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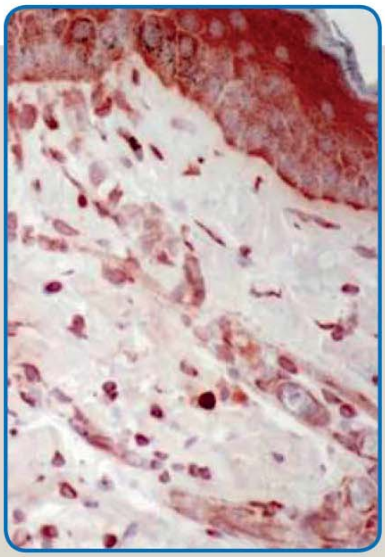
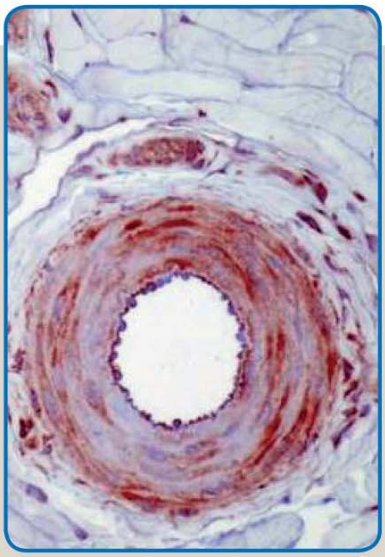
Herausgegeben von
Karsten Feige, Peter Stadler,
Harald Sieme, Bernhard Ohnesorge

Christiane Liliane Schnabel

Acute immune response of healthy horses to linear DNA encoding Interleukin 12 and Interleukin 18 complexed with SAINT-18





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University of Veterinary Medicine Hannover

Acute immune response of healthy horses to linear DNA encoding Interleukin 12 and Interleukin 18 complexed with SAINT-18

Thesis

Submitted in partial fulfilment of the requirements for the degree

-Doctor of Veterinary Medicine-

Doctor medicinae veterinariae

(Dr. med. vet.)

by

Christiane Liliane Schnabel

Wuppertal

Hannover 2015

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Academic supervision:	Prof. Karsten Feige, Clinic for Horses of the University of Veterinary Medicine Hannover, Foundation

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Abstract:

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Abbreviations

#	horse Identification (letters)
AEC 3	amino-9-ethylcarbazole
AIM2	absent in melanoma 2
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ASC	apoptosis-associated speck-like protein containing a carboxy-terminal CARD
BSA	bovine serum albumin
cDNA	complementary DNA
cGAMP	cyclic GMP-AMP
cGAS	cyclic GMP-AMP synthase
ctrl	control skin samples, locally treated with PBS
CMV	cytomegalovirus
CTL	cytotoxic T lymphocytes
CXCL	chemokine (C-X-C motif) ligand
DB	Dot Blot
DMEM	Dulbecco's modified Eagle’s medium
DNA	deoxyribonucleic acid
Dpap	papillary dermis
Dret	reticular dermis
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
e. g.	for example
ELISA	enzyme linked immunosorbent assay
Ep	epithelium
ER	endoplasmic reticulum
FOV	fields of view
FSC	forward scatter
GM-CSF	Granulocyte Macrophage Colony stimulating factor
Gp	glycoprotein

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H&E	Haematoxylin and Eosin
Hct	haematocrit
i.d.	intradermal
i.m.	intramuscular
IFN	interferon
IHC	immunohistochemistry
IL	Interleukin
ILRAP	IL-1beta receptor antagonist protein
IRAK	Interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
LAVES	Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit
LB	Lysogeny broth (medium)
LC/MS	Liquid chromatography-mass spectrometry
LPS	lipopolysaccharide
mAb	monoclonal antibody
MIDGE	minimalistic immunologic defined gene expression
MIDGE-Th1	MIDGE vector with nuclear localisation sequence
mRNA	messenger ribonucleic acid
mv	multivariate
MyD88	myeloid differentiation primary response gene (88)
NCBI	National Center for Biotechnology Information
NFκB	nuclear factor kappa B
NGS	normal goat serum
NK	natural killer (cell)
ODN	oligodeoxynucleotides
pAb	primary antibody
PAMPs	pathogen associated patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate

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PRE	Pura Raza Española
PVDF	Polyvinylidenedifluoride
qPCR	quantitative PCR
RIG-I	retinoic acid-inducible gene I
RNA	ribonucleic acid
Rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
RT	rectal temperature
rt	room temperature
SAA	serum amyloid A
sAb	secondary antibody
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	side scatter
SNP	single nucleotide polymorphism
STING	stimulator of IFN genes
t(hours)	time post-treatment
t-(hours)	time prae-treatment
t0	time immediately prior to treatment
TBK	TANK-binding kinase
TBS	Tris buffered saline
T_H	T helper
ThB	Thoroughbred
TLR	Toll-like-receptor
TNF	Tumour necrosis factor
TPP	total plasma protein
TRAF	TNF receptor-associated factor
treat	locally treated skin samples
uv	univariate
WB	Western Blot
WBC	white blood cell count
WBl	Warmblood

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1 Introduction

1.1 Equine melanoma

1.1.1 Overview

Melanoma is a common, spontaneously occurring, usually progressive neoplastic skin disease with high prevalence in aging grey horses (Cavalleri et al., 2014; Cotchin, 1977; Jeglum, 1999; M`Fadyean, 1933; Valentine, 2006).

Genetic predisposition linked to the grey phenotype (genetically determined by a 4.6-kb duplication in intron 6 of syntaxin 17) evidently increases melanoma incidence, along with other mutations (e.g. a loss-of-function mutation in agouti signalling protein), (Pielberg et al., 2008). However, to date the underlying mechanisms of melanoma development in individual horses has not been completely understood (Cavalleri et al., 2014; Phillips et al., 2012; Pielberg et al., 2008).

Therapy of melanoma is usually not sufficient for complete remission of all tumours present in the affected horse. Most commonly, either no therapy is conducted or surgical excision is performed depending on localization and dimension of the tumour (Jeglum, 1999; Moore et al., 2013). Surgical therapy is usually limited to local treatment of early stage melanomas without eliminating metastases. Intra- and peritumoural chemotherapy with cisplatin is used experimentally with variable outcome and is usually limited to local treatment as well (Hewes and Sullins, 2006; Spugnini et al., 2011; Théon et al., 2007).

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1.1.2 Immunotherapy

Based on the theory of immune-escape mechanisms leading to establishment and progression of melanomas, experimental immunotherapy is an emerging field of research on equine melanoma therapy. In comparison to locally restricted therapies, it has the advantage of systemic effects (Cavalleri et al., 2014; Phillips and Lembcke, 2013). In addition to autologous tumour vaccines (Jeglum, 1999; MacGillivray et al., 2002), DNA vaccines are experimentally used for immunotherapy of equine melanoma. Specific immunization with vectors encoding melanoma antigens, such as tyrosinase and glycoprotein (gp) 100, has been used (Lembcke et al., 2012; Mählmann et al., 2015; Phillips and Lembcke, 2013). Beyond that, antigen unspecific attempts are employed to (re-) induce mechanisms activating the antitumoural immune response of the host. These attempts are most commonly based on gene therapy with DNA coding for cytokines, such as Interleukin (IL)-12, IL-18 or combinations of these cytokines with antigen immunization (Heinzerling et al., 2001; Mählmann et al., 2015; Müller et al., 2011a; Müller et al., 2011b). The encoded recombinant cytokines are thought to induce tumour remission by inhibiting melanoma immune-escape mechanisms and by (re-) inducing an immune response against tumour cells via T-helper (T_H)1-biased specific immune responses to tumour antigens, enhanced cytotoxic T cell (CTL) and natural killer (NK) -cell activity, improved antigen presentation and inhibition of angiogenesis (Bael and Gollob, 2007; Del Vecchio et al., 2007; Shizuo, 2000; Tizard, 2004; Trinchieri, 1995a, 2003).

