Wissenschaftliche Reihe der Klinik für Pferde

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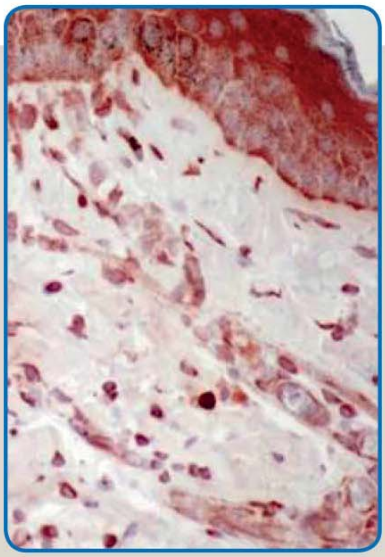
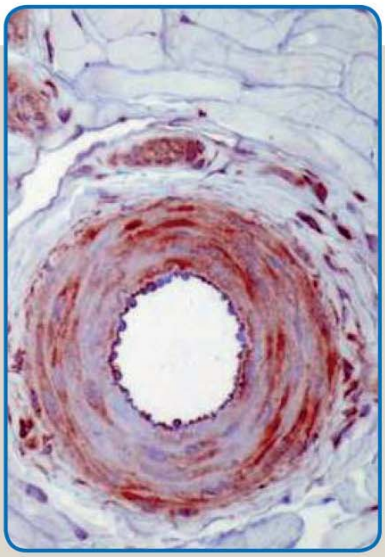
Acute immune response of healthy horses to linear DNA encoding Interleukin 12 and Interleukin 18 complexed with SAINT-18





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

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-Doctor of Veterinary Medicine-

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(Dr. med. vet.)

by

Christiane Liliane Schnabel

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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

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

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

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).

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).

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

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).

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.

2 Manuscript I

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

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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.

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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Research paper

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

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ABSTRACT

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1. Introduction

To study cellular immune reactions, the characterization of cells and their secreted products upon activation is of major interest. Cytokines are not only indicators of cellular immunity but can also reveal the activation status of cells (Splitter, 1997; Steinbach et al., 2002a; Swaggerty et al., 2008). Thus, they are commonly used for the characterization and comparison of immune responses (Whiteside, 1994; Swiderski et al., 1999; Wattrang et al., 2005; Ntrivalas et al., 2006; Svansson et al., 2009; Hamza et al., 2010; Wagner et al., 2010; Hostetter, 2012).

Research focusing on cytokines at the protein level is impacted by the lack of reliable target detection tools for many species (Scheerlinck, 1999; Pedersen et al., 2002; Steinbach et al., 2002b). Some bioassays have been used (Hawkins et al., 1995; Kato et al., 1997; Furr and Pontzer, 2001; McMonagle et al., 2001; Steinbach et al., 2002b, 2005; O'Donovan et al., 2004; Wu et al., 2004; Wattrang et al., 2005, 2012), but immunological detection of equine cytokines is not broadly available. However, only a few validated mAbs targeting equine cytokines are available for horses (Hawkins et al., 1995; Gutmann et al., 2005; Wagner et al., 2006, 2008a, 2008b; Lin et al., 2008; Tong et al., 2010; Noronha et al., 2012). But often the creation of specific immunological detection assays such as bead-based assays, necessitates specific (monoclonal) antibodies (Vignali, 2000; Kellar et al., 2001; Wagner and Freer, 2009; Duran et al., 2013). Consequently, many studies evaluating equine cytokines relied on the detection of mRNA (Ainsworth et al., 2003; David et al., 2007; Vick et al., 2007; Riihimaki et al., 2008; Miller et al., 2010; Heimann et al., 2011; Lembcke et al., 2012) rather than proteins taking the loss of lacking evidence of translation, processing and/or secretion of the respective cytokines.

Generating new specific mAbs is often challenging and time consuming. However, the evaluation of commercially available antibodies for reactivity or cross-reactivity with equine cytokines can overcome some of the problems and identify appropriate reagents to enable cytokine detection in horses (Wagner et al., 2005; Breathnach et al., 2006).

Herein we analysed various monoclonal antibodies for their reactivity with recombinant equine cytokines in Dot Blot (DB) and Western Blot (WB) analysis and evaluated their reactivity with endogenously expressed equine cytokines in Immunohistochemistry (IHC) and flow cytometric analysis.

2. Materials and methods

2.1. Monoclonal antibodies

Fifteen monoclonal antibodies against IL-1β, IL-6, IL-8, IL-12, IL-18 and Granulocyte Macrophage Colony stimulating factor (GM-CSF) were tested for reactivity with the equine cytokines (Table 1).

Two antibodies, one against equine IL-1β (Clone 1D4) and one against ovine GM-CSF (Clone 8D8), respectively, were chosen due to their previously described reactivity with the equine cytokines (Pedersen et al., 2002; Martoriati and Gerard, 2003). Three additional antibodies against equine IL-1β (Clones 8B25, 608714, 424823) were chosen because of their predicted species specificity. Antibodies with documented evidence of reactivity against the remaining cytokines of our interest were not available. Thus, ten antibodies were selected due to protein homology between their target protein (cytokines of other species) and the corresponding equine cytokine. No information was available on the specific epitopes against which the chosen antibodies were directed.

2.2. Dot Blots and Western Blots

2.2.1. Recombinant equine cytokines

Recombinant equine cytokines were used as positive controls for the (primary) antibodies tested (Table 2). Commercially available cytokines (IL-1β, IL-6, IL-8 and GM-CSF) were expressed in *Escherichia coli* or yeast (*e.g. Pichia pastoris*). Lyophilized recombinant cytokines were reconstituted according to the manufacturers' instructions. The mAbs targeting IL-1β, IL-6, IL-8 and GM-CSF were also tested for reactivity with cytokines expressed in eukaryotic cells (Chinese Hamster Ovary Cells, CHO) comparatively. Due to the purification technique, mammalian cell expressed cytokines were produced as fusion proteins containing an equine IL-4 tag as previously described by one of the authors (BW) (Wagner et al., 2005, 2012). It was expected that the secondary and tertiary structure of the recombinant cytokines synthesized in mammalian cells would be more similar to endogenous equine cytokines than that of those produced in bacteria or yeast.

Equine IL-12 and IL-18 were not commercially available and were thus produced in mammalian cells as described below. Protein purification was not performed. Cell lysates and cell culture supernatants containing recombinant IL-12 and IL-18 were directly used as positive controls for the Blots.

The IL-4 tagged cytokines (diluted in cell culture media), supernatants and lysates containing IL-12 or -18, respectively, were concentrated with the Vivaspin 15 centrifugal concentrator system (Sartorius AG, Göttingen, Germany) with a molecular weight cut off (MWCO) of 10,000 Da, according to the manufacturer's instructions, estimating 10- to 20-fold concentration. For the IL-4 tagged cytokines, final concentrations of between 2 and 2.36 μg/ml were achieved (Table 2).

2.2.2. Expression of IL-12 and IL-18

Equine IL-12 was expressed in a canine mammary cell line (MTH53A) transfected with plasmid DNA encoding equine IL-12. The plasmid contained cDNAs of the p35 and p40 IL-12 subunits (p35: Acc. No. Y11129; p40: Acc. No. Y11130) separated by an IRES element, as previously described (Duran et al., 2011). The pIRES-hrGFPII-rHMGB1 control expression plasmid was used with FuGENE HD (FHD, Roche, Mannheim, Germany). Protein expression was verified by intracellular staining using immunofluorescence with polyclonal IL-12 antibodies specific for the IL-12 subunits p35 and p40 (data not shown).

For the production of equine IL-18 the plasmids PCIproeqIL18 and PCImateqIL18 were used, encoding for the propeptide and the mature form of equine IL-18,

Table 1
Monoclonal antibodies tested.

Target	Host	Immunogen	Clone	Producer/distributor	Catalogue no.	Cross-reactivity with equine stated by	Protein homology	
IL-1β	Rat	Rec. equine IL-1β	8B25	USbio, Swampscott, MA, USA	I7663-20A.100	Fact sheet, USbio	100%	Equine: ref |NP_001075995.1|
IL-1β	Mouse	Rec. ovine IL-1β	1D4	AbDSerotec, Puchheim, Germany	MCA 1658	(Martoriati and Gerard, 2003)	63%	Equine: ref |NP_001075995.1| Ovine: gene ID: 443539 (precursor)
IL-1β	Mouse	Rec. equine IL-1β	608714	RnD Systems, Wiesbaden-Nordenstadt, Germany	MAB 33401	Fact sheet, RnD Systems	100%	Equine: ref |NP_001075995.1|
IL-1β	Rat	Rec. equine IL-1β	424823	RnD Systems, Wiesbaden-Nordenstadt, Germany	MAB 3340	Fact sheet, RnD Systems	100%	Equine: ref |NP_001075995.1|
IL-6	Rat	Rec. human IL-6	MQ2-13A5	AbDSerotec, Puchheim, Germany	1012001	Homology	61%	Equine gb |AAB87703.1| Human NP_000591.1
IL-6	Mouse	Bovine IL-6	CC310	AbDSerotec, Puchheim, Germany	MCA 2109	Homology	58%	Equine gb |AAB87703.1| Bovine (precursor) NP_776348.1
IL-8	Mouse	Rec. human IL-8	B-K8	AbDSerotec, Puchheim, Germany	MCA 1109	Homology	71%	Equine gb |AAO37764.1| Human NP_000575.1
IL-8	Mouse	Rec. ovine IL-8	8M6	AbDSerotec, Puchheim, Germany	MCA 1660	Homology	81%	Equine gb |AAO37764.1| Ovine NP_001009401.1
IL-12	Mouse	Rec. bovine	CC326	AbDSerotec, Puchheim, Germany	MCA 2173 Z	Homology	p35 83%	Equine gb |AAT92222.1| Bovine NP_776780.1
IL-12	Mouse	Rec. bovine	CC301	AbDSerotec, Puchheim, Germany	MCA 1782 EL		P40 91%	Equine p40 gb |AAT92225.1| Bovine NP_776781.1
IL-18	Mouse	Rec. porcine	7-G-8	AbDSerotec, Puchheim, Germany	MCA 2093	Homology	90%	Equine Q9XSQ7
IL-18	Mouse	Rec. porcine	5-C-5	AbDSerotec, Puchheim, Germany	MCA 2094			Porcine ABF55514.1
GM-CSF	Mouse	Rec. Ovine	10B2340	USbio, Swampscott, MA, USA	G8951-07E.2	Fact sheet, USbio	84%	Equine ref |NP_001075351.1|
GM-CSF	Mouse	Rec. Ovine	8D8	AbDSerotec, Puchheim, Germany	MCA 1923	(Pedersen et al., 2002)		Ovine NP_001009805.1
GM-CSF	Mouse	Rec. Ovine	3C2	AbDSerotec, Puchheim, Germany	MCA 1924	Homology		

Table 2
Rekombinant equine cytokines used as positive controls in DB and WB.

Cytokine	Expressed in	Producer/distributor	Catalogue no.	Molecular mass (kDa)	Concentration (μg/ml)	Applied in Dot Blots (ng)	Applied in Western Blots (ng)
IL-1β	*E. coli*	RnD Systems, Wiesbaden-Nordenstadt, Germany	3340-EL-010(/CF)	17	10	20	280
IL-1β/IL-4	CHO cells	Dr B. Wagner,Ithaca, NY, USA		47–57	2	4	56
IL-6	*E. coli*	RnD Systems, Wiesbaden-Nordenstadt,Germany	1886-EL-025/CF	21 kDa	100	200	2800
IL-6/IL-4	CHO cells	Dr B. Wagner, Ithaca, NY, USA		51–61	2.36	4.72	66
IL-8	Pichiapastoris	AbDSerotec, Puchheim, Germany	PEP005	8.5	40	80	1120
IL-8/IL-4	CHO cells	Dr B. Wagner, Ithaca, USA		38.5–48.5	2.35	4.7	66
IL-12	MTH53A	C. Duran		70	Not determined		
Pro IL-18	AAV 293	DNA vector provided by Dr L. Nicolson		24	Not determined		
Mat IL-18	AAV 293	DNA vector provided by Dr L. Nicolson		18	Not determined		
GM-CSF	Yeast	Biomol GmbH, Hamburg, Germany	RP0022E-005	15.2	100	200	2800
GM-CSF/IL-4	CHO cells	Dr B. Wagner, Ithaca, USA		45.2–55.2	2.1	4.2	59

respectively. The plasmids (Acc. Nr. NM_001082512) were kindly provided by Dr. Lesley Nicolson (University of Glasgow, UK).

For the amplification and verification the IL-18 encoding vectors were cloned into the thermo competent bacteria of the *E. coli* strain DH5α (#18265-017, Invitrogen, Darmstadt, Germany) according to the 'Subcloning Efficiency DH5α Competent Cells' protocol (Invitrogen, Darmstadt). Transformed bacteria were plated on 2% Select Agar YT medium (Invitrogen, Darmstadt, Germany) plates containing 0.1% ampicillin (Applichem, Darmstadt, Germany) and cultured overnight at 37 °C. Positive clones were subsequently cultured in YT medium (Invitrogen, Darmstadt, Germany) supplemented with 0.1% ampicillin at 37 °C, overnight. From these cultures, plasmids were isolated with the PureYield Plasmid Miniprep System (Promega, Mannheim, Germany) according to the manufacturer's instructions. DNA-sequencing of both clones was performed by SeqLab Sequence Laboratories Göttingen GmbH. The analysed sequence matched equine IL-18 by DNA-DNA-BLAST (NCBI).

For the production of recombinant IL-18 3×10^5 AAV 293 cells (derived from human HEK 293 cells) were seeded in each well of a 6-well plate and cultured overnight in 2 ml Dulbecco's MEM medium (DMEM) (Biochrom AG, FG 0445) supplemented with 10% heat-inactivated foetal calf serum (PAA Laboratories GmbH, Pasching, Austria) in a humidified atmosphere at 37 °C and 5% CO_2. The transfection was performed according to the manufacturer's protocol (Roche, Mannheim, Germany) using X-tremeGENE HP DNA transfection reagent, OptiMEM (Invitrogen, Darmstadt, Germany) and 2 μg plasmid DNA. Twenty-four hours after transfection, the medium was changed and the cells were incubated for an additional 48 h. Subsequently, culture medium was aspirated, cells were detached by incubating with Trypsin (TrypLE Express, Invitrogen, Darmstadt, Germany), and pelleted by centrifugation at $350 \times g$, at room temperature (RT), for 3 min. The remaining supernatant was decanted and the pellet was lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% Sodiumdecholat, 1% Nonidet, one cOmplete Ultra Tablet, Roche, Mannheim, Germany). To remove cell debris, the cell lysates were centrifuged at 14,000 rpm at 4 °C for 10 min. The supernatants were stored at −20 °C until further analysis.

2.2.3. Preparation of naturally derived equine cytokines

Two mAbs (clone 1D4, clone 8D8) known to cross-react with equine IL-1β or GM-CSF, respectively, were additionally tested against endogenous equine cytokines. Analyses were carried out as reported in the literature (Pedersen et al., 2002; Martoriati and Gerard, 2003).

Regarding to GM-CSF, stimulated PBMC served as a source of equine cytokines as described by Pedersen et al. (2002) for clone 8D8. Briefly, PBMC of a healthy gelding were isolated by density gradient centrifugation over a lymphocyte separation medium (LSM 1077, PAA Laboratories GmbH, Cölbe, Germany), 2×10^6 cells were cultured in 1 ml RPMI with 10% FCS in the presence of 20 ng/ml PMA, 1 μg/ml Ionomycin and 10 μg/ml Brefeldin A for 4 h in a humidified atmosphere at 37 °C and 5% CO_2. Cells were pelleted by centrifugation and lysed as described for the IL-18 transfected AAV 293 cells.

In the case of IL-1β follicular fluid from a 33 mm diameter follicle of a healthy mare served as a source of equine IL-1β as described by Martoriati and Gerard (2003) for the clone 1D4.

Samples were stored at −80 °C until further analysis.

2.2.4. Dot Blots

Polyvinylidenedifluoride (PVDF) membranes (Immobilon P, Millipore, Schwalbach; Germany) were activated in 100% methanol for 15 s. Diluted recombinant cytokines (4–200 ng/ml, Table 2) were applied directly onto membranes in dots with a volume of 5 μl and allowed to dry for

5–10 min at RT. The membranes were activated again in 100% methanol for 15 s followed by incubation in blocking buffer [containing 5% non-fat dry milk in Tris buffered saline (TBS; 10 mM Tris–HCL, 150 mM NaCl) with 0.05% Tween 20, pH 8] at RT on a shaker for 1 h to prevent additional protein binding. Subsequently, respective primary antibodies were added to the blocking buffer to a final concentration of 1 μg/ml, and incubated at RT on a shaker for 2 h or at 4 °C overnight. The membranes were washed three times with TBS and incubated afterwards with a secondary antibody (Goat Anti-Mouse or Goat Anti-Rat, AP-Conjugate, Promega, Mannheim, Germany) diluted in TBS with 0.05% Tween 20 at RT on a shaker for 1 h. Afterwards, the membranes were washed twice with freshly prepared AP buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) and each membrane was incubated in 10 ml of AP buffer with 100 μl of NBT/BCIP substrate (NBT stock solution, Roche, Mannheim, Germany) protected from light, at RT on a shaker for 10 min. To stop the alkaline phosphatase reaction, membranes were washed with water once and dried in the dark at RT overnight before evaluation of results.

A distinct purple staining of the dots was interpreted as positive reactivity of the primary Ab tested with the recombinant cytokine applied to the membrane.

Controls were included as additional dots. The efficacy of the AP was verified by applying secondary antibodies directly onto activated membranes, resulting in dots when the AP reacted with the substrate. The specificity of the secondary antibodies was confirmed by applying primary antibodies onto the membranes, displaying dots after binding of the secondary antibodies and AP-reaction. Empty membrane parts served as negative controls for unspecific binding of antibodies to the membrane.

As negative controls of cross-reactivity of the secondary antibodies with the recombinant cytokines, cytokines were applied in dots, incubated with AP-conjugated secondary antibody and afterwards with substrate. The absence of dots of cytokines excluded cross-reactivity of the secondary antibodies with the cytokines.

Antibodies with positive or inconclusive results in the DB were further tested by WB.

2.2.5. SDS-PAGE

Electrophoresis of denatured cytokines was performed using gels containing 10% SDS and 15% acrylamid/bisacrylamid (37.5:1) (both Applichem, Darmstadt, Germany). Each cytokine solution was mixed 1:5 with 5-fold Laemmli buffer (final concentration: 50 mM Tris/HCl, 2% β-mercaptoethanol, 2% (w/v) SDS, 4%, 0.025% bromophenol blue) and was denatured at 95 °C for 5 min and put on ice afterwards. A molecular weight marker (PageRuler Prestained Protein Ladder or Spectra Multicolor Broad Range Protein Ladder, Fermentas, St. Leon-Rot, Germany) was included on each gel. The electrophoresis was performed in running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) at 20 mA for about 80 min at RT.

For the analysis of the amino acid sequence of proteins, the respective PA-gel was stained with coomassie-blue after PAGE, the relevant area was cut out and analysed by liquid chromatography/mass-spectrometry (LC/MS) by the Department of Toxicology, Hannover Medical School, Hannover, Germany, Prof. Andreas Pich.

2.2.6. Western Blotting

After PAGE, the SDS-gels were incubated in transfer buffer (25 mM Tris base, 192 mM glycine, 1 mM $MgCl_2$, 20% methanol, pH 8.3) at RT for 10 min. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Schwalbach, Germany) in a tank blot. The blotting was conducted in transfer buffer at a constant current of 0.35 A for 30 min.

2.2.7. Immunoblotting

After Western Blotting, the membranes were treated as described for the DB, following the steps of PVDF membrane activation, blocking of the membrane, incubation with primary antibody, secondary antibody, NBT/BTIP substrate, washings and drying.

2.3. Experiments with naturally derived equine cytokines

MAbs with positive or doubtful results in DB and WB were subsequently tested in IHC with naturally derived cytokines in equine tissues known to express target cytokines and in flow cytometric analysis on equine *in vitro* stimulated PBMC.

2.4. Immunohistochemistry

2.4.1. Preparation of tissues

Formaldehyde-fixated Paraffin-embedded equine tissue samples were used for analyses. Slices of 3 μm were cut and dried on salined glass slides (Histobond, Marienfeld, Lauda-Königshofen, Germany) at 60 °C overnight. The sections were deparaffinised in xylene and rehydrated in a series of alcohols of descending grades. Endogenous peroxidase was blocked in 0.6% hydrogen peroxide in 80% ethanol for 30 min at RT. Sections were rinsed three times in PBS at RT for 5 min.

For each mAb, subsequent reactions were performed (a) after heat pre-treatment in citrate-buffer and (b) without pre-treatment.

2.4.2. Pre-treatment

The sections were incubated in 0.01 M citrate-buffer (pH 6.0) at 96–99 °C for 15 min and allowed to cool down in the buffer, until reaching 65 °C. Following, the sections were rinsed three times in PBS at RT for 5 min.

2.4.3. Reaction

To block unspecific protein binding sections were incubated with heat-inactivated normal goat serum diluted 1:5 in PBS (NGS), for 20 min at RT in a moist chamber.

NGS was decanted and the sections were covered with mAbs as primary antibodies (pAbs) diluted 1:50, 1:100, 1:500 and 1:1000 in PBS with 1% BSA and incubated at 4 °C overnight. Negative controls were incubated with PBS/BSA only. On the following day, sections were rinsed three times in PBS for 5 min at RT (negative controls were handled separately).

The sections were incubated with biotinylated secondary Ab (sAb) (Anti-Mouse-Biotin, Vector, Burlingame, Canada or Anti-Rat-Biotin, Bethyl, Montgomery, USA) diluted in PBS in a moist chamber at RT for 45 min followed by rinsing in PBS and signal amplification with avidin-biotin-complex according to the manufacturer's instructions (Vectastain ABC Kit Elite, Vector, Burlingame, Canada).

After rinsing the sections three times in PBS for 5 min, visualization was performed with the chromogen, 3-amino-9-ethylcarbazole (AEC; Peroxidase-Substrat-Kit AEC, Biologo, Kronshagen, Germany) which was applied according to the manufacturer's instructions. After incubation for 10 min at RT in a moist chamber, the slides were rinsed in PBS for 5 min and in slowly running tap water for 10 min.

To facilitate identification of specific tissue components, individual sections were counterstained in Delafield's haematoxylin for 2 s and rinsed in running tap water for 10 min.

Slides were mounted with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany) and coverslips and left to dry at RT.

Sections were viewed with a Zeiss Axioskop (Carl Zeiss Jena GmbH, Jena, Germany) and images were captured using an Olympus DP Soft Camera (Olympus Deutschland GmbH, Hamburg, Germany).

Distinct red staining of cells was interpreted as a positive reaction of the mAb given that control slides showed no such staining.

2.5. Flow cytometric analysis

PBMC were isolated from heparinized blood of four clinical healthy, adult (5–19 years of age) Thoroughbred horses by density gradient centrifugation (Ficoll-PaqueTM Plus, Amersham Bioscience, Piscataway, NJ). A total of 3×10^6 PBMC were incubated for 4 h in medium (DMEM containing 10% (v/v) FCS, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 50 mg/ml gentamycin) or in medium supplemented with PMA (25 ng/ml) and ionomycin (1 mM) or with LPS (12.5 mg/ml; all from Sigma, St. Louis, MO, USA). To block the secretion of intracellular protein during stimulation, Brefeldin A (10 mg/ml; Sigma, St. Louis, MO, USA) was added to all cultures. Afterwards, the cells were washed in PBS and fixed in 2% formaldehyde for 20 min at room temperature.

Intracellular staining was performed in saponin buffer (PBS, supplemented with 0.5% (w/v) BSA, 0.5% (w/v) saponin and 0.02% (w/v) NaN3). Monoclonal antibodies were used for intracellular staining. An isotype control was included in the procedure using an aliquot of the PBMC. Staining of around 1×10^6 fixed PBMC with 2 μg/ml mAb (1:250 dilution for the clones 8B25, 608714, 424823 and 1:500 for the others) was performed for 15 min at RT. After washing in PBS, as secondary antibodies DyLight 649 conjugated goat anti-mouse IgG (H + L) or DyLight 649 conjugated F(ab)2 Fragment goat anti-Rat IgG (H + L) (both Jackson ImmunoReasearch, West Grove, PA, USA) were used at 7.5 μg/ml (1:200 dilution). Subsequently, cells were washed twice with saponin buffer and resuspended in PBS/BSA (PBS, supplemented with 0.5% (w/v) BSA and 0.02% (w/v) NaN3) and measured by flow cytometry using a FACS Canto II (BD Biosciences, San Diego, CA, USA). A total of 30.000 events were measured per sample.

2.6. Statement of ethical approval

Follicular fluid used in this study was collected as parts of routine procedures from a healthy mare during preparations for embryo transfer. Blood was collected of healthy horses in the course of blood testing prior to elective surgical procedures.

3. Results

3.1. Dot Blots and Western Blots

Five out of the 15 monoclonal antibodies tested with recombinant equine cytokines reproducibly reacted with recombinant equine IL-1β or equine GM-CSF in DB and WB applications (Table 3, Figs. 1–3).

The two mAbs that were described to cross-react with naturally derived equine IL-1β in follicular fluid (Martoriati and Gerard, 2003) or equine GM-CSF in the lysate of stimulated PBMC (Pedersen et al., 2002) did not react with these in WBs in the current study.

The mAbs raised against IL-6 and IL-8 did not cross-react with recombinant equine cytokines in DB (Figs. 4 and 5) and were not evaluated further.

Two additional antibodies against IL-18 reacted in DBs with native lysates of AAV 293 cells transfected with plasmid encoding either the propeptide or the mature form of equine IL-18. In the WB only one IL-18 mAb (Clone 7-G-8) cross-reacted with the propeptide of IL-18 ectopically expressed in AAV-293 cells (Fig. 6).

For the mAb clones 8B25, 608714, 424823, 10B2340 and 3C2, the molecular size detected by WB corresponded to the expected molecular weights of the cytokines (Table 3). In the case of recombinant equine IL-1β derived from *E. coli* detected by mAb clones 8B25 and 424823, a second band

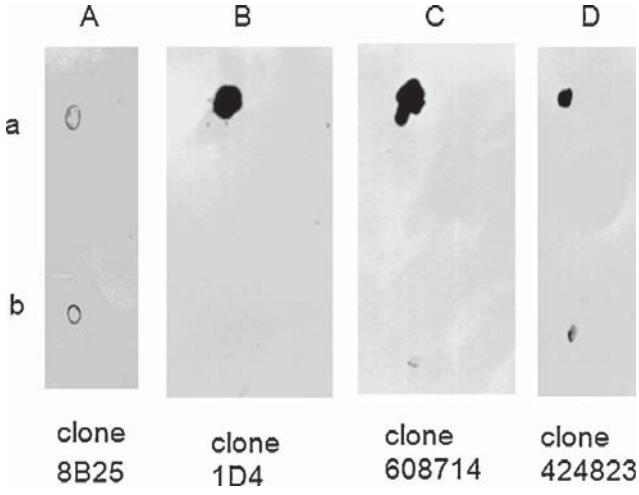

Fig. 1. Dot Blots prepared with mAbs detecting equine IL-1β: (a) primary Ab (pAb) applied as positive control; (b) tested equine rIL-1β produced in *E. coli*. Reactivity was detected for clones 8B25, 608714 and 424823.

