \text{width}^2 \times 0{,}5$$

with “width” representing the smaller diameter. For ultrasound measurements the formula

$$\text{volume} = \text{length} \times \text{width} \times \text{depth} \times 0.5$$

was used for the calculation of the tumor volume (EUHUS et al. 1986; TOMAYKO a. REYNOLDS 1989; HEINZERLING et al. 2001; STÄHLI 2005). Relative values to each timepoint (in %) were calculated in reference to day one

volume in % on day *x* = (tumor volume on day *x* (cm^3) x 100%) / tumor volume on day *1* (cm^3).

For day 120 the mean value of the relative volumes of all non-locally treated melanomas in one horse was calculated for each horse. Statistical calculations were done with these values, representing the response to treatment of all untreated melanoma in each horse. This was used as a method to rule out a statistical bias because melanomas of one horse could not be statistically treated as independent variables.

According to the guidelines for the evaluation of the response to treatment for solid tumors (THERASSE et al. 2000), the greatest diameter of the melanomas was also documented for both methods. The sum of all greatest diameters for all melanoma measured in one horse was calculated at each time point. The relative values to each time point (in %) were calculated in reference to day one

sum of greatest diameters of all measured melanomas in one individuum in % on day *x* = (sum of greatest diameters of all measured melanomas in one individuum on day *x* (cm^3) x 100%) / sum of greatest diameters of all measured melanomas in one individuum on day *1* (cm^3).

A positive response to treatment was defined for an individual as a decrease of more than 30 % of the sum of greatest diameters, taking the baseline as reference (100%). In this study the definition of the target lesion proclaimed by THERASSE et al. (2000) which included all measurable lesions but only a maximum of five lesions per organ and ten lesions in total, was modified to maximal nine measured lesions per horse which were defined as target lesions.

3.8.7 Photographic documentation

To document the melanomas, photographs were taken at each time of injection (G11, Canon, Tokio, Japan). Visibly pigmented areas of the skin (i.e. nostrils, mouth, eyes) were photographed to evaluate systemic depigmentation as a potential side effect.

3.9 Cell culture and transfection of human embryonic kidney cells (HEK 293)

For the measurements of specific antibodies against hgp100 and htyr, HEK293 cells were transfected with DNA vectors coding for these tumor antigens to provide the antigens for a cell based antibody assay.

HEK293 cells were grown in HEK-culture medium containing Minimal Essential Medium Eagle (MEM), supplemented with 10 % heat-inactivated fetal calf serum (FCS), 1 % non-essential amino acids, 1 % pyruvate, and penicillin (100 international units (I.U.)/ml) as well as streptomycin (100 µg/ml) (all PAA Laboratories GmbH, Pasching, Austria) and maintained at 37 °C with 5 % CO_2. Medium was changed and cells were passaged according to requirements. HEK293 cells were used in the fourth passage. HEK293 cells were transfected with pMCV1.4 htyr or pMCV1.4 hgp100 plasmid using Lipofectamine LTX® (Invitrogen, Darmstadt, Germany) as transfection reagent according to the manufacturer's instructions. After removal of medium and washing with an equal amount of PBS, cells were dissociated using Accutase® (PAA Laboratories GmbH, Pasching, Austria) and 2 x 10^6 cells were seeded in 5 ml HEK-culture medium into 25 cm^2 culture flasks (Nunc GmbH&Co KG, Langenselbold, Germany) and incubated at 37 °C and 5 % of CO_2. Cells were transfected on the following day after reaching a confluence of 50-80 %. Ten µg DNA was diluted in 1.5 ml Opti-MEM® (Invitrogen, Darmstadt, Germany) and 20 µl Lipofectamine LTX® was added. After 30 minutes of incubation at room temperature, DNA-Lipofectamine-complexes were added to the cells. Cells were incubated for 24 hours before the assay was performed. The cells were dissociated as described above, counted and resuspended in FACS-buffer (Phosphate buffered saline (PBS)

(Dulbecco's PBS, Biochrom AG, Berlin, Germany), 2.5 % bovine serum albumin (BSA) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 500 µl NaAcid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)) to a final concentration of 10^6 cells/ml. The cell suspension was subsequently used for the measurement of the humoral immune response induced by vaccination against hgp100 and htyr.

3.10 Measurement of the humoral immune response induced by vaccination

To measure specific antibodies against hgp100 and htyr in the sera of the patients a cell based, flow cytometric antibody assay was performed.

For this purpose 18 ml blood were collected from the jugular vein of all patients on days 1, 22, 78 and 120 using 20 G hypodermic needles (BD Vacuette®, greiner bio-one, Kremsmünster, Austria) and serum tubes (BD Vacuette®, Serum tubes, 10 ml, greiner bio-one, Kremsmünster, Austria).

The serum was allowed to clot for 30 to 40 minutes, centrifuged (3500 *g*, 10 min), and frozen at -80 °C. Immediately before testing, the serum was thawed to room temperature.

For each staining and FACS-analysis, 200 µl cell suspension, containing 2 x 10^5 cells, were plated in every well of a round-bottom 96-well-plate (Gechno Plastic Products, TPP®, Trasadingen, Switzerland). Cells were fixed with 200 µl of 4 % paraformaldehyde (Carl Roth GmbH&Co.KG, Karlsruhe, Germany) per well for 30 minutes at room temperature (RT) and washed three times. Therefore, PBS (200 µl) was added to each well followed by centrifugation (1500 *g*, 4 min at room temperature (RT)) and removal of supernatant.

Sera were diluted in saponin-buffer (FACS-buffer, 0.5 % saponin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)) 1:1600, 1:3200 and 1:6400. Fifty µl of each dilution were added to the fixed cells and incubated for 30 minutes at RT. Each serum was separately tested for antibodies against hgp100 and htyr using the hgp100 and htyr expressing HEK293 cells. Cells were washed three times with saponin-buffer as described above.

To visualize bounded antibodies, the fluorescein isothiocyanate (FITC) marked anti horse antibody (goat anti horse FITC (Jackson ImmunoResearch Laboratories, West Grove, USA)), was added, followed by incubation for 30 min at RT and three washing steps with saponin-buffer as described above (figure 1: 1.). Monoclonal antibodies specific for hgp100 and htyr (Monoclonal MouseAnti-Human Melanosome Clone HMB45, Monoclonal Mouse Anti-Human Tyrosinase Clone T311, both Dako North America Inc., Carpinteria, CA, USA), diluted 1:200 in saponin-buffer were used as positive control. The monoclonal antibody stained cells were also used to determine the amount of transfected cells and, corresponding to that, the quantity of antigene produced (figure 1: 2.) Transfected cells were therefore also visualized by bright field microscopy and a 460-490 nm excitation filter (Zeiss Axiovert 200 M Fluorescence microscope, Carl Zeiss AG, Oberkochen, Germany, Digital camera Olympus DP70, Olympus, Hamburg, Germany). As negative controls sera of healthy horses (figure 1: 3.), transfected cells only incubated with the secondary antibody (figure 1: 4.) and untransfected cells incubated with the tested sera and secondary antibodies (figure 1: 5.), were used. Staining was performed as described above.

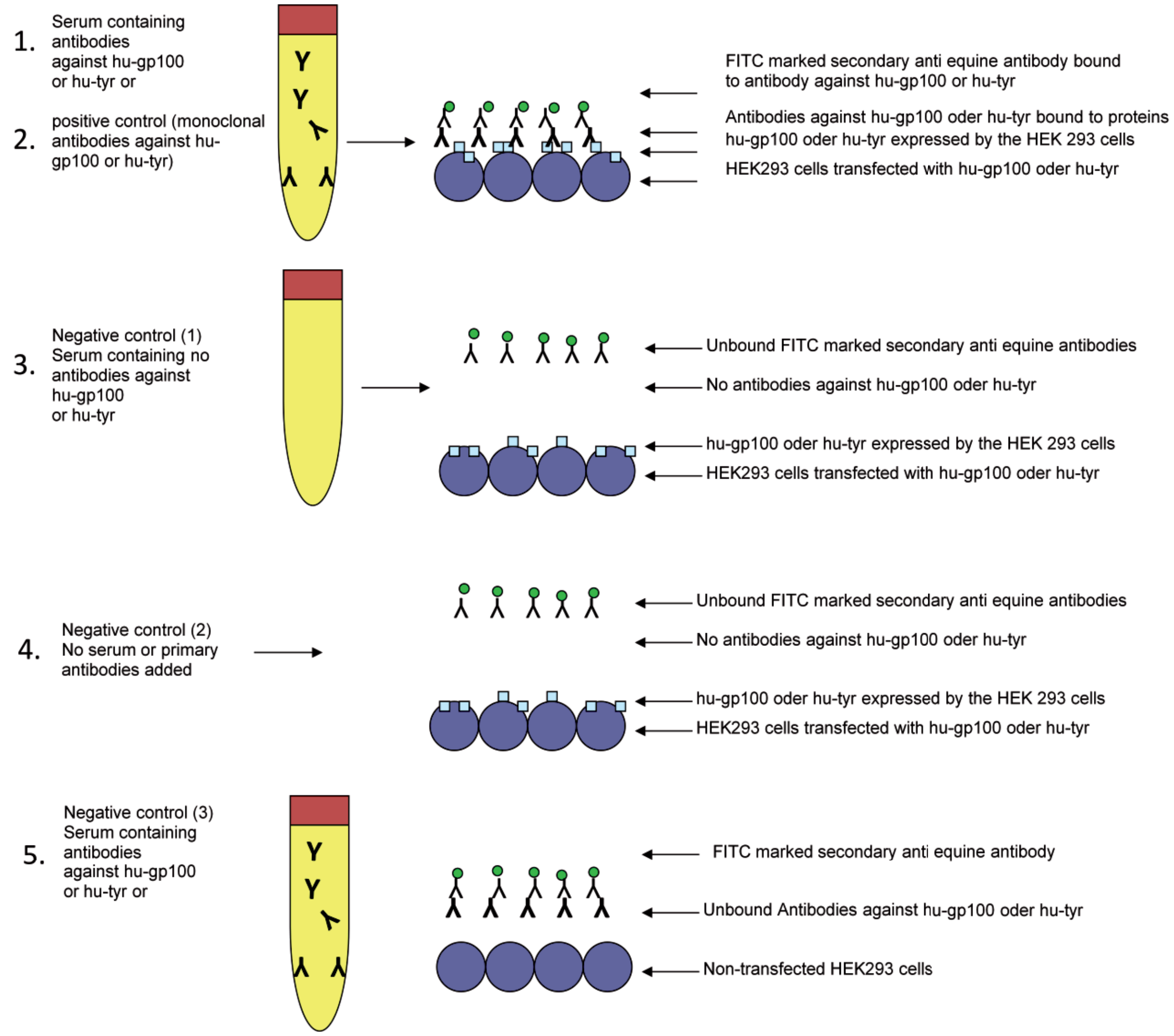


Figure 1: Cell based antibody assay used for detection of specific antibodies against gp100 or tyr.

Stained cells were washed twice with saponin-buffer and resuspended in 200 µl of Sheath-buffer. Cells were evaluated by FACS analysis to detect antigene bound specific antibodies against hgp100 and htyr. Therefore 10.000 events were counted (FACScan®, CellQuest™ Pro 4.0.2, Becton Dickinson, Heidelberg, Germany). FcsExpress software (De Novo Software Los Angeles, CA, USA) was used to analyze the amount of stained cells. For the detection of specific antibodies against hgp100 and htyr in the sera of vaccinated horses, values from negative controls (untransfected cells incubated with serum and secondary antibodies) were subtracted from results of transfected cells incubated with sera and secondary antibodies. Mean at baseline was calculated out of all values before vaccination. Standard deviation (SD) to this timepoint was calculated and multiplied with 3. The mean and the threefold SD before vaccination were subtracted from the positive stained cells to each timepoint.

At any time post-vaccination a humoral response was considered positive when fluorescence increased ≥ 3 standard deviations over the mean value at baseline and had an absolute value > 0.1 %.

3.11 Indirect evaluation of cytotoxic response by IFNγ production of specifically stimulated PBMCs using flow cytometry

In order to measure specific cytotoxic T-cells against hu-gp 100 and htyr, an indirect cell based assay was used. Autologous equine dermal cells were transfected with the DNA vectors coding for hgp100 or htyr in order to produce the proteins and to present the epitopes in Major histocompatibility-I complexes (MHC-I)-complexes. Peripheral blood mononuclear cells (PBMCs) were incubated with these cells or stimulation of specific cytotoxic T-cells to produce interferon γ (IFN γ).

3.11.1 Dermal cell isolation, culture and cryopreservation

Equine dermal cell (EDC) cultures were established from every horse about four weeks prior to the first vaccination. For this purpose, three punch biopsies were harvested at one side of the neck near the mane (Biopsy Punch 0.8 cm, Stiefel, GlaxoSmithKline GmbH & Co. KG, Munich, Germany) after five minutes of aseptical preparation of the clipped skin with chlorhexidin containing soap (HibiScrub, Regent

Medical, Manchester, UK). Biopsies were transferred into 5 ml fibrocyte culture medium (RPMI1640 with HEPES and glutamine (3 %), PAA Laboratories GmbH, Pasching, Austria; fetal calf serum (20 %) and penicillin (100 international units (I.U.)/ml) as well as streptomycin (100 µg/ml)) in sterile 50 ml centrifuge tube (Corning Incorporated, Corning, NY, USA) and transported to the laboratory at room temperature. Fat was removed and biopsies were cut into pieces of 1 mm^3 by scalpel (Surgical Disposable Scalpel, B. Braun Melsungen AG, Tuttlingen, Germany). The tissue was incubated for eight hours with 1 mg/ml (0.15 U/ml) collagenase A (Roche Holding GmbH, Germany, Mannheim, Germany) and 2 mg/ml (1.6 U/ml) dispase I (neutral protease, grade I) (Roche Holding GmbH Deutschland, Mannheim, Germany) diluted in a total of 5 ml RPMI1640 in a 50 ml centrifuge tube in an incubator (37 °C, 5 % CO_2). Tubes were agitated every two hours. Afterwards, preparations were centrifuged (450 *g*/10 min, 37 °C), supernatant discharged, and pellet resuspended in 5 ml fibrocyte culture medium and cultivated in 25 cm^2 tissue culture flasks at 37 °C and 5 % CO_2. Medium was changed and cells were passaged according to requirements. Cells were frozen after the third or fourth passage in fibrocyte culture medium with 10 % of dimethylsulfoxide (DMSO, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in 3.5 ml cryotubes (Carl Roth GmbH&Co. KG, Karlsruhe, Germany) and stored at -96 °C. Approximately two weeks prior to use, cells were thawed and cultivated in fibrocyte culture medium. Cells were subsequently transfected with the DNA vectors coding for vaccination antigenes and used as stimulator cells for PBMCs in the indirect cytotoxic T-cell assay.

3.11.2 Transfection of Equine Dermal Cells

For each test for specific IFNγ producing cells, the EDCs (1 x 10^6) of the individuum to be tested were seeded in four of the six wells of 6-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) to reach 50-80 % confluence on the following day. EDCs were transfected with pMCV1.4 hgp100, htyr or eGFP, respectively, using lipofectamine™ LTX and PLUS™ reagent according to the manufacturer's instructions. Cells of one well were left untransfected (control). Per well, 3 µl DNA were solved in 500 µl OptiMEM. 6 µl PLUS™ reagent (Invitrogen, Darmstadt, Germany) was added and the solution was mixed gently. After five minutes of incubation at room temperature, 6 µl lipofectamine™ LTX were added, mixed gently and incubated for 30 minutes at room temperature. The solution was added to the EDCs. EDCs were incubated for at least 24 hours to express the proteins. These transfected EDCs were subsequently used in the indirect detection of specific cytotoxic T-cells against hgp100 and htyr by IFNγ detection after specific stimulation. The EDCs were used as stimulator cells for this assay.

EGFP transfected cells were evaluated for each patient and each test by fluorescence microscopy or FACS analysis to prove transfection.

To verify the functionality and efficiency of transfection and expression of the hgp100 and htyr vectors in EDCs, transfected cells were stained and evaluated exemplarily. Transfected EDCs were fixed in the plates with 500 µl of chilled methanol/acetone for approximately 5 minutes. Cells were then washed three times with PBS and 500 µl monoclonal antibodies, in a dilution of 1:100, specific for hgp100 or htyr (Monoclonal MouseAnti-Human Melanosome Clone HMB45, Monoclonal Mouse Anti-Human Tyrosinase Clone T311, both Dako North America Inc., Carpinteria, CA, USA) were added and incubated for 30 minutes at RT. After three times of washing with PBS, 50 µl of the 1:100 diluted secondary antibody (polyclonal goat anti mouse PE, Dianova GmbH, Hamburg) was added to the cells for another 30 minutes at room temperature. Cells were washed three times with PBS. Transfection of EDCs was visualized using a confocal microscope (Leica TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany). The quantity of transfected EDCs was estimated by superimposition of a bright field image and one with a 529 - 534 nm excitation filter.

3.11.3 Isolation and culture of peripheral blood mononuclear cells

The amount of specific cytotoxic T-cells in the blood of the patients was measured by an indirect assay by measurement of IFNγ after simulation with the previously transfected EDCs (3.12.2). Therefore blood samples were collected in sodium-heparin tubes (Vacuette® NH, 9ml, greiner bio-one, Kremsmünster, Austria) on days 1, 22, 81 and 120. Erythrocytes were allowed to settle for approximately half an hour. Peripheral Blood Mononuclear Cells (PBMCs) were obtained by density gradient centrifugation. Plasma from heparinised blood samples was overlaid in a ratio of 1:1

over the separation medium (Lymphocyte Separation Medium LSM 1077; PAA Laboratories GmbH, Pasching, Austria) at RT in a 50 ml centrifuge tube (Corning Incorporated, Corning, NY, USA). After centrifugation (1000 *g*, 30min, without brake at 4 °C), interphase was harvested and resuspended in 40 ml PBS. Three centrifugation steps followed at 600 *g*, 300 *g*, 150 *g* at 4 °C with brake to remove thrombocytes. After each centrifugation step, the supernatant was discharged and the cell pellet was resuspended in 40 ml PBS. After the last centrifugation, cells were resuspended in complete medium (RPMI1640 with HEPES and glutamine (3 %), PAA Laboratories GmbH, Pasching, Austria, fetal calf serum (10 %) and penicillin (100 international units (I.U.)/ml) as well as streptomycin (100 µg/ml)) at a concentration of 2 x 10^6 /ml. PBMCs were subsequently incubated with the transfected EDCs for indirect detection of cytotoxic T-cells by measurement of IFNγ production.

3.11.4 IFNγ staining

For the indirect detection of cytotoxic T-cells by their IFNγ production after specific stimulation of these cells with the transfected EDCs, 1 x 10^6 PBMCs were added to each well of transfected EDCs and incubated for 20 hours. To hinder protein secretion, Brefeldin A (Sigma Aldrich Chemie, GmbH, Taufkirchen, Germany) was added to a final concentration of 10 µg/ml nine hours before staining.