1.1.2.1 Minimalistic immunologic defined gene expression (MIDGE)-Th1 vectors

Complexed MIDGE-Th1 vectors have been employed in experimental immunotherapy of grey horse melanoma resulting in partial tumour remission (Mählmann et al., 2015). MIDGE-Th1 vectors are linear double-stranded DNA molecules, which are covalently closed with single-stranded hairpin loops at both ends. The vectors are rather short as they only contain a promoter, the coding sequence to be transfected and a polyadenylation site (Lopez-Fuertes et al., 2002; Moreno et al., 2004). A nuclear localization sequence peptide covalently bound to one of the ends triggers an improved humoral and cellular response and directs it towards T_H1 type (Schirmbeck et al., 2001; Zheng et al., 2006). *In vivo* transfection is improved by the DNA complexation with cationic lipids, such as SAINT-18 (Audouy et al., 2002; Endmann et al., 2010).

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1.2 Immune effects of DNA

Effects of DNA applied *in vivo* are primarily ascribed to expression of their transgene product exerting its (physiological) effects. However, effects of randomly or additionally included CG motifs are to be considered as well as the effects of the structure of the DNA, independent of its sequence.

DNA is known to exert immunological effects in mammals usually attributed to unmethylated CG motifs recognized by Toll-like receptors (TLR) 9 and activating their downstream pathways of the innate immune system (Hacker et al., 2002; Hacker, 2000; Mutwiri et al., 2003). Immunostimulatory effects of DNA via TLR-9 have been proven in horses (Leise et al., 2010; Wattrang et al., 2005; Wattrang et al., 2012; Zhang et al., 2008). These effects are employed in the use of oligodeoxyribonucleotides (ODN) as vaccine adjuvants and in experimental immunotherapies (Bordin et al., 2012; Klier et al., 2012; Klier et al., 2011; Liu et al., 2009; Weiner et al., 1997). Antitumour effects of CG motifs have been demonstrated in mammals (humans and mice) (Brown et al., 2006; Hafner et al., 2001; Hofmann et al., 2008; Miconnet et al., 2002; Molenkamp et al., 2007; Olbert et al., 2009).

The mammalian response to different types of ODN seems to be evolutionarily conserved in general. There are, however, cell-type and species-specific components of these responses, as seen for instance in the pattern of induced cytokines by different (classes of) CG motifs (Booth et al., 2007; Klier et al., 2011; Mutwiri et al., 2003; Rankin et al., 2001; Scheule, 2000; Wattrang et al., 2012; Werling et al., 2004). These class differences have also been demonstrated in horses (Klier et al., 2011; Wattrang et al., 2012).

Little is known about immunological effects of DNA independent of CG motif content in horses. In other mammalian species some general mechanisms are suggested: Double-stranded (ds) DNA activates different DNA sensors (with cyclic GMP-AMP synthase, absent in melanoma 2, and RNA polymerase III being best defined), when reaching intracellular compartments (Unterholzner, 2013). Signal transduction either acts via STING (stimulator of IFN genes) or RIG-I (retinoic acid-inducible gene 1). These lead to the increased transcription of type I interferons (IFNs), pro-inflammatory cytokines and chemokines involved in antiviral immune defence. Another pathway is the inflammasome activation leading to maturation and secretion of IL-1β and IL-18 (Hornung et

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al., 2009; Hornung et al., 2014; Ishii et al., 2006; Unterholzner, 2013; Unterholzner et al., 2010; Wu and Chen, 2014). These mechanisms of CG-motif-independent DNA effects have recently been experimentally used in DNA vaccines to improve immunogenicity (Coban et al., 2011). Such effects, demonstrated in human cell cultures and in mice, remain to be confirmed in horses. However, due to general homology between mammalian species, it may be assumed that mechanisms sensing DNA independent of its sequence exist in horses as well and may lead to similar immune responses in this species.