Table 3
Reactivity of monoclonal antibodies with recombinant equine cytokines in DB and WB.

Monoclonal antibody	Clone	Rec. protein expressed in	Mol. mass	Dot Blot	Band(s) in Western Blot
Rat anti-equine IL-1β	8B25	*E. coli*	17 kDa	+	15 kDa, 35 kDa
		CHO	47–57 kDa	+	35 kDa
Mouse anti-ovine IL-1β	1D4	*E. coli*	17 kDa	–	–
		CHO	47–57 kDa	–	–
Mouse anti-equine IL-1β	608714	*E. coli*	17 kDa	+	15 kDa
		CHO	47–57 kDa	+	40 kDa
Rat anti-equine IL-1β	424823	*E. coli*	17 kDa	+	15 kDa, 35 kDa
		CHO	47–57 kDa	+	35 kDa
Rat anti-human IL-6	MQ2-13A5	*E. coli*	21 kDa	–	n.d.
		CHO	51–61 kDa	–	n.d.
Mouse anti-bovine IL-6	CC310	*E. coli*	21 kDa	–	n.d.
		CHO	51–61 kDa	–	n.d.
Mouse anti-human IL-8	B-K8	*Pichia pastoris*	8.5 kDa	–	n.d.
		CHO	38.5–48.5 kDa	–	n.d.
Mouse anti-ovine IL-8	8M6	*Pichia pastoris*	8.5 kDa	–	n.d.
		CHO	38.5–48.5 kDa	–	n.d.
Mouse anti-bovine IL-12	CC326	MTH53A	70 kDa	n.d.	–
Mouse anti-bovine IL-12	CC301	MTH53A	70 kDa	n.d.	–
Mouse anti-porcine IL-18	7-G-8	AAV 293 pro-IL18	24 kDa	(+)	20 kDa
		AAV 293 mat-IL18	18 kDa	(+)	–
Mouse anti-porcine IL-18	5-C-5	AAV 293 pro-IL18	24 kDa	(+)	–
		AAV 293 mat-IL18	18 kDa	(+)	–
Mouse anti-ovine GM-CSF	10B2340	Yeast	15.2 kDa	+	–
		CHO	45.2–55.2 kDa	+	55 kDa
Mouse anti-ovine GM-CSF	8D8	Yeast	15.2 kDa	–	–
		CHO	45.2–55.2 kDa	–	–
Mouse anti-ovine GM-CSF	3C2	Yeast	15.2 kDa	n.d.	50 kDa
		CHO	45.2–55.2 kDa	+	50 kDa

with an approximate size of 35 kDa was detected, which is about twice the expected molecular mass (Fig. 2).

Coomassie-staining of the PA-gel with this recombinant equine IL-1β displayed only one band at about 20 kDa (Fig. 7). This band and the area of about 35 kDa in which the second band had been visible in the WBs were cut out and analysed using LC/MS. LC/MS identified equine IL-1β in both samples with a Mascot score of 293 and three peptides (false discovery rate of 0.05 on peptide level) in the region around 35 kDa. Thus, the band in doubt was interpreted as consisting of IL-1β dimers, which are detected by the clones 8B25 and 424823 but not by clone 608714.

Cross-reactivity of the secondary antibodies with recombinant cytokines was not observed.

The results of the seven mAbs with positive or inconclusive results in DB and WB (Table 3), further tested in IHC and flow cytometric analysis, are stated below.

3.2. Immunohistochemistry

All seven mAbs showing positive or inconclusive results in DB and WB reacted with equine cytokines in paraffin-embedded tissues (Figs. 8–10). Admittedly, two mAbs, anti-porcine IL-18 (clone 7-G-8) and anti-ovine GM-CSF (clone 3C2), showed only weak reactivity. Optimum conditions in the current study were specified (Table 4).

Negative controls without primary antibodies showed no cross-reactivity of secondary antibodies or detection reagents.

3.3. Flow cytometric analysis

Two of the seven monoclonal antibodies with positive or inconclusive results in DB and WB detected the naturally derived cytokines produced by stimulated equine PBMC without staining non-stimulated cells (Table 5, Fig. 11).

The anti-equine IL-1β mAb, clone 608714, showed a small population of positive cells after stimulation with PMA and ionomycin. This positive population increased after stimulation with LPS (Fig. 12B), whereas the staining of non-stimulated cells was identical to the isotype control staining (data not shown). This IL-1β mAb (clone 608714) detected stimulated PBMC with a high side scatter which is consistent with the morphological characteristics of monocytes. For the other mAbs against IL-1β (clone 8B25; clone 424823) the stainings of stimulated PBMC were weak and overlapped broadly with non-stimulated cells (Fig. 12A and C). Thus, the latter two mAbs do not seem to be useful in this application.

The two mAbs specific for porcine-IL-18 did not result in any convincing staining of equine PBMC by flow cytrometric analysis. The mAb clone 7-G-8 displayed no signal on stimulated or non-stimulated equine PBMC, whereas clone 5-C-5, resulted in almost identical staining pattern for cells stimulated with LPS and non-stimulated cells (Fig. 13).

Of two mAbs against GM-CSF, one mAb (clone 3C2) clearly identified a population of PBMC after stimulation with PMA and ionomycin, but not with LPS. Non-stimulated cells remained unstained with this mAb (Figs. 11 and 14B). The other GM-CSF mAb, clone 10B2340, did result in a

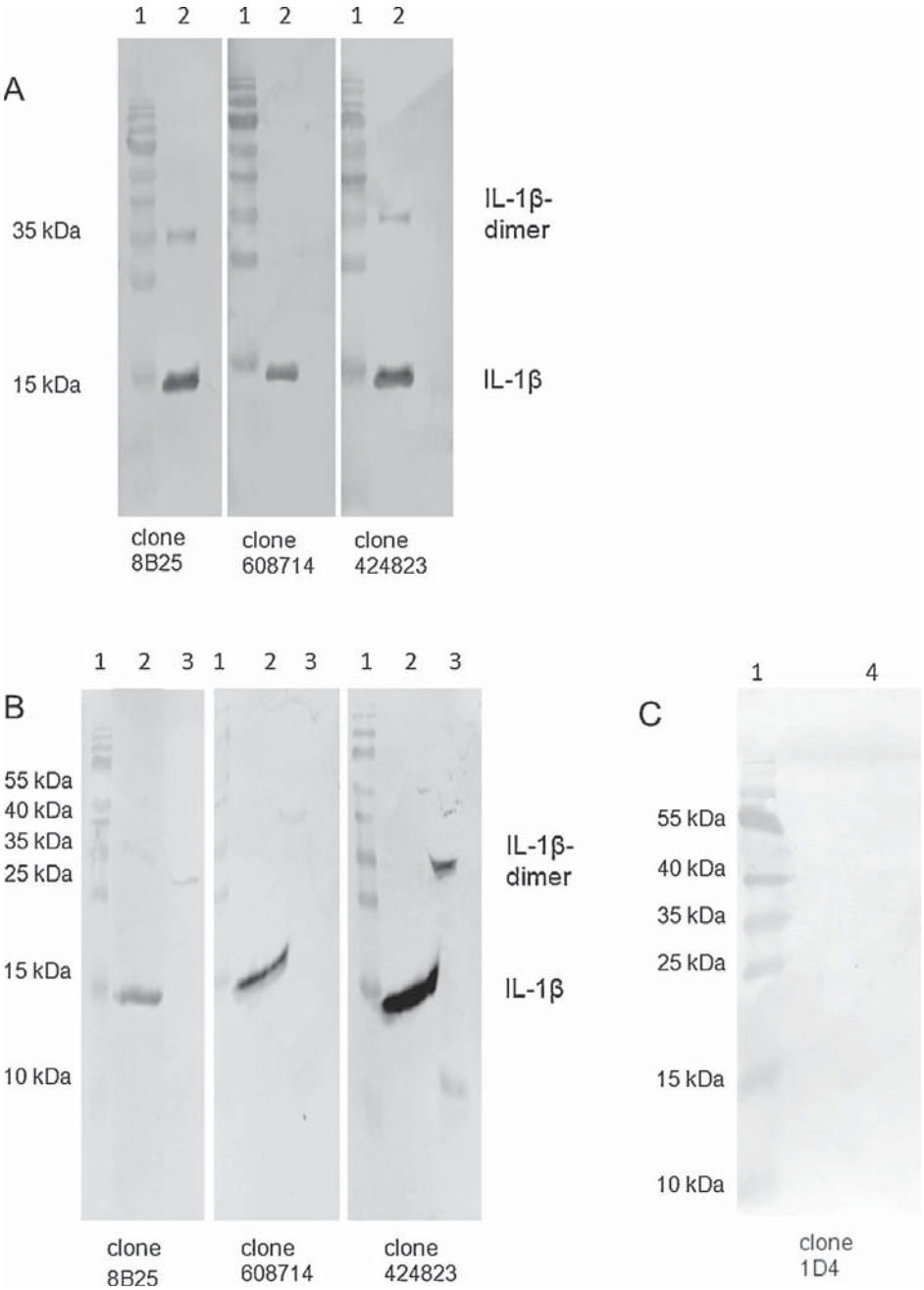

Fig. 2. Western Blots with mAbs detecting equine IL-1β: (A) Blots with rIL-1β produced in *E. coli*; (B) Blots with rIL-1β produced in *E. coli* and Chinese hamster Ovary Cells (CHO); reactivity was detected for clones 8B25, 608714 and 424823 with equine rIL-1β produced in both, *E. coli* or CHO. (C) Blot prepared with equine follicular fluid presumably containing natural equine IL-1β (lane 4): this anti-IL-1β clone 1D4 was described in the literature to react with IL-1β in equine follicular fluid. No reactivity was detected in this experiment. (1) Marker; (2) equine rIL-1β produced in *E. coli*; (3) equine rIL-1β-IL-4 produced in CHO; (4) equine follicular fluid.

higher background and only slightly elevated staining in PBMC from two horses after PMA and ionomycin stimulation (Fig. 14A).

3.4. Summary of different applications

Seven of the 15 mAbs evaluated in the current study (cross-)reacted with recombinant equine cytokines by DB or WB. All of these clones reacted with equine tissues by IHC and two mAbs were additionally able to detect a positive cell population in stimulated equine PBMC in flow cytometry (Table 6).

4. Discussion

The goal of this work was to test different mAbs directed against cytokines in various species for their cross-reactivity with the respective horse cytokines. The antibodies were tested against recombinant cytokines by DB and WB. They were also tested on equine tissue sections by IHC and on equine PBMC by flow cytometry to confirm their specificity for native equine IL-1β and GM-CSF.

Three antibodies raised against equine IL-1β (clones 8B25, 608714, 424823) reacted with their target cytokine in WB (as stated in the respective data sheets for WB) and IHC.

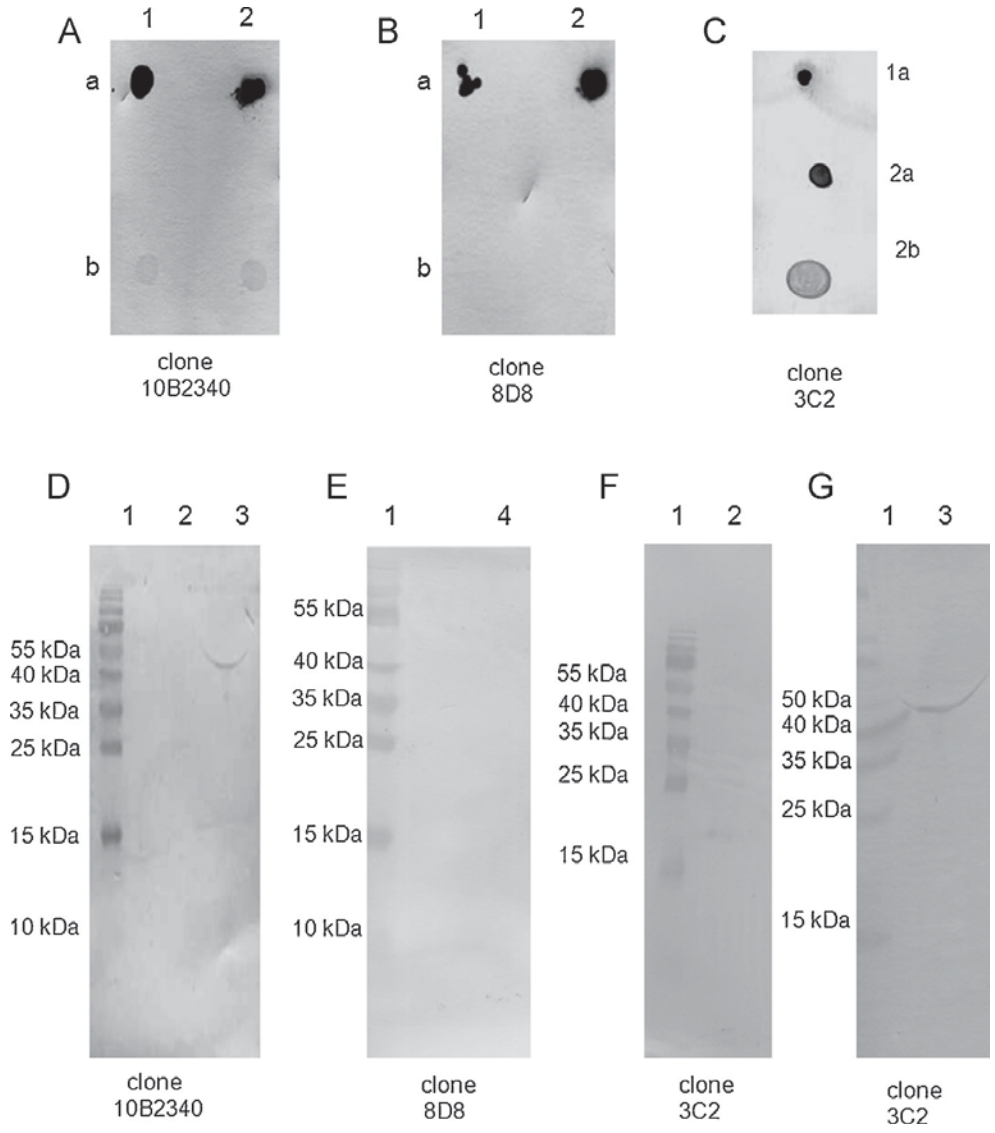

Fig. 3. Dot Blots and Western Blots with mAbs detecting equine GM-CSF: (A-C) Dot Blots with mAbs as pAb: (a) applied as positive control (1a) pAb and (2a) sAb; (b) tested equine rGM-CSF produced in (1b) yeast or (2b) CHO; (D–G) Western Blots with mAbs as pAb: (1) Marker; (2) rGM-CSF produced in yeast; (3) rGM-CSF-IL-4 produced in CHO; (4) lysate of PBMC cultured in the presence of PMA, ionomycin and Brefeldin A, presumably containing natural equine GM-CSF. (E) The anti-GM-CSF clone 8D8 was described in the literature to react with equine GM-CSF in the lysate of stimulated PBMC. No reactivity was detected in this experiment. (D, F, G) Reactivity was detected for clones 10B2340 and 3C2 with rGM-CSF produced in both yeast or CHO.

One mAb (clone 608714) additionally detected a small cell population in equine PBMC by flow cytometric analysis. To the best of our knowledge, this is the first report describing reactivity for these mAb with equine IL-1β using IHC and flow cytometric analysis.

Two antibodies raised against ovine GM-CSF (clones 10B2340 and 3C2) cross-reacted with equine GM-CSF in WB, IHC and one mAb reacted in flow cytometric analysis. For both antibodies, cross-reactivity with equine GM-CSF has not yet been described. However, cross-reactivity was somewhat expected due to a protein homology of 84% between the ovine and equine targets.

Two antibodies, against ovine IL-1β and ovine GM-CSF (clones 1D4 and 8D8) respectively, for which cross-reactivity in WB had been described previously, reacted neither with recombinant cytokines derived from *E. coli* or yeast nor with the IL-4-tagged cytokines derived from mammalian cells in our procedures. Descriptions of the antibodies' cross-reactivities by WB refer to the use of anti-ovine GM-CSF on endogenous cytokines in lysates from equine PBMC stimulated with PMA and ionomycin (Pedersen et al., 2002) or anti-ovine IL-1β on endogenous cytokines in equine follicular fluids (Martoriati and Gerard, 2003). Both procedures were also performed in our approach to characterize these mAbs. The reported results could not be reproduced with the experimental settings in the current study. The observed lack of reactivity with recombinant cytokines may be a result of structural differences between native and recombinant proteins, even if the latter are produced in mammalian cells. However, this would not explain the herein reported discrepancy of results obtained with naturally derived cytokines when using similar methods as previously described (Pedersen et al., 2002; Martoriati and Gerard, 2003). The reason for the difference in antibody reactivity in the previous and our approaches remains speculative. However, it can be

Table 4
Reactivity of monoclonal antibodies with equine cytokines in IHC.

Monoclonal antibody (mAb)	Clone	Reactivity	Equine tissue used as positive control	Reactivity towards tissue/cells	Optimum pre-treatment	mAb optimum dilution (concentration)	Secondary antibody, dilution
Rat anti-equine IL-1β	8B25	+	Tonsil	Lymphatic tissue	Citrate, heat	1:50 (10 μg/ml)	Anti-rat-biotin, 1:500
Mouse anti-equine IL-1β	608714	+	Lymph node	Lymphatic tissue	Citrate, heat	1:50 (10 μg/ml)	Anti-mouse-biotin, 1:200
Rat anti-equine IL-1β	424823	+	Tonsil	Lymphatic tissue	Citrate, heat	1:50 (10 μg/ml)	Anti-rat-biotin, 1:500
Mouse anti-porcine IL-18	7-G-8	(+)	Colon	Cells in *Lamina propria mucosae*	None	1:100 (5 μg/ml)	Anti-mouse-biotin, 1:200
Mouse anti-porcine IL-18	5-C-5	+	Colon	Cells in *Lamina propria mucosae*, lymphatic tissue	None	1:50 (10 μg/ml)	Anti-mouse-biotin, 1:200
Mouse anti-ovine GM-CSF	10B2340	+	Lung	Respiratory epithelium, various interstitial cells	Citrate, heat	1:100 (10 μg/ml)	Anti-mouse-biotin, 1:200
Mouse anti-ovine GM-CSF	3C2	(+)	Lung	Respiratory epithelium	None	1:50 (20 μg/ml)	Anti-mouse-biotin, 1:200

Legend: reactivity: + reactive; (+) weakly reactive; – not reactive.

Fig. 4. Dot Blots with mAbs detecting equine IL-6: (A and B) incubated with mAbs as pAb: (1) applied as positive control (1a) pAb and (1b) secondary Ab (sAb); (2) tested equine rIL-6 produced in (1b) *E. coli* or (2b) CHO; (C and D) negative control incubated with buffer instead of pAb: (1) rIL-6 applied; (2) pAb applied. No reactivity was detected for these mAbs.

concluded that the mAbs against ovine IL-1β (clone 1D4) and ovine GM-CSF (clone 8D8) gave discrepant results and were not easily repeatable by different investigators.

All protein bands detected by the different antibodies in WB corresponded to the expected molecular masses of the targeted cytokines or of IL-1β dimers. The authors are thus confident about their specificity to the corresponding equine cytokines. Previous studies concluded that mAbs' reactivities appeared to be specific for the target in most applications if cross-reactivity between antibodies against

Table 5
Reactivity of monoclonal antibodies with native equine cytokines by flow cytometric analysis.

Monoclonal antibody (mAb)	Clone	Suitable in FACS
Rat anti-equine IL-1β	8B25	No
Mouse anti-equine IL-1β	608714	Yes
Rat anti-equine IL-1β	424823	No
Mouse anti-porcine IL-18	7-G-8	No
Mouse anti-porcine IL-18	5-C-5	No
Mouse anti-ovine GM-CSF	10B2340	No
Mouse anti-ovine GM-CSF	3C2	Yes

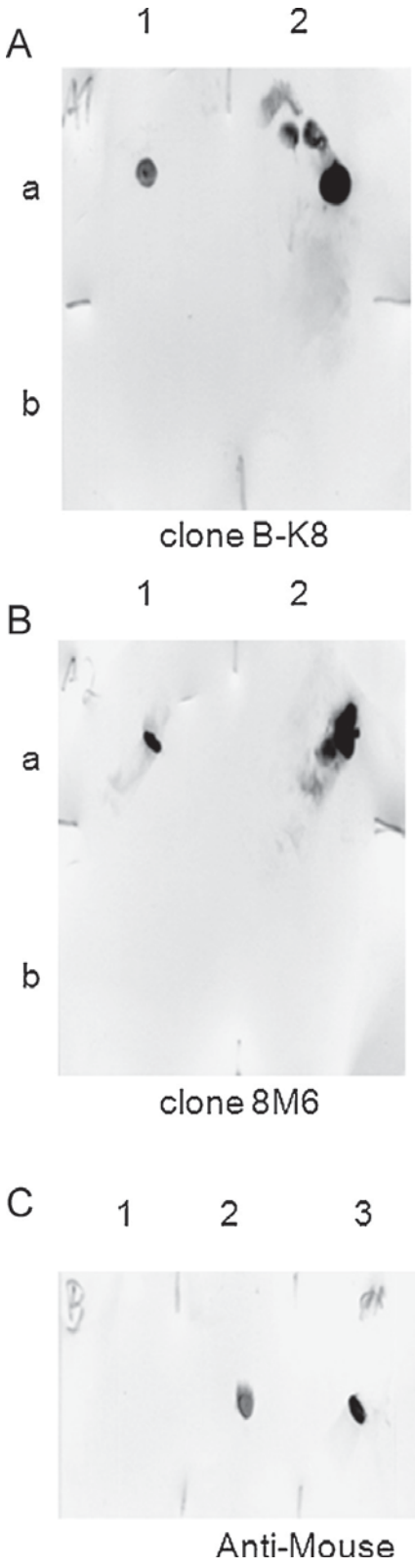

Fig. 5. Dot Blots with mAbs detecting equine IL-8: (A and B) incubated with mAbs as pAb: (1) applied as positive control (1a) pAb and (1b) sAb; (2) tested equine rIL-8 produced in (2a) *E. coli* or (2b) CHO; (C) negative control incubated with buffer instead of pAb: (1) rIL-8 produced in *E. coli*; (2) pAb clone B-K8, (3) pAb clone 8M6, (4) rIL-8-IL-4 produced in CHO; no reactivity was detected for these mAbs.

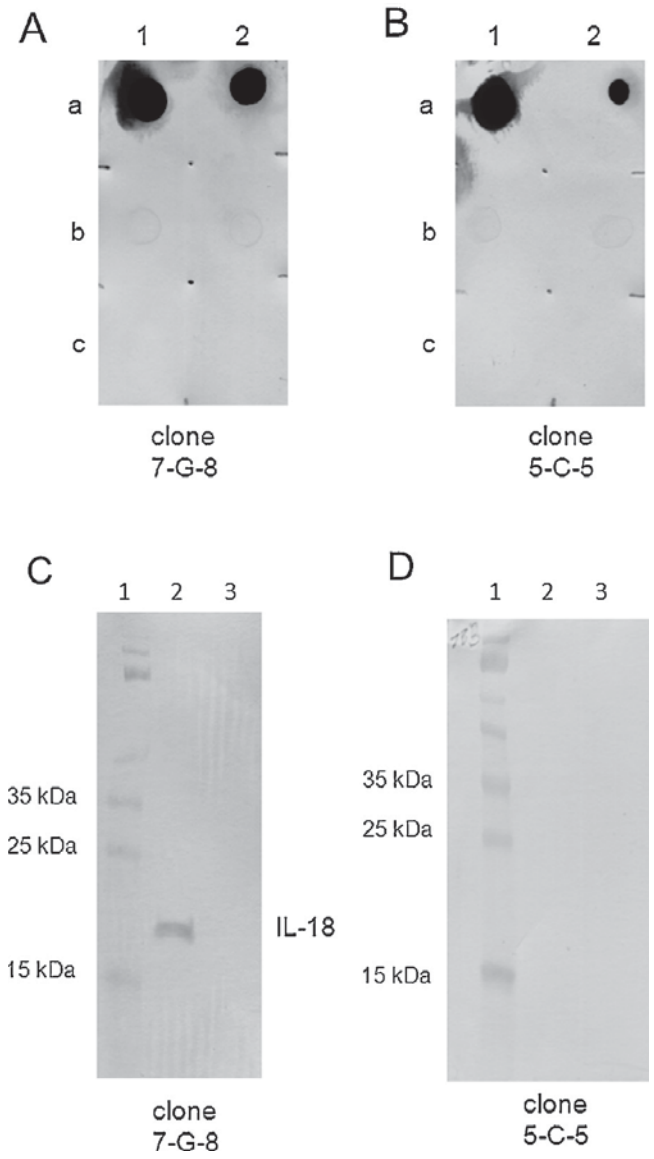

Fig. 6. Dot Blots and Western Blots with mAbs detecting equine IL-18: (A and B) Dot Blots with mAbs as pAb: (a) applied as positive controls (1a) pAb and (2a) sAb; (b) tested cell lysates of AAV-293 transfected with equine IL-18 (1b) propeptide or (2b) mature; (c) tested 10x concentrated supernatants of AAV-293 transfected with equine IL-18 (1c) propeptide or (2c) mature; ± reactivity with cell lysates containing equine IL-18 (C and D) Western Blots with mAbs as pAb: (1) marker; (2) cell lysate of AAV-293 transfected with equine pro-IL-18; (3) cell lysate of AAV-293 transfected with equine mat-IL-18; reactivity was detected for clone 7-G-8 with equine pro-IL-18.

cytokines of different species is observed (Pedersen et al., 2002).