Stimulation with 12-myristat 13-acetat (PMA, final concentration 25 ng/ml) and Ionomycin (final concentration 1µM) for nine hours served as positive control, and secretion was avoided using Brefeldin A (10 µg/ml) added simutaneousely to the PMA and Ionomycin.

As negative control, PBMCs incubated with fibrocytes or cultured with medium only were used.

After 20 hours of incubation the medium, containing the PBMCs, was removed. It was centrifuged (350 *g*, 10 min, RT), the supernatant discarded, and cells were resuspended in FACS-buffer. Intended for flow cytometry, 2x10^5cells were plated in a well of a 96-well round-bottom plate. Cells were washed three times with FACS-buffer and were incubated with 50 µl of antibody against equine CD8 (CVS8 undiluted, obtained by the AG immunology, University of Veterinary Medicine Hannover, Foundation) or anti equine CD4 antibody (diluted 1:10 in FACS buffer, 25 µl of the final solution) (mouse anti horse, CVS4, IgG1, AbD Serotec, Kidlington, UK), for 30 min at RT and washed for three times with FACS buffer. Rhodamine phycoerythrin (PE) conjugated goat-anti-mouse IgG + IgM (dianova, Hamburg, Germany), the secondary antibody, was diluted 1:100 in FACS buffer and 25 µl of the final solution were incubated with the cells for 30 min at RT. Cells were washed for three times with FACS buffer. For double fluorescence, cells were fixed in 4 % paraformaldehyde prior to detection of intracellular equine IFNγ using a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody, for which specificity for equine IFNγ had previously been proven by PAILLOT et al. (2005) (mouse anti bovine, CC302, IgG1, AbD Serotec, Kidlington, UK), diluted 1:100 in permeabilisation-buffer. cells were incubated with the antibodies for 30 min at RT. Afterwards cells were washed for three times with permeabilisation-buffer. For the detection of background staining cells were incubated without primary antibody and PE conjugated goat-anti-mouse IgG + IgM (1:100, 25 µl) and human anti CD61 FITC conjugated antibody (mouse anti human, Y2/51, IgG1, AbD Serotec, Kidlington, UK) only. Cells were resuspended in FACS tubes (Beckton Dickinson, Heidelberg, Germany) in 200 µl Sheath (sterile filtered PBS). For analysis 50.000 events were counted using a live gate for the lymphoid cell fraction.

The amount of IFNγ positive cells was calculated by subtracting the background staining control (cells only incubated with the secondary antibody PE conjugated goat-anti-mouse IgG + IgM (1:100, 25 µl) and human anti CD61 FITC conjugated antibody (mouse anti human, Y2/51, IgG1, AbD Serotec, Kidlington, UK).

After this substraction the cells stained positive for CD4 or CD8 in combination with IFNγ (double positive cells) and cells stained only positive for IFNγ (single positive cells) were summed up to get the total amount of IFNγ positive cells. Mean at baseline was calculated out of all values before vaccination. Standard deviation (SD) was calculated from the values before vaccination and multiplied with 3. The sum of the mean and the threefold SD was subtracted from the total amount of IFNγ positive cells to each timepoint (YUAN et al. 2010).

At any time post-vaccination a T-cell response was considered positive when fluorescence increased ≥ 3 standard deviations over the mean value at baseline and had an value > 0.1 % (YUAN et al. 2010).

3.12 Statistical analysis

All data were documented using excel tables (Microsoft Excel 2003, Microsoft Deutschland GmbH, Unterschleißheim, Germany).

An Analysis of Variance (ANOVA) was performed to identify statistical significant influences of the parameters documented in this study on the relative volume at day 120. Parameters evaluated were treatment group, locally treated versus non-locally treated melanomas and examination method (ultrasonography, calliper).

A multivariate ANOVA with Tukey-Kramer multiple comparison test was performed to analyse the size development of melanomas.

Correlation of ultrasonographic measurements and calliper measurements was evaluated using the Pearson correlation test.

The development of temperature and white blood cell count was evaluated by ANOVA and Tukey-Kramer multiple comparison test.

Calculations for the indirect assay for specific cytotoxic T-cells and specific antibodies were calculated as described above.

A p – value < 0.05 was considered as significant.

Statistical analyses were performed using the statistic software JMP 8.0 (SAS Institute Inc., Cary, NC, USA) and NCSS (NCSS, Kaysville, Utha, USA).

4 Results

4.1 Control of *in vivo* transfection

Horses were injected intradermally (n = 3) and intramuscularly (n = 1) with MIDGE-Th1® coding for firefly-luciferase complexed with SAINT-18.

General examination revealed that horses were bright, alert and responsive during the time of the study. All clinical parameters were in normal ranges for 24 hours after injection, except temperature. Increased body temperature was observed in all horses with an onset after 6 hours for at least 6 hours. Maximal temperatures were 38.8 ± 0.47 °C.

Biopsies of transfected skin and muscles emitted variable but higher amounts of fluorescence compared to the negative control. Only in the one skin biopsy, which was collected after two weeks, and in one muscle biopsy, expression was low. As no calibration of the assay was performed, results are qualitative. Intra assay variability was low, as shown by duplicate measurements (table 8).

Table 8: Luciferase assay: relative light units (RLU) emitted during ten seconds of luminescence measurement. Higher values of RLU indicate a higher concentration of luciferase.

horse, localisation (sample number)	days after application	relative light units	
		measurement 1	measurement 2
negative control, skin		110	114
negative control, muscle		94	139
horse 1, skin (1)	1	38.795	35.928
horse 1, skin (2)	1	4.047	3.952
horse 1, skin (3)	1	64.524	64.536
horse 2, skin (1)	1	141.198	129.057
horse 2, skin (2)	1	37.364	37.591
horse 3, skin (1)	14	263	304
horse 3, muscle, ventral tail (1)	5	539	487
horse 3, muscle, ventral tail (2)	5	25.850	28.084
horse 3, muscle, dorsal tail (1)	5	148	155
horse 3, muscle, dorsal tail (2)	5	1.194	1178
horse 4, muscle, face (1)	5	534	609
horse 4, muscle, face (1)	5	21.735	27.108

4.2 Therapy Control

4.2.1 Clinical examination of treatment groups IL12/18, gp100 and tyr

Prior to vaccination and during the study period from day one to day 120, patients were bright, alert and responsive. All vital parameters were in normal ranges except temperature. No diverge from normal values were evaluated for hematology and blood biochemistry.

Rectal temperature increased over 38.2°C from 37.4 ± 0.26 °C prior to treatment to 38.7 ± 0.59 °C in 19/27 horses and in 40/81 injections on the day after vaccination. Fever with rectal temperatures over or equal to 38.6°C was observed after 20 injections (39.2 ± 0.51 °C). However, eight horses developed no increased body temperature. The temperature of all horses taken together on the day after injection was significantly higher (38.0 ± 0.69 °C) compared to the temperature on the other days of hospitalisation (Tukey-Kramer Multiple-Comparison Test, p=0.05, figure 2).

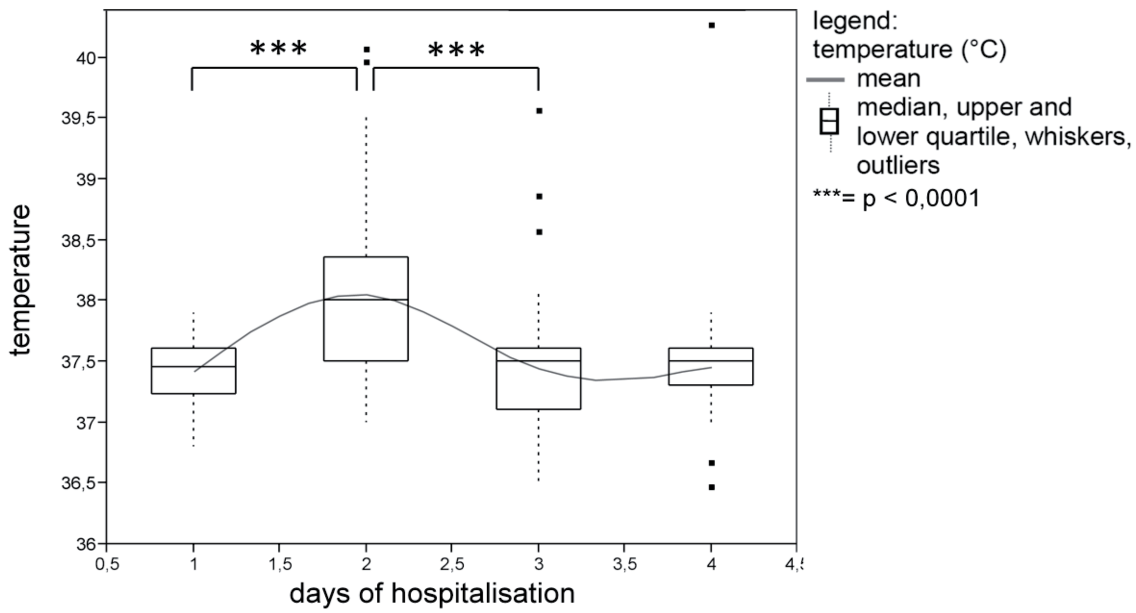


Figure 2: Daily rectal temperatures (in °C) measured in the horses (n=27) on the days of each injection and on days 2-4 after each injection.

There was a significant increase (Tukey-Kramer Multiple Comparison Test) in the temperature on the day after injection.

In seven horses, increased body temperature was present on the day after each of the three injections (IL12/18: n=2; gp100: n=3; tyrosinase n=2). Six horses showed increased body temperature on the day after two of the three injections (days 2 and 23: n=1 (IL12/18), days 23/79: n = 3 (tyr n=2 and IL12/18: n=1) and days 2 and 79: n = 2 (gp100: n=1 and tyr: n=1) and three horses after one injection (day 2 (tyr), day 23 (tyr) and day 79 (IL12/18). Furthermore increased body temperature was recognized in one horse on days 3, 4, 5 and 80 (gp100, horse 3), in one horse on days 2 and 24 (gp100, horse 9), in one horse on days 2 and 3 (IL12/18, horse 18) and in one horse on days 79 and 80 (tyr, horse 27).

No differences in temperature could be seen between the groups (ANOVA, p= 0.9818).

Horse 3 had fever for three days after the first vaccination and additionally showed signs of an acute viral pulmonary infection.

4.2.2 Clinical examination of horses treated for evaluation of unspecific side effects (group SAINT-18/luc, group SAINT-18/- and group -/IL12/18)

Five horses were injected singularly i.m. and i.d. with a combination of MIDGE-Th1®-luciferase and SAINT-18, MIDGE-Th1®-IL12/18 only, or SAINT-18 only.

All five horses in group SAINT-18/luc developed an elevated temperature with an onset at 6 hours after injection. Increased body temperature persisted for about 6 hours.

Horses in group -/IL12/18 and group SAINT-18/- remained normothermic (figure 3)

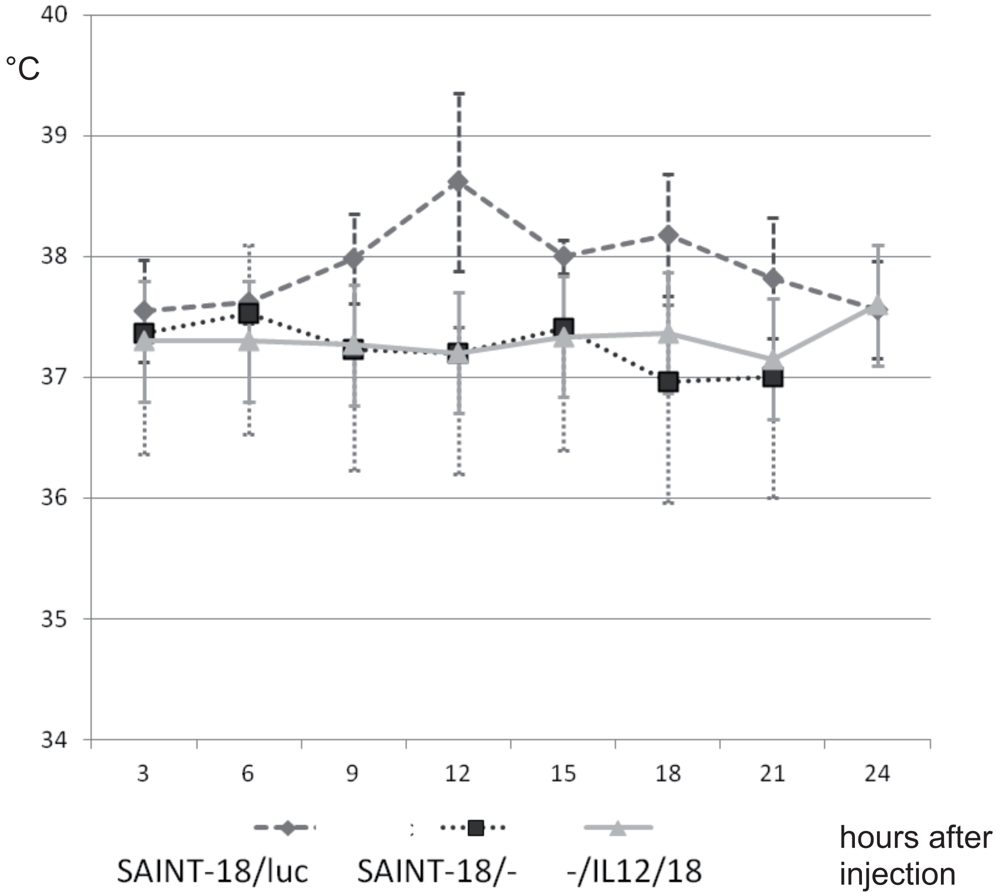


Figure 3: Rectal temperatures (°C) measured in the horses used for evaluation of unspecific side effects from the day of injection on. There was an increase in the temperature in the group SAINT-18/luc whereas the other groups SAINT-18/- and -/IL12/18 stayed normothermic.

Signs of local inflammation (reddening, swelling, increased temperature) were seen at the injection sites from three days after injection on in the luciferase/SAINT-18 group but not in the others.

4.2.3 Macroscopic evaluation and photographic documentation

After 81 injections (27 horses, 3 injections each), horses showed mild subcutaneous swelling (81 / 81), reddening (10 / 81), exsudation (7 /81) and ulceration (2 / 81). These signs of acute inflammation occurred within the first three days after injection during the time of hospitalisation. At re-evaluation on days 22, 79 or 120 these signs were not obvious anymore except in horse 11 were reddening, exsudation and ulceration persisted until day 22 after the first injection. The inflammatory signs were seen focally at the injection sites. At the injection sites all horses except one showed depigmentation. The depigmentation was firstly observed after 22 days in 23 horses and in 3 horses after 81 days. The depigmentation had decreased (2 horses), remained stable (12 horses) or progressed (12 horses). The depigmentation was mild in 13 horses on day 22, 15 horses on day 78 and 12 horses on day 120. The depigmentation was moderate in 7 horses on day 22, 5 horses on day 78 and 8 horses on day 120. The depigmentation was severe in 3 horses on day 22, 5 horses on day 78 and 6 horses on day 120. Horses showed no signs of depigmentation in examined pigmented regions distant from injected melanomas. Local reactions are exemplarily shown for horse No. 11 (figure 4).

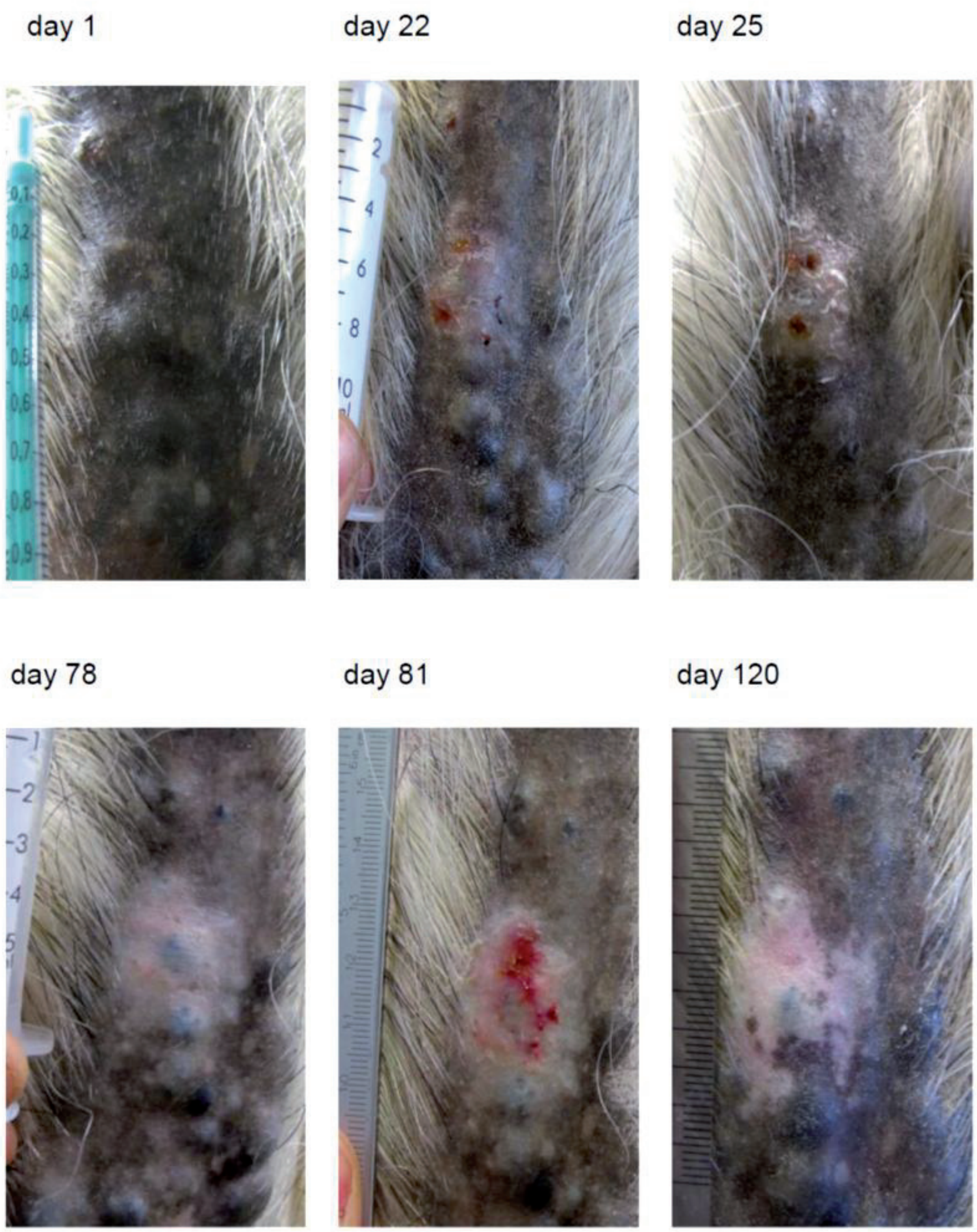


Figure 4: Local inflammation (reddening, ulceration, exsudation) and depigmentation at the injection site (No.11) during the study period.