1.3 Immunological biomarkers in horses

To study immunological effects, which are known to be species specific, it is essential to employ valid assays as well as suitable specific and sensitive biomarkers for the mechanism examined. At present only few assays for the detection of equine immunological biomarkers are available. Antibodies against cytokines (as key signalling molecules in immunological processes) are often not suitable for the favoured application or for the sample to be analysed. Thus, the basis for the determination of biomarkers for specific immunological effects in horses is the establishment of valid assays for the detection of candidate molecules.

Furthermore, mechanisms suspected by homology with other species such as humans and laboratory animals, which are extensively studied for immunological effects, must be carefully evaluated in horses. Species differences have for example been demonstrated in circadian rhythms (Murphy et al., 2006; Murphy, 2010; Piccione et al., 2005a; Piccione et al., 2005b), responses to TLR agonists (Jungi et al., 2011; Mauel et al., 2006; Mutwiri, 2012), and tumour immunology (Block et al., 2011).

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1.4 Aims

The primary aim of the present research was (1) to elucidate immunological effects caused by *in vivo* application of DNA complexed with SAINT-18 in horses and (2) to identify which component of DNA-based immunotherapy is probably responsible for the previously observed antitumour immune effects in grey horses bearing melanoma.
First of all, (3) suitable assays for potential immunological biomarkers in horses were to be established and (4) validated for the *in vivo* model in order to enable close examination of the immunological effects which were of interest.
Moreover, (5) possible biases or influencing factors of horses (age, sex, breed), sampling (time of day) and analysis (methods) relating to these biomarkers were to be investigated in order to gain valid results and to achieve correct interpretations.

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2 Manuscript I

Evaluation of the reactivity of commercially available monoclonal antibodies with equine cytokines

Veterinary Immunology and Immunopathology 2013, 156; 1-2, 1-19;
DOI: 10.1016/j.vetimm.2013.09.012

C.L. Schnabel, S. Wagner, B. Wagner, M.C. Durán, S. Babasyan, I. Nolte, C. Pfarrer, K. Feige, H. Murua Escobar, J.-M.V. Cavalleri

Abstract

Research on equine cytokines is often performed by analyses of mRNA. For many equine cytokines an analysis on the actual protein level is limited by the availability of antibodies against the targeted cytokines. Generation of new antibodies is ongoing but time consuming. Thus, testing the reactivity of commercially available antibodies for cross-reactivity with equine cytokines is of particular interest.

Fifteen monoclonal antibodies against IL-1ß, IL-6, IL-8, IL-12, IL-18 and Granulocyte Macrophage Colony stimulating factor (GM-CSF) of different species were evaluated for reactivity with their corresponding equine cytokines. Dot Blot (DB) and Western Blot (WB) analyses were performed using recombinant equine cytokines as positive controls. Immunohistochemistry (IHC) was carried out on equine tissue and flow cytometry on equine PBMC as positive controls.

As expected, three equine IL-1β antibodies detected equine IL-1β in DB, WB and IHC. For these, reactivity in IHC has not been described before. One of them was also found to be suitable for intracellular staining of equine PBMC and flow cytometric analysis. Two antibodies raised against ovine GM-CSF cross-reacted with equine GM-CSF in DB, WB and IHC. For these anti-GM-CSF mAbs this is the first experimental description of cross-reactivity with equine GM-CSF (one mAb was predicted to be cross-reactive in WB in the respective data sheet). The other clone additionally proved to be appropriate in flow cytometric analysis. Two mAbs targeting porcine IL-18 cross-reacted in IHC, but did not show specificity in the other applications.

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No reactivity was shown for the remaining five antibodies in DB, although cross-reactivity of two of the antibodies was described previously.

The results obtained in this study can provide beneficial information for choosing of antibodies for immunological tests on equine cytokines.

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