One limitation of the current study was the missing standardization of cytokine amounts applied in DB and WB. Thus the obtained results cannot be analysed quantitatively. However, this approach served the overall purpose of the study to obtain qualitative results and deliver data about the potential value of the mAbs for detecting equine cytokines. When using these mAbs with naturally derived cytokines, it should be considered, that concentrations in body fluids may differ significantly from the rather high concentrations of cytokines used for DB and WB in this study. Further, background matrixes are

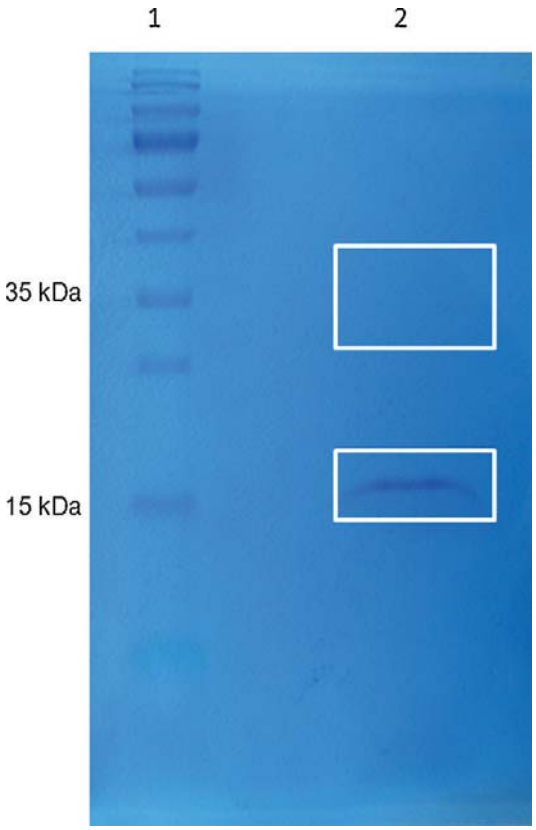

Fig. 7. Coomassie stained PA-gel loaded with equine rIL-1β produced in *E. coli*; one band at about the expected size for IL-1β of 17 kDa is visible. The second expected band (visible in WB with clones 8B25 and 424823) cannot be detected; (1) Marker; (2) equine rIL-1β produced in *E. coli*; (Frames) areas cut out for LC/MS.

different and recombinant and naturally derived cytokines can considerably vary in tertiary and quaternary structure, glycosylation pattern and other modifications. Thus, the herein obtained results describe the mAbs' general reactivity. This may not always be reproducible if smaller concentrations of cytokines, different solvents, or cytokines of different source (native or recombinant) are used (Absolom and van Oss, 1986; Morgan et al., 1998).

In general, recombinant proteins can differ from naturally derived proteins. Consequently, results obtained with mAbs using recombinant proteins in DB or WB cannot absolutely predict their reactivity with native cytokines or their efficacy for other experimental methods. However, data on recombinant protein reactivity delivers valuable information for pre-selection of mAbs for further experimental approaches.

Herein, the DB staining of cell lysates containing equine IL-18 with two anti-porcine-IL-18 mAbs (clones 7-G-8, 5-C-5) is suspected to be non-specific, as the results appeared not to be consistent with the WB results, although DB and WB are the two most comparable applications evaluated in this study. It is remarkable that one antibody (clone 7-G-8) did react with the propeptide of equine IL-18 expressed in AAV293 cells, but not with the mature form. The other anti-IL-18 antibody (clone 5-C-5) did not show any reactivity in the WBs.

There are some hypotheses potentially explaining the differences between the herein generated DB and WB results with equine IL-18: (1) The antibodies may only react with native forms of their target and thus, reactivity is impaired or absent after reduction and denaturation of the proteins for WBs which change the epitopes the mAbs are directed against. (2) Staining in DB may be false positive as many protein-rich fluids, like lysates of non-transfected cells or 1% bovine serum albumin in PBS, showed weak staining of the dots applied (data not shown). (3) The epitope detected by the antibody could be located in the precursor region of IL-18, which may be cleaved during protein maturation. Consistent to the latter, a reaction with the mature form of IL-18 in WB did not occur. Unfortunately, there was no information available which part of IL-18 is detected by antibody 7-G-8 or whether it was produced with the propeptide of porcine IL-18 as immunogen. The latter could offer an explanation for the sole detection of the propeptide of equine IL-18. (4) Secretion of pro-IL-18 could be impaired in the human AAV 293 cells when cleavage by IL-1β-converting enzyme is compromised (Gu et al., 1997; Fantuzzi et al., 1999) resulting in accumulation of the recombinant IL-18 in the cells and higher concentration in the lysates tested. (5) It cannot be excluded that expression

Table 6
Reactivity of monoclonal antibodies with equine cytokines in different applications.

Monoclonal antibody	Clone	DB	WB	IHC	FACS
Anti-equine IL-1β	8B25	+	+	+	–
Anti-ovine IL-1β	1D4	–	–	n.d.	n.d.
Anti-equine IL-1β	608714	+	+	+	+
Anti-equine IL-1β	424823	+	+	+	–
Anti-human IL-6	MQ2-13A5	–	n.d.	n.d.	n.d.
Anti-bovine IL-6	CC310	–	n.d.	n.d.	n.d.
Anti-human IL-8	B-K8	–	n.d.	n.d.	n.d.
Anti-ovine IL-8	8M6	–	n.d.	n.d.	n.d.
Anti-bovine IL-12	CC326	n.d.	–	n.d.	n.d.
Anti-bovine IL-12	CC301	n.d.	–	n.d.	n.d.
Anti-porcine IL-18	7-G-8	(+)	+	(+)	–
Anti-porcine IL-18	5-C-5	(+)	–	+	–
Anti-ovine GM-CSF	10B2340	+	+	+	–
Anti-ovine GM-CSF	8D8	–	–	n.d.	n.d.
Anti-ovine GM-CSF	3C2	n.d.	+	(+)	+

Legend: DB, Dot Blot; WB, Western Blot; IHC, immunohistochemistry; FACS, flow cytometry; reactivity: +, reactive; (+), weakly reactive; –, not reactive; n.d., not determined.

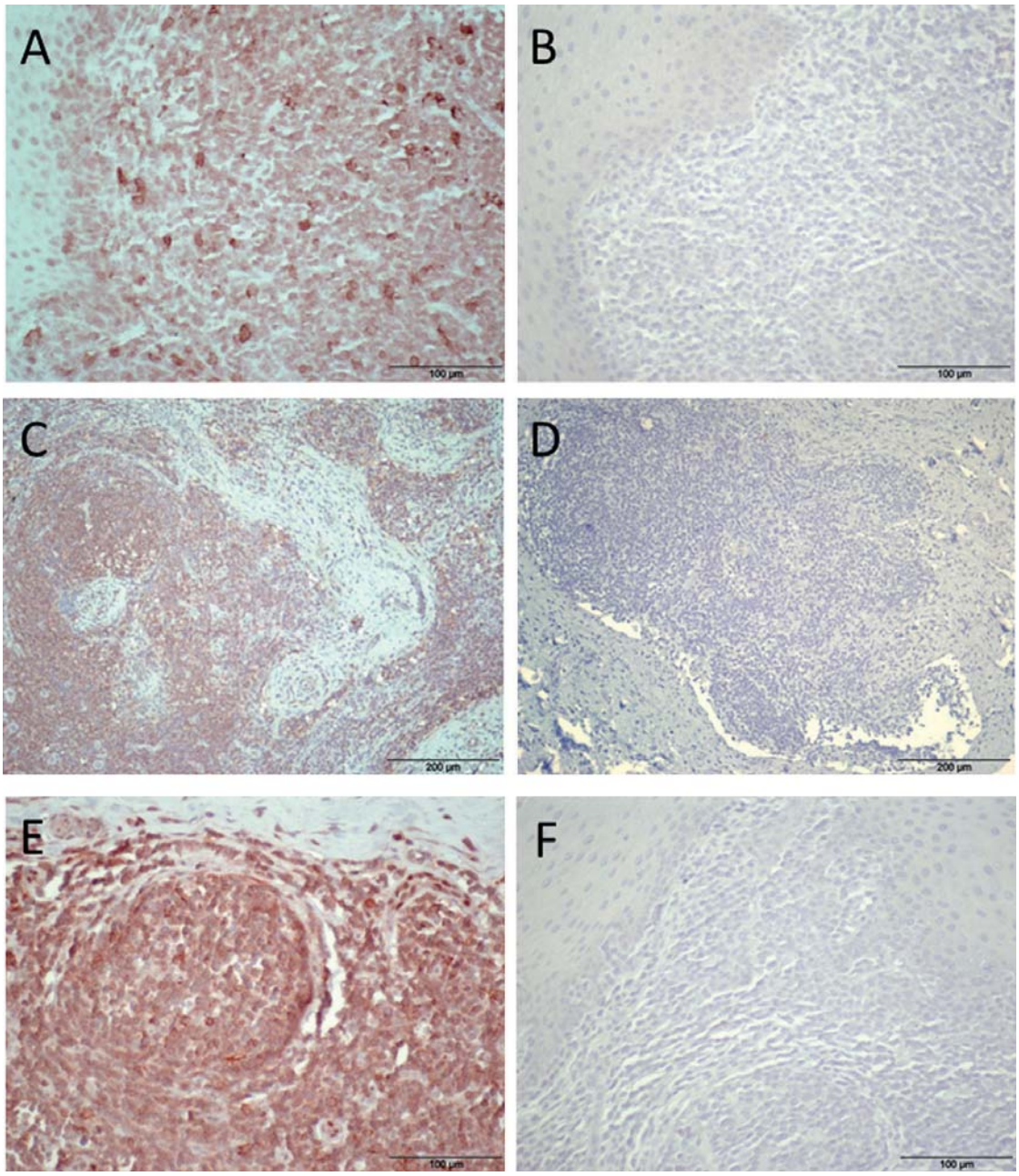

Fig. 8. Immunohistological staining of IL-1β in equine lymphatic tissues. Lymphatic tissues show reactivity with mAbs (A, C, E) visible as red staining of cells. Sections were pre-treated with heat, citrate, and counterstained with haematoxylin. (A and B) Tonsil. (A) Rat-anti-equine-IL-1β clone 8B25 as pAb, diluted 1:50. (B) Negative control incubated with buffer instead of pAb. (C and D) Lymph node. (C) Mouse-anti-equine-IL-1β clone 608714 as pAb, diluted 1:50. (D) Negative control incubated with buffer instead of pAb. (E and F) Tonsil. (E) Rat-anti-equine-IL-1β clone 424823 as pAb, diluted 1:50. (F) Negative control incubated with buffer instead of pAb.

of the mature equine IL-18 in AAV 293 has failed, leading to a negative result in the WB. As there were no other positive controls of equine IL-18 or reactive anti-equine-IL-18 antibodies available for comparison in WB, thus these results could not be investigated further and remain to be determined.

In IHC both mAbs for IL-18 were reactive with equine colon tissue. Clone 7-G-8 only reacted weakly, but with the same pattern of staining as the other. The difference in staining intensity in IHC makes clone 5-C-5 more suitable for IL-18 detection in this application. Stained cells are likely to be macrophages, known to be localized in the *lamina propria mucosae* of the colon (Donnellan, 1965). In addition, cells in the gut associated lymphatic tissue were positive for IL-18 in the current study for a lymph follicle. Production of IL-18 in lymphatic tissues has previously been reported in pigs (Foss et al., 2001).

However, clone 5-C-5 did not detect IL-18 by WB. This was in contrast to clone 7-G-8 showing only weak staining in IHC, but detecting (pre-)IL-18 in WB. None of the mAbs was suitable for IL-18 detection in flow cytometric analysis, although stimulation by LPS should be capable of inducing

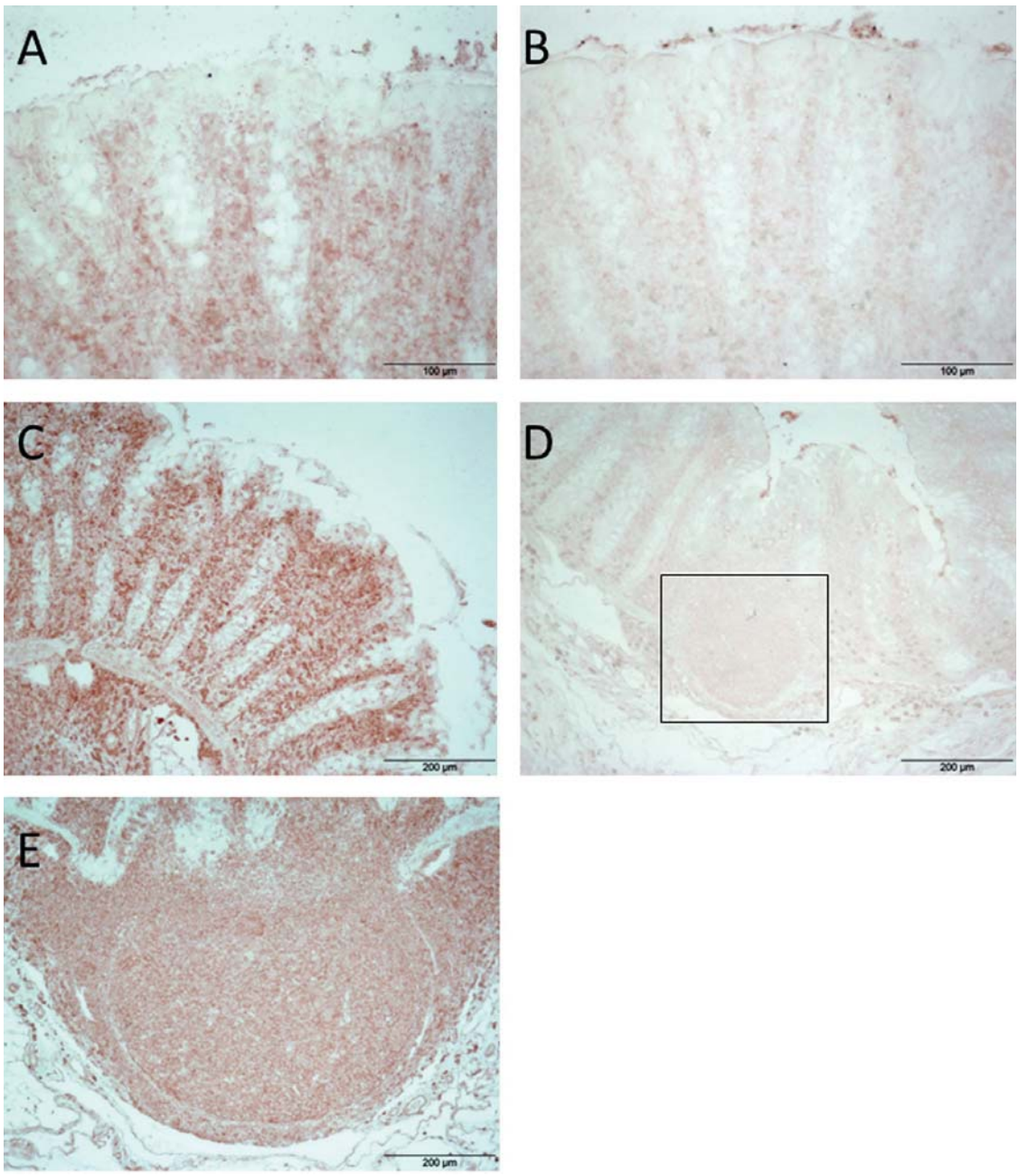

Fig. 9. Immunohistological staining of IL-18 in equine colon. Cells in the *Lamina propria mucosae* (A and C) and a solitary lymph follicle (E) show reactivity with mAbs visible as red staining of cells. Immunohistology was performed without pre-treatment and counterstaining. (A) Mouse-anti-porcine-IL-18 clone 7-G-8 as pAb, diluted 1:50 (weak reactivity). (B) Corresponding negative control incubated with buffer instead of pAb. (C) Mouse-anti-porcine-IL-18 clone 5-C-5 as pAb, diluted 1:100. (D) Negative control with buffer instead of pAb. Frame contains solitary lymph follicle. (E) Same pAB as in (C) magnified area from D shows positive cells in solitary lymph follicle.

IL-18 production (Puren et al., 1999). Taken together, the two mAbs seem to cross-react with an equine protein by IHC, but results in different applications are inconsistent.

IL-1β was detected in IHC by all three mAbs reactive in DB and WB. Lymph node sections served as positive controls. Sections displayed cells with varying intensity of staining within the lymphoid tissues. A minority of cells stained most intensively can likely be interpreted as macrophages as the major source of IL-1β. The remaining majority of less intensively stained cells possibly represent lymphocytes producing less IL-1β (Tizard, 2004). Usually, IL-1β is detected in lymphatic tissues after stimulation or infection (Ruco et al., 1989; Rothel et al., 1997; Miller et al., 2010). Therefore, it is surprising that by IHC lymph nodes without pathologic changes in histomorphology were distinctively positive for IL-1β in the current results. For GM-CSF both mAbs with positive results in DB and WB showed reactivity in IHC with equine lung samples, but clone 3C2 was only weakly positive. The staining pattern was identical for both mAbs, but for clone 3C2 the diagnostic value in further approaches seems doubtful because of the weak staining. GM-CSF production in respiratory epithelium has previously been described for other species (Blau et al., 1994). The stained interstitial cells could

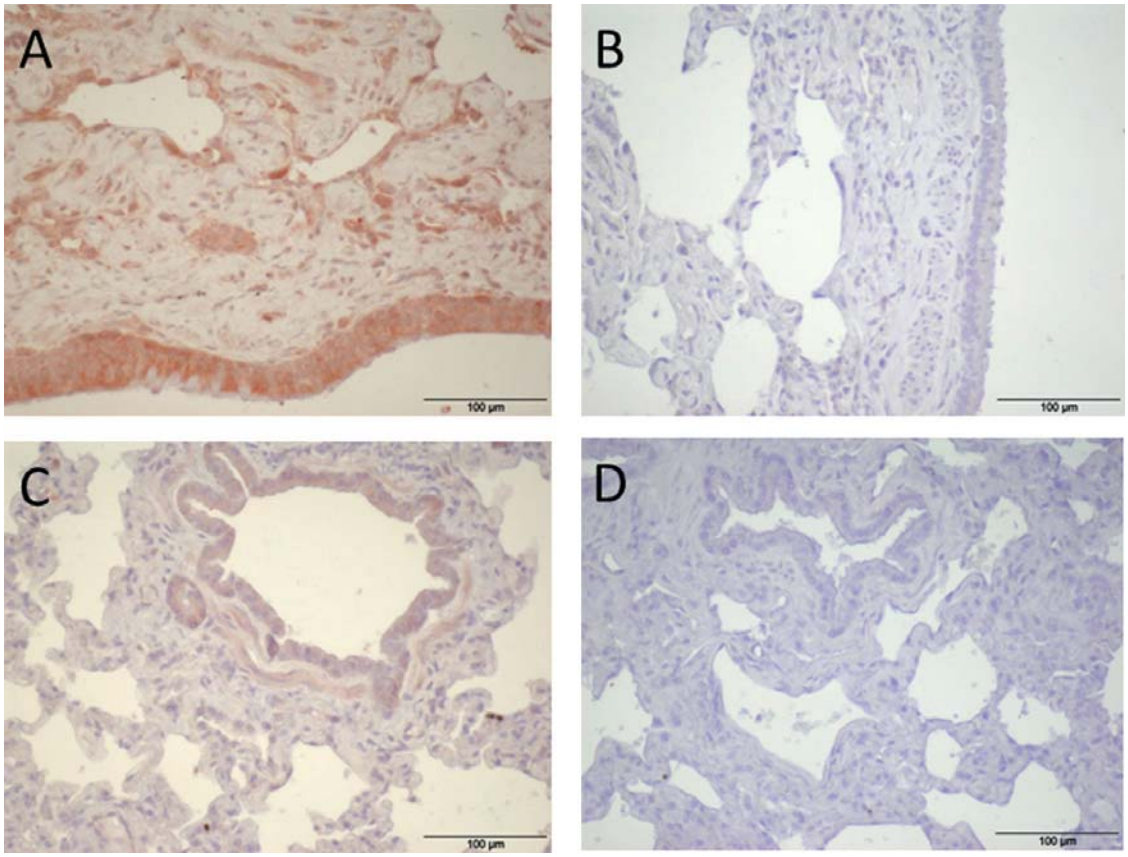

Fig. 10. Immunohistological staining of GM-CSF in equine lung. Respiratory epithelium (A and C) and various interstitial cells (A) show reactivity with mAbs visible as red staining of cells. Sections were counterstained with haematoxylin. (A and B) Sections pre-treated with heat, citrate. (A) Mouse-anti-ovine-GM-CSF clone 10B2340 as pAb, diluted 1:100. (B) Negative control incubated with buffer instead of pAb. (C and D) Sections without pre-treatment. (C) Mouse-anti-ovine-GM-CSF clone 3C2 as pAb, diluted 1:50 (weak reactivity). (D) Negative control incubated with buffer instead of pAb.

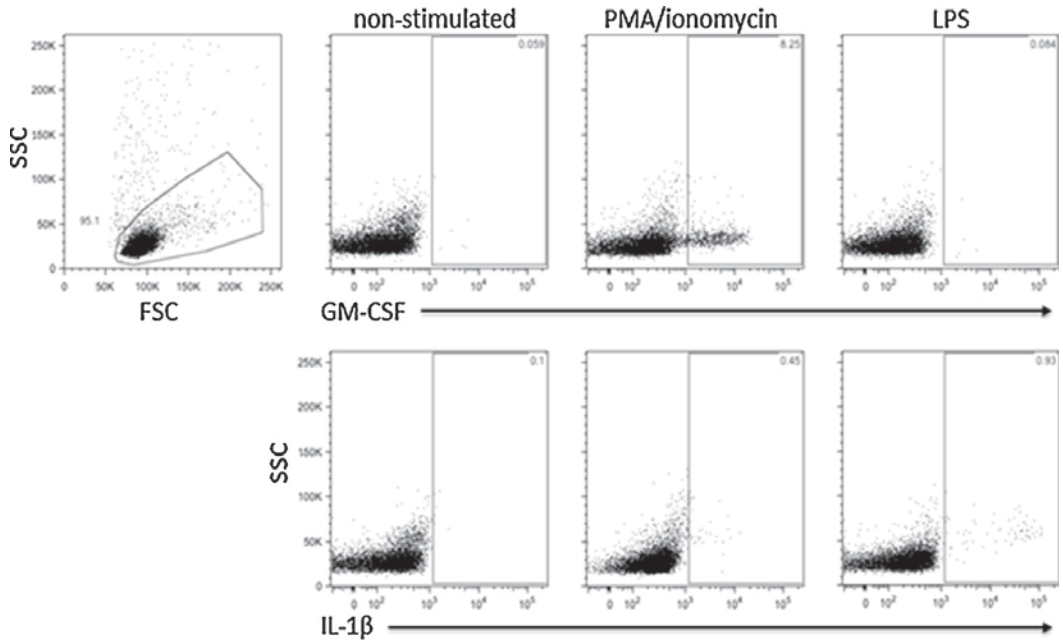

Fig. 11. Flow cytometric analysis of equine PBMC after stimulation with PMA and ionomycin or LPS and intracellular staining with anti-GM-CSF or anti-IL-1β antibodies. PBMC were cultured for 4 h in medium with or without stimuli and the secretion blocker Brefeldin A. Cells were fixed and intracellular staining was performed with anti-ovine GM-CSF, clone 3C2 (upper panel) or anti-equine IL-1β, clone 608714 (lower panel). The upper left blot show the morphology of the cells (SSC/FSC) and the PBMC gate used for analysis of GM-CSF and IL-1β positive cells.

be endothelial cells, fibroblasts or alveolar macrophages, described to produce GM-CSF (Griffin et al., 1990; Holldack et al., 1992; Tizard, 2004).

The (cross-)reactivities with naturally derived equine cytokines in IHC and flow cytometric analysis was expected as mAbs were chosen due to their potential reactivity estimated by homology or earlier descriptions, *e.g.* for flow cytometry (Pedersen et al., 2002).

For all mAbs tested by IHC with equine tissue samples, reactivity on paraffin-sections has not yet been described. The presented IHC results suggest reactivity of seven mAbs with endogenous equine cytokines. However, two mAbs with weak staining (clone 7-G-8 and 3C2) should be critically evaluated for their use in future studies. For one anti-IL-18 mAb (Clone 7-G-8) non-reactivity is stated in the fact sheet, while weak reactivity was observed here. This indicates that results may differ when different methods are applied.

For the two mAbs to IL-1β and GM-CSF staining subpopulations of equine PBMC by flow cytometry, reactivity with equine cells has not yet been reported. These mAbs provide useful tools for future studies to characterize protein expression of IL-1β and GM-CSF in PBMC *in vitro*.

As stated in the literature, PMA and ionomycin were able to induce the production of IL-1β and GM-CSF in equine PBMC (Kato et al., 1995; Mihara et al., 1996; Pedersen et al., 2002; Ci et al., 2008). LPS induced the production of IL-1β by a population of PBMC with larger side and forward scatter characteristics (Eggesbo et al., 1994; Kato et al., 1995). These cells likely correspond to equine monocytes which are known to be capable of the production of IL-1β (Figueiredo et al., 2008).

The results of the current study support the use of amino acid homology as a suitable tool for selecting cross-reactive mAbs for IHC, although the evaluation of four mAbs cannot provide sufficient information to draw general conclusions.

Although two antibodies against ovine GM-CSF did cross-react with equine GM-CSF in WB and IHC (84% homology of equine and ovine GM-CSF), six antibodies against different equine cytokines chosen by the homology criterion did not react in DB and WB, even if protein homology was as high as 83% to 91% (for IL-12). Thus, great homology of the primary protein-structure of the targets alone is generally not a reliable indicator of Ab cross-reactivity in DB and WB. This has also been

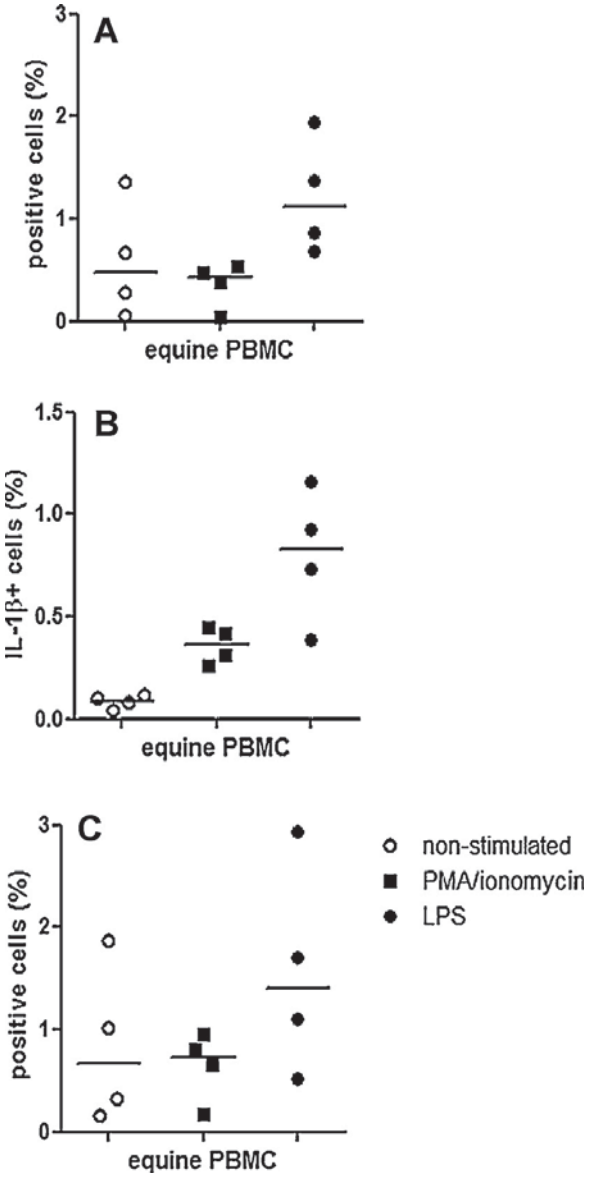

Fig. 12. Flow cytometric analysis of equine PBMC from four adult horses after intracellular staining with three anti-IL-1β antibodies. PBMC were cultured for 4 h in medium with or without stimuli and the secretion blocker Brefeldin A. (A) anti-equine IL-1β, clone 8B25; (B) anti-equine IL-1β, clone 608714; (C) anti-equine IL-1β, clone 424823. Clone 608714 is suitable for this application detecting equine IL-1β.