4.2.4 Hematology and blood biochemistry analysis

Standard blood analysis on days 1, 4, 22, 25, 78, 81 and 120 showed neither pathological changes nor significant shifts in leucocyte differential counts after therapy.

4.2.5 Absolute tumor volume

4.2.5.1 Differences between the treatment groups

A significant difference of absolute tumor volumes in calliper measurements, between the tyr and the gp100 (days 1 ($p = 0.0452$), 22 ($p = 0.0329$), 78 ($p = 0.0365$)) and between the tyr and the IL12/18 group (days 22($p = 0.0396$), 78($p = 0.0482$), 120 ($p = 0.0455$)) (Student's T-test) was found. On day one there was a trend for difference between IL12/18 and tyr ($p = 0.0637$), as well as on day 120 for gp100 and tyr ($p = 0.0553$). There were no differences of the absolute tumor volumes found between the treatment groups in ultrasonographic measurements.

The melanomas causing the differences in calliper measurements were too large for ultrasonographic measurements and were therefore only measured by calliper (horse No.4: melanom (mel) 7, mel 9, horse No. 19: mel 8, horse No.20: mel 1, horse No.21: mel 9, horse No. 26: mel 1.

4.2.6 Relative tumor volume

In the analysis of variance the influences of the treatment group, locally treated versus non-locally treated melanomas and examination method on the relative tumor volumes on day 120 were analyzed. The factor influencing the tumor volume on day 120 significantly was the examination method ($p < 0.001$) whereas treatment (locally treated versus non-locally treated melanomas) ($p = 0.47$) and treatment group (IL12/18, gp100 and tyr) ($p = 0.52$) had no influence on the relative end volume.

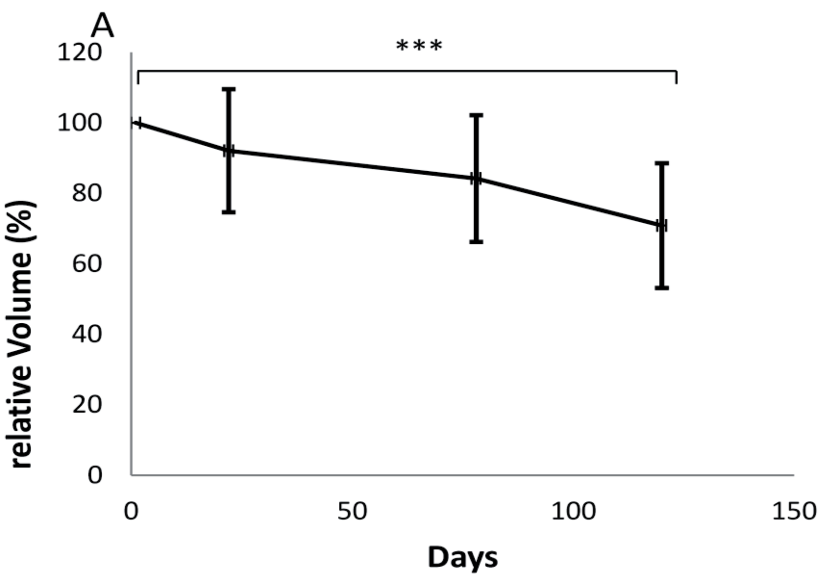
4.2.6.1 Calliper measurements

Calliper measurements were performed on days 1, 22, 78 and 120 for all melanomas (locally treated and non-locally treated melanomas)(IL12/18: $n = 42$, gp100: $n = 50$, tyr: $n = 44$) and additionally on days 4, 25 and 81 for locally treated melanomas (n =

27). Relative volumes and relative greatest diameters were calculated in reference to day one.

There were no statistically significant differences detected between the treatment groups in non-locally treated melanomas. Relative volume of non-locally treated melanomas in all treatment groups taken together decreased significantly from 100% to 70.4 ± 11.50 % ($p < 0.001$, t-test). In the different treatment groups the mean volume decreased to 72.1 ± 13.24 % in group IL12/18, to 68.1 ± 7.44 % in group gp100, and to 72.5 ± 14.14 % in group tyr (figure 5a).

In locally treated melanomas there were no statistically significant differences detected between the treatment groups. Volumes of locally treated melanomas in all treatment groups taken together decreased also significantly ($p < 0.001$, t-test) to 67.2 ± 29.89 %. In the different treatment groups the mean volume decreased to 71.4 ± 16.90 % in group IL12/18, to 72.5 ± 32.40 % in group gp100, and to 66.1 ± 30.24 % in group tyr (figure 5b). The changes in the tumor volume between day 1 and 4 as well as 78 and 81 were not significant. The volume increased significantly between days 22 and 25 ($p = 0.016$, ANOVA).



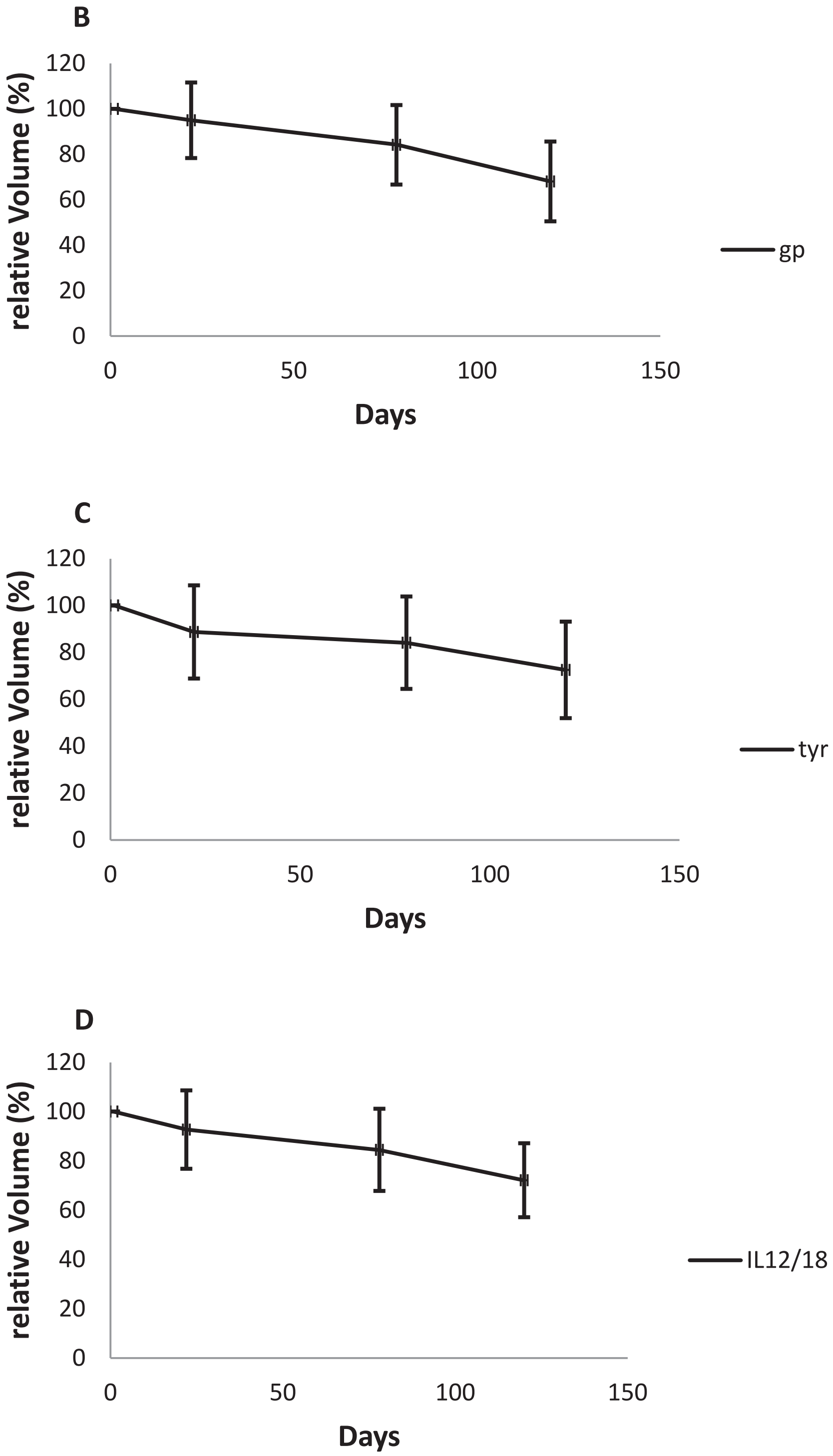
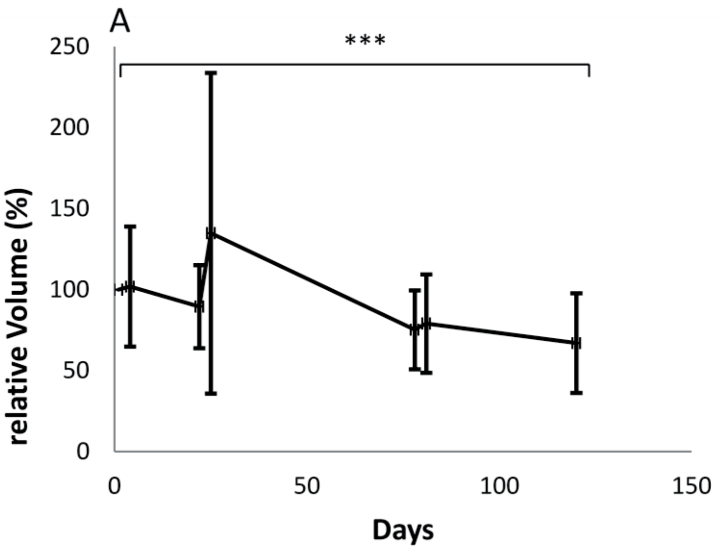


Figure 5a: Relative volumes (%) of non-locally treated melanomas calculated by caliper measurements on days 0, 22, 78 and 120. A: all non-locally treated melanomas, B: non-locally treated melanomas group gp100, C: non-locally treated melanomas group tyr, D: non-locally treated melanomas group IL12/18



A
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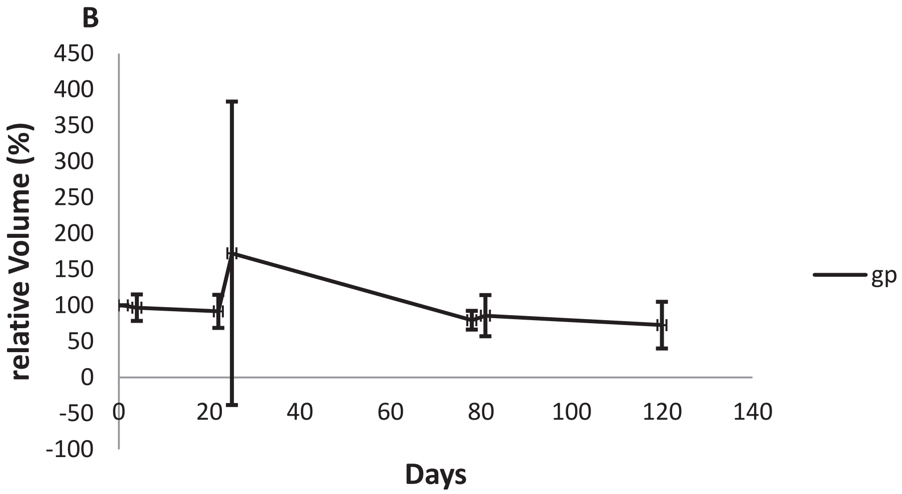

B
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relative Volume (%)
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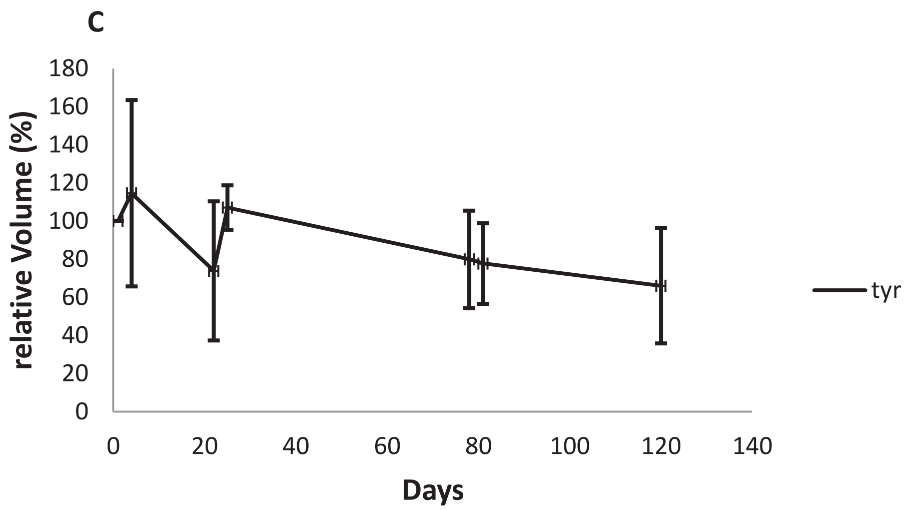
20
40
60
80
100
120
140
Days
gp



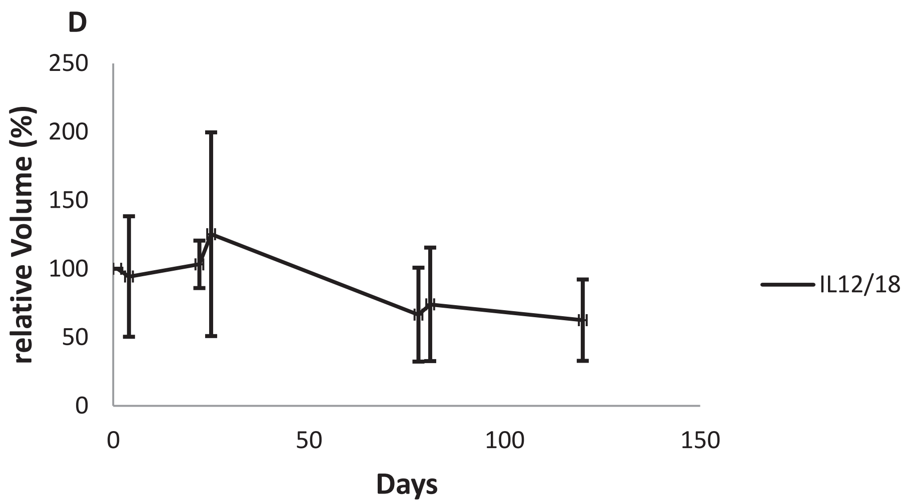


Figure 5b: Relative volumes (%) of locally treated melanomas calculated by caliper measurements on days 0, 22, 78 and 120. A: all locally treated melanomas, B: locally treated melanomas group gp100, C: locally treated melanomas group tyr, D: locally treated melanomas group IL12/18

4.2.6.2 Ultrasonographic measurements

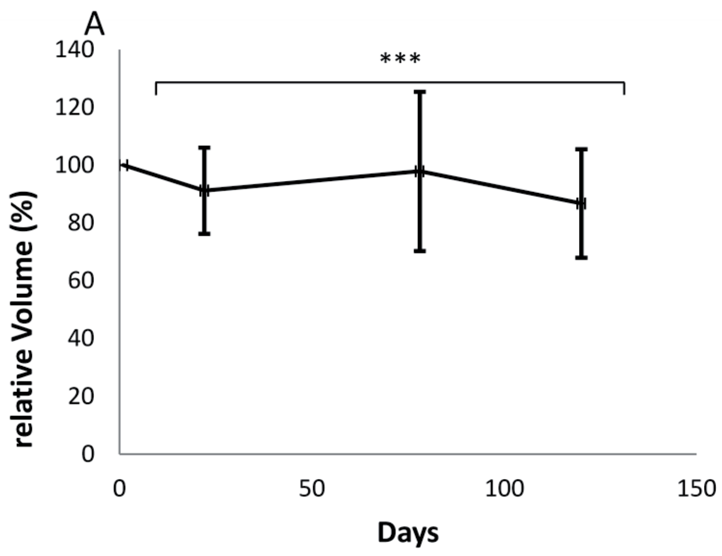
Ultrasonographic images were made synchronously with calliper measurements of the largest diameter in sagittal (length) and transversal (width) plain. A total of 130 (examiner 1: IL12/IL18: n = 42, gp100: n = 47, tyr: n = 41) and 121 (examiner 2: IL12/IL18: n = 33 gp100: n = 47, tyr: n = 41) melanomas were measured.

Melanomas were hypoechoic, surrounded with a hyperechoic line. In some melanomas, hyper- and also hypoechogenic regions, mainly in the centres, were seen.

There were no statistically significant differences detected between the treatment groups in non-locally treated melanomas. Relative volume of all non-locally treated melanomas decreased significantly to 86.7 % ± 19.81 ($p < 0.002$, t-test). In the different treatment groups the mean volume decreased to 79.0 ± 23.34 % in group IL12/18, to 94.8 ± 16.11 % in group gp100, and to 86.5 ± 18.31 % in group tyrosinase (figure 6a).

Relative volume of all locally treated melanomas decreased significantly to 84.0 % ± 25.42 ($p = 0.002$, t-test) on day 120. In the different treatment groups the mean volume decreased to 76.7 ± 66.14 % in group IL12/18, to 93.7 ± 21.67 % in group gp100, and to 81.5 ± 18.01 % in group tyrosinase (figure 6b). There were no statistically significant differences detected between the treatment groups.

The changes in the tumorvolume between day 1 and 4 as well as 78 and 81 were not significant. The volume increased significantly between days 22 and 25 ($p = 0.016$, ANOVA).