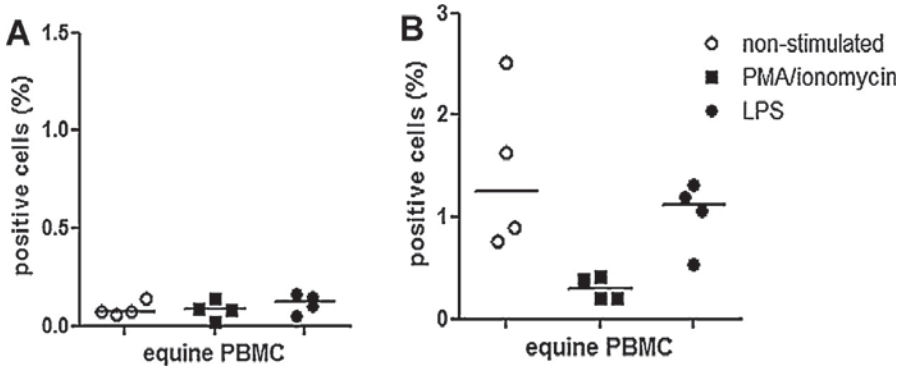

Fig. 13. Flow cytometric analysis of equine PBMC from four adult horses after intracellular staining with three anti-IL-18 antibodies. PBMC were cultured for 4 h in medium with or without stimuli and the secretion blocker Brefeldin A. (A) anti-porcine IL-18, clone 7-G-8; (B) anti-porcine IL-18, clone 5-C-5.

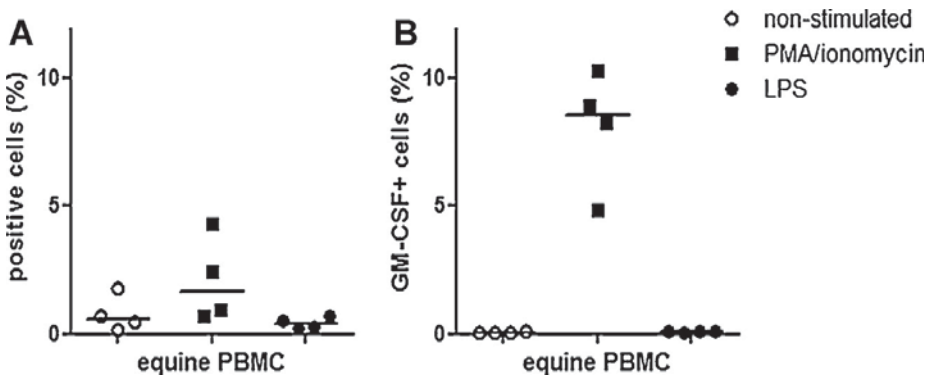

Fig. 14. Flow cytometric analysis of equine PBMC from four adult horses after intracellular staining with two anti-GM-CSF antibodies. PBMC were cultured for 4 h in medium with or without stimuli and the secretion blocker Brefeldin A. (A) anti-ovine GM-CSF, clone 10B2340; (B) anti-ovine GM-CSF, clone 3C2. Clone 3C2 is suitable for this application detecting equine GM-CSF.

shown previously for different immunological applications detecting equine IL-12 (Duran et al., 2013). These results are in contrast to the relatively consistent prediction of biological cross-reactivity by homology (Scheerlinck, 1999).

Failure of antibodies to (cross-) react with homologue equine cytokines in DB and WB or flow cytometric analysis could be due to the specificity of a tested antibody to its epitope which may not be identical between immunogen and the native equine cytokine even if total protein homology is approximately 100%. Another reason for the lack of cross-reactivity can be a different 3D confirmation between recombinant and native proteins which potentially causes steric inhibition of the antibody's paratope binding to the epitope of the protein or modifies conformational epitopes detected by the antibody.

Our results were also not consistent between applications. Thus cross-reactivity in one method cannot prove that the mAb detects the targeted equine cytokine in general, it only applies to the respective application. Hence, reactivity of mAbs needs to be confirmed for each experimental approach or method.

5. Conclusion

The herein shown results indicate that reactivity of monoclonal antibodies varies depending on experimental conditions and applications. In particular, different sources of proteins and different immunogens during generation of the antibody influence the ability to detect the corresponding protein. Cross-reactivity cannot be easily predicted by protein homology alone or by results obtained in other immunological applications tested. Consequently, antibody specificity should be confirmed in the development of any new immunological test and application utilized and by including tests and controls that show proof for specific detection of the naturally derived protein to avoid false negative or false positive results.

We confirmed that seven of 15 tested monoclonal antibodies react with equine cytokines by Western Blotting, IHC or flow cytometric analysis. These antibodies may be used for the development of immunological assays for the detection of equine IL-1β, equine IL-18 and equine GM-CSF. Thus, data of the current study can support the reader in his or her choice of antibodies (targeting equine cytokines) for various immunological applications.

Conflict of interest

The authors state that there is no conflict of interest.

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3 Manuscript II

Influences of Age and Sex on Leukocytes of Healthy Horses and their *ex vivo* Cytokine Release

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Abstract

Leukocytes and their functional capacities are used extensively as biomarkers in immunological research. Commonly employed indicators concerning leukocytes are as follows: number, composition in blood, response to discrete stimuli, cytokine release, and morphometric characteristics. In order to employ leukocytes as biomarkers for disease and therapeutic monitoring, physiological variations and influencing factors on the parameters measured have to be considered. The aim of this report was to describe the ranges of selected leukocyte parameters in a sample of healthy horses and to analyse whether age, sex, breed, and sampling time point (time of day) influence peripheral blood leukocyte composition, cell morphology and release of cytokines *ex vivo.* Flow cytometric comparative characterization of cell size and complexity in 24 healthy horses revealed significant variance. Similarly, basal release of selected cytokines by blood mononuclear cells also showed high variability [TNFα (65 – 16624 pg/ml), IFNγ (4 – 80 U/ml), IL-4 (0 – 5069 pg/ml), IL-10 (49 – 1862 pg/ml), and IL-17 (4 – 1244 U/ml)]. Each animal's age influenced leukocyte composition, cell morphology and cytokine release (TNFα, IL-4, IL-10) *ex vivo*. Geldings showed smaller monocytes and higher spontaneous production of IL-10 when compared to the mares included. The stimulation to spontaneous release ratios of TNFα, IL-4 and IL-17 differed in warmblood and Thoroughbred types. Sampling time influenced leukocyte composition and cell morphology. In summary, many animal factors – age being the dominant one – should be considered for studies involving the analysis of equine leukocytes. In addition, high inter-individual variances argue for individual baseline measurements.

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Research paper

Influences of age and sex on leukocytes of healthy horses and their *ex vivo* cytokine release

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Leukocytes and their functional capacities are used extensively as biomarkers in immunological research. Commonly employed indicators concerning leukocytes are as follows: number, composition in blood, response to discrete stimuli, cytokine release, and morphometric characteristics. In order to employ leukocytes as biomarkers for disease and therapeutic monitoring, physiological variations and influencing factors on the parameters measured have to be considered. The aim of this report was to describe the ranges of selected leukocyte parameters in a sample of healthy horses and to analyse whether age, sex, breed, and sampling time point (time of day) influence peripheral blood leukocyte composition, cell morphology and release of cytokines *ex vivo*. Flow cytometric comparative characterisation of cell size and complexity in 24 healthy horses revealed significant variance. Similarly, basal release of selected cytokines by blood mononuclear cells also showed high variability [TNFα (65–16,624 pg/ml), IFNγ (4–80 U/ml), IL-4 (0–5069 pg/ml), IL-10 (49–1862 pg/ml), and IL-17 (4–1244 U/ml)]. Each animal's age influenced leukocyte composition, cell morphology and cytokine release (TNFα, IL-4, IL-10) *ex vivo*. Geldings showed smaller monocytes and higher spontaneous production of IL-10 when compared to the mares included. The stimulation to spontaneous release ratios of TNFα, IL-4 and IL-17 differed in Warmblood and Thoroughbred types. Sampling time influenced leukocyte composition and cell morphology. In summary, many animal factors – age being the dominant one – should be considered for studies involving the analysis of equine leukocytes. In addition, high inter-individual variances argue for individual baseline measurements.

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1. Introduction

Blood leukocyte counts, their proportions and properties have been widely used as indicators for the so-called immunological status of an individual. Proliferative capacities and cytokine production of peripheral cells in horses have been employed as biomarkers in different areas of research, such as laminitis (Hurley et al., 2006; Wray et al., 2013), hypersensitivities (McKelvie et al., 1999; Cordeau et al., 2004; Hamza et al., 2010; Lavoie-Lamoureux et al., 2012b), infections (Edmonds et al., 2001; Hubert et al., 2004; Breathnach et al., 2005; Brault et al., 2010; Soboll Hussey et al., 2011), sepsis (Burton et al., 2009), and aging (Adams et al., 2008; Katepalli et al., 2008).

Activity and reactivity of immune cells are reflected in the cytokines they secrete (de Jager, 2009). The morphologic characteristics of the cells are further indicators of their reactivity and proliferative capacity. Their size and granularity change during proliferation, variations in metabolism, degranulation and/or phagocytosis, *e.g.* after *in vitro* stimulation (Schuberth et al., 2000). Flow cytometric methods have been employed to determine the size and granularity/complexity of immune cells to evaluate morphological changes. Even subtle effects on circulating cells can be detected by this method *ex vivo* and *in vitro* (Webster et al., 1995; Schuberth et al., 2001; Böhmer et al., 2011).

When using blood cell morphometric parameters and cytokine production as biomarkers for health and disease or to monitor response to treatment, it is necessary to identify the variables affecting these parameters in healthy animals (Schuberth et al., 2001) and to prove whether diurnality must be considered for comparisons (Piccione and Giannetto, 2011; Benito et al., 2014).

Influences of age, breed and sex on hematological parameters have been described for different species (Schalm et al., 1975; Mayr et al., 1979; Allen et al., 1984; Goto and Nishioka, 1989; Kieferndorf and Keller, 1990; Shiga et al., 1999; Čebulj-Kadunc et al., 2002, 2003; Satué et al., 2009; Mikniene et al., 2014; Novotny et al., 2014), as well as for lymphocyte subset composition (Goto and Nishioka, 1989; Ohtsuka et al., 2002; Satué et al., 2010; Robbin et al., 2011; Macedo et al., 2013), innate immunity parameters (Krasimira et al., 2013), and cytokine serum/plasma levels or secretion *in vitro* (Bruunsgaard et al., 2001; Marik and Zaloga, 2001; Silberer et al., 2008; Fujiwara et al., 2012; Giraldo et al., 2013; Wray et al., 2013). Furthermore, circadian differences have been described for secreted cytokine levels (Pollmächer et al., 1996). Most research in this area has been performed in humans and laboratory animals.

A range of applications have been utilised for cytokine determinations in leukocytes of healthy horses, including mRNA-expression analyses of cytokines (Edmonds et al., 2001; Ainsworth et al., 2003; Vick et al., 2007; Adams et al., 2008, 2009; Svansson et al., 2009), intracellular immunofluorescence (Adams et al., 2008, 2009; Hamza et al., 2010), and ELISA or bead-based analyses of cell culture supernatants (Desjardins et al., 2004; Adams et al., 2008, 2009; Hamza et al., 2010) or serum (Vick et al., 2007; Lavoie-Lamoureux et al., 2012a,b). In general, reference values for the cytokines expressed are lacking and comparability is hampered by the differences in experimental conditions and assay systems used. Thus, individual baselines may be favorable (Friedrichs et al., 2012) to detect effects within a sample of horses in prospective studies.

Morphometric measurements have been commonly employed to gate leukocyte subsets in flow cytometry in horses (Adams et al., 2008, 2009; Hamza et al., 2010; Satué et al., 2010), but evaluation of morphological features and their changes have not been performed so far.

For baseline determinations in the course of a study on DNA effects leukocyte properties of 24 healthy horses displayed high interindividual variances. In order to determine the calculation methods for individual baselines, influences of age, sex, breed and time of sampling were analysed. The aim of the present explorative report was to determine variances for the morphological parameters of blood leukocytes and PBMC-secreted cytokines *ex vivo* in healthy horses and to describe the influencing potential of age, breed/type, sex, and time of day of drawing samples.

2. Materials and methods

All procedures were carried out according to the ethical guidelines of the law on animal welfare (Tierschutzgesetz) and approved by the "Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit" (LAVES) in AZ 33.9-42502-04-11/0399.

2.1. Animals

Twenty-four clinically healthy horses (six mares, 15 geldings and three stallions) aged between two and 21 years (mean 10.6 years) with a body weight of 425–680 kg (mean 572 kg) were included in the study. There were six Thoroughbred (ThB) type horses (two Arabians, two Arabian-mix and two English Thoroughbreds) and 18 Warmblood (WBl) type horses (nine Hanoverian Warmbloods, one Hessian Warmblood, one Polish Warmblood, two Pura Raza Espanolas (PRE), one PRE-Mix, one Standardbred, one Oldenburg Warmblood, and oneWestfalian Warmblood).

The animals were kept in the Clinic for Horses, University of Veterinary Medicine, Hannover, Foundation, housed in standard single boxes on straw or wood shavings under a natural light-dark cycle of German summer (15–16.3 h of light, sunrise between 05:12 and 05:44, light from large windows or adjacent paddocks). The horses were fed hay and concentrates twice a day (at 07:00 and 18:00) according to their body weight and had access to water *ad libitum*. Horses had access to an outdoor 20 × 60 m sand area once a day, in the late afternoon or early evening, with a minimum of 2 h rest prior to examination and sampling. Before starting the experiment, the horses were allowed to acclimatise for at least three days and were trained by classical and operant conditioning for blood sampling to minimise stress during blood drawings.

As the measurements herein were part of internal controls before injection of different test substances, horses were injected with sterile PBS under sterile conditions into the skin of the neck and into the *M. pectoralis* with 0.5 ml per injection site after the first sampling at 09:00.

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2.2. Clinical examinations and blood sampling

Horses underwent a general clinical examination twice a day and measurement of rectal temperatures four times a day (at 03:00, 09:00, 15:00, and 21:00).

Blood samples were drawn from the jugular vein at 09:00, 15:00 and 21:00, after disinfection with ethanol, by use of a vacutainer system (Vacuette, greiner bio-one, Frickenhausen, Germany) with a 20 G single-use cannula. Blood was collected in EDTA-coated tubes for flow cytometric analysis of whole blood leukocytes and in heparinized (sodium-heparin) tubes for the isolation of PBMC.

Additional samples were taken at each sampling time for analysis by routine clinical laboratory measurements to monitor total plasma protein (TPP), hematocrit (Hct), white blood counts (WBC), and differential haemograms by a manual refractometer (TPP), a Sysmex kx-21N (Sysmex Deutschland GmbH, Norderstedt, Germany) (Hct, WBC) and an ADVIA120 (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) (haemograms), respectively.

2.3. Preparation of blood leukocytes

Erythrocytes were lysed in 100 μl EDTA-blood and nucleated cells were fixed by the addition of BD FACS lysing solution (BD Bioscience, Heidelberg, Germany), according to the manufacturer's instructions. Cells were washed once in cell wash (BD Bioscience) and suspended in 200 μl of FACS Flow (BD Bioscience).

2.4. Isolation of PBMC

PBMC were isolated from heparinized blood by density gradient separation. Briefly, after sedimentation at room temperature (RT) for 1 h, leukocyte-rich plasma was layered onto LSM 1077 (density gradient solution, PAA, Pasching, Austria) and centrifuged (30 min, 1000 × *g*, RT). Interphase PMBC were harvested and washed three times in PBS (10 min, 500/250/150 × *g*, RT). Cells were counted by using the Cellometer Auto T4 cell counting system (Nexcelom Bioscience, MA, USA).

2.5. Cell culture

A total of 2×10^6 PBMC per well were seeded in sterile cell culture 12-well plates and incubated for 12 h in a humidified atmosphere at 37 °C (5% CO_2 in air) in 1 ml culture medium [(RPMI, Biochrom AG, Berlin, Germany) supplemented with 10% heat-inactivated FCS (PAA Laboratories GmbH, Pasching, Austria) and penicillin 100 U/ml, streptomycin 0.1 mg/ml (PAA)], or in 1 ml medium containing PMA (50 ng/ml) and Ionomycin (1.34 μM) [2 μl of Cell stimulation cocktail (500×, eBioscience, Frankfurt, Germany) containing PMA 40.5 μM and Ionomycin 670 μM ad 1 ml]. Medium settings were used to evaluate spontaneous cytokine release and PMA/Ionomycin stimulation to assess cytokine induction. This concentration of stimuli was used in recent studies on human PBMC (Nagendran et al., 2013), T cells (Rohlman et al., 2012), murine T cells (Edwards et al., 2014), murine lung cells (Mays et al., 2013) and natural killer T cells (Ren et al., 2014) and had proven useful to induce all targeted cytokines (except IFNα) in preliminary experiments with equine PBMC (data not shown). After incubation, cell-free supernatants were obtained and stored at −80 °C until further analysis. Thawing was performed at 37 °C immediately prior to the cytokine determinations.

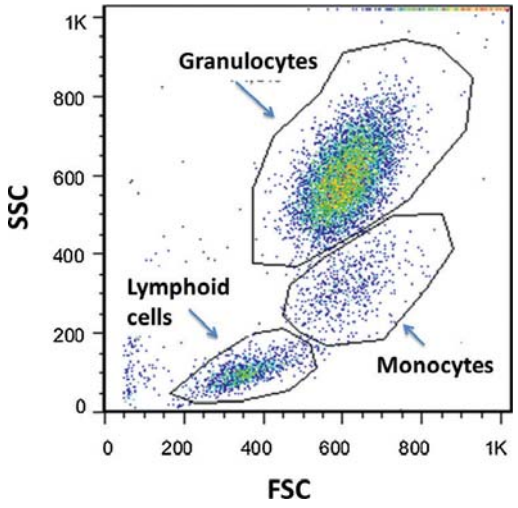

Fig. 1. Gating strategy for the identification of granulocytes, monocytes and lymphoid cells among equine peripheral blood leukocytes. Leukocytes were flow cytometrically assessed after whole blood lysis. In forward scatter (FSC) *versus* side scatter (SSC) dot plots, major cell populations were identified based on their characteristic size and granularity.

2.6. Flow cytometric analysis

Flow cytometric analysis was performed using a FACScalibur (BD Bioscience). At least 10,000 events were acquired for each determination. Instrument and analysis settings were kept constant for blood and PBMC of individual horses. Data analysis was performed with FlowJo 7.6.1 software (Celeza GmbH, Olten, Switzerland). Leukocyte populations were gated manually by morphometric criteria, *i.e.* forward scatter (FSC) and side scatter (SSC) (Fig. 1). Fractions of each population were calculated as percentages of all gated cells.

2.7. Determination of equine cytokines in cell culture supernatants

TNFα was measured by a sandwich ELISA for equine TNFα (Duo Set DY 1814, RnD, Wiesbaden, Germany), performed according to the manufacturer's protocol with the exception that coating was carried out at 4 °C overnight, as described previously (Lavoie-Lamoureux et al., 2010). Dilutions of standards and supernatant samples (if necessary due to exceeding upper detection limit) were made with PBS containing 1% BSA (# P3688, Sigma Aldrich). Absorption was measured by a Synergy2 instrument (BioTek Instruments GmbH, Bad Friedrichshall, Germany) and data was analysed by Gene 5 1.11 software (BioTek Instruments GmbH). The lower and upper limits of detection of the assay were 31.2–2000 pg/ml.

Two samples were excluded from further analysis as the differences measured between TNFα duplicates were excessive.

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IFNα, IFNγ, IL-4, IL-10, and IL-17 were determined with a bead-based multiplex assay using equine-specific monoclonal antibodies on a Luminex Instrument (Luminex, Austin, TX, USA), as described previously (Wagner and Freer, 2009). Limits of detection were as follows: IFNα (12–30,000 pg/ml), IFNγ (10–5000 U/ml), IL-4 (40–80,000 pg/ml), IL-10 (15–35,000 pg/ml), and IL-17 (10–10000 U/ml).

2.8. Statistical analysis

Statistical analysis was performed with SAS Analytics Pro (SAS Institute Inc, Cary, NC, USA) version 9.3 or higher. p-values < 0.05 were considered significant.

Cytokine data were log-transformed for statistical analyses due to log-normal distribution of the values. The ratio of IFNγ to IL-4 was calculated as ratio (U/pg) = IFNγ (U/ml)/IL-4 (pg/ml). Stimulation ratios (times release) were calculated for each cytokine as $\text{cytokine}_{\text{PMA/Ionomycin}}/\text{cytokine}_{\text{medium}}$. The influences of age, sex and breed on all parameters measured and calculated were each analysed in a multivariate (mv) and a univariate (uv) ANOVA. The horses' individual influences were considered by inclusion of the horses as a random factor in the model. Interactions of different influencing parameters were not analysed herein. Results were considered significant if p-values were <0.05 in both models (mv and uv). Where significant influences of age were found, linear regression was calculated by GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

ANOVA was employed for the analysis of the influence of sampling times on all parameters measured and calculated, with the time of day as a fixed factor and horses as random factors considering the interaction of the time of day and horses.

3. Results

3.1. Distributions and variances

Cell size, cellular complexity and blood cell composition (except monocyte percentages) showed a Gaussian distribution (data not shown). Cytokine concentrations were usually log-normally distributed (Table 1). Interindividual variances of secreted cytokine concentrations and morphometric parameters were high across all animals tested (Tables 1 and 2) enabling analysis of variances for influencing factors. All cytokines tested were detectable in supernatants of unstimulated and stimulated PBMC, except IFNα, which was usually not detectable in supernatants of unstimulated PBMC and only weakly inducible. Therefore, IFNα was not analysed with respect to different influences. Stimulation by PMA/Ionomycin resulted in strong stimulation for the other cytokines [means of stimulation ratios (cytokine, times release): TNFα 53; IFNγ 112; IL-4 3263; IL-10 104; and IL-17 1143].

3.2. Influence of age

Lymphocyte percentages among blood leukocytes decreased with age ($p = 0.0029$, slope = −0.3583), while granulocyte and monocyte percentages increased (Fig. 2). Mean FSC values of granulocytes and lymphocytes increased significantly with age. The trend of a decreasing complexity (lower SSC) of granulocytes with age was not significant (Fig. 2).

Both spontaneous and PMA/Ionomycin-induced releases of TNFα were positively correlated with age. Only the spontaneous release correlated positively with age for IL-4 and IL-10 (Fig. 3). The tendency for a lower spontaneous but higher induced release of IFNγ with age was not significant. A non-significant trend of positive influence of age was also visible for PMA/Ionomycin-induced release of IL-17 (Fig. 3). Age furthermore significantly lowered the IFNγ/IL-4 ratio in supernatants of unstimulated PBMC ($p = 0.0012$, slope = −1.655). The stimulation ratio decreased with age for TNFα and IL-4 (Supplementary Fig. 1).

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.02.011.

3.3. Influence of breed

In comparison to ThB type horses, WBl type horses showed up with lower lymphocyte SSC values (mv

Table 1
Concentrations of cytokines in unstimulated and stimulated PBMC supernatants.

Cytokine	Release	Mean ± SD	Min	Median	Max	Distribution of values
IFNα (pg/ml)	Spont.	0.98 ± 3.03	0.0	0.00	18.0	–
	Induced	15.58 ± 14.82	0.0	12.50	89.0	–
TNFα (pg/ml)	Spont.	2122 ± 2560	65	1411	16,624	Log-normal
	Induced	36,422 ± 23,474	431	34,143	93,311	Log-normal
IFNγ (U/ml)	Spont.	13.15 ± 12.17	4.0	10.00	80.0	Log-normal
	Induced	961 ± 319	4	10,700	1380	Normal
IL-4 (pg/ml)	Spont.	553 ± 813	0	231	5069	Log-normal
	Induced	124,519 ± 73,181	7	129,780	304,383	Normal
IL-10 (pg/ml)	Spont.	466 ± 297	49	402	1862	Normal
	Induced	33,813 ± 30,178	260	26,283	145,551	Log-normal
IL-17 (U/ml)	Spont.	154 ± 224	4	71	1244	Log-normal
	Induced	27,779 ± 13,950	16	28,893	52,487	Normal

Spont.: spontaneous release; Induced: PMA/Ionomycin-induced release.

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Table 2
Blood cell composition and flow cytometrically assessed size and complexity of cells are influenced by the time of day of blood sampling.

Cell type	Parameter	09:00			15:00			21:00			Overall			*p*	
		Mean[1,2]	Min	Max	Mean[1,2]	Min	Max	Mean[1,2]	Min	Max	Mean	Min	Max	[1]	[2]
Lymphocytes	% of leukocytes	21.48	11.0	33.0	22.33^{C}	12.0	34.0	20.79^{c}	9.0	33.0	21.52	9.0	34.0	Cc	0.0406
Granulocytes	% of leukocytes	72.96	62.0	84.0	72.13	62.0	83.0	73.50	61.0	85.0	72.89	61.0	85.0		
Monocytes	% of leukocytes	5.67^{b}	4.0	9.0	5.67	3.0	10.0	5.83^{B}	3.0	10.0	5.71	3.0	10.0	Bb	0.0395
Lymphocytes	Forward scatter	346^{B}	273	409	346^{C}	316	380	$336^{b,c}$	231	379	344	231	409	Bb	0.0013
														Cc	0.0041
	Side scatter	82^{A}	57	99	$79^{a,b}$	66	94	82^{B}	64	143	81	57	143	Aa	0.0010
														Bb	0.0080
Granulocytes	Forward scatter	$589^{a,B}$	489	667	$597^{A,C}$	550	663	$579^{b,c}$	400	678	589	400	678	Aa	0.0418
														Bb	0.0073
														Cc	0.0002
	Side scatter	$550^{a,b}$	430	657	$561^{A,c}$	497	634	$576^{B,C}$	492	825	560	430	825	Aa	0.0368
														Bb	<.0001
														Cc	0.0144
Monocytes	Forward scatter	611^{a}	484	720	$625^{A,C}$	557	686	602^{c}	418	694	612	418	720	Aa	0.0018
														Cc	<.0001
	Side scatter	274^{b}	201	355	276^{c}	227	344	$284^{B,C}$	228	477	277	201	477	Bb	0.0017
														Cc	0.0217

[1] Superscripts, annotations for *p*-values "Aa, Bb, Cc", significantly different means between sampling times with the same letters in each row.
[2] Fat values represent means significantly different from at least one other sampling time.

$p = 0.057$, uv $p < 0.001$). This trend was also visible for the other subtypes (granulocytes and monocytes; Supplementary Table 1). Stimulation ratios of IL-4 and IL-17 were higher in WBls than in ThBs (IL-4 mv $p = 0.049$, uv $p = 0.034$; IL-17 mv, $p = 0.039$, uv $p = 0.037$; Supplementary Fig. 2). The IFNγ/IL-4 ratio in supernatants of unstimulated PBMC was significantly higher in WBls (mv$p = 0.0175$, uv $p = 0.0193$; Supplementary Fig. 3).

Supplementary Table S1 and Figs. S1 and S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.02.011.

3.4. Influence of sex

Stallions were excluded from statistical analysis due to their low number. In comparison to mares, geldings had significantly higher monocyte FSC values (mv $p = 0.018$, uv $p = 0.008$; Supplementary Table 2) and showed up with significantly higher spontaneous *ex vivo* release of IL-10 by PBMC (mv$p = 0.047$, uv $p = 0.030$; Supplementary Table 3).

Supplementary Tables S2 and S3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.02.011.

3.5. Influence of sampling time of day

Leukocyte percentages showed significant differences between sampling times of day with the highest percentages of lymphocytes at 15:00. Monocyte percentages were lowest at 09:00 with a significant difference to samples taken at 21:00. Granulocyte percentages did not significantly differ between times of sampling (Table 2).

FSC geometric means showed a minimum for granulocytes, lymphocytes and monocytes at 21:00, indicating significantly smaller cell sizes of all leukocyte subtypes at that time. SSC geometric means of granulocytes appeared mildly but significantly reduced when blood was sampled at 09:00, whereas lymphocyte SSC reached a nadir at 15:00 and monocyte SSC a peak at 21:00 (Table 2).

No influence of sampling times on the cytokine concentrations in PBMC supernatants was observed. Stimulation ratios were subtly influenced by sampling time for IFNγ and IL-17, with the lowest ratios at 09:00, and for IL-4, with the highest ratios at 15:00 (Supplementary Fig. 4).

Supplementary Fig. S4 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.02.011.

3.6. Correlation between cytokine release and morphometric parameters of blood leukocytes

The morphometric properties (FSC and SSC means) of blood lymphocytes were significantly positively correlated with basal PBMC release of TNFα, IL-4 and IL-10 (Fig. 4). Mean FSC and SSC values of blood granulocytes were negatively correlated with stimulated PBMC release of TNFα, IFNγ, IL-10 (with FSC only), and IL-17 (Supplementary Fig. 5). The FSC and SSC means of blood monocytes were negatively correlated with stimulated PBMC release of TNFα. The SSC of monocytes was further negatively correlated with the induced release of IFNγ (Fig. 5).

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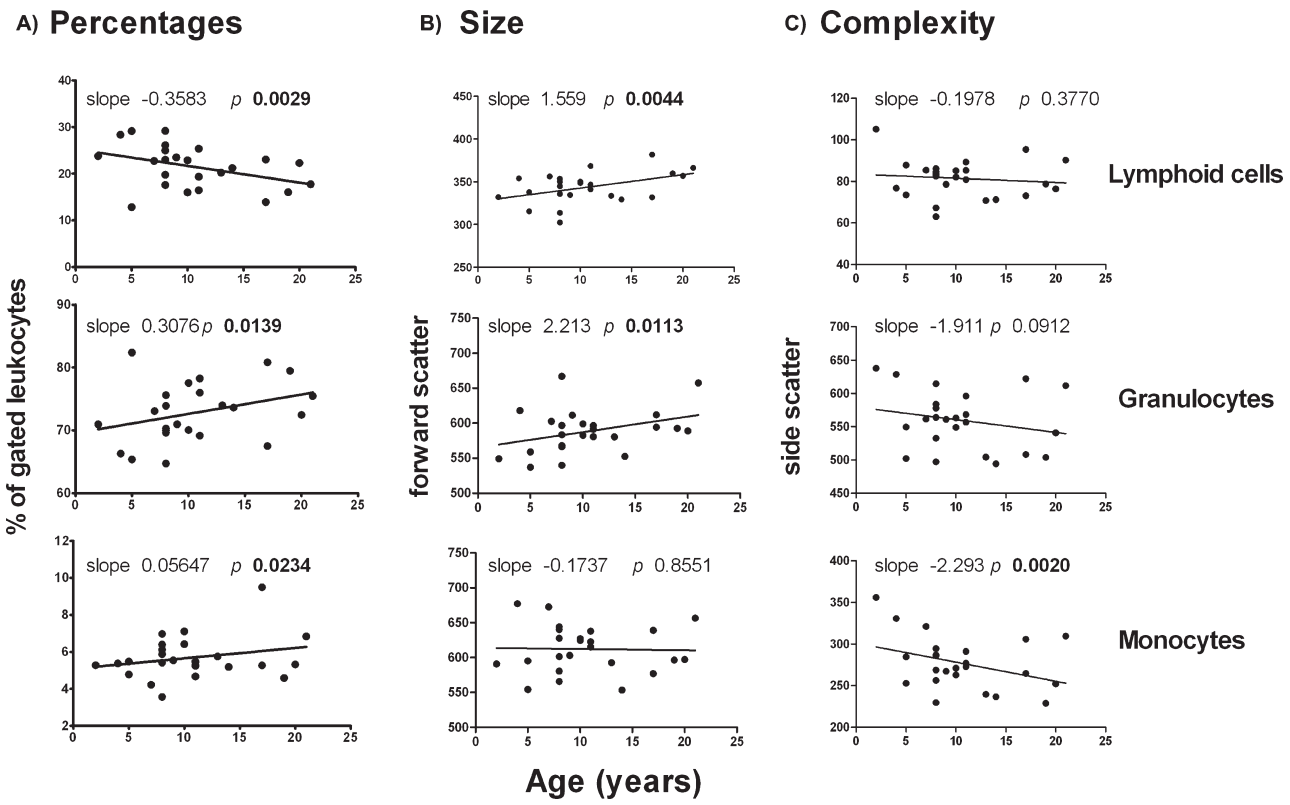

Fig. 2. Influence of age on leukocyte composition and morphological parameters of major cell types. Percentages(A) of leukocyte subpopulations, their apparent size (B) and complexity (C) after flow cytometric assessment were correlated with the age of the animals. Slopes after linear regression are given above each graph.

Supplementary Fig. S5 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2015.02.011.

4. Discussion

4.1. Variances of leukocyte properties in healthy horses

Since most cytokines are not detectable or present at very low levels in the serum of healthy individuals, as known for other species (Bienvenu et al., 1998), the authors herein focused on equine cytokine determinations in supernatants of freshly isolated PBMC after *in vitro* culture under standardised conditions. Proved advantages are high detection rates and usually good reproducibility for individual horses in examinations of *ex vivo* PBMC release. Cytokines, except IFNα, were usually detectable herein in supernatants of non-stimulated PBMC. The lack of constitutive IFNα expression and secretion may be due to the absence of antiviral or antitumour responses in the horses utilised (Van Reeth, 2000; Pasquali and Mocellin, 2010; Goodman et al., 2013).

The high variability of secreted cytokine amounts is paralleled by previous reports in men (Wong et al., 2008; Tarrant, 2010) and horses (Hamza et al., 2010; Lavoie-Lamoureux et al., 2012b; Lembcke et al., 2012) on spontaneous and stimulated cytokine releases. This indicates that individual animals, although clinically healthy, show up with a different baseline activation or priming of circulating cells. This kind of variability was also reflected by a considerable variation in the size and granularity of circulating leukocytes, which may relate to *in vivo* encountering of modulating molecules, as shown in other contexts (Webster et al., 1995; Schuberth et al., 2001; Böhmer et al., 2011).

The presence of high interindividual variances within the examined horse cohort leads the authors to an investigation of variables possibly influencing the results of immunological studies.

4.2. Morphometry correlates with functionality ex vivo

Morphometric parameters of blood leukocytes correlated with secreted cytokines, confirming the use of these measurements for the estimation of leukocyte functionality. Regarding spontaneously secreted TNFα, IL-4 and IL-10, correlation with lymphocyte morphometry was high, confirming flow cytometric measurements as indicators of lymphocyte functionality.

The morphometric properties (FSC and SSC) of monocytes in blood correlated negatively with stimulated cytokine release by PBMC. Monocytes contribute only a small amount to the total PBMC, but may produce a relevant amount of cytokines upon stimulation.

Flow cytometric measurements were herein conducted in whole blood without stimulation. Thus, morphologic changes should be indicators of the milieu *in vivo* which influenced both morphologies of all circulating cells and

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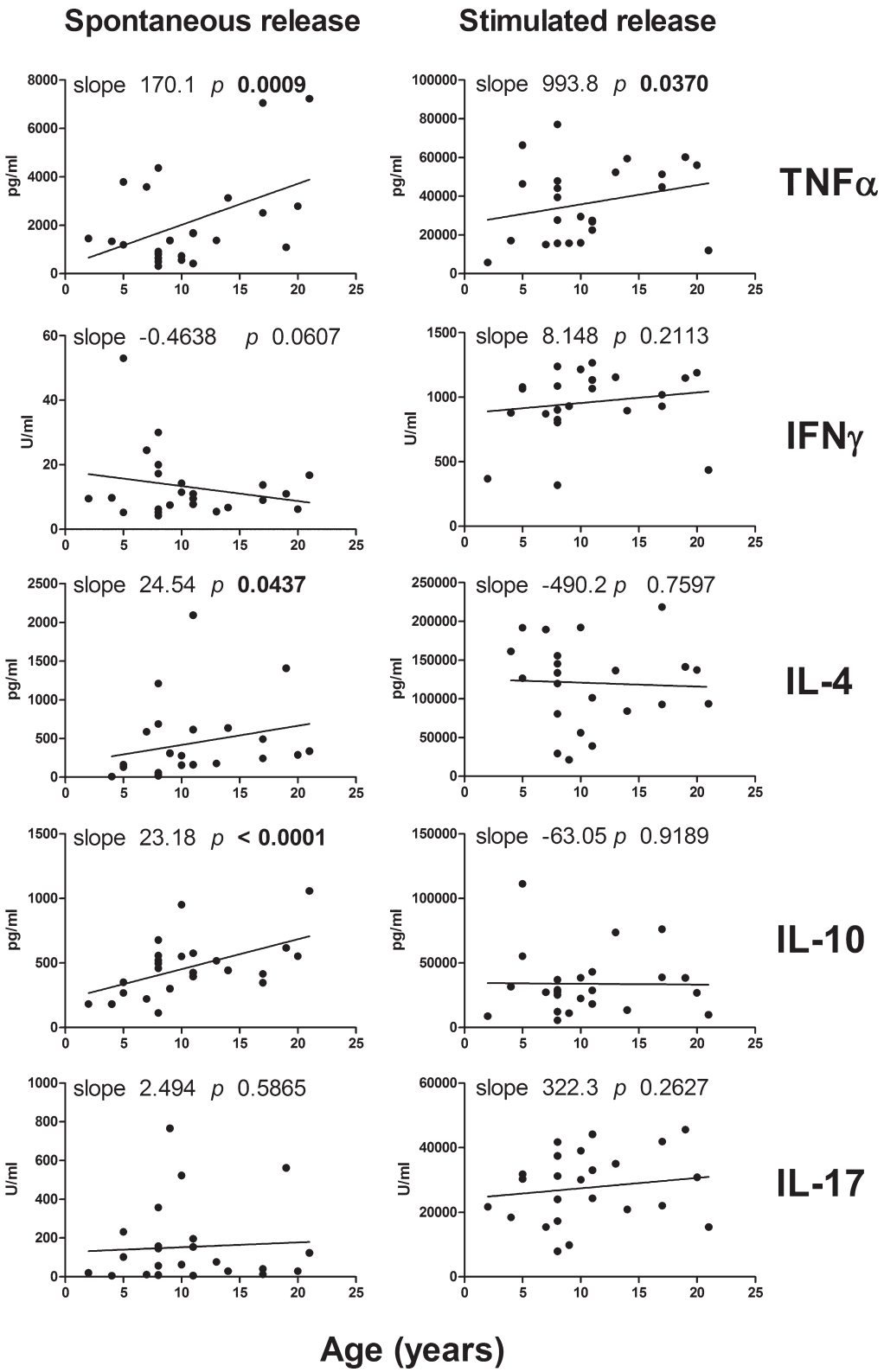

Fig. 3. Influence of age on PBMC cytokine release. The spontaneous or the stimulation (PMA/Ionomycin)-induced release of cytokines indicated was correlated with the age of the animals. Slopes after linear regression are given above each graph.

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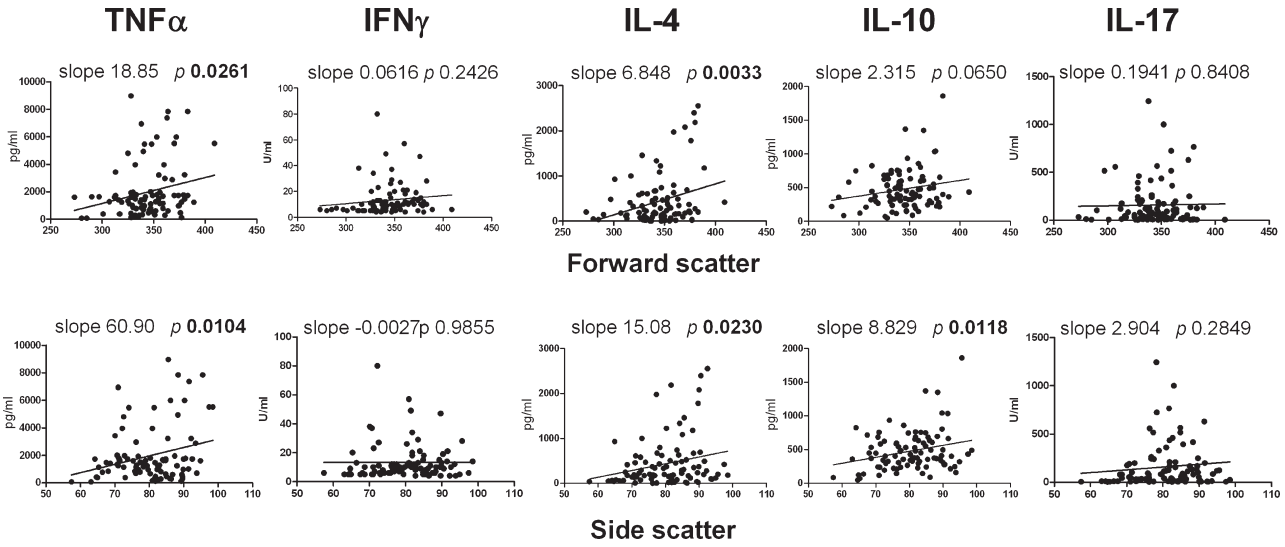

Fig. 4. Correlation between morphological parameters of lymphocytes and spontaneous cytokine release *in vitro*. Forward and side scatter values of lymphocytes were determined after whole blood lysis. Cytokine concentrations in supernatants of unstimulated mononuclear cells were determined after 12 h of *in vitro* culture. Slopes after linear regression are given above each graph.

the *ex vivo* reactivity of PBMC after isolation. Further research is needed to identify the exact relationships between morphometry and cytokine release in horses beyond the notification of correlations.

4.3. Age is relevant for leukocyte activity

The tendency of lymphocytes and granulocytes with higher FSC values in older horses may match the theory of *inflamm-aging* (Adams et al., 2008; Katepalli et al., 2008). Since increased cell sizes are indicators of leukocyte activation (Scharsack et al., 2000; Schuberth et al., 2001), higher levels of circulating pro-inflammatory and cell-activating cytokines (Harris et al., 1999; Bruunsgaard et al., 2001) may account for the altered leukocyte size in older individuals. The significantly higher spontaneous *ex vivo* release of TNFα, IL-4 and IL-10 in older horses supports this hypothesis. The findings for IL-4 and IL-10 are also in accordance with findings in aged mice (Sharma et al., 2013; Garg et al., 2014). A shift in the response type of PBMC between younger and older horses also became apparent when analysing the stimulus-induced release of cytokines. The concentrations of TNFα and tendentiously of IFNγ and IL-17 in PBMC supernatants increased with age. This is partially in line with a higher intracellular expression of TNFα and IFNγ after equine PBMC stimulation of older horses (Adams et al., 2008; Katepalli et al., 2008; Hansen et al., 2013) and findings in humans, in which increased T_H17-cells and IL-17 expression without stimulation in aged individuals were found; however, after stimulation with

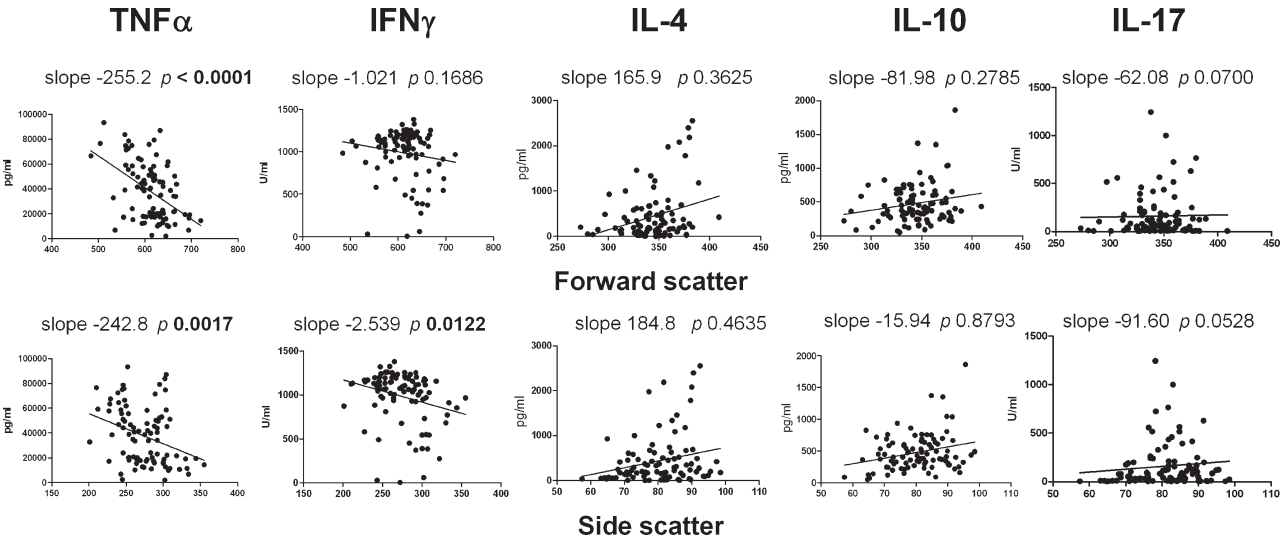

Fig. 5. Correlation between morphological parameters of monocytes and stimulated cytokine release *in vitro*. Forward and side scatter values of monocytes were determined after whole blood lysis. Cytokine concentrations in supernatants of PMA/Ionomycin-stimulated mononuclear cells were determined after 12 h of *in vitro* culture. Slopes after linear regression are given above each graph.

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PHA, secretion was enhanced in IL-10, but not in IL-17, in the elderly (Schmitt et al., 2013).

Differences in the amounts of secreted cytokines found herein could be explained by different compositions of cell subsets in PBMC. The amount of large cells within PBMC, meant to be reactive lymphocytes, monocytes and CTL (Brault et al., 2010), was relatively constant herein and did not influence cytokine concentrations in PBMC supernatants with any statistical significance (data not shown). Still, cells gated into the same fraction by morphometric criteria can be of different phenotypes. The composition of phenotypes *e.g.* varying in $CD4^+/CD8^+$ ratios by age might be the source of the observed variances (McFarlane et al., 2001). Beyond such differences of lymphocyte subset composition, altered activity statuses of cell subtypes could underlie the different amounts of cytokines in supernatants of a standardised number of PBMC. This has been observed for different types of T cell subsets in horses (Adams et al., 2008; Wagner et al., 2010; Robbin et al., 2011; Hansen et al., 2013). Such altered activity profiles in a relevant portion of cells could lead to the variances of secreted cytokines by PBMC of horses of different age found in the present study. In parallel to the herein employed methods cell surface staining of phenotypical markers and intracellular staining of cytokines in PBMC in prospect studies will hopefully further elucidate relationships of cell phenotypes, their morphologic properties and cytokine secretion in closer detail including single cell cytokine expressions.

4.4. Sex is relevant for some immunological parameters

A further parameter influencing immunological parameters herein was sex. Mares showed larger monocytes and lower spontaneous release of IL-10 than geldings. A tendency of higher values of TNFα and IFNγ was also present. This may indicate a trend of more pro-inflammatory and less regulatory cytokines in females. This is still a controversial issue in horses (Hamza et al., 2008; Uner et al., 2013; Wray et al., 2013), but is in line with higher plasma levels of TNFα found in mares than in geldings of mixed breeds and ages (Suagee et al., 2013).

4.5. Breed is relevant for some immunological parameters

WBls showed lower lymphocyte SSC values and higher stimulation ratios of TNFα, IL-4 and IL-17 than ThBs. The findings of breed or type differences parallel results previously found for routine hematology (Gerber et al., 1975; Kieferndorf and Keller, 1990; Pomorska, 2013), leukocyte subsets (Macedo et al., 2013), differential haemograms, platelet-rich plasma production of growth factors (Giraldo et al., 2013), and inflammatory mediators, such as TNFα and IL-1 in response to *in vivo* stimuli (Wilmink et al., 2003). The two types of horses compared in the present study summarised different numbers of individuals of different breeds. This partially limits the generalisation of the implications mentioned. However, differences in leukocyte properties between types or breeds of horses should be carefully kept in mind when comparing immunological studies conducted with different breeds.

4.6. Time of sampling has no distinct effect

The lack of robust diurnality of *ex vivo* cytokine release in the present study is in contrast to other species (human, rat, mouse) in which diurnal or circadian rhythms of cytokines have been regularly documented (Petrovsky et al., 1994, 1998; Pollmächer et al., 1996; Petrovsky and Harrison, 1998; Arjona and Sarkar, 2008; Keller et al., 2009). It is, however, in line with a report by Murphy et al. (2006), who found evidence of an oscillating peripheral clock in an equine fibroblast cell line and adipose tissue, but not in peripheral blood. Whether dissimilarities to other species are due to differences of rhythmicity in blood cells was not addressed (Murphy et al., 2006). Studies focusing on circadian rhythms usually employ shorter sampling intervals than in the present study, which may hamper the detection of feeble differences, as short peaks and nadirs may be just between the selected sampling times. However, if diurnal oscillations were robust, even with a few samples, detection of significant differences could be possible, as proved in other studies on cytokine levels employing only two sampling times throughout the day (Takata et al., 2002; Benito et al., 2014).

Influences of the time of day of sampling were present for morphometric parameters, leukocyte percentages and for some cytokine stimulation ratios. However, absolute differences between the sampling times employed were low and variances of data were large, thus, clinical relevance is unlikely at the moment. Nevertheless, the existence of circadian differences in selected parameters shown herein should be regarded in longitudinal studies using lymphocyte properties as biomarkers. Attention should be paid to the time of sampling blood for valid comparisons and bias by different sampling times should be excluded.

5. Conclusion

The determination of equine leukocyte morphometry and *ex vivo* secreted cytokines employed herein is a prerequisite for a valid monitoring of disease and treatment effects on blood leukocytes. Variances of healthy individuals were in the detection range, showed sufficient consistency for most individual horses and can, thus, be used as potential biomarkers fulfilling these criteria determined by Benito et al. (2014). The measurements herein provide orientation about ranges of a sample of healthy horses. Further research identifying exact interrelationships of the influences of age, sex, horse type, and sampling time linked to cell subsets or phenotypes and activation statuses is desirable. Regarding the presented influences of animal factors, baseline measurements for each individual seem more meaningful compared to fixed reference values.

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Conflicts of interest

The authors state that there are no conflicts of interest.