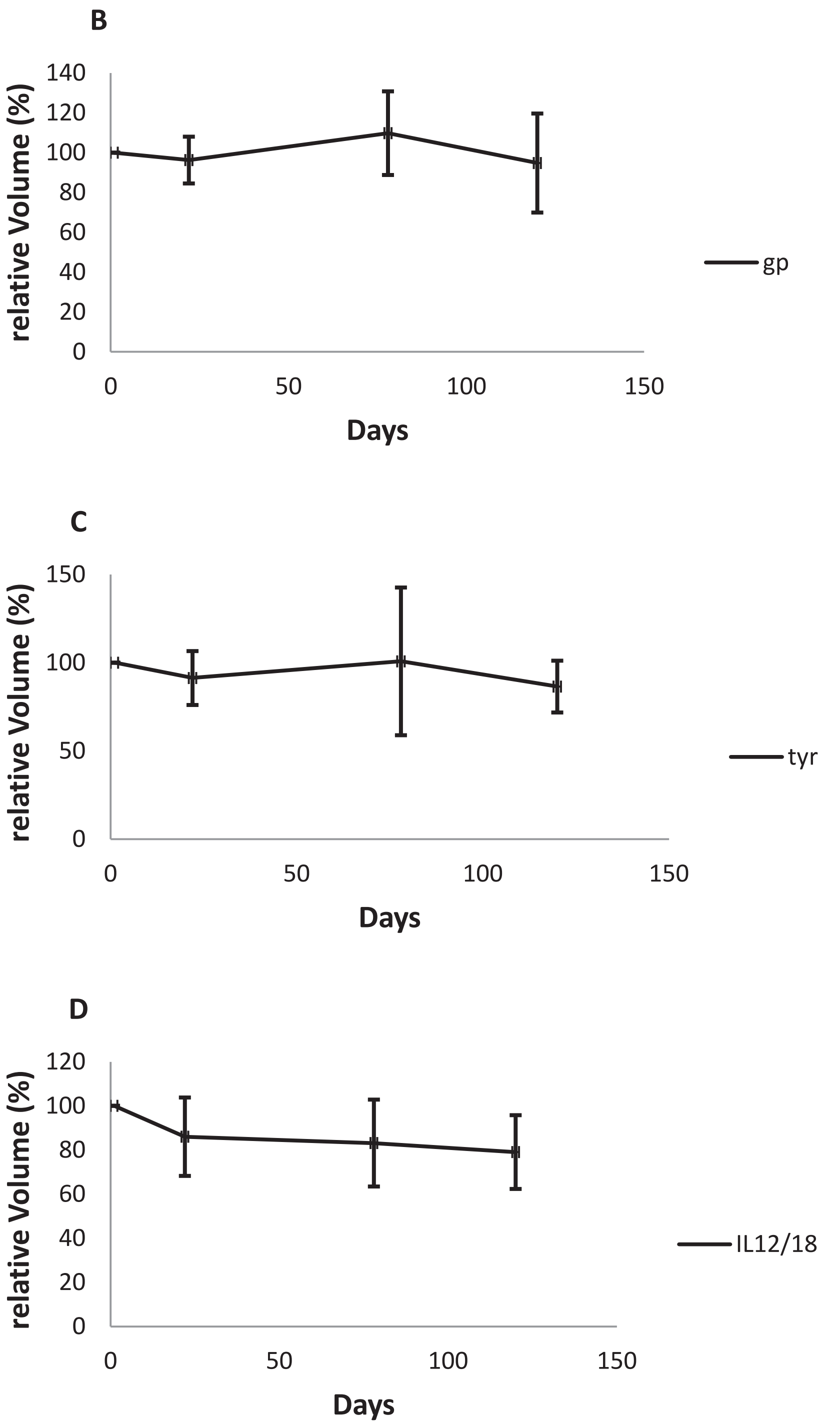
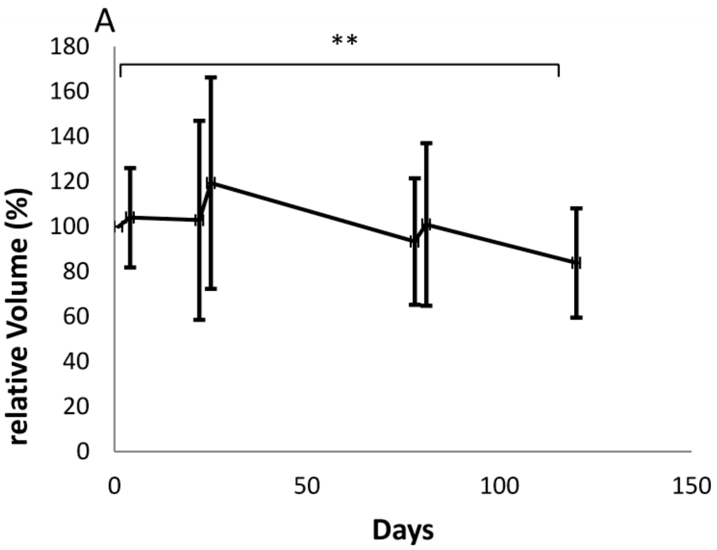


Figure 6a: Relative volumes (%) of non-locally treated melanomas calculated by ultrasonographic measurements on days 0, 22, 78 and 120. A: all non-locally treated melanomas, B: non-locally treated melanomas group gp100, C: non-locally treated melanomas group tyr, D: non-locally treated melanomas group IL12/18


A
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180
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140
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relative Volume (%)
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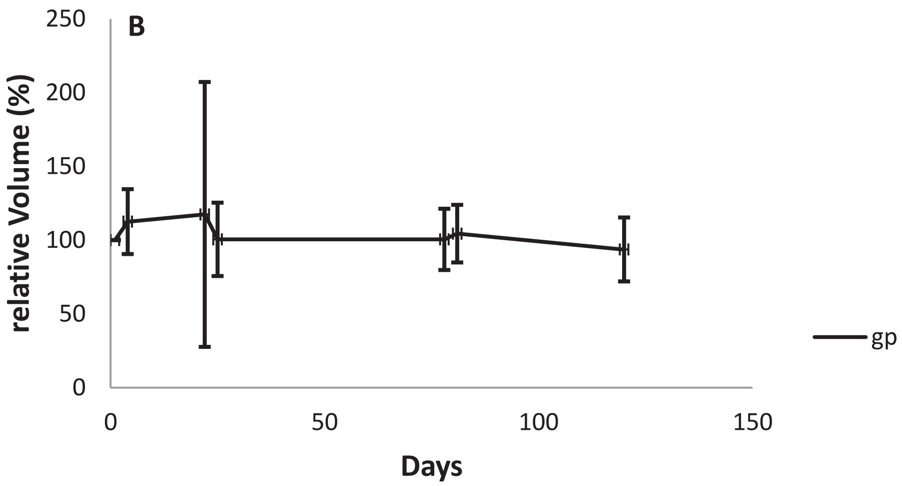
B
250
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relative Volume (%)
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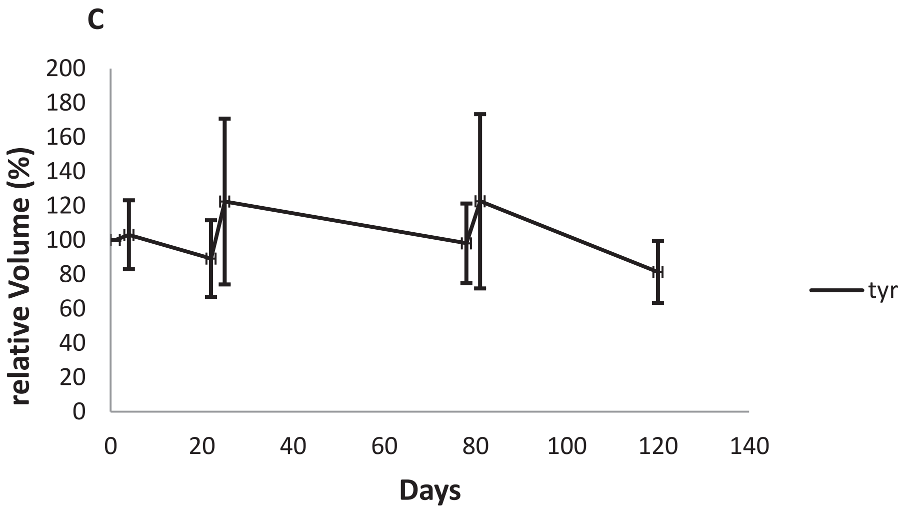
Days
gp



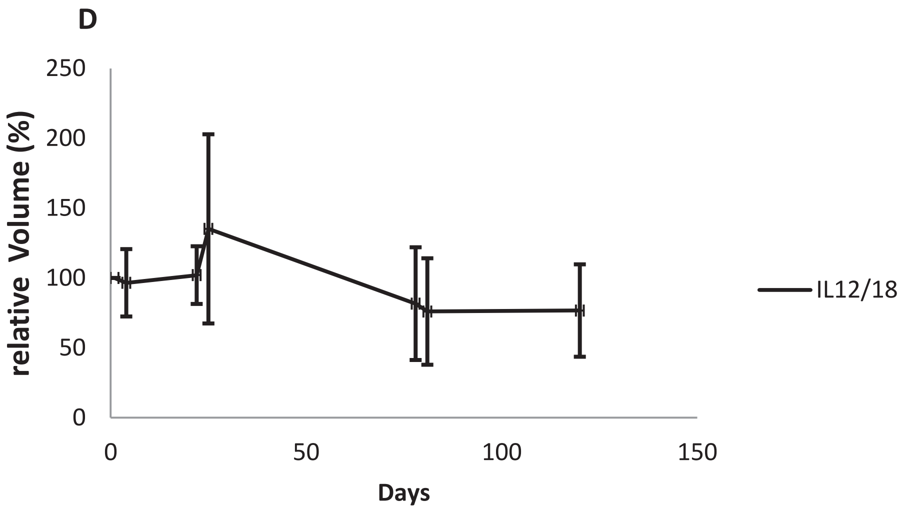


Figure 6b: Relative volumes (%) of locally treated melanomas calculated by ultrasonographic measurements on days 0, 22, 78 and 120. A: all locally treated melanomas, B: locally treated melanomas group gp100, C: locally treated melanomas group tyr, D: locally treated melanomas group IL12/18

4.2.7 Correlations between measurement methods and examiners

The absolute values for length, width (for calliper and ultrasound measurements) and depth (only for ultrasonographic measurements) were correlated between ultrasound examiners. Correlations were 0.9069 for length 0.8201 for width and 0.7064 for depth (table 9). After exclusion of the forementioned melanomas only measured by calliper because they were to large to be measured by ultrasound, the absolute values for length, width were correlated between calliper and ultrasonographic measurements.

The calliper measurements showed a good correlation with the ultrasound measurements for length (0.8556) and width (0.9232) (table 9). However the absolute volumes calculated by length and width for calliper and by length, width and depth for ultrasound measurements showed a better correlation between the ultrasonographic measurements 0.8716) compared to the measurements by ultrasound examiner 1 and calliper measurements (0.8078) (table 9). The relative values calculated by calliper measurements differed significantly (p=0,0002, ANOVA, Tukey-Kramer Multiple-Comparison Test) from the values calculated by ultrasonographic measurements.

Table 9: Correlation coefficients of the absolute values of measurements for length, width and depth measured by calliper and ultrasound (examiner 1 and 2).

	Ultrasound examiner1 /Ultrasound examiner2	**Calliper/ Ultrasound examiner1**
length	0,9069	0,8556
width	0,8201	0,9232
depth	0,7064	
absolute volume	0.8716	0.8078

4.2.8 Evaluation of the response to treatment according to the RECIST criteria

Responding to treatment was defined according to RECIST criteria (THERASSE et al. 2000) as a ≥ 30% decrease of the sum of all greatest diameters of target lesions of one individual. According to these criteria, only one responder (horse 14) was detected (table 10).

Table 10: The sums of the greatest diameters of all measured melanoma in one horse were calculated on day 120 (Dia %). Relative values were calculated in relation to day 1 (100 %). Responder according to the RECIST criteria (decrease of > 30 % compared to baseline) are marked grey.

Horse_ID	diameter (%) (calliper measurement)	diameter (%), ultrasound examiner 1	diameter (%) ultrasound examiner 2
1	93,8347865	102,523534	
2	85,7808858	79,5818607	91,5954416
3	112,054329	99,2670157	86,6990291
4	87,0010002	95,9622208	88,0556567
6	82,7020424	97,2228723	88,7289853
7	82,8419995	98,4984262	87,9443804
8	90,8883725	88,512868	91,0739281
9	91,3138401	86,9993993	90,3141375
10	90,0740486	94,390568	111,059689
11	83,104734	90,2033174	84,4020015
12	79,2195305	102,91833	94,4615136
13	83,5856784	94,9139388	89,9388176
14	48,5294118	40,9638554	37,3684211
15	80,9151636	89,934507	93,7980579
16	88,9189201	93,8398599	94,0272117
17	95,679967	86,971831	
18	96,270779	77,6033953	94,2888399
19	88,9516998	92,3385484	93,0786779
20	94,4515494	98,7059829	129,182662
21	92,1220332	96,9394096	96,4204473
22	89,1891892	93,0930931	103,773585
23	92,8571429	95,7487034	97,9744136
24	91,5254237	95,8441558	100,587084
25	93,9632789	95,3707718	99,55853
26	93,6191764	92,0970359	90,3143471
27	106,521739	96,4412811	90,4761905
28	85,2354332	96,5786923	99,2996125

4.3 Measurement of the humoral response induced by vaccination

The transfection efficiancy of the genes hgp100 and htyr was approximately 30 % in Hek293 cells as proven by monoclonal antibodies and visualized by fluorescence microscopy and flow cytometry (figure 7 a, b). Therefore, it was considered that enough antigens had been produced for the cell based antibody assay.

Because of considerable autofluorescence of the sera, dilutions of 1:1600, 1:3200 and 1:6400 were prepared. No specific antibodies against human tyrosinase or gp100 were detected in equine sera.

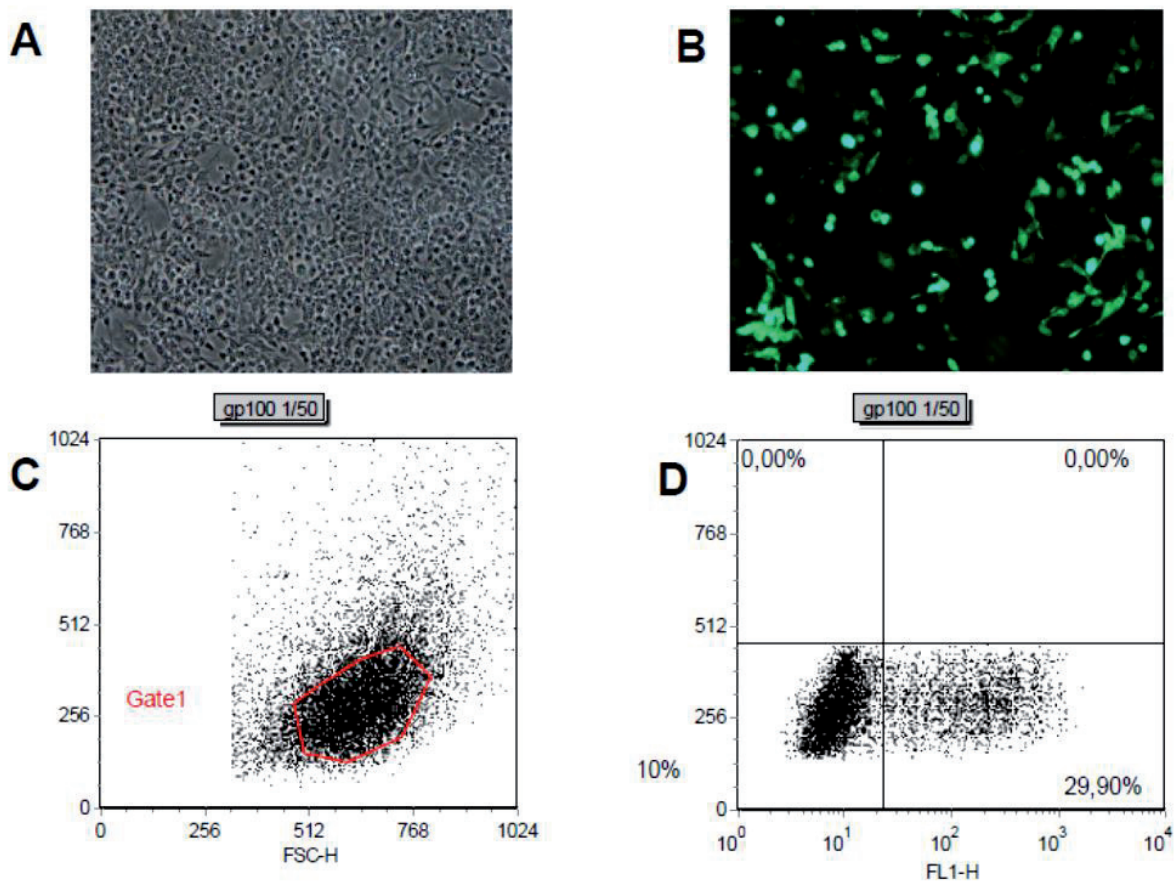


Figure 7a Human embryonic kidney cells (Hek) 293 cells transfected with pMCV1.4hgp100 plasmid. Cells expressed the antigen with an transfection efficacy of approximately 30 % as visualized after immunostaining (Monoclonal MouseAnti-Human Melanosome Clone HMB45, Dako North America Inc., Carpinteria, CA, USA; polyclonal Goat Anti Mouse FITC, Dianova GmbH, Hamburg) in fluorescence microscopy and flow cytometry (**A**: HEK293 cells without filter, **B**: HEK cells with filter (521 nm), **C**: HEK293 cells forward scatter (FSC), side scatter (SSC), **D**: HEK293 cells SSC, FL1 (521 nm).

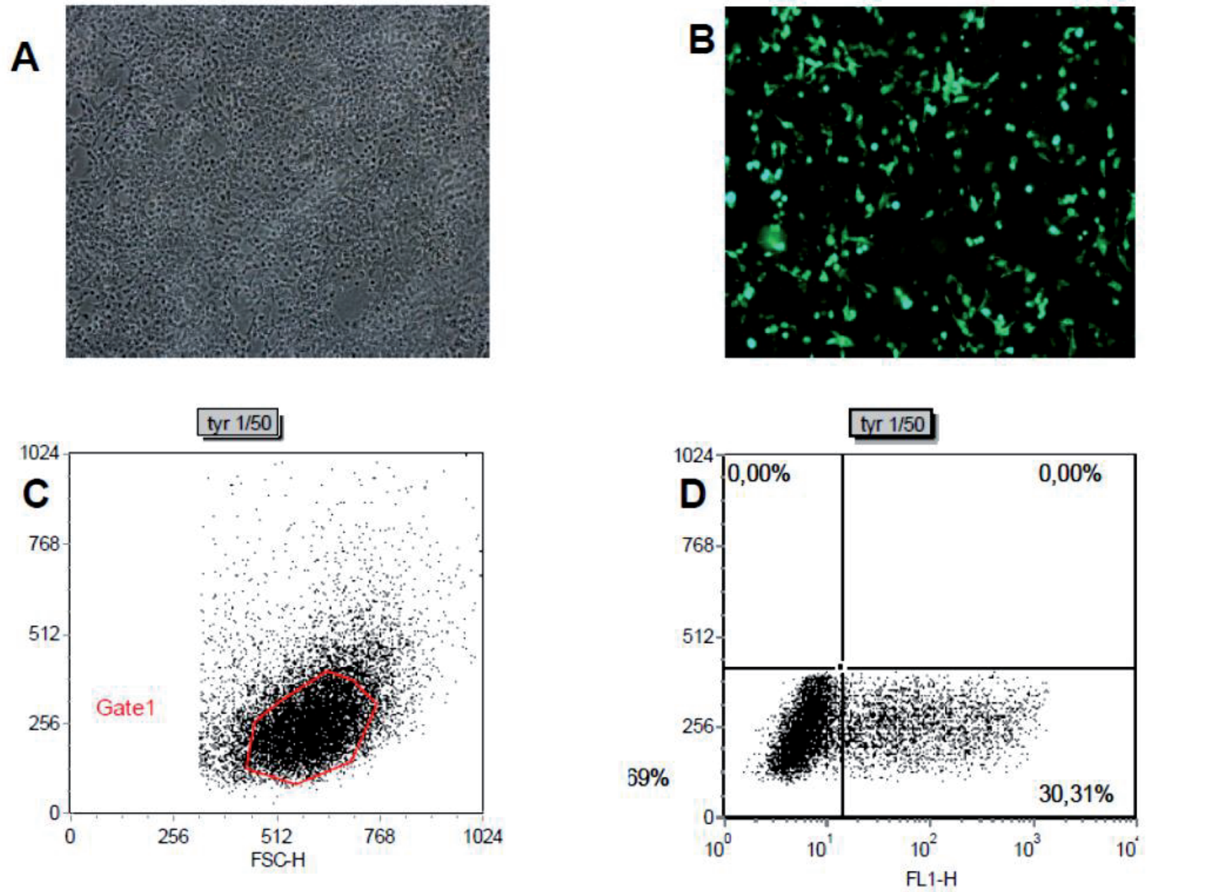


Figure 7b Hek cells transfected with pMCV1.4htyr. Cells expressed the antigen with an transfection efficacy of approximately 30% as visualized after immunostaining (monoclonal Mouse Anti-Human Tyrosinase Clone T311 (Dako North America Inc., Carpinteria, CA, USA; polyclonal Goat Anti Mouse FITC, Dianova GmbH, Hamburg) in fluorescence microscopy and flow cytometry (**A**: HEK293 cells without filter, **B**: HEK293 cells with filter (521 nm), **C**: HEK293 cells FSC, SSC, **D**: HEK293 cells SSC, FL1 (521 nm).