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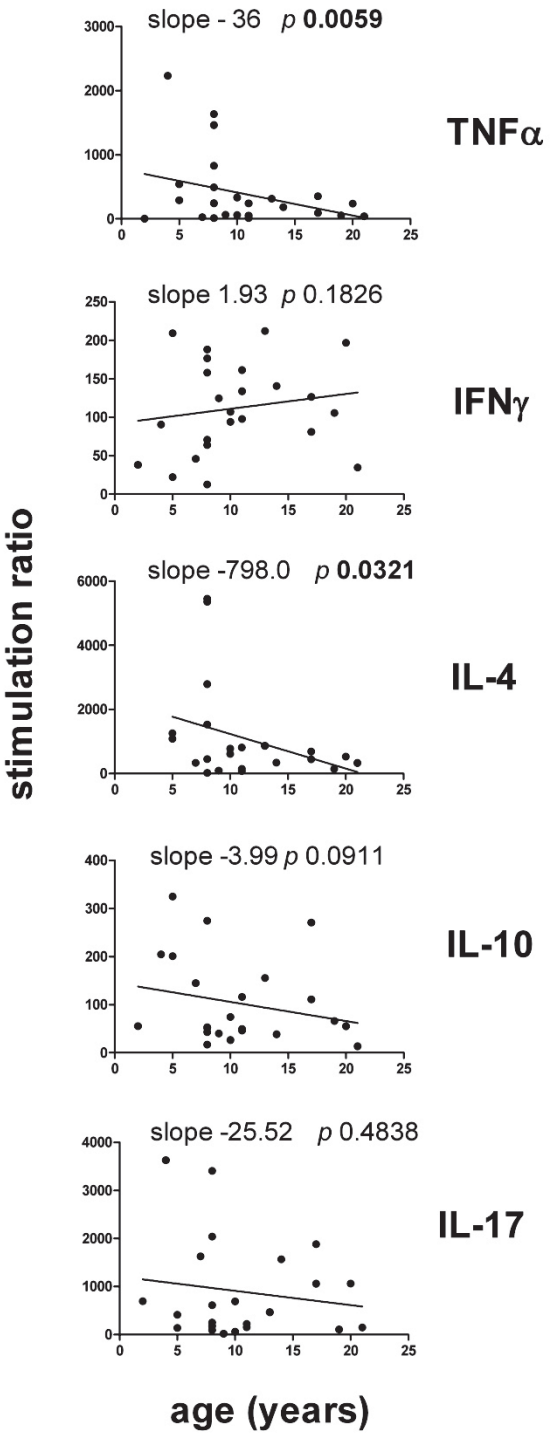
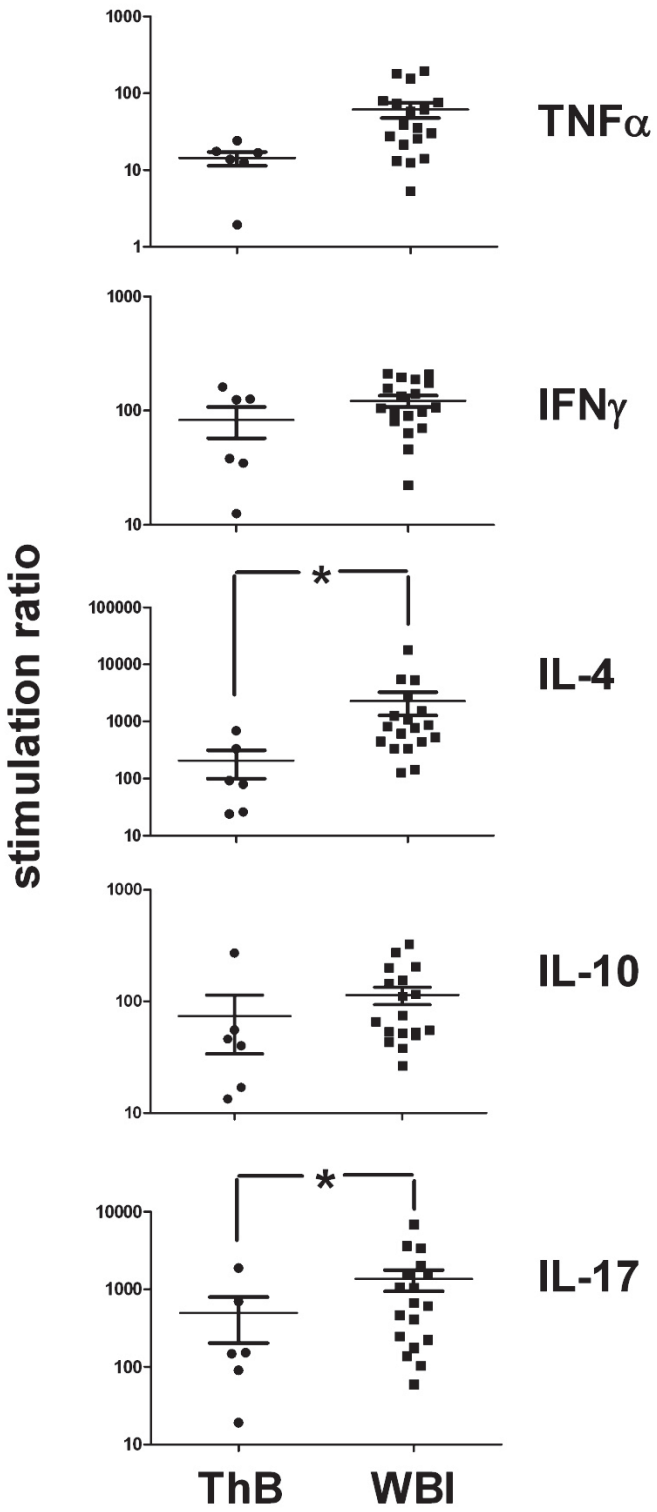
Supplemental data

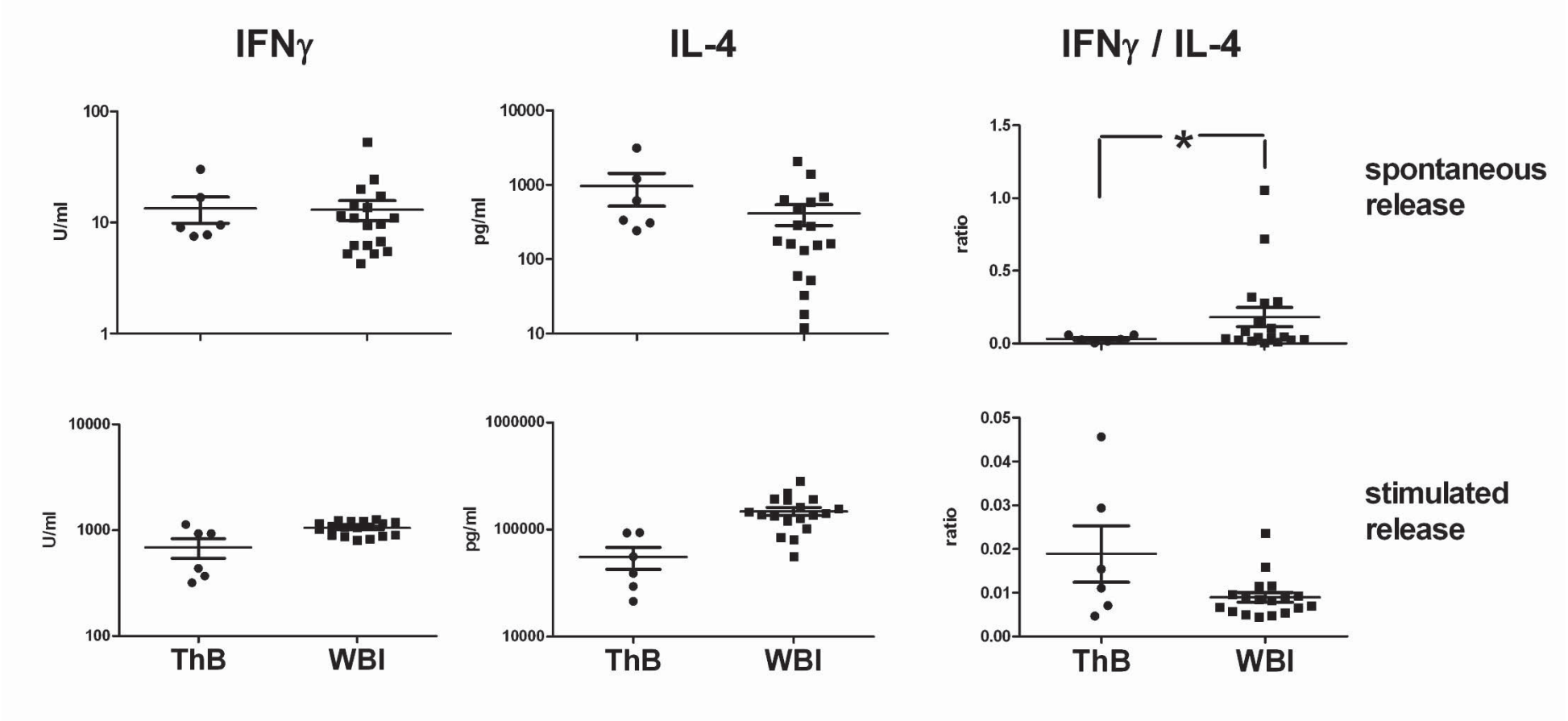


Suppl. Fig. 1. Influence of age on PBMC cytokine stimulation ratios. Cytokine stimulation ratios (PMA/Ionomycin-induced versus spontaneous release) are plotted against the age of the animals. Slopes and *p*-values of linear regressions are given above each graph.



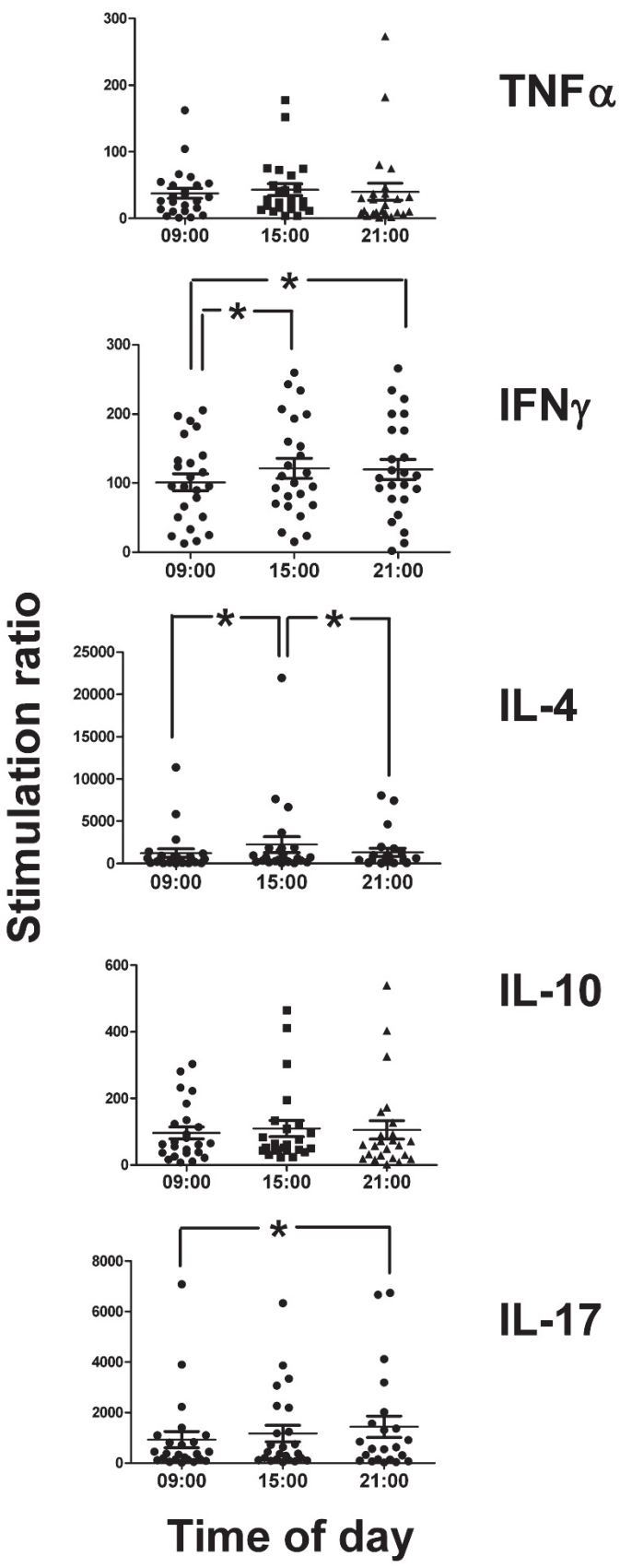
Suppl. Fig. 2. Influence of breed on PBMC cytokine stimulation ratios.

Stimulation ratios of *ex vivo* released cytokines plotted in histograms for Thoroughbred (ThB) and warmblood (WBl) horses. Horizontal bars represent mean ± SEM (asymmetric when log scale is used). Stimulation ratios of IL-4 and IL-17 were significantly influenced by breed: IL-4 mv $p = 0.049$, uv $p = 0.034$; IL-17 mv, $p = 0.039$, uv $p = 0.037$.

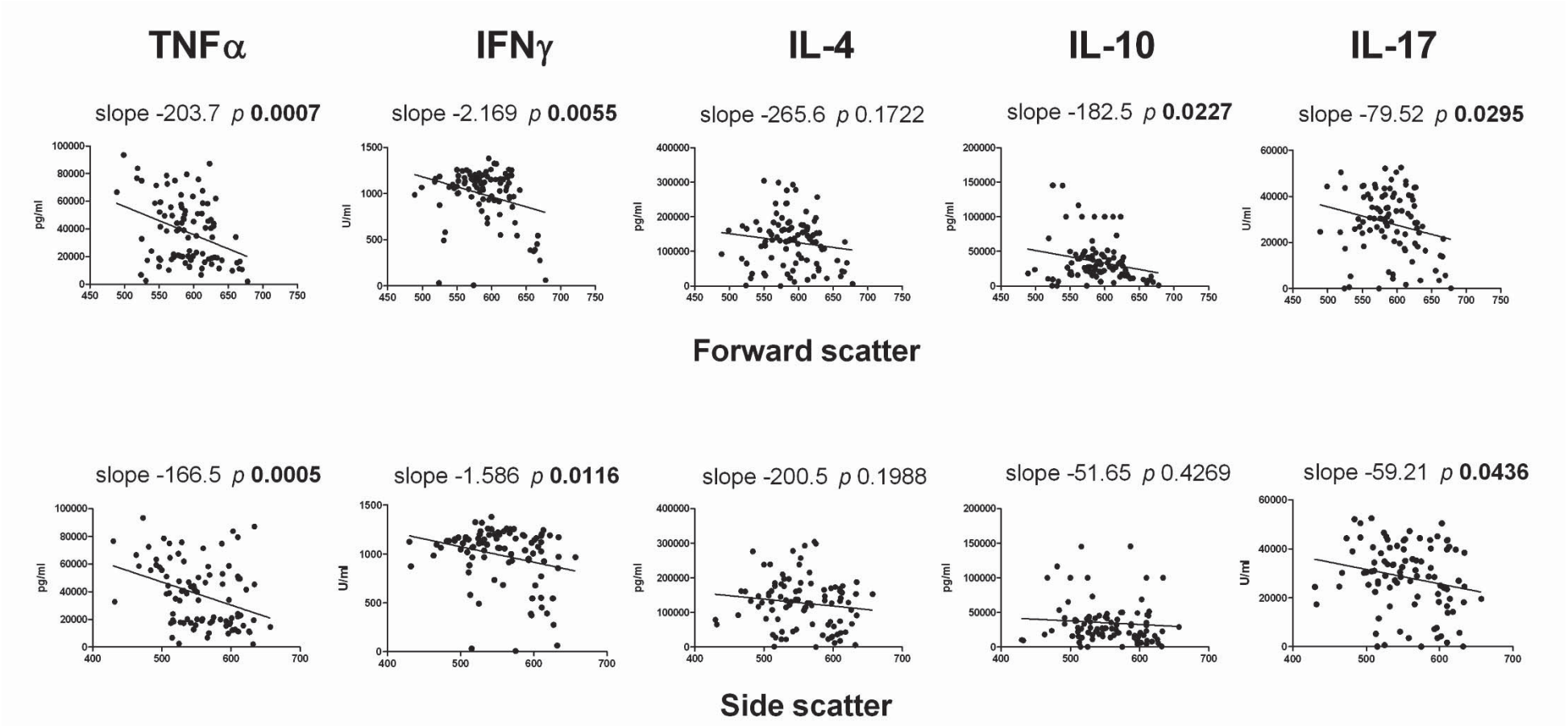


Suppl. Fig. 3. Influence of breed on IFNγ/IL-4 ratios.

Ex vivo released cytokines of PBMC (spontaneous versus PMA/Ionomycin-induced) and IFNγ/IL-4 ratios (U IFNγ/pg IL-4) are shown for Thoroughbred (ThB) and Warmblood (WBl) horses. Horizontal bars represent mean ± SEM (asymmetric when log scale is used).



Suppl. Fig. 4. Influence of time of day of sampling on PBMC cytokine stimulation ratios. Stimulation ratios (PMA/Ionomycin-induced versus spontaneous release, mean ± SEM) of *ex vivo* released cytokines from PBMC were plotted against sampling time of day.



Suppl. Fig. 5. Correlation of granulocyte forward and side scatter values with stimulated cytokine release of PBMC.
PMA/Ionomycin-induced cytokine release of PBMC was plotted against the mean forward (upper row) or the mean side scatter of granulocytes. Slopes of linear regression are given above each graph.

Supplement 1

Table S1 Blood cell composition and flow cytometrically assessed size and complexity of cells are influenced by breed.

Cell type	**Parameter**	**ThB[1] (n = 6)**			**WBl[2] (n = 18)**			**mv** ***p***	***uv*** **p**
		mean	*min*	*max*	*mean*	*min*	*max*		
Lymphocytes	% of Leukocytes	21.2	12.4	32.5	21.6	9.5	34.1	0.0699	0.7990
Granulocytes	% of Leukocytes	72.4	62.0	80.8	72.9	61.3	85.2	0.1732	0.8394
Monocytes	% of Leukocytes	6.4	4	9.8	5.5	3.3	7	0.1007	**0.0037**
Lymphocytes	Forward scatter	352	231	409	341	273	383	0.0830	0.0944
	Side scatter	89	76	143	79	57	95	0.0567	**<.0001**
Granulocytes	Forward scatter	613	400	678	580	489	634	0.1732	0.8394
	Side scatter	603	517	825	545	430	657	0.2878	**<.0001**
Monocytes	Forward scatter	626	418	695	608	484	720	0.3502	0.0876
	Side scatter	301	254	477	269	201	355	0.5692	**0.0001**

1) ThB, Thoroughbred; 2) WBl, Warmblood;

Fat p-values represent significant differences.

Supplement 2

Table S2 Blood cell composition and flow cytometrically assessed size and complexity of cells are influenced by sex.

Cell type	Parameter	Mares n = 6			Geldings n = 15			Stallions n = 3			mv *p*[1]	*uv* p[1]
		mean	*min*	*max*	*mean*	*min*	*max*	*mean*	*min*	*max*		
Lymphocytes	% of Leukocytes	21.3	11.3	34.1	21.9	9.5	33.0	19.6	12.9	32.5	0.3697	0.4697
Granulocytes	% of Leukocytes	72.6	62.1	84.1	72.5	61.3	85.2	74.8	62.0	81.2	0.4640	0.8592
Monocytes	% of Leukocytes	6.1	3.3	9.8	5.6	3.7	7.6	5.5	4.0	7.3	0.8248	0.1422
Lymphocytes	Forward scatter	351	273	409	341	231	383	342	307	389	0.7041	0.2773
	Side scatter	84	65	99	80	57	143	82	76	91	0.0781	0.4757
Granulocytes	Forward scatter	610	489	678	578	400	634	597	550	641	0.1246	**0.0069**
	Side scatter	593	463	657	545	430	825	564	517	606	0.7547	**0.0009**
Monocytes	Forward scatter	635	484	720	602	418	696	618	574	668	**0.0178**	**0.0082**
	Side scatter	299	245	355	270	201	477	268	253	292	0.3400	**0.0023**

1) Fat p-values represent significant differences.

Supplement 3

Table S3 Spontaneous and PMA/Ionomycin-induced cytokine release of equine PBMC is influenced by sex.

Cytokine	**Release**[1]	**Mares** **n = 6**			**Geldings** **n = 15**			**Stallions** **n = 3**			**mv** ***p***[2]	***uv*** **p**[2]
		mean	*min*	*max*	*mean*	*min*	*max*	*mean*	*min*	*max*		
TNFα	Spont.	3150	419	16624	1912	65	8967	1199	129	1920	*0.0803*	0.1682
(pg/ml)	Induced	31701	1921	87022	40866	0	93311	24014	12235	43756	0.3399	0.1827
IFNγ	Spont.	15	4	47	13	4	80	10	5	20	**0.0491**	0.4245
(U/ml)	Induced	756	61	1184	1016	4	1380	1094	677	1325	0.2625	0.1715
IL-4	Spont.	331	0	1782	679	7	5069	360	27	1178	**<.0001**	0.2247
(pg/ml)	Induced	108224	5694	196986	148172	7	304383	38843	11837	80591	0.2810	**0.0242**
IL-10	Spont.	414	49	1862	468	85	935	559	211	1371	**0.0471**	**0.0298**
(pg/ml)	Induced	34636	471	145551	36782	260	145279	17323	6323	33340	0.2543	0.2743
IL-17	Spont.	66	4	249	121	5	766	495	83	1244	0.4623	**<.0001**
(U/ml)	Induced	19918	88	43536	32197	16	52487	21412	4259	38168	0.8657	0.2394

1) 1) Spont.: spontaneous release; Induced: PMA/Ionomycin-induced release

2) Fat p-values represent significant differences.

4 Manuscript III

Colour matters: immunological responses of grey and non-grey horses to DNA constructs complexed with a cationic transfection reagent

Biomed Central Veterinary Research, *under revision*

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Abstract

Background

Deoxyribonucleic acid (DNA) vaccines are used for experimental immunotherapy of equine melanoma. The injection of complexed linear DNA encoding interleukin (IL)-12 / IL-18 induced partial tumour remission in a clinical study including 27 grey horses. To date, the detailed mechanism of the anti-tumour effect of this treatment is unknown.

Results

In the present study, the clinical and cellular responses of 24 healthy horses were monitored over 72 h after simultaneous intradermal and intramuscular application of equine IL-12/IL-18 DNA (complexed with a transfection reagent) or comparative substances (transfection reagent only, nonsense DNA, nonsense DNA depleted of CG).

Although the strongest effect was observed in horses treated with expressing DNA, horses in all groups treated with DNA showed systemic responses. In these horses treated with DNA, rectal temperatures were elevated after treatment and serum amyloid A increased. Total leukocyte and neutrophil counts increased, while lymphocyte numbers decreased. The secretion of tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) from peripheral mononuclear blood cells *ex vivo* increased after treatments with DNA, while IL-10 secretion decreased. Horses treated with DNA had significantly higher myeloid cell numbers and chemokine (C-X-C motif) ligand (CXCL)-10 expression in skin samples of the intradermal injection sites compared to horses treated with transfection reagent only, indicating an inflammatory response to DNA treatment.

In horses treated with expressing DNA, however, local CXCL-10 expression was highest and immunohistochemistry revealed most IL-12-positive cells when compared to the other treatment groups.

In contrast to non-grey horses, grey horses showed fewer effects of DNA treatments on blood lymphocyte counts, TNFα secretion and myeloid cell infiltration in the dermis.

Conclusion

Treatments with complexed linear DNA constructs induce an immunologic response independent of the coding sequence and of CG motif content. Expressing IL-12/IL-18 DNA locally induces expression of the downstream mediator CXCL-10.

Based on the weaker systemic response after injection, the grey horses included lack a significant immune response to DNA treatment, although grey horses bearing melanoma responded to this treatment with partial tumour remission. Whether the different immunological reactivity compared to other horses may contribute to the melanoma susceptibility of grey horses remains to be elucidated.

Keywords:

equine melanoma; grey horse; MIDGE vector; immune response; cytokines; CpG, IL-12; IL-18; transfection; transfection reagent; DNA vaccine;

Background

Melanoma is one of the most common equine neoplasms of the skin (Cavalleri et al., 2014; Cotchin, 1977; Valentine, 2006). Prevalence is up to 80 % in grey horses older than 15 years, while horses of other colours are rarely affected by this disease (Jeglum, 1999; M`Fadyean, 1933). To date, there is no satisfactory therapeutic approach available in advanced cases of grey horse melanoma (Cavalleri et al., 2014; Jeglum, 1999; Phillips and Lembcke, 2013). One experimental *in vivo* approach is immunotherapy with melanoma antigens and/or immune modulating cytokines encoded by Deoxyribonucleic acid (DNA) vectors taking advantage of systemic effects on metastases (Heinzerling et al., 2001; Müller et al., 2011b; Phillips et al., 2012).

A moderate decrease in the volume of tumour melanomas could be demonstrated by Mählmann et al. (2015) during a study period of 120 days after immunotherapy of 27 grey horses with intramuscularly (i.m.) and peritumourally intradermally (i.d.) administered mixed linear DNA vectors (minimalistic immunologic defined gene expression (MIDGE)-Th1) encoding for equine interleukin (IL)-12 and IL-18 complexed with the transfection reagent SAINT-18.

MIDGE-Th1 vectors are minimalistic linear double-stranded DNA molecules lacking plasmid backbone sequences. The vectors are covalently closed with single-strand hairpin loops at both ends. They contain the expression cassette only, i.e. a promoter, the coding sequence of interest and a polyadenylation site (Lopez-Fuertes et al., 2002; Moreno et al., 2004). One of the ends is covalently bound to a nuclear localisation signal peptide, triggering an improved humoral and cellular response of a T_H1 phenotype (Schirmbeck et al., 2001; Zheng et al., 2006). *In vivo* transfection is established and can be facilitated by the complexation of the DNA vectors with cationic lipids, such as SAINT-18 (Audouy et al., 2002; Endmann et al., 2010).

Signs of a systemic immune response, i.e. the development of fever 12 h after administration of MIDGE-Th1 formulated with SAINT-18 and the size reduction of metastases which had not been treated locally, were observed in a study of Mählmann and colleagues (2015). Local immunological responses were indicated by signs of acute inflammation (swelling, reddening, etc.) and depigmentation of the skin at the site of injection. The exact mechanism of this anti-tumour therapy is still unknown.

Effects of DNA vectors are usually explained by their transgene products (Endmann et al., 2010; Heinzerling et al., 2006; Lopez-Fuertes et al., 2002; Phillips et al., 2012), by immunologic effects caused by CG motifs randomly contained in the vector constructs activating toll-like receptor (TLR-)9 pathways (Hafner et al., 2001; Lopez et al., 2006; McCluskie et al., 2000), by reactions triggered by interaction with cytosolic receptors for dsDNA (Hornung et al., 2009; Hornung et al., 2014; Ishii et al., 2006; Unterholzner, 2013; Unterholzner et al., 2010), or by combinations of these effects.

The mechanisms of the immunotherapeutic effects of MIDGE-Th1 encoding equine IL-12 and IL-18 demonstrated by Mählmann et al. (2015) in grey horses affected with melanoma were to be elucidated in order to improve the anti-tumour treatments directed towards clinical use. Therefore, the aim of this study was to investigate various candidate immune parameters on the systemic and local level in healthy horses in comparison to internal controls (Figure 1). Furthermore, potential immunologically active components were analysed comparatively in four treatment groups, i.e. transfection reagent, SAINT-18, as a placebo without DNA (represented in group A), or DNA complexed with SAINT-18 (groups B – D). Of the latter, B represents the inclusion of effects by transgene products (receiving SAINT-18 and MIDGE-Th1 vectors coding for equine IL-12 and IL-18), while C and D received non-expressing complexed nonsense DNA (without start codon). The DNA applied in C contained as many CG sequences, thus potential CG motifs, as the vector mix applied in B. Group D received complexed nonsense DNA in which CG were additionally inverted to GC.

Figure 1 Procedure

Test procedure illustrated in course of time.

The horses were left to acclimatise for at least three days. At t-24, the horses were injected with PBS (grey symbols) i.m. (0.5 ml) and i.d. (2 x 0.5 ml). Baseline samples (blood) as internal controls for systemic parameters were acquired from t-48 – t0.

At t0, horses were injected with treatments A – D (purple symbols) i.m. (0.5 ml) and i.d. (0.5 ml) and contralaterally with PBS (grey symbol, 0.5 ml). From t6 – t72, post-treatment samples (blood) of systemic parameters were acquired.

At t24, skin biopsies were acquired of treatment (purple) and PBS control (grey) sites.

Eleven days post-treatment (t264), one long-term sample was acquired at the home stable of each horse. General examinations were performed at all sampling times.

Blood samples were used to determine WBC, haemograms, SAA, cytokine mRNA and *ex vivo* cytokine secretion by PBMC. Skin samples were used to perform histological examinations, IHC of IL-12, IL-18 and calprotectin, and qPCR of cytokine and chemokine mRNA.