4.4 Evaluation of cytotoxic response by IFNγ detection using flow cytometry

4.4.1 EDC isolation, culture and cryopreservation; Transfection of EDCs

Equine dermal cell cultures were successfully established in 25 out of 27 horses in order to be transfected with the DNA coding for htyr and hgp100 and to be used as stimulator cells in the indirect test for specific cytotoxic T-cells after vaccination. In the cultures of the EDCs of horses 24 and 27 contamination was noted. In successfully established cell cultures, cells had a fibroblast appearance and a spindle shaped morphology. The cells showed variable growth characteristics concerning the speed of growth. This was evident in cultures of different horses and even in different passages of the cells.

A culture period of an average time of two weeks was needed to get enough cells ($5x10^6$) for the assay. Cells were transfected between the third and the sixth passage. After transfection, as proven exemplarily by monoclonal antibodies, cells expressed gp100 and tyr (figure 8 a, b).

For each experiment, cells were transfected with eGFP to prove functionality of transfection. The equine dermal cells showed transfection efficiencies of approximately 1.5 % (absolute cell count per well), evaluated by fluorescent microscopy or FACS analysis (Figure 9c). These results showed a successful but relatively inefficient transfection of EDCs.

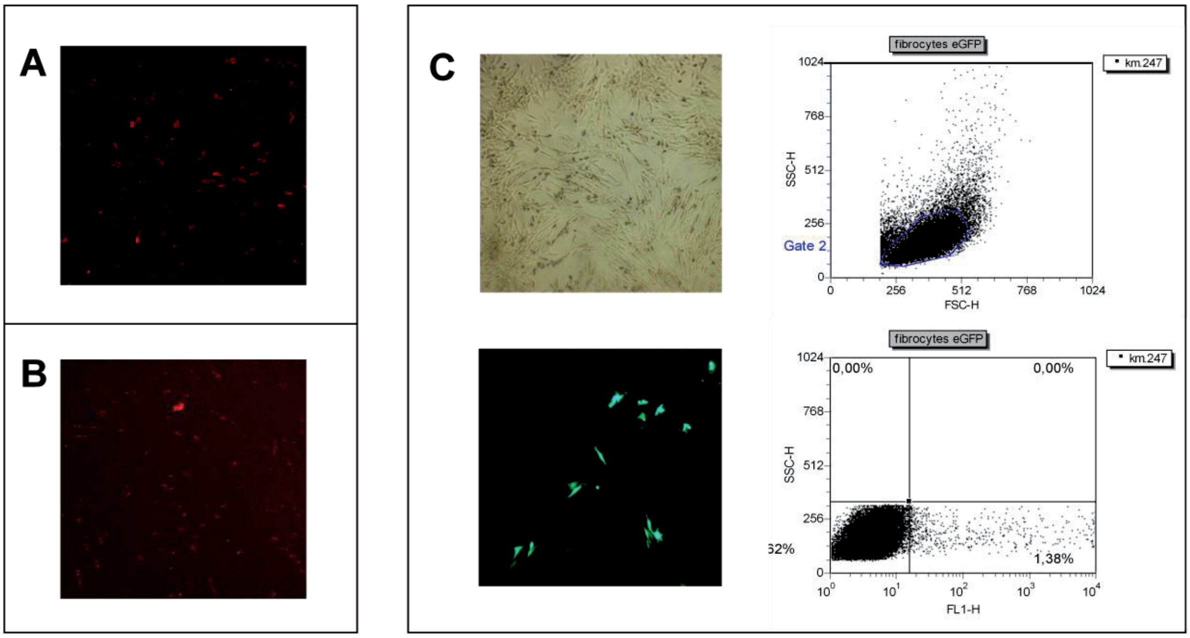


Figure 8 Equine dermal cells transfected with pMCV1.4hgp100 (**A**: Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany), Monoclonal Mouse Anti-Human Melanosome Clone HMB45 (Dako North America Inc., Carpinteria, CA, USA) and polyclonal goat anti mouse PE, Dianova GmbH, Hamburg), pMCV1.4htyr (**B**, confocal microscopemonoclonal, Mouse Anti-Human Tyrosinase Clone T311 (Dako North America Inc., Carpinteria, CA, USA) and polyclonal goat anti mouse PE) or pMCV1.4eGFP (**C**, 1a without filter, 2a with filter (521 nm); fluorescence microscope (Zeiss Axiovert 200M fluorescence microscope; Zeiss, Jena, Germany) and FACS analysis (C 1b forward scatter (FSC) side scatter (SSC) , 2b SSC and fluorescence 1(FL1) (521-nm) FACScan® Beckton Dickinson, Heidelberg, Germany) with a transfection efficacy of approximately 1,5 %.

4.4.2 IFNγ staining

Stimulation of equine PBMCs with PMA and Ionomycin on each time of the CTL-assay induced IFNγ production in an average of 13.7 ±10.26 % (n=160) of cells (figure 10). Unstimulated cells and stimulated cells stained with an IgG1 isotype antibody used as negative control showed no or low numbers of positive events (figure 10). None of the PBMC samples collected from probands showed positive IFNγ-staining after co-incubation with equine dermal cells transfected with pMCV1.4 htyr and hgp100.

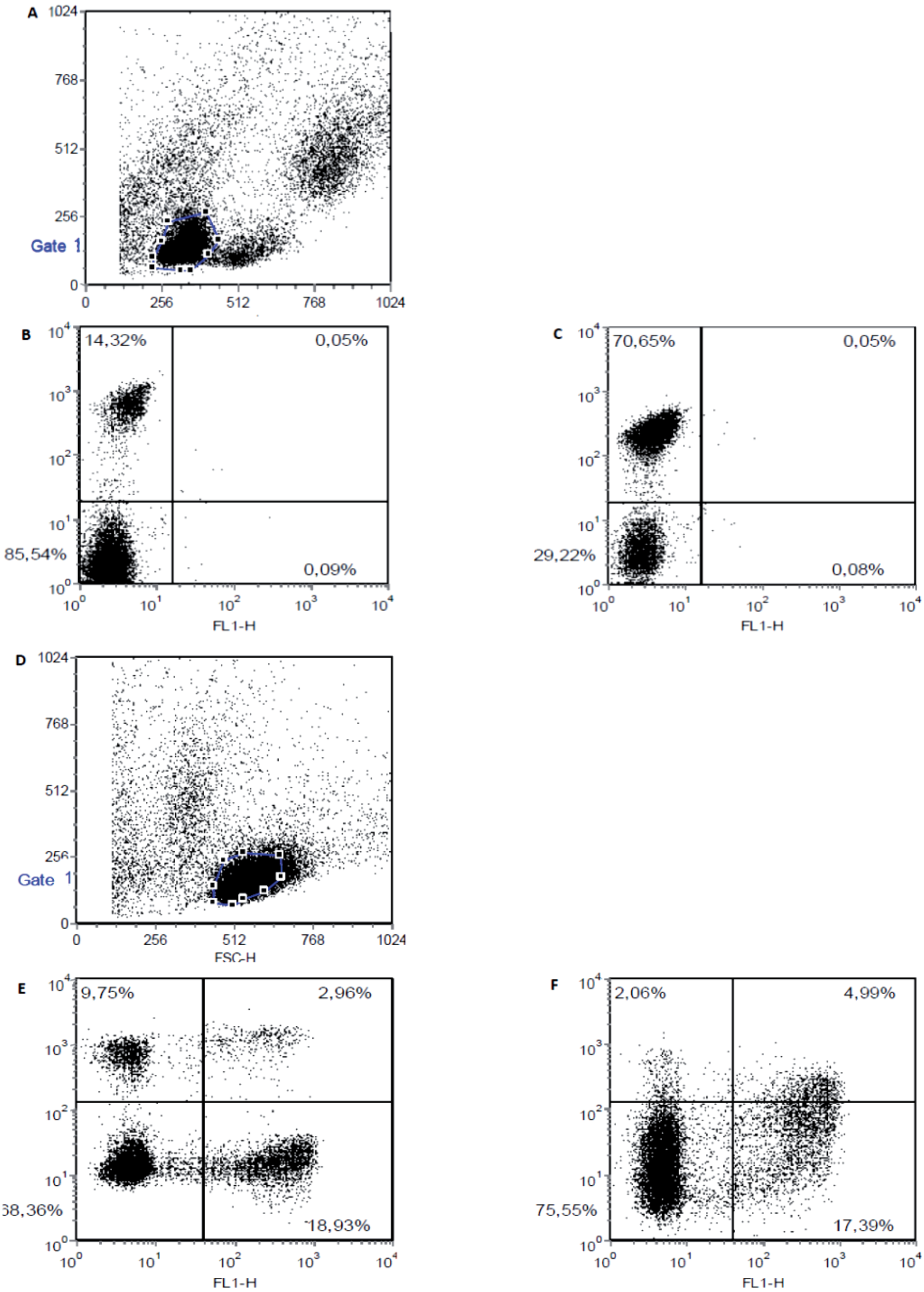


Figure 10 Stimulation of equine PBMC with PMA and Ionomycin lead to IFNγ production in CD4 and CD8 positive cells (here shown for horse No.10).

A: unstimulated PBMC (SSC/FSC)

B: unstimulated PBMC, CD8 (CVS8 undiluted, AG immunology, University of Veterinary Medicine Hannover, Foundation, Rhodamine phycoerythrin (PE) conjugated goat-anti-mouse IgG + IgM (dianova, Hamburg, Germany), mouse anti bovine, CC302, IgG1(AbD Serotec, Kidlington, UK)(FL-1/FL-2)

C: unstimulated PBMC, CD4 (mouse anti horse, CVS4, IgG1, AbD Serotec, Kidlington, UK, PE conjugated goat-anti-mouse IgG + IgM)(FL-1/FL-2)

D: stimulated PBMC (SSC/FSC)

E: stimulated PBMC, CD8 (FL-1/FL-2)

F: stimulated PBMC, CD4 (FL-1/FL-2)

5 Discussion

Cytokines administered as allogenic DNA have been shown to have clinical effects in the therapy of melanomas (HEINZERLING et al. 2001; KISHIDA et al. 2001; MÜLLER et al. 2011). The use of DNA coding for xenogenic gp100 and tyrosinase has been proven to be effective against melanomas in mice, dogs and humans (OVERWIJK et al. 1998; ZHOU et al. 1999; HAWKINS et al. 2000; BERGMAN et al. 2003; GOLDBERG et al. 2005; BERGMAN et al. 2006; WOLCHOK et al. 2007; YUAN et al. 2009). In the present study gene therapy with the equine cytokines IL12 and 18 alone was compared to a combined therapy with xenogenic DNA vaccination with hgp100 and htyr DNA.

5.1 Antitumoral effect: possible influencing factors

5.1.1 Antitumoral effect of IL 12 and 18 DNA

In the recent study a decrease of the relative volume of melanomas in grey horses with natural occurring melanomas after three treatments with MIDGE-Th1® IL12 and eqILRAP-IL18 alone or in combination with human MIDGE-Th1®gp100 or MIDGE-Th1®tyr in combination with the cationic transfection agent SAINT-18 could be measured. Decrease of volume was significant in all groups. Since there were no differences shown between the three groups and all groups received MIDGE-Th1® coding for eqIL 12 and eqILRAP-IL18 complexed with the amphiphilic transfection agent SAINT-18, it can be assumed that the application of IL12/18 constructs and SAINT-18 is responsible for the therapeutic effect. Both cytokines have been used in cancer therapy alone or in combination and have shown antitumoral effects (COUGHLIN et al. 1998; HEINZERLING et al. 2001; KISHIDA et al. 2001; MÜLLER et al. 2011; CHANG et al. 2007; DAUD et al. 2008).

In the treatment of human melanomas using IL12 DNA, antitumoral effects were shown (SCHULTZ et al. 1999). The effect in melanoma treatment and also in other tumors in mice was shown to be mediated by NK, CD8 and CD4 cells (ZITVOGEL et al. 1995; SCHULTZ et al. 1999). In humans the same immune cells were found after

IL12 DNA therapy (DAUD 2008) and also antigen specific immunity against MAGE-1 and MART-1 was established in one study (HEINZERLING et al. 2005).

IFNγ mRNA as well as peritumoral and intratumoral infiltrates of $CD4^+$ and $CD8^+$ cells could be detected in equine melanomas after injection of hIL12 DNA (HEINZERLING et al. 2001). After injection of eqIL12 in only one out of 40 tumor samples, lymphocytes could be detected (MÜLLER et al. 2011). Murine IL12, expressed after muIL12 DNA injection in mice, triggered the endogenous IL12 production whereas hIL12 DNA could not induce this effect, which supports the species specifity of the effect of the cytokines in therapy (SCHULTZ et al. 2000).

Additionally IL18 was used in the present study. IL18 causes antitumoral effects by enhancing T and NK cell maturation, cytotoxicity and cytokine production (DAO et al. 1996; TSUTSUI et al. 1996). Various cytokines IL4, IL6, IL10, IL13, IFNγ and TNFα like can be induced (HOSHINO et al. 1999; HOSHINO et al. 2000; NETEA et al. 2000; YOSHIMOTO et al. 2000). Antiangiogenesis induced by IL18 is mediated by up regulation of antiangiogenic chemokines like interferon inducible protein 10 and monokine induced by IFNγ, and the down regulation of angiogenin (NAGAI et al. 2002). After intratumoral injection of IL18 DNA in mice, numbers of effector T and NK cells increased in the spleen and in peripheral blood (CHANG et al. 2007) indicating a systemic effect of the treatment.

Effects of intratumorally injected equine IL18 and IL12 DNA in equine melanomas were compared by MÜLLER et al. (2011). Both interleukins caused a significant size reduction of spontaneously developing melanomas in horses in the local treated melanoma. Vascularisation and perfusion measured by power-doppler ultrasonography were low before and after therapy with IL18 DNA in grey horses (STÄHLI 2005).

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). Combinations of IL12 and IL18 DNA were shown to prolong survival and reduction of melanoma size in mouse models (KISHIDA et al. 2001), collateral tumor growth was suppressed (TAMURA et al. 2003) and also the growth of distant tumors was reduced (COUGHLIN et al. 1998).

In the present study for the first time the combination of equine DNA coding for IL12 and 18 was used in the treatment of equine melanoma. The treatment provoked a significant size reduction in locally treated and non-locally treated melanoma. Therefore it can be assumed that not only a local effect on the melanoma was achieved but the treatment had also a systemic effect. In other studies equine IL18 and IL 12 DNA have already been used separately in the therapy of equine melanomas by intratumoral injection. Both interleukins induced a significant size reduction in locally treated melanoma (MÜLLER et al. 2011). Compared to the study done by MÜLLER et al. (2011) a systemic effect of the treatment could be seen in the present study. Also in the treatment of equine sarcoids IL12 and 18 DNA was used injected intralesionally. An effect on size could not be detected (GERMANN 2006). In contrary to the trials done by MÜLLER et al. (2011) and GERMANN (2006), the interleukins were not injected separately but together. As synergistic effects of IL12 and 18 were shown in experimental studies in mice (COUGHLIN et al. 1998; HARA et al. 2000; KISHIDA et al. 2001) the improved effect of the treatment used in the present study might be caused by the combination of IL12 and IL18.

In the present study the plasmid DNA was administered in combination with the cationic-amphiphilic transfection agent SAINT18 to improve *in vivo* expression. Transfection efficiency might be enhanced by using a transfection agent compared to DNA only, leading to higher levels of effective cytokines and/or prolonged expression of genes as complexes of DNA with cationic transfection agents have been reported to improve transfer of DNA in cells and to protect DNA from degradation and therefore improve transfection efficiency and immune responses (AUDOUY et al. 2002). The efficacy of *in vivo* transfection was shown for the used combination of MIDGE-Th1® vectors by the luciferase assay. In all samples of skin and muscle evaluated, expression of the enzyme was recognized. Therefore it can be assumed that also the interleukins and the xenogeneic proteins were expressed.

In the present study the IL18 gene was modified with the gene sequence coding for the ILRAP peptide. Ligation of the IL-1beta receptor antagonist protein (ILRAP)-signal sequence to the original gene has been proven to lead to an augmented secretion of the cytokine (O'DONOVAN 2004). As in the studies from GERMANN (2006) and MÜLLER et al. (2011) the IL18 gene was used without the ILRAP gene sequence, the addition of this sequence might also be a cause for the improved antitumoral effect.

The size reduction in this study can be explained by treatment, as no spontaneous regression of equine melanomas is reported. In former studies melanomas treated with empty plasmid or gene in reverse orientation progressed or did not change in size during the time of study (HEINZERLING et al. 2001; MÜLLER et al. 2011).

5.1.2 Further factors possibly influencing the antitumoral effect

5.1.2.1 The antitumoral effect of the combination of DNA and transfection agent

In the present study the DNA was administered in combination with the cationic-amphiphilic transfection agent SAINT18 to improve *in vivo* expression. The combined injection of DNA and transfection reagent can explain local and systemic inflammation and antitumoral effects. In the present study local inflammation with swelling, reddening and exsudation of the injection sites was seen, which was also most probably responsible for the measured increase in size of locally treated melanoma between days 22 and 25 (STÄHLI 2005). Representative for a systemic effect of the treatment, horses showed fever on the day after injection. The relative volume of melanomas decreased significantly in all treatment groups without statistical significant differences between the groups or between treated and non treated melanomas. During the evaluation of unspecific side effects it was demonstrated that the combination of MIDGE-Th1® vectors coding for luciferase in combination with SAINT-18 (group SAINT-18/luc) caused increased body temperature and a local inflammation similar to the one observed in the vaccination groups (IL12/18, gp100 and tyr). The treatment with MIDGE-Th1® vectors coding for IL12/18 alone (group -/IL12/18) or the injection of SAINT-18 alone (group SAINT-18/) did not provoke these reactions. Studies demonstrated that i.v. lipid-protamine plasmid DNA injections resulted in local inflammation of the lung and other organs (DOW et al. 1999a; DOW et al. 1999b, LI et al. 1999; WHITMORE et al. 1999). Increased levels of cytokines like IL-12, IFNγ and TNFα as well as infiltration with immune cells could be seen. As in accordance with the present study, for administration of DNA or transfection agent alone inflammatory effects were not observed (DOW et al. 1999a; DOW et al. 1999b, LI et al. 1999; WHITMORE et al. 1999). Antitumoral effects were documented in studies using lipoplexes with non-coding DNA. This was suggested to be a result of unspecific immune activation, which manifested in cytokine production and immune cell activation (DOW et al. 1999a; DOW et al. 1999b). DOW et al. (1999a, 1999b) showed that lipoplexes with DNA encoding for IL12 induced a more effective antitumor response then lipoplexes with non-coding plasmid DNA and had an effect even on established metastasis. In consideration of the results of these studies and as increased body temperature and local inflammation was also seen after injection of MIDGE-Th1® luciferase and SAINT-18 in the present study, it is most likely that the acute systemic and local inflammation as well as antitumoral effects as a reaction to treatment were at least partially induced by the combination of DNA and transfection agent independent of the expressed proteins.

5.1.2.2 The antitumoral effect of unmethylated CpG-motifs

In the present study MIDGE-Th1® vectors were used because of their reduced backbone DNA. 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). The MIDGE-Th1® vectors used in the present study, even only containing the DNA sequences necessary for expression, contained CpG-motifs (IL12: 134, IL18: 51, gp100: 69, tyr: 68). Therefore an inflammatory response and also an antitumotral effect could have been provoked also by these CpG motifs in the present study.

The induction of inflammation and antitumoral effects by CpG motifs was shown by SCHEULE et al. (2000). Methylation of plasmid DNA partially reduced the inflammatory effect of lipid-protamine plasmid DNA injections (LI et al. 1999; WHITMORE et al. 1999). The comparison of methylated MIDGE-Th1® vectors to the ones used would be necessary to evaluate part of unspecific immune stimulation by CpG motifs.

5.2 Absent antitumoral effect of DNA coding for xenogenic melanoma antigens

As mentioned above size reductions of melanotic lesions were observed in all groups of the present study. Based on this observation we can conclude that the administration of DNA coding for the differentiation antigens hgp100 and htyr had no additional effect in this vaccination regime. In previous studies the use of xenogenic DNA was able to overcome autotolerance and lead to a response against melanomas (YUAN et al. 2009, WOLCHOK et al. 2007).

5.2.1 *In vivo* transfection

Efficacy of DNA gene therapy is determined by several basic events which have to be optimized (SRINIVASAN a. WOLCHOK 2004). The first prerequisite for successful vaccination is the *in vivo* transfection of the DNA and the expression of the antigen. We showed the functionality of the expression system used with MIDGE-Th1® DNA coding for luciferase and SAINT-18. The muscle and skin biopsies transfected *in vivo* with a combination of MIDGE-Th1® vectors coding for luciferase and SAINT-18 showed expression of the luciferase, indirectly measured by emission of RLUs.

5.2.2 Influence of the dosage

A possible reason for failure of xenogenic vaccination might be that the dosage of the administered DNA might not have been ideal in the present study. All horses received a total of 200 µg MIDGE-Th1® IL12 and ILRAP-IL18 each and two groups received additionally 500 µg hgp100 or htyr MIDGE-Th1® respectively. In other studies dosages of 100, 500 and 1500 µg plasmid DNA for gp100 in human (YUAN et al. 2009) and tyrosinase in dogs (LIAO et al. 2006) were used. Dogs treated with the 100 µg and the1500 µg dosage produced antibodies (LIAO et al. 2006), and in humans for all dosages (YUAN et al. 2009) an induction of cytotoxic T-cells could be shown. With an approximately 10 to 50 fold higher body mass of the horses the chosen dosage might have been to low. However, plasmid DNA dosage cannot be compared to MIDGE-Th1® DNA. The effective dose of MIDGE®-DNA is higher compared to plasmid DNA as no unnecessary backbone DNA is content of the vector (ENDMANN et al.2010). In mice MIDGE-Th1® vectors have been used to vaccinate against hepatitis B virus surface antigen (HBsAg). Here 0.5 µg MIDGE-Th1® vectors were sufficient to induce specific antibodies but there was a dose depended increase for 10 and 20 µg per mouse (ENDMANN et al. 2010). For a horse with approximately 500 kg these would have been dosages of 1 g to 20 – 40 g. According to this result, even if the dosage might not be necessarily proportional to the bodyweight, the dosage of the vectors might have been insufficient in this study. Further investigations to define the ideal dosage of MIDGE-Th1® vectors for horses and genes used are needed.

5.2.3 Influence of the proteins chosen for vaccination

Another critical point for the functionality of the vaccination is the proteins chosen for vaccination. As shown by SELTENHAMMER et al. (2004) gp100 and tyrosinase are expressed on equine melanomas. The lesions classified as malignant express higher levels of gp100 and tyrosinase compared to benign forms (SELTENHAMMER et al. 2004). In the present study most horses (18 / 28) had more than seven melanomas and therefore classification as “malignant dermal grey horse melanoma” according to the definition of SELTENHAMMER et al. (2004) is probable. Also HEINZERLING et al. (2001) could detect the gp100 protein in equine melanoma tumor samples. However, SUTTON and COLEMAN (1997) failed to stain melanotic lesions with HMB 45 antibody, specific for gp100. In this study also the positive controls, canine malignant melanoma samples, were not stained positive. A methodological problem might have been present as canine melanoma stain mostly positive for HMB-45 (SULEIMON et al. 2002). Recently tyrosinase expression was proven in equine melanocytic tumors by RT-PCR (PHILLIPS et al. 2012). It is most likely that the melanoma in the present study contained tyr and gp100 in consideration to the results of HEINZERLING (2001), SELTENHAMMER et al. (2004) and PHILLIPS et al. (2012). Therefore it was assumed that the proteins can be used as targets for anti-melanoma vaccination.

5.2.4 Influence of the grade of similarity

In previous studies xenogenic DNA vaccination was able to overcome autoimmunity caused by the difference of the allogenic and xenogenic antigens (OVERWIJK et al. 1998; WEBER et al. 1998; ZHOU et al. 1999; GOLD et al. 2003; BERGMAN et al. 2006, LIAO et al. 2006). The comparison of the antigens showed a homology of aminoacid sequences between the equine and human proteins of 83 % for gp100 and 84 % for tyrosinase. These results are little different to the proteins of mice compared to human for gp100 (75.5%) (ZHOU et al. 1999) and comparable of canine tyrosinase to that of mouse and human (LIAO et al. 2006) (84.4% and 87.5%). In the quoted studies DNA coding for xenogenic melanoma associated antigens was able to overcome autoimmunity and induced an immune response. Therefore it can be assumed that the difference in the aminoacid sequence between the human and the equine antigens is adequate.

5.2.5 Influence of the vaccination interval

The vaccination interval used in this study might not have been appropriate to induce a sufficient antigen specific immunity. The vaccination interval used is comparable to immunisation strategies for infectious diseases in which a sufficient immunity is induced with this protocol. However vaccination against tumors elects a weaker immunity (ROMERO et al. 1997) and therefore a more frequent vaccination might be superior. In other studies more frequent vaccination schemes for therapeutical antitumor vaccines were used (LIAO et al. 2006; WOLCHOK et al. 2007; YUAN et al. 2009) and also in cytokine gene therapy a more frequent administration of DNA every 3 days was more effective than the 6 or 9 days intervals (HEINZERLING et al. 2006). In the study performed by LEMBCKE et al. (2012) healty horses were vaccinated with human tyrosinase DNA every two weeks for four times. IFNγ producing cells could be induced successfully. The successful induction of a specific immune response in the studies of LIAO et al. (2006), WOLCHOK et al. (2007) and YUAN et al. (2009) would also support the hypothesis that a more frequent vaccination scheme could be helpful for inducing a specific immune response. It has been reported that gene expression in the muscle takes place over months and therefore an antigenetic stimulus after DNA vaccination is existent for a longer time compared to administration of proteins (WOLFF et al. 1992). SCHULTZ et al. (2000) showed that after injection of muIL12 plasmid in mice highest plasma levels of IL12 were reached 20 days after injection, but an antimetastatic effect was still seen when B16 tumor cells were injected 30 days after treatment with IL12 plasmid. These results would justify the vaccination schedule of the present study. The luciferase assay used for the verification of *in vivo* transfection was performed after 24 hours in two horses and after 2 weeks in one horse. The RLUs emitted, representing the amount of luciferase expression, were lower after two weeks but the number of horses is too low to make pharmacokinetic conclusions. The kinetics of the expression of the used MIDGE-Th1® DNA and SAINT-18 combination are not investigated until now. This would be necessary to establish an evidence based vaccination interval.

5.2.6 Influence of the route of administration

In the present study horses were vaccinated by i.d. and i.m. injection. The route and form of administration as well as the formulation of the vaccine are factors influencing the transfection efficacy and the efficacy of immune response (ENDMANN et al. 2010).

The intradermal administration was reported to be efficient for vaccination (WAGNER et al. 2000). It has been reported that intradermal gene gun delivery leads to direct transfection of DC in the skin which are subsequently transported to lymphatic organs to present the epitopes to immune cells (PORGADOR et al. 1998). A recent study reports effective immunisation using HBs-Ag MIDGE-Th1® vectors in combination with SAINT-18 injected intradermally. This combination was most potent for the induction of specific antibodies compared to i.m. vaccination with SAINT-18, i.d. injection of naked DNA, subcutaneous injection with SAINT-18 and i.m. injection of naked DNA (ENDMANN et al. 2010). In another study intradermal electroporation of plasmid DNA induced inflammation with cellular infiltration and higher gene expression. Both factors were supposed to enhance immune response to the DNA vaccination (BABIUK et al. 2004).

Intramuscular injection combined with electroporation induced better immunisation against spontaneous human epidermal growth factor receptor 2 (HER2/*neu*) positive tumor development compared to i.d. injection. The immune response was humoral and cellular, but Th1 weighted (SMORLESI et al. 2006).

By comparison of i.m. injection and particle-mediated epidermal delivery of gp100 DNA, a comparable efficacy has been shown with a trend towards increased IFNγ producing $CD8^+$ T-cell generation. For intradermal vaccination a significantly lower dose of DNA was needed (GINSBERG et al. 2010).

After intramuscular injection of plasmid DNA in mouse muscles, DNA uptake and gene expression as well as immunogenicy has been recognized. Hydrostatic pressure by high injection volumina has been supposed to increase uptake, and authors suggested that this might be a cause of lesser effectiveness of DNA vaccines in larger animals (DUPUIS et al. 2000).

Injection of plasmid DNA into lymphnodes has been 100- to 1000-fold more effective for the induction of a CTL response than intradermal and intramuscular delivery (MALOY et al. 2001).

In former studies intratumoral injection of DNA coding for interleukins was performed (HEINZERLING et al. 2001; MÜLLER et al. 2011). We decided not to use this way of delivery, because problems have been reported concerning the constant volume applied with no regard to the tumor size (MÜLLER et al. 2011).

In the present study, the vector was injected in combination with an amphiphilic cationic transfection agent intradermally and intramuscularly. Gene expression took place in the skin as well as the muscle which was shown using the luciferase assay.

In this study it was not differentiated between delivery routes because the main goal was to evaluate safety of treatment and compare efficacy of the genes administered. So we decided to use two routes, intradermal and intramuscular to get adequate gene expression (WAGNER et al. 2000; SMORLESI et al. 2006). The local treatment was also done to see if therapy has an additional local effect. Although i.d. injection was easy to perform, the i.m. route might be more practicable. On the other hand it was reported that for intradermal vaccination less DNA was needed which might be a good argument for i.d. vaccination in large animals (GINSBERG et al. 2010). Inflammation at the injection site was observed in the present study after i.d. injection. Inflammation has been mentioned to enhance DNA uptake in cells, antigen processing and presentation of epitopes as well as migration of antigen presenting cells to lymphoid organs (WEIDE et al. 2008).

The increased induction of CTL response by injection of DNA into lymphnodes (MALOY et al. 2001) might also be interesting for a following study.

Expressed protein could be detected exemplarily after injection of luciferase MIDGE-Th1® DNA complexed with SAINT-18 after intradermal and intramuscular injection. No adequate number of horses was treated and no quantification of expressed luciferase was done to compare the quantity of expression for i.d. and i.m. transfection and no evaluation of the cell type expressing the protein was performed, two factors, which might be crucial for vaccination efficacy (HAUPT 2002, McCLUSKIE 1999, GURUNATHAN 2000).

For optimization of the the treatment intervals, dosage and route of administration an assessment of the pharmacokinetics and –dynamics of the applied therapeutics used in the current study should be performed in the future.

5.3 Correlation of measurements between measurement methods and examiners

Absolute values measured for length, width (calliper and ultrasound measurements) and depth (only ultrasound measurements) correlated well between the examination methods and ultrasound examiners in this study.

In this study ultrasonographic measurements were performed by two examiners. Correlation between the length, width and depth was good. It was concluded that ultrasound is a well comparable method to evaluate size development in equine melanomas. Length showed the best correlation in our study whereas width and depth had lower correlation coefficients. One possible explanation might be that during the length measurement the transducer had better contact than during the width measurement because of the convex surface of the tail, where most melanomas are localized, and therefore poorer defined boundaries might be seen in ultrasonographic pictures. The correlation of depth was even lower compared to the other diameters. It was assumed that this might be a result of intra-melanomal reflection lines which sometimes caused different measurement points. In contrast the findings in the present study it has been described that the measurement of depth by A-scan ultrasonography in uveal melanomas in humans shows very low inter- and intraobserver variations (HARITOGLOU et al. 2002).

The absolute calliper measurements showed a good correlation with the ultrasound measurements for length (0.8556) and width (0.9232). However the absolute volumes calculated by length and width for calliper and by length, width and depth for ultrasound measurements showed a better correlation between the ultrasonographic measurements 0.8716) compared to the measurements by ultrasound examiner 1 and calliper measurements (0.8078). The relative values differed significantly from the ultrasonographic values. This can be explained by the systematic error caused by volume calculation formula which included the depth for ultrasonographic measurements only. In the calliper measurements the smallest diameter was squared and might have caused this difference in this calculated value. With calliper measurements also the skin was measured and not only the melanoma (STÄHLI 2005). That might also have lead to a different value of the volume. Difference might also be due to the different localisation of the melanomas in reference to the skin as melanomas can be localized more superficial or profound. So the diameter which can be measured from outside (calliper) might differ to ultrasound measurements. In ultrasonographic measurements, deviation from true tumor volume measured after resection and inter-rater differences were lower compared to calliper measurements (AYERS et al. 2010). Calliper measurement volume variance was 1.3- fold higher compared to ultrasound (AYERS et al. 2010). These results indicate that ultrasound might be the more reliable method for the evaluation of the tumor volume, especially if subtile changes in volume are expected. In the present study the correlation was good and therefore both methods can be considered as useful.

Methods like magnetic resonance imaging (MRI), X-ray computed tomography (CT) and positron emission tomography (PET) have been used in experimental trials for the measurement of tumors (THERASSE et al. 2000; WEISSLEDER 2002) but the use is impossible in equine medicine, especially for the locations of equine melanomas, due to physical restrictions, availability and costs. For CT and MRI examinations in most cases general anaesthesia is required in horses. These methods are only reported for melanomas of the head region (GERLACH et al. 2007; SASAKI et al. 2007). The possible advantage of more precision does not outweight the related risk. Melanomas measured in this study were almost all located not in the head region. The tail region of the horse cannot be scanned because of the gantry diameter of computer tomographs.

For this reason a non-invasive, cost effective imaging technique that allowed rapid examination without risk for the patient was chosen in the present study.

5.3.1 Evaluation of the response to treatment according to the RECIST criteria

Response Evaluation Criteria in Solid Tumors have been defined previously (THERASSE et al. 2000). According to these criteria there was only one responder (horse number 14) found in this study, whereas the other horses had stable disease. This Shetland pony had only three melanomas with a mean size of 0.45 cm x 0.4 cm (calliper measurement). The locally treated melanoma disappeared completely. The melanomas were small compared to those of the other probands. And the pony had only three melanomas. These criteria might have influenced the therapeutic effect. Another influence might be the body mass of this proband, increasing the relative dosis. At least, ponies and horses have been shown to develop different kinetics in inflammation (WILMINK et al. 1999a; WILMINK et al. 1999b; WILMINK et al. 2001; WILMINK et al. 2002; WILMINK et al. 2003; WILMINK a. VAN WEEREN 2005) which also might influence outcome of immunotherapy.

Due to the nature of equine melanomas, which in general are slow growing tumors with low vascularisation and a pseudo capsule, the regression of the melanomas might be less fast. Also tumor response criteria are originally made for therapies with the intention to complete regression of a malignant tumor. New therapy approaches like immunotherapy might not reach this efficacy (THERASSE et al. 2000). It was concluded that therapy efficacy as intended for human medicine is not reached in the present study.

5.4 Measurement of the humoral response induced by vaccination

No specific antibodies against hgp100 and htyr could be detected in this study with the cell based, flow cytometric antibody assay. Since the clinical results show that there were no differences between the groups no specific immunity might have been induced by the treatment. In other studies a humoral reaction could be induced by vaccination with DNA coding for xenogenic proteins (ZHOU et al. 1999; GOLDBERG et al. 2005). Criteria discussed above (5.2) can be responsible for this the lack of development of specific antibodies. Also the influence of expressed Th1 cytokines (IL12 and 18) could have reduce the humoral answer. The MIDGE-Th1® vectors were shown to be more effective in electing a cellular immunity (ENDMANN et al. 2010).

5.5 Evaluation of cytotoxic response by IFNγ detection using flow cytometry

An IFNγ production after specific stimulation *in vitro* could not be achieved in PBMCs of horses vaccinated with xenogenic DNA coding for melanoma differentiation antigens in this study. As groups show no clinical differences and no detectable amounts of specific antibodies by the method used in this study, it is possible that no specific immunity was induced or was relevant for size reduction of melanomas as mentioned above. Nevertheless, it was already reported that clinical and immunological effects might be paradox and sometimes no clinical effects are observed whether immunity is induced or not (GAJEWSKI 2000; NIELSEN a. MARINCOLA 2000). Also the development of an immunologic response might develop without clinical effect (MARCHAND 1999).

In the present study it was shown that equine dermal cells expressed proteins of interest as demonstrated exemplarily by the staining with monoclonal antibodies against hgp100 and htyr. Unspecific stimulation of PBMCs with PMA and Ionomycin *in vitro* led to IFNγ production by the PBMCs and bovine anti IFNγ antibody could detect equine IFNγ which was already shown before by PAILLOT et al. (2005). These results are basic elements for this test used in this study. The functionality of a similar test for the detection of cytotoxic T-cells was also shown in a previous study by PAILLOT et al. (2005).