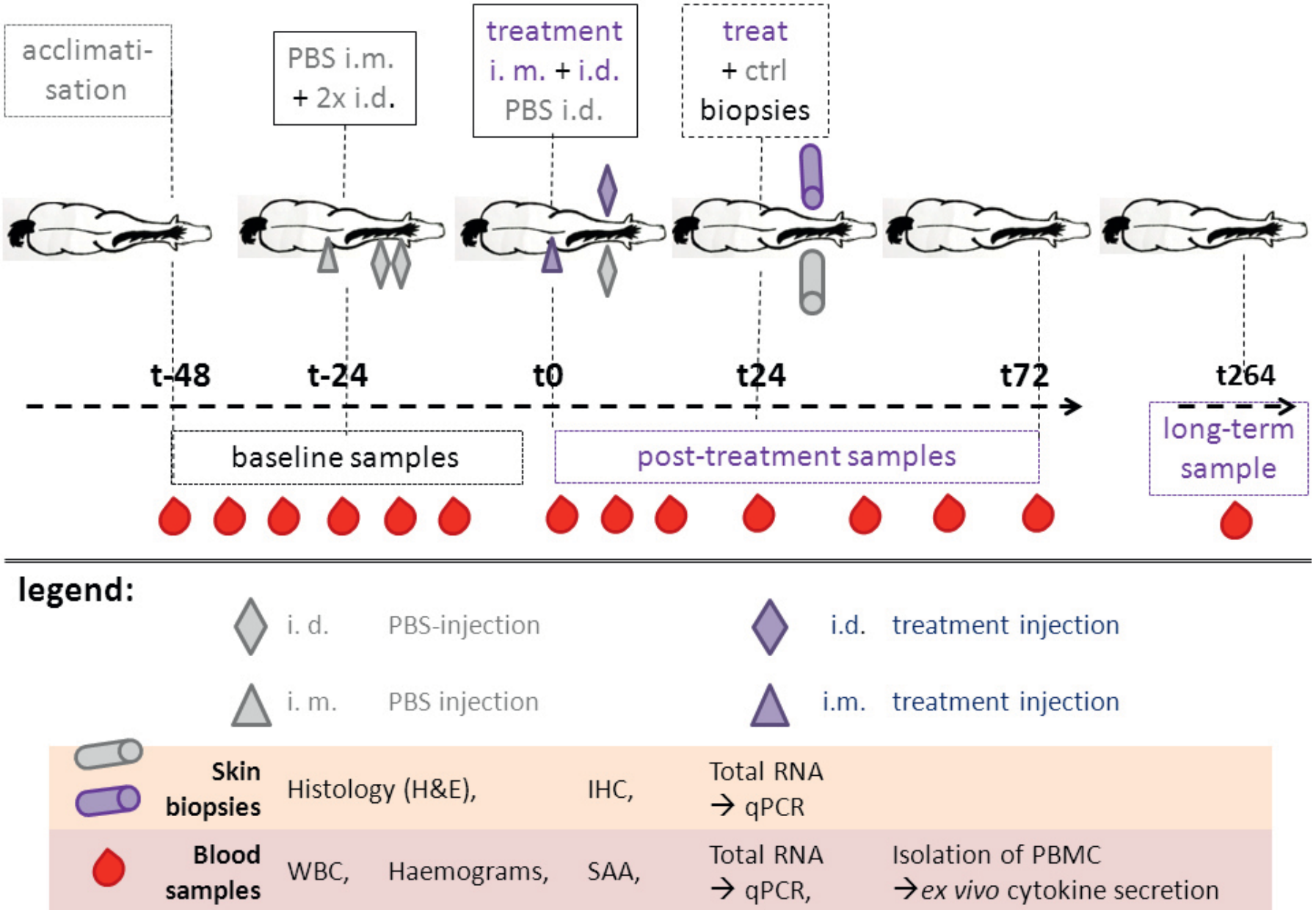


Figure 1 Procedure

Results and discussion

Changes of measured systemic parameters were induced by DNA containing treatments (B, C, D) and occurred within 24 h after injection for all parameters measured, except for serum amyloid A (SAA), an acute phase protein, which was used as a long-term indicator demonstrating effects by 72 h after injection.

In summary, after treatment with DNA complexed with SAINT-18 (B, C, D), horses showed elevated rectal temperatures (RT) and increased white blood cell counts (WBC), with the increase mainly caused by granulocytes. These effects were interpreted as signs of a mild systemic inflammatory reaction (MacKay, 2000; Reed et al., 2009).

Clinical findings

Treatment with SAINT-18 and MIDGE-Th1 were well tolerated in all horses. Clinical parameters of all horses included herein were within normal ranges after treatments, except for elevated RT exceeding 38 °C in seven horses. This matches the findings described for this treatment in horses bearing melanoma (Mählmann et al., 2015). Thus, safety of the treatment can be considered as approved.

In comparison to individual time-of-day matched baselines, RT of horses in groups B to D were elevated between 12 and 18 h after treatments. This difference reached statistical significance in group B (t-test, $p = 0.0467$). However, these elevations of RTs were not significantly different between different treatments (analysis of covariance: ANCOVA) (Figure 2).

Rectal temperature increases or development of fever (RT > 38.5 °C) in the healthy horses included were far less frequently observed and tended to last for a shorter time compared to the previous study by Mählmann et al. (2015) of horses affected by melanoma. As injection of MIDGE-Th1 complexed with SAINT-18 coding for luciferase in five healthy horses had also led to fever in that study, this difference cannot be easily explained by the mere presence or absence of melanoma. The cause of this variance between studies remains to be elucidated.

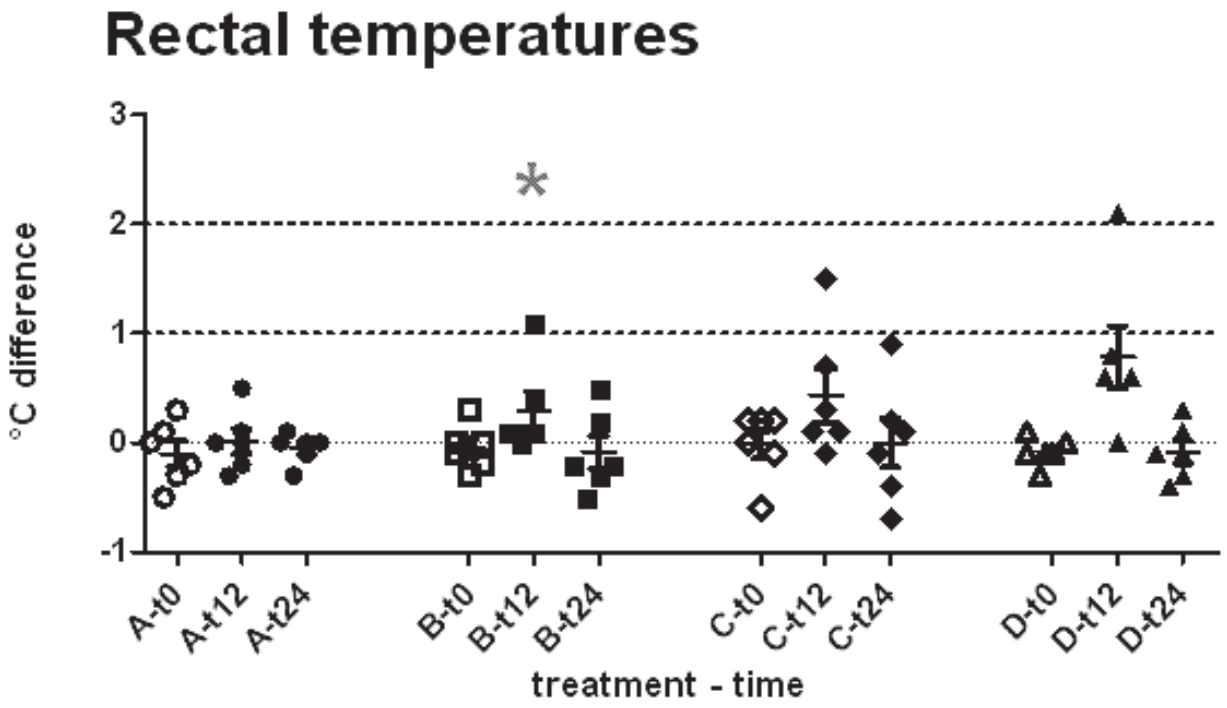


Figure 2 Rectal temperatures

Rectal temperature differences to individual time-of-day matched baselines are plotted in histograms for t0, t12 and t24. Datasets are marked as (group)-(hours post-treatment) at the X-axis. Horizontal bars represent mean and SEM. Grey asterisk (*) represents significant differences from baseline in the respective dataset. Group B was significantly different from baseline at t12 (t-test).

Blood counts and haemograms

All haematologic values measured remained within physiological ranges during all observations, with a tendency towards signs of an acute systemic inflammatory response (Reed et al., 2009), as follows: WBC increased in comparison to individual time-of-day matched baselines in horses of groups B, C and D between 12 and 36 h after treatment. A statistical trend towards significance compared to baseline was visible at t12 in group C (t-test, $p = 0.0541$). Treatment effects in group B differed statistically significantly from all other treatments [analysis of variance: ANOVA univariate (uv) and multivariate (mv) $p < 0.05$]. For single comparisons, treatment effects on WBC induced by B and D at t24 were significantly higher than in A (ANCOVA, $p = 0.0494$; 0.0227) (Figure 3a).

Neutrophils increased after all treatments, similar to the WBC effects. The overall effect of treatment on neutrophil numbers was significant (ANOVA, uv and mv $p < 0.05$). However, comparisons of neutrophil numbers to individual baselines showed statistically significant differences 24 h after treatment in group B (t-test, $p = 0.0406$) only. In group C and D, trends were visible without reaching statistical significance. Group A showed statistically significantly fewer treatment effects than B, C or D (ANCOVA; $p = 0.0167$; 0.0497; 0.0184, respectively) (Figure 3b).

Treatment effects on neutrophils were also found in the lymphocyte numbers, but with opposite orientations and lower amplitudes than neutrophils. Lymphocyte numbers decreased after treatments B, C and D. In comparison to individual baselines, lymphocyte numbers decreased significantly after treatment B at t12 (t-test, $p = 0.0399$) and t24 (t-test, $p = 0.0111$), and after treatments C (t-test, $p = 0.0005$) and D (t-test, $p = 0.0276$) only at t24 (Figure 3c).

The decrease of lymphocyte numbers after the treatments can be explained in the context of an acute systemic inflammatory state of the horses treated with DNA, which may occasionally include lymphopenia, as seen after endotoxin challenge (Peiró et al., 2010). Decrease in lymphocytes and increase in neutrophils has been demonstrated in mice after systemic administration of complexed plasmid DNA paralleling the present findings in horses (Tousignant et al., 2000). Lymphocyte decrease may be due to extravasation following endothelial activation, as indicated in histologic examinations of skin samples in the present study.

Figure 3 Haemograms

Leucocyte quantities in peripheral blood measured by automated haematology systems are plotted as differences to individual time-of-day matched baselines for t0, t12 and t24. Datasets are marked as (group)-(hours post-treatment) at the X-axes. Horizontal bars represent mean and SEM. Grey asterisks (*) represent significant differences from baseline in the respective dataset. Asterisks (*) with brackets (⌐ ¬) represent significantly different comparisons.

a) Increases of WBC induced by B and D at t24 were significantly higher than in A (ANCOVA).

b) The increase in neutrophils was significantly different from baseline in B (t-test) at t24. Group A showed significantly less treatment effects than B, C and D (ANCOVA) at t24.

c) In comparison to individual baselines, lymphocyte numbers decreased significantly after treatment B at t12 and t24 (t-test) and at t24 in C and D (t-test).

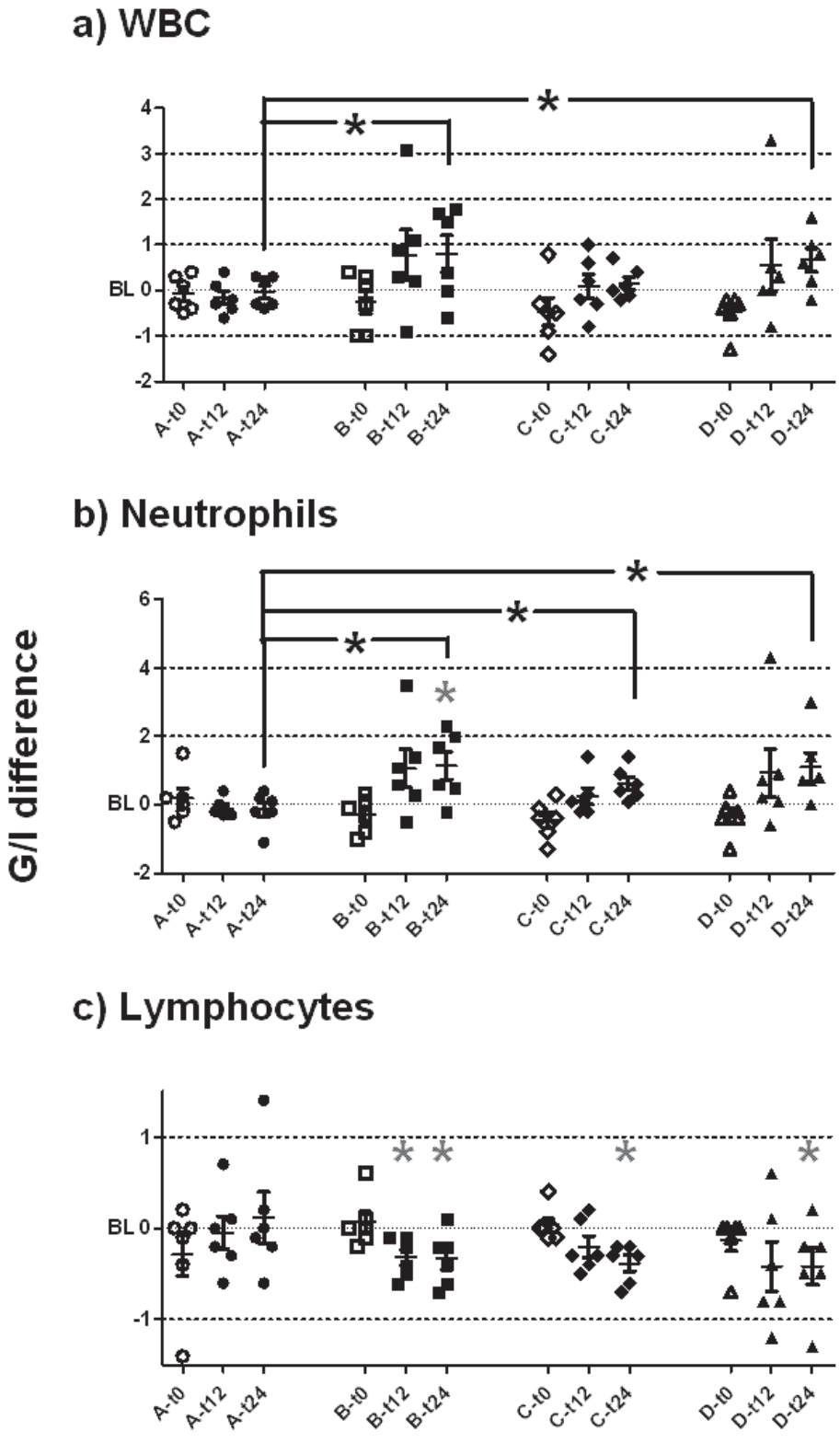


Figure 3 Haemograms

Serum Amyloid A

The horses included usually showed physiological levels of SAA below the lower limit of detection of 5 µg/ml before treatment. Levels of SAA after treatment were highly variable between individual horses of the same treatment group. Despite not reaching statistical significance, SAA in some horses in groups B to D clearly increased 24 – 72 h after treatment. Group B showed statistically significantly higher values after treatment compared to group A (ANOVA uv p = 0.0176; mv p = 0.0197) (Figure 4).
All horses had SAA levels below 7 µg/ml at t264, except for one horse (horse # H), which had shown no increase by t72, but had a history of infectious diseases in the herd of its home stable to which it returned at t96.

The increase of SAA indicates an acute phase response, which is sensitively detected by distinct increases in SAA in horses (Jacobsen and Andersen, 2007). This again suggests a systemic inflammatory reaction to the DNA treatment in individual horses. The reaction seems to resolve in a short time, as measurements at t264 no longer displayed increased SAA values.

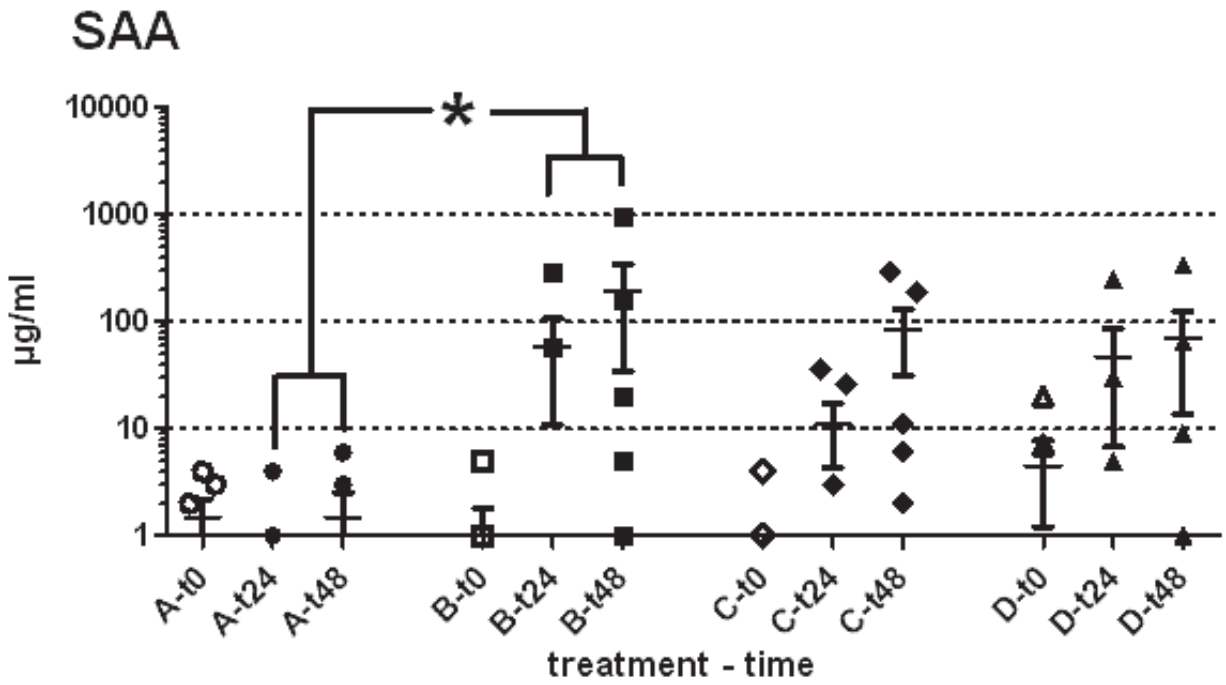


Figure 4 Serum amyloid A

Serum amyloid A levels measured by an immunoturbimetric latex agglutination test are plotted for t0, t24 and t48 (logarithmic scale). Datasets are marked as (group)-(hours post-treatment) at the X-axis. Horizontal bars represent mean and SEM. Asterisk (*) with brackets (⌜ ⌝) represents significantly different comparisons. Group B showed significantly higher values after treatment compared to group A (ANOVA).

Cytokine messenger ribonucleic acid (mRNA) in blood samples

Cytokine expression in peripheral blood samples, determined by qPCR, was not significantly altered between controls (t0) and treatment (t12) in any treatment group or between different treatments. Interferon-γ transcripts could not be detected at all (Table 1).

Table 1 cytokine cDNA copy numbers (qPCR)

Tissue	Blood								Skin							
Treatment	A		B		C		D		A		B		C		D	
Cytokine	t0	t12	t0	t12	t0	t12	t0	t12	*ctrl*	*treat*	*ctrl*	*treat*	*ctrl*	*treat*	*ctrl*	*treat*
IL-12p35	1,715	1,425	852	2,028	2,656	2,007	1,452	1,645								
IL-12p40	45	113	123	132	118	119	97	87								
IL-18	391	526	1,349	2,889	1,899	2,345	1,484	2,070	4256	4444	3,911	9,217	5,123	10,548	5,991	13,317
IFNγ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CXCL-10	1,079	515	726	1,884	2,108	2,674	1,297	4,281	2,167	626	3,045	1,167,155	845	120,600	3,099	230,462

Equine cytokine expression measured with absolute quantification by qPCR after transferring mRNA into cDNA. Medians of copy numbers given per µl; t0: before treatment; t12: 12 h after treatment; *ctrl*: local PBS control sample; *treat*: locally treated (A – D) sample; n.d. = not detectable (<100 cDNA copies/µl).

Equine cytokines in cell culture supernatants

Cytokines were usually detectable by enzyme linked immunosorbent assay (ELISA) or bead-based assay in supernatants of *ex vivo*-cultured peripheral blood mononuclear cells (PBMC, with and without stimuli) of all horses and displayed great interindividual variances. Interferon-α was hardly detectable, even after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin and was, thus, excluded from further analysis (Table 2).

There were no treatment effects detectable in individual concentrations and alterations of IL-4 and IL-17 in supernatants of cells cultured without stimuli compared to individual baselines.

Stimulation ratios (e.g. IFNγ PMA-ionomycin/IFNγ medium) showed high interindividual variances in all cytokines, but no treatment effects were detectable.

Tumour necrosis factor alpha (in medium settings) increased in single horses of all treatment groups compared to individual baselines. *Ex vivo* lipopolysaccharide (LPS) stimulation of TNFα secretion was mild (mean stimulation ratio = 3.717 times secretion). Changes post-treatment were usually parallel in medium and LPS settings. The increase of TNFα after treatments was statistically significant in groups B, C and D.

Treatment effects in groups C and D were higher than in A (details given in Figure 5).

Interferon-γ increased without *ex vivo* stimuli within 24 h after treatments B, C and D with statistically significant differences from group A only in B. Four out of six horses in group B displayed an increase of IFNγ (details given in Figure 6). Lipopolysaccharide stimulation of PBMC *ex vivo* resulted in only mild induction of IFNγ (mean stimulation ratio = 2.041 times secretion). Changes after treatments were similar to those in medium settings, but did not reach statistical significance.

Interleukin-10 in medium settings decreased 6 h after treatment B and 12 h after all other treatments. In general, LPS mildly induced IL-10 secretion *ex vivo* (mean stimulation ratio = 2.821 times secretion). The stimulated IL-10 secretion in PMA and ionomycin settings decreased 12 h after treatments in groups B, C and D (details given in Figure 7).

Table 2 *Ex vivo* secreted cytokines

		All		**A**		**A**	**A**	**B**		**B**	**B**	**C**		**C**	**C**	**D**		**D**	**D**
Cyto-kine	*setting*	**BL mean**	*BL STD*	**BL mean**	*BL STD*	t12 mean	t24 mean	**BL mean**	*BL STD*	t12 mean	t24 mean	**BL mean**	*BL STD*	t12 mean	t24 mean	**BL mean**	*BL STD*	t12 mean	t24 mean
IFNα (pg/ml)	*medi-um*	**1**	*3*	**2**	*5*	1	8	**0**	*1*	0	2	**0**	*1*	2	0	**1**	*2*	0	3
IFNα (pg/ml)	*PMA /ionom ycin*	**16**	*15*	**13**	*10*	10	12	**16**	*12*	14	18	**17**	*22*	11	15	**16**	*13*	10	11
TNFα (pg/ml)	*medi-um*	**2122**	*2560*	**2686**	*2938*	3785	3192	**2881**	*3457*	3926	7443	**1471**	*1365*	3325	4367	**1421**	*1610*	1364	3170
TNFα (pg/ml)	*LPS*	**4435**	*3874*	**4168**	*4107*	5694	4761	**6081**	*4924*	7581	1103 9	**3970**	*2762*	8966	7762	**3460**	*2926*	7563	6623
TNFα (ng/ml)	*PMA /ionom ycin*	**36.4**	*23.5*	**33.6**	*26.3*	24.4	36.3	**44.8**	*22.2*	41.2	48.2	**33.3**	*21.4*	20.2	37.7	**33.9**	*23.1*	19.4	24.9
IFNγ (U/ml)	*medi-um*	**13**	*12*	**9**	*5*	10	12	**20**	*19*	13	42	**7**	*2*	9	14	**17**	*10*	10	30
IFNγ (U/ml)	*LPS*	**25**	*32*	**13**	*7*	13	28	**40**	*38*	31	76	**10**	*6*	15	32	**36**	*43*	16	70
IFNγ (U/ml)	*PMA/ iono-mycin*	**961**	*319*	**926**	*264*	831	906	**1052**	*178*	899	1049	**932**	*384*	566	1052	**932**	*400*	723	946

IL-4 (pg/ml)	*medium*	**552**	*813*	**280**	*305*	308	194	**448**	*551*	563	665	**1030**	*1335*	1334	586	**452**	*466*	281	267
IL-4 (ng/ml)	*PMA/ iono-mycin*	**124.5**	*73.2*	**111.7**	*53.0*	96.2	106.8	**133.1**	*66.7*	109.5	135.9	**119.4**	*90.0*	86.1	142.8	**134.0**	*79.9*	101.6	118.9
IL-10 (pg/ml)	*medium*	**466**	*297*	**552**	*386*	411	521	**459**	*303*	426	415	**435**	*230*	301	370	**417**	*244*	282	416
IL-10 (pg/ml)	*LPS*	**1053**	*679*	**1128**	*935*	808	1078	**1067**	*640*	955	1069	**959**	*375*	748	960	**1059**	*683*	944	1409
IL-10 (ng/ml)	*PMA/ iono-mycin*	**33.8**	*30.2*	**32.0**	*26.7*	33.8	59.5	**55.2**	*43.3*	35.4	103.3	**22.8**	*14.4*	11.0	26.8	**25.2**	*16.8*	14.3	27.6
IL-17 (U/ml)	*medium*	**154**	*224*	**248**	*314*	243	196	**240**	*252*	180	212	**53**	*80*	30	46	**74**	*68*	38	71
IL-17 (1000 U/ml)	*PMA/ iono-mycin*	**27.8**	*14.0*	**25.6**	*13.7*	22.3	24.2	**29.7**	*13.5*	23.0	30.2	**27.4**	*13.8*	16.8	30.9	**28.5**	*15.3*	19.4	22.1

Equine cytokines in PBMC supernatants after 12 h of cell culture: mean concentration (STD);

BL: Baseline (mean t-24 – t0); t12: 12 h post-treatment; t24: 24 h post-treatment; Detection limits: IFNα (12 – 30,000 pg/ml), TNFα (31.2 – 2,000 pg/ml), IFNγ (10 – 5,000 U/ml), IL-4 (40 – 80,000 pg/ml), IL-10 (15 – 35,000 pg/ml), and IL-17 (10 – 10,000 U/ml).