In the present study EDCs were used as stimulator cells. In assays evaluating immune responses to infectious diseases, infected autologous cells like equine dermal cells or PBMCs were used before as target or stimulator cells (PAILLOT et al. 2007). Equine dermal cells were shown to express MHC-I complexes on their surface (CASTILLO-OLIVARES et al. 2003). They were used as target cells in a ^{51}Cr-release assay to detect equine arteritis virus (EAV) (CASTILLO-OLIVARES et al. 2003) and EIV (PAILLOT et al. 2007) specific T-cells after culture amplification with EIV-infected autologous PBMC (PAILLOT et al. 2007). It is important to use autologous stimulator cells because of MHC-I restriction. CD8$^+$ cells would not be stimulated by cells of a different MHC-I haplotype. It could be demonstrated that PBMC do not lyse heterologous equine dermal cells in a ^{51}Cr-release assay (CASTILLO-OLIVARES et al. 2003). These studies showed the functionality of autologous EDCs as stimulator cells. It can be concluded, that it was an appropriate choice to use autologous EDCs as stimulator cells in the present study. Recently, equine PBMCs were stimulated with a tyrosinase peptide pool after vaccination of the horses with human tyrosinase DNA. PBMCs could be successfully stimulated and IFNγ production, measured by qRT-PCR, increased during the study (LEMBCKE et al. 2012).

The effector to target ratio was shown previously to be an important factor for the stimulation of CD8$^+$ T-cells (ROMERO et al. 1998a). In the present study the ratio of antigen producing cells and PBMC was approximated depending on transfection efficacy at 1:50 to 1:100. The fraction of CD8 positive cells was at an average of 17.89 % of all PBMCs. Therefore a ratio of 1:10 to 1:20 of stimulator cells to CD8$^+$ cells was realized. Equine dermal cells worked well as targets in an effector (PBMC) to target (EDC, infected) ratio of 1:50 - 1:100 in a ^{51}Cr assay (PAILLOT et al. 2007). This was reported for cells previously stimulated *in vitro*. Therefore it was concluded that the effector to target ratio used in the present study was adequate.

IFNγ producing CD8 positive cells could be detected in horses after vaccination with DNA against infectious diseases (BREATHNACH et al. 2005; PAILLOT et al. 2006). Frequencies of IFNγ producing cells were 1.24-6.25 % (PAILLOT et al. 2006) and 0.77 % (BREATHNACH et al. 2005). Immunity induced against cancer is often weak because of targeting self antigens (HOUGHTON 1994; NAFTZGER et al. 1996).

For neoplasia, especially melanoma, specific T-cell answers have been measured after vaccination by GOLDBERG et al. (2005) in mice with 0.125 % specific CD8 positive cells, by LEWIS et al. (2000) with 1 specific cell in 2000 CD8 positive cells in a melanoma patient and by YUAN et al. (2009) with 0.16 % specific IFNγ producing cells in one of 18 melanoma patients after previous *in vitro* expansion of the cells. These studies show that frequencies of specific $CD8^+$ cells in positively tested post-vaccination samples ranged from 0,025 % (in $CD8^+$ preselected cells (LEWIS et al. 2000)) to 0,16 % (PBMCs, *in vitro* expansion for 10 days (YUAN et al. 2009). These frequencies are very low compared to frequencies measured in infectious diseases (1.24-6.25 % (PAILLOT et al. 2005)). In the present study no *in vitro* expansion and no preselection of $CD8^+$ cells were performed and 50000 events were counted. The method used in the present study might have been too insensitive for the detection of low numbers of specific T-cells.

5.6 Evaluation of unspecific side effects

5.6.1 Increased body temperature

In the present study increased body temperature was a common symptom on the day after treatment. It was supposed that the increased body temperature was caused by the vaccine formulation because of the early onset after treatment. To evaluate this hypothesis horses were injected intradermally and intramuscularly with the combination of MIDGE-Th1® firefly luciferase gene and SAINT-18. They developed fever on the day after injection like horses treated with the interleukin DNA. To differentiate if symptoms were induced by one of the components (IL12/18-MIDGE-Th1® or SAINT-18) horses were treated separately with these. Horses treated with SAINT-18 or MIDGE-Th1® alone did not show any symptoms. So it was concluded, that increased body temperature was a reaction to a direct immune-stimulatory effect of the formulation.

Cationic liposomes led to an influx of T and NK cells in the lung after systemic administration (LI et al. 1999). In dogs this treatment induced fever with an onset of two hours after injection (DOW et al. 2005). Immune reaction could be seen after local and systemic administration of lipoplexes. The reaction was characterized by systemic release of proinflammatory cytokines such as IL6, IL12, TNFα and IFNγ. An infiltration of NK- and T- cells could be seen in the lung. Components of the lipoplexes (DNA, transfection agent) injected alone showed no toxic reaction (AUDOUY et al. 2002). Inflammation and influenza like symptoms have also been described in mice and humans due to administration of plasmid DNA containing CpG-motifs (SCHWARTZ et al. 1997; ALTON et al. 1998).

In the present study MIDGE® vectors were used. Although unmethylated CpG sequences should be rare in these vectors there are, as mentioned above, several CpG motifs in these vectors which might be enough to influence the immunoreactions leading to increased body temperature.

Another cause for increased body temperature could have been due to expressed cytokines. In the present study all treatment groups received MIDGE-Th1® vectors coding for IL 12 and 18. The transcription and translation of the proteins took already place in the first 24 hours as shown by the transfection of the luciferase gene under the same conditions. Increased body temperature has been observed in humans after i.v. injection at a 3 ng/kg dose level of rhIL12, occurring 8-12 h after administration. Following subcutaneous injection of 0.1 µg/kg, 0.5 µg/kg and 1 µg/kg recombinant allogenic interleukin, human cancer patients developed fever after the first injection (MOTZER et al. 1998). In addition, administration of DNA coding for IL12 has been related to fever and influenza like symptoms before (HEINZERLING et al. 2005). Horses with melanomas which received 250 µg human plasmid DNA coding for IL12 or equine plasmid DNA coding for IL12 or IL18 intratumorally showed no fever (HEINZERLING et al. 2001; MÜLLER et al. 2011). In the present study the mean temperatures induced by the vaccine formulations were 38.05 ± 0.69 °C and 38.8 ± 0.47 °C by the MIDGE-Th1®/luc combination. It was concluded, that the interleukins transfected in groups IL12/18, gp100 and tyr had no additional effect on the temperature compared to the MIDGE-Th1®/luc combination.

5.6.2 Local skin reactions

The local skin reactions seen in the present study were swelling, reddening, exsudation, ulceration and depigmentation. Another skin reaction is depigmentation which is a reported effect of immunisation against melanoma self-proteins in mice and dogs and has been shown for xenogenic DNA vaccination against tyrosinase (BERGMAN et al. 2006) or gp75 (WEBER et al. 1998) and allogenic gp100 (HAWKINS et al. 2000). Mice vaccinated with an adenovirus vector coding for human tyrosinase related protein 2 (Adh-Trp2) showed also depigmentation only following i.d., but not i.m. injection, whereas tumor protection was reached by both application paths. Intradermal injection of incomplete Freund´s adjuvant or a control adenovirus vector subsequent to immunisation with Adh-Trp2 also caused depigmentation. The author hypothesized that an inflammatory insult of the skin is required to recruit Trp-2 specific effector cells into the skin (LANE et al. 2004). In the present study, depigmentation was obvious in all groups, indicating that no specific effect of the administered xenogenic DNA coding for tumor proteins was responsible for this effect. The reactions were not seen in the SAINT-18 or IL12/18 treated horses. The combination of DNA and transfection agent was shown to cause inflammation (DOW et al. 1999a; DOW et al. 1999b) and might be responsible for this effect in the skin of horses in the present study. This might lead to depigmentation after melanocyte antigen delivery after necrosis or by unspecific inflammation.

Dogs, comparable to horses in the present study, developed local wheals after receiving tyrosinase plasmid injection at the injection site (BERGMAN et al. 2003).

The increase in volume of locally treated melanomas after injection was not significant. These measurements might have been influenced by local inflammation which caused swelling and reddening at injection site. The local reaction with swelling of the melanomas and the surrounding skin was reported for intratumorally treated melanomas before (STÄHLI 2005).

5.7 Conclusions limitations and outlook

In the present study, MIDGE-Th1® vaccines encoding for equine (eq) IL12 and eqILRAP-IL18 alone or in combination with human gp100 (hgp100) or human tyrosinase (htyr) complexed with the transfection agent SAINT-18 were tested in 27 grey horses with natural occurring melanoma. The horses showed no abnormalities in the general examination, except fever, at all times of the study. Therefore the treatment can be assumed to be safe. Following administration in 40 out of the 81 injections (27 horses, 3 injections each), increased body temperature over 38.2 °C (38.7 ± 0.59 °C) was recorded and usually lasted for one day (in two horses for two days). Local inflammatory reactions such as redness, exudation, ulceration and depigmentation were observed in all groups starting at day 3 after the first application. These symptomes of local and systemic inflammation are most probably not mediated by the proteins expressed but by the formulation of the vaccine with the DNA-transfection agent complex and the contained CpG-motifs as the same reaction could be observed after injection of a combination of SAINT-18 and MIDGE-Th1® luciferase.

The treatment with allogeneic DNA encoding eqIL12 and eqILRAP-IL18 in combination with SAINT-18 showed a systemic antitumor effect against grey horse melanoma. The relative tumor volume (%) was significantly reduced (calliper measurement: 71.5 ± 29.73%, ultrasound examiner 1: 87.0 ± 24.42%, ultrasound examiner 2: 85.0 ± 33.19%) compared to the initial value (100%). There were no significant differences detected between the treatment groups or peritumorally treated versus non local treated melanoma. There was no additional clinical or immunological effect observed by vaccination with MIDGE-Th1® vectors encoding the differentiation antigens hgp100 and htyr. By applied research methods for the detection of specific cytotoxic $CD8^+$ T-cells and specific antibodies against hgp100 and htyr, neither a cellular nor a humoral immune response against the vaccine antigens could be detected.

It remains unclear whether the antitumoral effect was caused by expressed interleukins, unmethylated CpG motifs or the induction of an unspecific immunereaction by the combination of DNA and transfection reagent.

Reasons for the absence of a specific immune response may be an inadequate dosage, or treatment interval. It is also possible that the sensitivity of immunoassays was too low to detect a subclinical immune response.

To evaluate the factors causing the response to treatment, different control groups to the eqIL12/eqILRAP-IL18MIDGE-Th1®/SAINT-18 treatment like noncoding MIDGE-Th1®, noncoding MIDGE®, noncoding plasmid, eqIL12/eqILRAP-IL18 MIDGE®, eqIL12/eqILRAP-IL18 plasmid all without or in combination with SAINT-18 and methylated or unmethylated as well as SAINT-18 alone would be necessary. This would be important to figure out the unspecific effect of DNA/transfection agent combination with the different DNA constructs, the effect of unmethylated CpG motifs and the effect of SAINT-18 alone. In this study only patients were used. For ethical reasons and since owner's compliance was vital, it was decided to treat no group with a placebo. The placebo treatment which has been performed a former study showed no therapeutical effect (MÜLLER et al. 2011). The unspecific therapeutical effect of the treatment used in the present study, as mentioned above, has to be evaluated. As there might be differences in susceptibility to lipoplexes in different species (DE KANTER 2002) the use of horses would be ideal to evaluate this effect which might have been important in this study.

Measurements were performed by calliper as well as by ultrasound. Measurements showed a good correlation and can be considered as feasible.

The evaluation of cytotoxic T-cells might not have been functional or sensitive enough in this study. A positive control to check the functionality of this assay in a system which was previously proved to be immunogenic is needed. Therefore, horses could be vaccinated with a DNA vaccine encoding for an EHV1 gene (gene 64) which has already been shown to be immunogenic (KYDD et al. 2006; PAILLOT et al. 2006). Equine dermal cells could be transfected with this gene intended for use in in-vitro stimulation of PBMC.

Further studies are required concerning ideal dose and treatment frequency. Therefore, not only treatment efficacy, but also measurements of cytokines in plasma and *in vivo* transfection efficacy have to be investigated. Also long-time effect should be evaluated in order to determine efficacy and safety of the treatment.

6 Summary

Mählmann, Kathrin:

Minimalistic immunologically defined gene expression T helper cell 1 (MIDGE-Th1®) vectors coding for Interleukin (IL) 12 and -18 in combination with the transfection agent SAINT-18 have systemic antitumoral effects on equine melanomas

Equine melanomas are the most common neoplasms in older grey horses. Until now, no curative therapy is established. Xenogenic DNA vaccination represents a promising therapeutic approach.

Goal of the present study was to evaluate the safety and clinical efficacy of vaccination of grey horses with natural occurring melanomas with MIDGE-Th1® vectors encoding for equine (eq) IL12 and eqILRAP-IL18 alone or in combination with human gp100 (hgp100) or human tyrosinase (htyr).

Three groups (each n=9) of adult grey horses with variable numbers of melanomas received three intramuscular and three intradermal peritumoral vaccinations. Each group was treated with MIDGE-Th1® vectors coding for eqIL12 and eqILRAP-IL18. One group (group gp100) was additionally treated with 500 µg hgp100MIDGE-Th1® and one group with 500 µg htyrMIDGE-Th1® (group tyr). The third group did not receive additional DNA (group IL12/18). Vectors were complexed with the transfection agent SAINT-18.The injections were performed on days 1, 22 and 78 intradermally around one selected melanoma (locally treated melanoma) and intramuscularly as a systemic treatment. A general examination of the horses and evaluation of local reactions at the injection site were performed daily during hospitalisation (on the day of each injection and 3 days after each injection as well as on day 120). In each horse the locally treated melanoma and up to 8 non-locally treated melanomas were measured before each injection and on day 120 by calliper and ultrasound. The relative volumes were calculated in relation to day 1 (100%).

For the evaluation of the generation of specific CD8+ cytotoxic T-cells against hgp100 and htyr, peripheral blood mononuclear cells (PBMCs) isolated before each injection and on day 120, were stimulated in vitro with autologous dermal cells transfected with hgp100 or htyr. Interferon γ (IFN γ) as a response to this specific stimulation was measured by intracellular immunofluorescence staining.

Specific serum antibodies against hgp100 and htyr were measured at equivalent time points as cytotoxic T-cells using a cell based flow cytometric assay. Human embryonic kidney cells, transfected with hgp100 or htyr served as a source for antigens.

The only consistent abnormal finding upon clinical examination of the treated horses was an increased body temperature. A significant increase in body temperature (38.7 ± 0.59 °C) was recorded after 40 of the 81 injections, and usually lasted for one day. Local inflammatory reactions and depigmentation were observed in all groups starting at day 3 after the first application.

The relative tumor volume (%) was significantly reduced to 71.5 ± 29.73% (calliper measurements) and 87.0 ± 24.42% (ultrasound measurements). There were no significant differences detected between groups (gp100, tyr and IL12/18). No difference was seen in reduction of tumor volume between locally treated and non-locally treated melanoma.

Neither a cellular nor a humoral immune response against the vaccine antigens could be detected.

In this study, treatment with eqIL12 and eqILRAP-IL18 DNA in combination with SAINT-18 showed a systemic effect against grey horse melanoma. There was no additional clinical or immunological effect observed by vaccination with MIDGE-Th1® vectors encoding hgp100 and htyr. It remains unclear whether the antitumoral effect was caused by the interleukins, unmethylated CpG motifs or the induction of an immune reaction by the combination of DNA and transfection reagent. Reasons for the absence of a specific immune response may be an inadequate selection of dosages or treatment interval. It is also possible that the sensitivity of the immunoassays was too low to detect a subclinical immune response. Further studies to characterize the antitumoral effects of the treatment used in this study and modifications of the treatment protocol to achieve a potential specific immune response are warranted.

7 Zusammenfassung

Mählmann, Kathrin:

Minimalistische immunologisch definierte Genexpression T Helferzell 1 (MIDGE-Th1®) Vektoren kodierend für Interleukin (IL) 12 und -18 in Kombination mit dem Transfektionsreagenz SAINT-18 haben systemische antitumorale Effekte gegen equine Melanome

Equine Melanome sind die häufigsten Neoplasien bei älteren Schimmeln. Bis heute ist keine kurative Therapie etabliert. Xenogene DNA Vakzination stellt einen vielversprechenden Therapieansatz dar.

Ziel der vorliegenden Studie war die Sicherheit der Anwendung, sowie klinische und immunologische Wirksamkeit einer Vakzination von Schimmeln mit natürlich vorkommenden Melanomen mit MIDGE-Th1® Vektoren kodierend für equines (eq) IL12 und eqILRAP-IL18 allein oder in Kombination mit humanem gp100 (hgp100) oder humaner Tyrosinase (htyr), zu zeigen.

Drei Gruppen adulter Schimmel (je n=9) mit unterschiedlich vielen Melanomen erhielten drei intramuskuläre und drei intradermale, peritumorale Impfungen. Jede Gruppe wurde mit je 200 µg MIDGE-Th1® Vektoren kodierend für eqIL12 und eqILRAP-IL18 behandelt. Eine Gruppe (Gruppe gp100) wurde zusätzlich mit 500 µg hgp100 MIDGE-Th1® und eine Gruppe mit 500 µg htyr MIDGE-Th1® (tyr) behandelt. Die dritte Gruppe (Gruppe IL12/18) erhielt keine zusätzliche DNA. Alle Vektoren wurden mit dem Transfektionsreagenz SAINT-18 gemischt. Die Injektionen wurden an den Tagen 1, 22 und 78 intradermal an ein ausgesuchtes Melanom (lokal behandeltes Melanom) und intramuskulär als systemische Behandlung durchgeführt. Eine klinische Allgemeinuntersuchung und eine Untersuchung lokaler Reaktionen an der Injektionsstelle wurden täglich während des Klinikaufenthaltes (am Tag jeder Injektion und drei Tage nach jeder Injektion, sowie am Tag 120) durchgeführt. Bei jedem Pferd wurden das lokal behandelte Melanom und bis zu acht weitere nicht lokal behandelte Melanome vor jeder Injektion und an Tag 120 mittels Schublehre

und Ultraschall gemessen. Die relativen Volumina wurden für jeden Untersuchungszeitpunkt in Relation zum ersten Tag (100%) errechnet.

Für die Evaluation der Bildung spezifischer zytotoxischer T-Zellen gegen hgp100 und htyr wurden Periphere Mononukleäre Blutzellen (PBMCs) vor jeder Injektion und an Tag 120 isoliert und *in vitro* mit hgp100 oder htyr DNA transfizierten autologen dermalen Zellen stimuliert. Interferon γ (IFN γ), als Antwort auf diese spezifische Stimulation, wurde mittels einer intrazellulären Immunfluoreszenz Färbung gemessen. Spezifische Serum Antikörper gegen hgp100 und htyr wurden zu äquivalenten Zeitpunkten wie die zytotoxischen T-Zellen mit einem Zell-basierten, durchflusszytometrischem Test gemessen. Humane embryonale Nierenzellen, transfiziert mit hgp100 oder htyr DNA, dienten als Antigenquelle.

Der einzig konsistente abnormale Befund in der Allgemeinuntersuchung der Pferde war eine erhöhte Körpertemperatur. Eine signifikante Steigerung der Körpertemperatur (38.5 ± 0.54 °C) wurde nach 40 der 81 Injektionen festgestellt und hielt normalerweise für einen Tag an. Lokale Entzündungsreaktionen und Depigmentation wurden in allen drei Gruppen ab dem 3. Tag nach der ersten Behandlung beobachtet. Die relativen Tumorvolumina reduzierten sich signifikant auf 71.5 ± 29.73 % (Schublehrenmessung) und 87.0 ± 24.42 % (Ultraschallmessung). Es konnten keine signifikanten Unterschiede zwischen den Behandlungsgruppen (gp100, tyr und IL12/18) festgestellt werden. Es wurde kein Unterschied in der Reduktion des Tumorvolumens zwischen lokal behandelten und nicht lokal behandelten Melanomen festgestellt. Es konnte weder eine zelluläre noch eine humorale Immunantwort gegen die Impfantigene festgestellt werden.

Die Behandlung mit eqIL12 und eqILRAP-IL18 DNA in Kombination mit SAINT-18 zeigte in dieser Studie einen systemischen Effekt gegen das equine Melanom, während die Vakzination mit hgp100 und htyr MIDGE-Th1® Vektoren keinen zusätzlichen klinischen oder immunologischen Effekt aufwies.

Es bleibt unklar, ob der antitumorale Effekt durch die Wirkung der Interleukine, durch unmethylierte CpG-Motive oder durch die Induktion einer Immunreaktion durch die Kombination von DNA und Transfektionsreagenz zustande kam.

Ursachen für eine fehlende spezifische Immunreaktion können eine ungeeignete Auswahl der Dosierungen oder des Therapieintervalls sein. Zudem ist es möglich, daß die angewandten immunologischen Testmethoden eine fehlende Sensitivität aufweisen, um eine subklinische Immunantwort zu detektieren.

Weitere Studien, um den antitumoralen Effekt der in dieser Studie angewendeten Behandlung zu charakterisieren und eine Modifizierung der Behandlung zur Induktion einer potentiellen spezifischen Immunreaktion zu erreichen, sind notwendig.

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Int Immunol 7, 1135-1145

9 Appendix

9.1 Materials

9.1.1 Instruments

Auctoclav Typ GE406	Getinge AB, Getinge, Sweden
BLAST-software	National Center for Biotechnology Information, National Library of Medicine Bethesda, MD, USA
Bunsen burner	Wartewing, Göttingen
calliper	Conmetall, Celle, Germany
cellcounting chamber, Bürker	Brand, Wertheim, Germany
CellQuest™ Pro 4.0.2	Becton Dickinson, Heidelberg, Germany
Centricon, Filtrationsystem	Amicon, Berverly, MA, USA
Centrifuge SORVALL	Heraeus, Hanau, Germany
centrifuge „ Multifuge“ 1S-R	Thermo scientific, Osterrode, Germany
centrifuge „Varifuge K“	Heraeus, Hanau, Germany
CO_2 flask for sterile filtration	Kohlensäurewerke Hannover, Hannover, Germany
delay line, SONOKIT soft	Sonogel Vertriebs GmbH, Bad Camberg, Germany
Digital camera Olympus DP70	Olympus, Hamburg, Germany
FACScan®	Becton Dickinson, Heidelberg, Germany
FcsExpress software	De Novo Software Los Angeles, CA, USA
Freezer -80°C	Kendro, Hanau, Germany
Freezer (BioFreezer)	Forma Scientific, Marietta, Ohio, USA

Gelelectrophorese (Gene ReadIR 4200)	LiCOR Biosciences, Lincoln, Nebraska, USA
GloMax® 20/20 Luminometer	Promega Corporation, Madison, Wisconsin, USA
ice mashine, type UBE 30-10	Ziegra, Isernhagen, Germany
Incubator	Infors (HT), Bottmingen, Switzerland
Incubator with CO_2 supply, serial number 5060	Heraeus, Hanau, Germany
JMP programme	SAS Institute Inc., Cary, NC, USA
fridge	Liebherr-International AG, Bulle, Switzerland
laboraotory weighing mashine BL310	Sartorius GmbH, Göttingen, Germany
laboratory fine weight	Mettler, Zurich, Switzerland
laminar flow workbank, Laminair HL2448	Heraeus, Hanau, Germany
Leica TCS SP2 confocal microscope	Leica Microsystems GmbH, Wetzlar, Germany
LogiQ P5, ultrasound machine	General Electrics, Connecticut, USA
Microplate-shaker AM69 Microshaker	Dynatec, Zug, Switzerland
Microsoft Excel 2003	Microsoft Deutschland GmbH, Unterschleißheim, Germany
Mikro 22R, centrifuge	Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany
NCSS	NCSS, Kaysville, Utha, USA
neoLab-mixer Vortex	neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg, Germany

forceps	Eickemeyer, Tuttlingen, Germany
pipette ”pipetman”, 1-20µl, 10-100 µl, 20-200 µl, 100-1000 µl	Gilson, Villers Le Belle, France
Pipette 1-10µl	Sarstedt, Frickenhausen, Germany
stainless steel pressure vessel	Alloy products, Wisconsin, USA
sterilisator type ST5050	Haereus, Harnau, Germany
T Personal 20, Thermocycler	Biometra Göttingen, Germany
TissueLyser II	Quiagen GmbH, Hilden, Germany
Tungsten Carbide Bead	Quiagen GmbH, Hilden, Germany
Zeiss Axiovert 200M Fluorescencemicroscope	Zeiss, Jena, Germany

9.1.2 Clinical materials

BD Microlance 3 Needles Black 27g x 0,75 Inch	Becton Dickinson, Heidelberg, Germany
BD Microlance 3 Needles Black 22g x 1.5 Inch	Becton Dickinson, Heidelberg, Germany
BD Microlance 3 Needles Pink 18G x 2 Inch	Becton Dickinson, Heidelberg, Germany
BD, Vacuette, Serum tube	Becton Dickinson, Heidelberg, Germany
BD, Vacuette, EDTA tube	Becton Dickinson, Heidelberg, Germany
BD, Vacuette, Sodium heparine tube	Becton Dickinson, Heidelberg, Germany

Biopsy Punch 0,8cm	GlaxoSmithKline GmbH & Co. KG, Munich, Germany
Contact gel for use in ultrasonic diagnostic, ultrasonic therapy, ECG and defibrillation	Sonogel Vertriebs GmbH, Bad Camberg, Germany
Stand off, SONOKIT soft	Sonogel Vertriebs GmbH, Bad Camberg, Germany
HibiScrub, Chlorhexidine gluconate 4%w/v	Regent Medical, Manchester, UK
LogiQ P5	General Electrics, Connecticut, USA
NeoLab-mixer Vortex	NeoLab Migge Laborbedarf-Vertriebs GmbH
PBS-Dulbecco w/o Ca2+ and Mg2+	Biochrom AG, Berlin, Germany
SAINT-18 (1-methyl-4-(cis-9-dioleyl) methyl-pyridinium-chloride, SAINT-18	Synvolux Therapeutics, Groningen, The Netherlands
Surgical Disposeable Scalpel	B. Braun Melsungen AG, Tuttlingen, Germany
Syringe, BD DISCARDIT 2,5,10ml	Becton Dickinson, Heidelberg, Germany
Syringe, Injekt 1ml	Braun, Melsungen, Germany
Vacuette® K3E, 4ml	Greiner bio-one, Kremsmünster, Austria
Vacuette® LH, 4ml	Greiner bio-one, Kremsmünster, Austria
Vacuette® NH, 9ml	Greiner bio-one, Kremsmünster, Austria

Monoclonal Mouse Anti-Human Tyrosinase	Dako North America Inc., Carpinteria, CA, USA
Vacutainer System, caps	Becton Dickinson, Heidelberg, Germany
Vacutainer Brand Luer adapter	Becton Dickinson, Heidelberg, Germany
MIDGE-Th1® luciferase DNA	Mologen AG, Berlin, Germany
MIDGE-Th1® gp100 DNA	Mologen AG, Berlin, Germany
MIDGE-Th1® tyrosinase DNA	Mologen AG, Berlin, Germany
MIDGE-Th1® IL12 DNA	Mologen AG, Berlin, Germany
MIDGE-Th1® IL18 DNA	Mologen AG, Berlin, Germany

9.1.3 Laboratory materials

25 cm^2 culture flasks	Nunc GmbH&Co KG, Langenselbold, Germany
3,5 ml cryotubes	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
15 ml centrifuge tube	Corning Incorporated, Corning, NY, USA
50ml centrifuge tube	Corning Incorporated, Corning, NY, USA
6-well plate	Greiner Bio-One GmbH, Frickenhausen, Germany
96-well round-bottom plate	Gechno Plastic Products, TPP®, Trasadingen, Switzerland
cell culture multiwellplate, 6 well, PS, with cover plate, sterile	Greiner Bio-One GmbH, Frickenhausen, Germany
Tube, 1,5ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Tube, 2ml	Greiner Bio-One GmbH, Frickenhausen, Germany
FACS-tubes, 5ml	Becton Dickinson, Heidelberg, Germany
round-bottom 96-well-plate	Gechno Plastic Products, TPP®, Trasadingen, Switzerland
Surgical Disposeable Scalpel	B. Braun Melsungen AG, Tuttlingen, Germany
Tungsten Carbide Bead	Quiagen GmbH, Hilden, Germany
Luciferase Assay System	Promega Corporation, Madison, Wisconsin, USA

NucleoSpin® 8 Extract II	Macherey-Nagel GmbH&Co.KG, Düren, Germany
Sterile filter, Sterifix 0,2µm	B. Braun Melsungen AG, Tuttlingen, Germany
pipette tips	Sarstedt, Frickenhausen, Germany
Combitips, 1,25ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Combitips, 2,5ml	Greiner Bio-One GmbH, Frickenhausen, Germany
microscope slide	Marienfeld, Lauda Königshofen, Germany
Coverglass	Schott AG, Grünenplan, Germany
Saugpipetten 10ml	Sarstedt AG & Co., Nümbrecht, Germany

9.1.4 Laboratory reagents

Accutase®	PAA Laboratories GmbH, Pasching, Austria
Albumine fraction V, bovine pulverisated 98%, Bovine serum albumine (BSA)	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
ATP	MBI Fermentas, Vilnius, Lithuania
Brefeldin A	Sigma Aldrich Chemie, GmbH, Taufkirchen, Germany
CMV-immediate-early enhancer/promoter	MOLOGEN AG, Berlin, Germany
Collagenase A	Roche Holding GmbH Deutschland, Mannheim, Germany
CVS8 undiluted	AG imunology, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany
Deoxynucleotide triphosphates, dNTPs	MBI Fermentas, Vilnius, Lithuania
Dispase I (neutral protease, grade I)	Roche Holding GmbH Deutschland, Mannheim, Germany
DMSO, Dimethylsulfoxide	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Eco31-I	MBI Fermentas, Vilnius, Lithuania
EcoR-I	MBI Fermentas, Vilnius, Lithuania

Ethanol 641, absolute, denatured	CG Chemicalien, Laatzen, Germany
Ethidiumbromide	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
FACS-buffer	500ml Dulbecco's PBS, 500µl NaAcid (10%), 2,5g BSA (all Carl Roth GmbH&Co. KG, Karlsruhe, Germany)
FCS (fetal calf serum)	Biochrom, Berlin, Germany
Fibrocyte culture medium	Roswell Park Memorial Institute Medium (RPMI1640), supplemented with 20% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin (all PAA Laboratories GmbH, Pasching, Austria)
Goat anti horse fluorescein Isothiocyanate (FITC)	Jackson ImmunoResearch Laboratories, West Grove, USA
G-Puffer	MBI Fermentas, Vilnius, Lithuania
Hairpinoligodeoxyribonucleotide	Eurogentec, Germany
HEK 293	courtesy of Dr. Jessika Müller, Hannover, Germany
HEK-culture medium	Minimal Essential Medium Eagle (MEM), supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% non-essential amino acids, 1% pyruvat, and 1% penicillin/streptomycin (all PAA Laboratories GmbH, Pasching, Austria)
Ilrap-1-fw	Microsynth, Balgach, Switzerland
CAT GGA AAT CTG CAG AGG CCT CCG CAG TCA CCT AAT CAC	

Ilrap-1-rv	Microsynth, Balgach, Switzerland
TTA GGT GAC TGC GGA GGC CTC TGC AGA TTT CCA TGG TAC	
Ilrap-2-fw	Microsynth, Balgach, Switzerland
TCT CCT CCT CTT CCT GTT CCA TTC AGA GAC GAT CTG CCT GC	
Ilrap-2-rv	Microsynth, Balgach, Switzerland
GCA GAT CGT CTC TGA ATG GAA CAG GAA GAG GAG GAG AGT GA	
Ionomycin	Merck KGaA, Darmstadt, Germany
Ampicillin	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
LB (lysogeny broth) selection agar	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
Ligase	MBI Fermentas, Vilnius, Lithuania
LigasePuffer	MBI Fermentas, Vilnius, Lithuania
Lipofectamine LTX	Invitrogen, Darmstadt, Germany
Luciferase Assay System	Promega Corporation, Madison, Wisconsin, USA
Lymphocyte Separation Medium LSM 1077	PAA Laboratories GmbH, Pasching, Austria
MEM	PAA Laboratories GmbH, Pasching, Austria
MgCl2	MBI Fermentas, Vilnius, Lithuania
MgCl2- buffer	MBI Fermentas, Vilnius, Lithuania
Monoclonal Mouse Anti-Human Tyrosinase Clone T311	Dako North America Inc., Carpinteria, CA, USA

Monoclonal MouseAnti-Human Melanosome Clone HMB45	Dako North America Inc., Carpinteria, CA, USA
mouse anti bovine, CC302, IgG1	AbD Serotec, Kidlington, UK
mouse anti horse, CVS4, IgG1	AbD Serotec, Kidlington, UK
Natrium Acid, $NaHCO_3$	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
non-essential amino acids	PAA Laboratories GmbH, Pasching, Austria
oligodeoxyribonucleotide (ODN)	Microsynth, Balgach, Switzerland
Opti-MEM®	Invitrogen, Darmstadt, Germany
paraformaldehyde	Carl Roth GmbH&Co. KG, Karlsruhe, Germany
PBS	Dulbecco's PBS, Biochrom AG, Berlin, Germany
pcDNA3 gp100	Dr. Robbins und Dr. Rosenberg, National Cancer Intitute, Bethesda, MD, USA
pciNeoIL18	courtesy L.Nicholson, University of Glasgow, Scotland
penicillin/streptomycin liquid, 100x	Invitrogen, Darmstadt, Germany
permeabilisation buffer	500ml Dulbecco's PBS, 500µl NaAcid (10%), 2,5g BSA (both Carl Roth GmbH&Co. KG, Karlsruhe, Germany), 0,5g Saponin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)
Phorbol 12-myristat 13-acetat, (PMA)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany

PLUS™ reagent	Invitrogen, Darmstadt, Germany
pMCV1.4	MOLOGEN AG, Berlin, Germany
pMCV1.4 gp100	MOLOGEN AG, Berlin, Germany
pMCV1.4 tyr	MOLOGEN AG, Berlin, Germany
pMCV1.4eGFP	MOLOGEN AG, Berlin, Germany
PMEL17li (5'-ATT ATA GAA TTC ATG GAT CTG CTA AAA AGA TG-3')	Microsynth, Balgach, Switzerland
PMEL17mut1re (5'-AAA CAA GGT CTC TCT TCT GGG ACC AAG AGC-3')	Microsynth, Balgach, Switzerland
PMEL17mut2li (5'-AAC AAC GGT CTC TGG GTG GAA ACC ACA GCT AG-3')	Microsynth, Balgach, Switzerland
PMEL17re (5'-TTA TAT GAG CTC AGA CCT GCT GCC CAC-3').	Microsynth, Balgach, Switzerland
PNK Polynukleotidkinase A	MBI Fermentas, Vilnius, Lithuania
PNK Polynukleotidkinase Puffer	MBI Fermentas, Vilnius, Lithuania
Polyacrylamide gel (5,5% KB-Plus)	LiCOR Lincoln, Nebraska, USA
Polyclonal goat anti mouse FITC	Dianova GmbH, Hamburg, Germany
Pyruvate	PAA Laboratories GmbH, Pasching, Austria

Rhodamine phycoerythrin (PE) conjugated goat-anti-mouse IgG+IgM	Dianova, Hamburg, Germany
RPMI1640 with HEPES and glutamine (3%)	PAA Laboratories GmbH, Pasching, Austria
SacI	MBI Fermentas, Vilnius, Lithuania
Saponin	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
SOB medium	(Carl Roth GmbH&Co. KG, Karlsruhe, Germany)
SOC medium	SOB medium substituted with 20% Glucose (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
T4Ligase	MBI Fermentas, Vilnius, Lithuania
T7 DNA Polymerase	MBI Fermentas, Vilnius, Lithuania
tango buffer	MBI Fermentas, Vilnius, Lithuania
TaqPolymerase LC	MBI Fermentas, Vilnius, Lithuania
XL1-Blue® chemo-competent E.coli (CC4, H1)	XL1-Blue® by Stratagene (Waldbronn, Germany); CC4 by Mologen (Berlin, Germany)

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Wissenschaftliche Reihe der Klinik für Pferde

Forschung ist die Grundlage des Gewinns neuer Erkenntnisse. Die Herausgeber beschäftigen sich seit vielen Jahren mit der wissenschaftlichen Bearbeitung von unterschiedlichen Aspekten der Pferdemedizin. Diese wissenschaftliche Reihe verfolgt das Ziel, Ergebnisse, die im Rahmen von Dissertationen an der Klinik für Pferde der Stiftung Tierärztliche Hochschule Hannover erarbeitet wurden, anderen Wissenschaftlern und einer interessierten Öffentlichkeit zugänglich zu machen. Damit wird kontinuierlich eine umfassende Darstellung aktueller wissenschaftlicher Themen veröffentlicht.

In the present study twenty-seven adult grey horses with variable numbers of melanomas were treated with MIDGE-Th1® vectors coding for equine IL12 and IL18 only or additionally with xenogenic human MIDGE-Th1® vectors coding for gp100 or tyrosinase. Melanomas were measured prior to each injection and on day 120 by calliper and ultrasound. Specific CD8+ cytotoxic T-cells and specific serum antibodies against hgp100 and htyr were measured using cell based flow cytometric assays. The relative tumor volume decreased significantly in all groups. No significant differences were detected between groups (gp100, tyr and IL12/18) or locally treated and non-locally treated melanomas. Neither a cellular nor a humoral immune response could be detected.

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