Detection of cytokine effects proved challenging, as the variance of ex vivo cytokine release was high even before treatments. This variance in immune parameters is not unexpected in outbred species such as the horse (Klier et al., 2011; Lembcke et al., 2012). However, in comparison to individual baselines, TNFα and IFNγ proteins were systemically induced by treatment with DNA (B, C and D), while IL-10 synthesis tended to decrease. These changes after each treatment containing DNA indicate a bias towards a pro-inflammatory immune state (Cavaillon, 2001; Dinarello, 2000; Kelso, 1998; Petrovsky et al., 1998; Wagner et al., 2008). It is conflicting that this effect could not be seen at the messenger ribonucleic acid (mRNA)-level of whole blood leukocytes. Potentially, the qPCR used here was not sensitive enough to detect low numbers of mRNA copies, which may, nevertheless, have been repeatedly translated to detectable amounts of cytokine proteins.

Figure 5 Tumor necrosis factor alpha

Tumour necrosis factor alpha differences to individual baselines in PBMC supernatants measured by ELISA are plotted in three cell culture settings [a) medium, b) LPS and c) PMA/ionomycin, vertical] for t0, t12 and t24. Datasets are marked as (group)-(hours post-treatment) at the X-axes. Horizontal bars represent mean and SEM. Grey asterisks (*) represent significant differences from baseline in the respective dataset. Asterisks (*) with brackets (⌜ ⌝) represent significantly different comparisons. The increase of TNFα could be statistically noticed in B (t12, medium and LPS; t-test; p = 0.0754 and p = 0.0162), in C (t12, LPS; t-test; p = 0.0220) and in D (t24, medium and LPS; t-test, p = 0.0501 and p = 0.0579). Treatment effects in LPS settings at t12 in C or D (ANCOVA, p = 0.0421 and 0.0774, respectively) were higher than in A. After *ex vivo* stimulation with PMA and ionomycin, mean TNFα secretion was lower than baseline after treatments A to D. This was significant after treatment A at t12 (t-test, p = 0.0042). Furthermore, TNFα in PMA/ionomycin settings was less decreased in B than in D at t12 (ANCOVA, p = 0.0372).

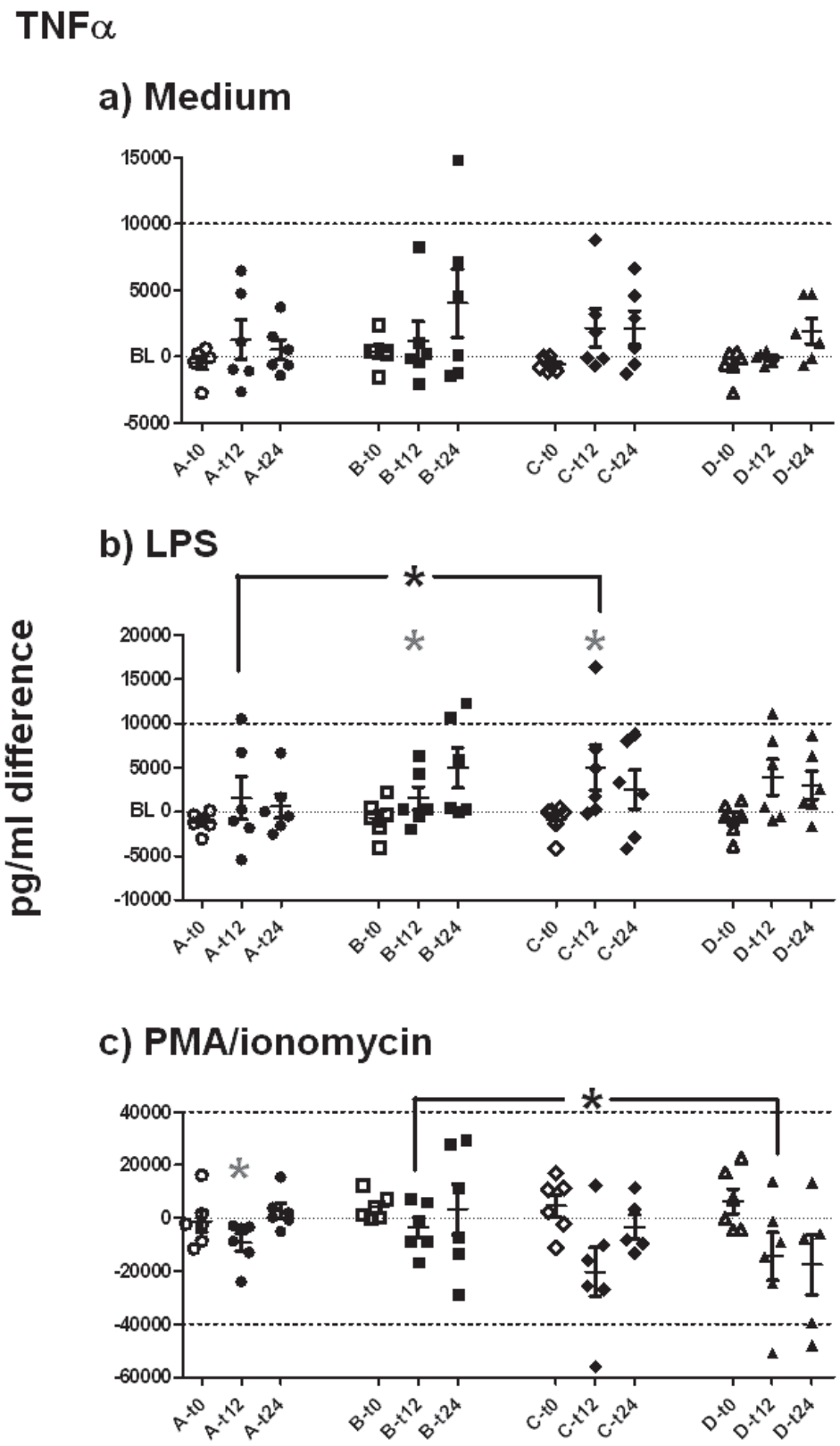


Figure 5 Tumor necrosis factor alpha

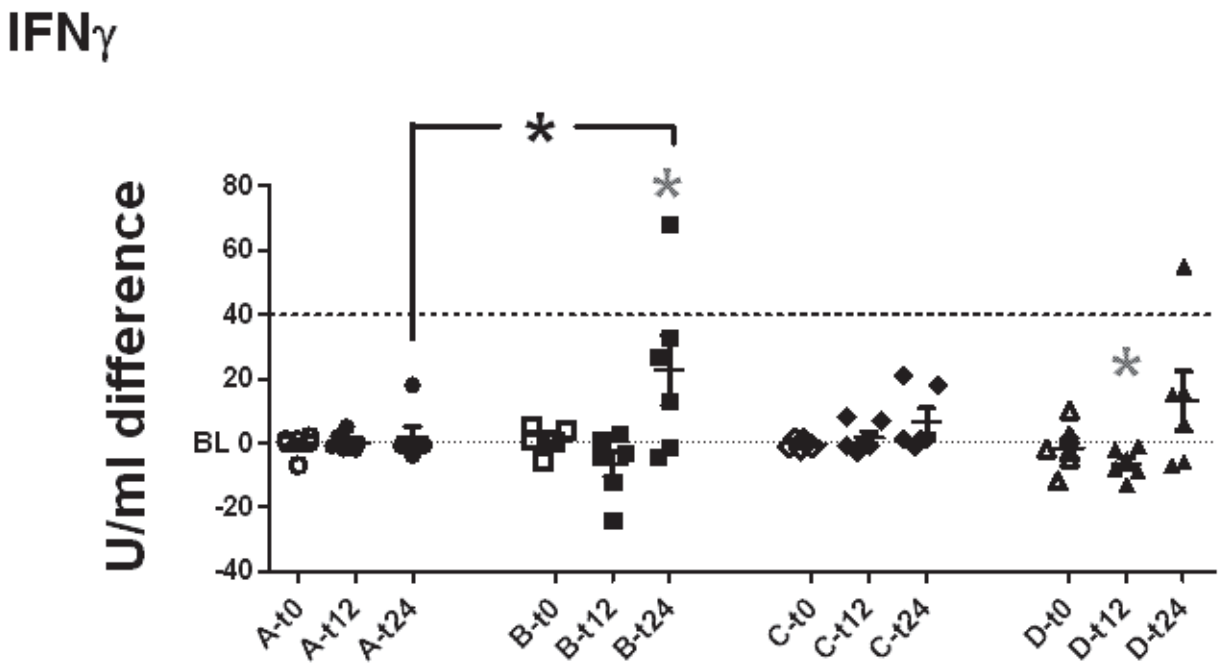


Figure 6 Interferon gamma

Interferon gamma differences to individual baselines in PBMC supernatants (medium settings) measured by a bead-based assay are plotted for t0, t12 and t24. Datasets are marked as (group)-(hours post-treatment) at the X-axis. Horizontal bars represent mean and SEM. Grey asterisks (*) represent significant differences from baseline in the respective dataset. Asterisk (*) with brackets (┌ ┐) represent significantly different comparisons. Interferon gamma was significantly increased compared to baselines in group B at t24 (t-test, p = 0.0377) and decreased in group D at t12 (t-test, p = 0.0112). Treatment effects at t24 differed significantly from group A in group B (ANCOVA, p = 0.0232) and with a trend toward significance in D (ANCOVA, p = 0.0611).

Figure 7 Interleukin 10

Interleukin 10 differences to individual baselines in PBMC supernatants are plotted in three cell culture settings (a) medium, b) LPS and c) PMA/Ionomycin). Interleukin 10 was measured by a bead-based assay. Data presented at t0, t12 and t24. Datasets are marked as (group)-(hours post-treatment) at the X-axes. Horizontal bars represent mean and SEM.
Asterisks (*) with brackets (⌐ ¬) represent significantly different comparisons.
Interleukin 10 decreased after all treatments in all settings. Treatment effects in LPS settings (b) in group D at t24 (increase) showed a trend towards significance compared to baselines (t-test p = 0.0503) and differed significantly from those in A, B and C (near baseline) (ANCOVA, p = 0.0347; 0.0276; 0.0105, respectively). In PMA and ionomycin settings, IL-10 secretion decreased clearly 12 h after treatments in groups B, C and D without reaching significance compared to individual baselines. Treatment effects in C (decrease) differed significantly from those in A (near baseline) at t12 (ANCOVA, p = 0.0495). The greatest interindividual variances were seen in responses in group B.

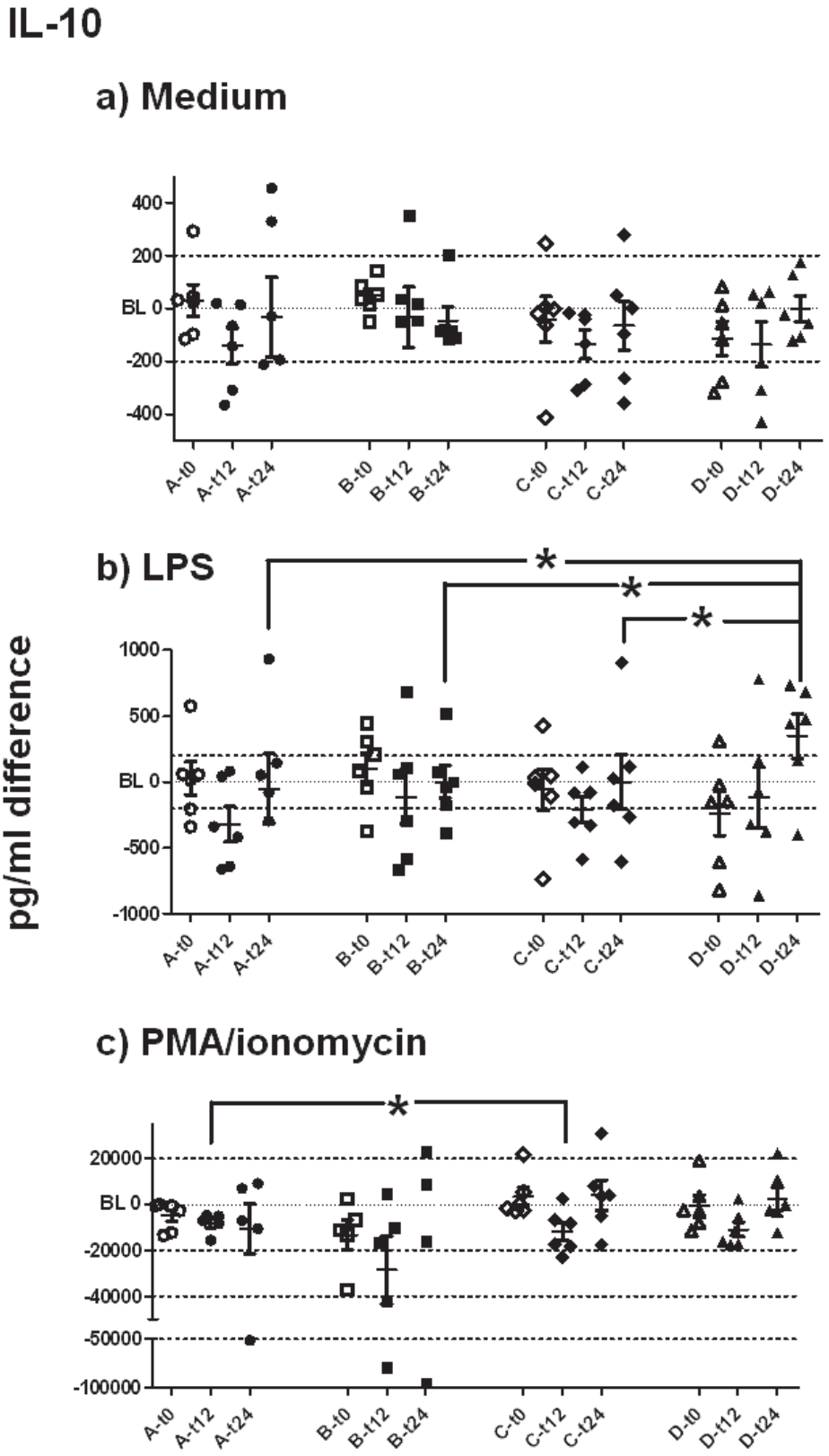


Figure 7 Interleukin 10

Systemic effects of DNA treatment

According to the evaluations of systemic parameters in the present study (RT, haemograms, SAA, cytokines in PBMC supernatants), simultaneous i.m. and i.d. treatment with DNA complexed with transfection reagent provokes a systemic immune reaction in horses in accordance with previous reports on inflammation induced by the combination of DNA and cationic transfection reagent in mice (Dow et al., 1999a; Dow et al., 1999b). The general effect herein was shown to be independent of transgene expression or CG motif content of the DNA injected, as differences between groups B – D were usually not statistically significant. It could, therefore, be hypothesised that the general stimulation of the immune system may be responsible for parts of the antimetastatic effects demonstrated by Mählmann et al. (2015). However, plasmids containing nonsense DNA alone were not effective in melanoma therapy in previous studies in horses (Heinzerling et al., 2001; Müller et al., 2011b).

Although most of the recent literature on DNA effects focuses on the immunological effects of CG motifs (Leise et al., 2010; Lopez et al., 2006; Wattrang et al., 2005; Wattrang et al., 2012), *in vivo* application of complexed DNA herein induced systemic and local effects independent of the CG motif content. It should be mentioned that the CG motif contents of the vectors have been estimated on the basis of counting CG without analysis of the flanking sequences. A detailed analysis was not possible as information on the exact sequences of stimulatory motifs or their allocation to classes are only known for a few oligodeoxynucleotides in the horse (Bordin et al., 2012; Klier et al., 2011; Wattrang et al., 2012) and have yet not been completely recognised. In comparison to plasmids, which also contain CG motifs in the backbone, MIDGE vectors only contain CG motifs in their expression cassettes (Moreno et al., 2004; Schakowski et al., 2007). According to the low CG motif content in the vectors utilised and the absence of statistically significant differences of treatment effects between DNA containing CG and depleted of CG, the authors conclude that signalling via TLR-9 receptors is not the primary mechanism of immunostimulatory action of *in vivo* applied complexed MIDGE-Th1 vectors in horses.

Effects of DNA, independent of CG motif and TLR signalling, stimulate innate immune responses, such as pro-inflammatory cytokine production via different pathways, as described more recently (Hornung et al., 2014; Ishii et al., 2006; Unterholzner, 2013;

Unterholzner et al., 2010). The signalling cascades employed are induced by intracellular double-stranded DNA and usually involve cGAS (cyclic GMP-AMP synthase) and STING (stimulator of IFN genes), AIM2 (absent in melanoma 2) and inflammasome activation or ribonucleic acid (RNA) polymerase III and RIG-I (retinoic acid-inducible gene), leading to pro-inflammatory and antiviral immune responses (Ablasser et al., 2009; Chiu et al., 2009; Hornung et al., 2009; Hornung et al., 2014; Unterholzner, 2013). To the best of the authors' knowledge, there are no reports of these mechanisms in horses. However, these signal cascades are phylogenetically highly conserved and very likely to exist in horses as well. Thus, although the causative molecular mechanism of the systemic and local immunostimulatory effect independent of the CG motif content of the DNA herein cannot be identified based on the present data, the stimulation of the pathways of the innate immune system by complexed DNA reaching cytosolic compartments described seems likely.

Table 3 Responders in systemic parameters

Treatment group horses	**A)**	# A	# E	# I	# N	# R	# W	**B)**	# B	# F	# K	# S	# O	# X	**C)**	# C	# G	# L	# P	# U	# Y	**D)**	# D	# H	# M	# Q	# V	# Z
Age (years) **(groups: mean)**	**11.1**	21	4	11	8	13	9	**10.6**	5	17	19	7	5	10	**10.3**	2	8	11	8	20	11	**10.9**	8	8	10	14	17	8
Sex		fe	fe	mc	mc	mc	mi		fe	mc	mc	fe	mc	mi		mc	mc	mc	mc	mc	mi		fe	fe	mc	mc	mc	mc
Type		ThB	WBl	WBl	WBl	WBl	ThB		ThB	WBl	WBl	WBl	WBl	WBl		ThB	WBl	WBl	WBl	WBl	ThB		ThB	WBl	WBl	WBl	WBl	WBl	WBl
Colour		g	g	o	o	o	o		g	o	o	o	o	g		g	o	g	o	o	o		o	g	o	o	g	o
period (h)	72							72							72							72						
thre-shold	10	-	+	-	-	-	-	10	+	-	+	+	+	-	10	+	-	+	-	+	+	10	+	-	+	-	-	+
Para-meter	**SAA** µg/ml)	-	+	-	-	+	-	**SAA** µg/ml)	-	-	+	+	+	-	**SAA** µg/ml)	+	-	-	-	+	+	**SAA** µg/ml)	+	-	+	+	-	+
	0		**3**					**3**							**3**							**5**						
RT °C)*	24	-	+	-	-	+	-	24	+	-	-	+	+	-	24	+	-	-	-	+	+	24	+	-	+	+	-	+
	3							**3**							**3**							**5**						
Neu G/l)*	24	-	-	-	-	-	-	24	+	-	-	+	+	-	24	-	-	-	-	+	+	24	+	-	+	+	-	+
	0							**3**							**2**							**2**						
Lymph G/l)*	24	+	-	-	-	-	-	24	-	-	+	+	+	+	24	-	-	-	-	+	+	24	+	-	+	+	-	+
	1							**4**							**2**							**5**						
TNFα pg/ml) LPS	24	+	-	+	-	-	-	24	-	-	+	+	+	-	24	-	-	+	-	+	+	24	+	-	+	+	-	+
	2							**3**							**3**							**4**						
IFNγ U/ml) med	24	-	-	-	-	-	-	24	+	-	+	-	-	+	24	-	-	-	-	-	-	24	+	-	+	+	-	+
	0							**3**							**1**							**2**						

Responder	**1**	**+**	-	-	-	-	-	**3**	-	-	**+**	**+**	**+**	-	**2**	-	-	-	-	**+**	**+**	**4**	**+**	-	**+**	**+**	-	**+**
Responses/ group parameters with response	**6**	3	1	1	0	1	0	**19**	2	0	5	5	5	2	**17**	1	0	2	0	5	6	**20**	6	1	3	3	0	6
Treatment group horses	A)	# A	# E	# I	# N	# R	# W	B)	# B	# F	# K	# S	# O	# X	C)	# C	# G	# L	# P	# U	# Y	D)	# D	# H	# M	# Q	# V	# Z

Numbers of horses that display values of ≥ threshold given in each cell of group columns;
response present (+, threshold reached) or absent (-, threshold not reached) given in each cell of individual horses (IDs); fe: female, mare; mc: male castrated, gelding; mi: male intact, stallion; Wbl: warmblood; ThB: thoroughbred; g: grey; o: other (non-grey); *: differences to individual time-of-day matched baselines; #: differences to individual baselines over all times of day; WBC: white blood cell counts; Neu: neutrophil counts; Lymph: lymphocyte counts; med: medium settings; LPS: LPS settings; responder: (response + in min. 3 parameters); response in groups: at least two horses +

Responder classification in systemic effects of treatments

The parameters included for responder classification were RT, SAA, neutrophil and lymphocyte counts, TNFα in LPS settings and IFNγ in medium settings. White blood cell counts, although influenced by treatments, were not included as this parameter is dependent on neutrophil and lymphocyte counts which were already being considered. Response periods up to t24 were usually chosen, except for SAA (until t72) (Table 3). A classification into responders and non-responders was not possible for local parameters evaluated in skin samples.

Responders were found in all groups. There were no noteworthy changes of SAA, neutrophil counts or IFNγ in group A. In groups B – D, there were more responses than in A, but no clear differences between types of DNA. Interestingly, not a single grey horse was classified as a responder after treatment with complexed DNA (B, C or D) (Table 3).

While some horses displayed systemic effects following DNA application (usually in RT, haematological parameters, acute phase proteins and cytokine secretion), others showed no effects at all. Similar to melanoma patients, responders and non-responders to DNA treatment in healthy horses seem to exist in accordance with systemic observations. In accordance with single parameters of systemic evaluations, significant differences between expressing (B) and non-expressing (C) or CG motif-free non-expressing (D) DNA could not be found by the responder classification.

Since no grey horse was classified as a responder, the authors cannot conclude reasonably on the predictive value of the parameters, thresholds and response periods chosen for the responder classification or on anti-tumour effects in grey horses bearing melanomas.

Evaluation of local treatment effects in clinical and histological examinations

The skin was unaffected and clinical scores were usually classified as grade 0 before treatment. Treatment (A – D injected i.d. locally treated skin samples: *treat*) and control (phosphate-buffered saline: PBS injected i.d. control skin samples: *ctrl*) sites showed mild oedema (grade 1 – 2) after treatments. Other alterations were very rare (painfulness: n = 2 in *treat* and *ctrl*; redness n = 2 in *treat* and *ctrl*; depigmentation = 2 in *ctrl* and *treat*). Clinical scores were not statistically different between control and treatment sites or between different treatments.

The evaluation of skin biopsies stained with haematoxylin and eosin (H&E) revealed ulceration of the epithelia (Supplement 1a – c) and perivascular mixed-cell dermatitis with some diffuse content, especially in the reticular dermis (*Dret*). Inflammatory cells were predominantly of lymphoid origin. Macrophages appeared perivascularly in subepithelial positions proximate to most ulcers and infrequently in diffuse patterns. Neutrophilic granulocytes were randomly seen intravascularly and sometimes found perivascularly and abundantly in a diffuse pattern in higher grade inflammation in *Dret* (Supplements 1a, c; 2a – c).

Scores of inflammation did not differ significantly between treatments and controls or between different treatments in any layer.

Shaving of the skin was performed to optimise the cutting of histological slices of biopsy samples after fixation, as hairs often cause disruption of slices and artefacts in microscopic views. An early time point (t-72) was chosen for shaving to facilitate gross recovery before treatment. Shaving and scrubbing alone led to ulcerative inflammation of the epithelium visible in histologic slides, as shown in six additional healthy horses which underwent shaving and scrubbing without further treatment (Supplement 11). This preparation of the skin may have hidden treatment effects on epithelia, but not on deeper skin layers where the main alterations in response to treatments A – D were expected. However, shaving immediately prior to taking biopsies might have been better. Nevertheless, since the effect of DNA was noted mainly in the dermis, this aspect of the study design may not be of relevance for interpretation of the data.

Immunohistochemistry results in the skin

Calprotectin stained by MAC387

The *strata spinosum, granulosum* and *lucidum*, and hairs' epithelial sheaths of the stratified squamous epithelium in all specimens were generally immunopositive for calprotectin. Ulcers in the epithelium usually did not stain, except for single infiltrated cells or debris (Supplement 3a, c). Calprotectin positive cells in the dermis were frequent in perivascular locations, but also occurred infrequently in other localisations in a diffuse pattern (Supplement 3b – d; 4a, c). Calprotectin, detected by MAC 387, is a marker for myeloid cells (Bjerke et al., 1993). The immunohistochemical staining pattern observed in the present skin samples corresponded well with neutrophilic granulocytes and macrophages identified by H&E staining.

Overall, *treat* specimens contained more calprotectin-positive cells than *ctrl*s in both layers. For single comparisons, this was significant in the *Dret* in A (Aa) and B (Bb). The *ctrl* samples of B, C and D contained more calprotectin-positive cells than *ctrl* samples of A without reaching significance. In the *Dret*, the *treat* samples of B contained statistically significantly more calprotectin-positive cells than the *treat* samples of A (details given in Figure 8).

Myeloid cell infiltration reveals that inflammation was induced in both the *treat* and *ctrl* samples. Thus, an acute inflammatory response was induced locally by i.d. injections in general, and by the transfection reagent, SAINT-18, which induced an influx of leukocytes and expression of the cytokines IL-12 and IL-18 (see below). The addition of DNA tended to enhance the inflammatory response, without reaching statistical significance by means of inflammation scores and IHC evaluation. This may be due to the overlapping of the systemic effects causing increased tissue reactivity and effects of the local stimulus by, for example, the pressure of the injection demonstrated in *ctrl* sites.

Figure 8 Calprotectin (MAC387) positive cells in immunohistochemistry

Calprotectin-positive cells per FOV plotted for the *Dpap* (a) and *Dret* (b) for each treatment (A – D) and site (*Ctrl*: control; *treat*: local treatment). Median, quartiles, minimum and maximum plotted for each dataset. Asterisks (*) with brackets (⌜ ⌝) and same letters (Aa, Bb) represent significantly different comparisons.

In the *Dpap* (a), *treat* specimens overall contained more calprotectin-positive cells than *ctrl*s (ANOVA, p = 0.0128). This was not significant for single treatments. In *Dret* (b), *treat* samples overall contained significantly more calprotectin-positive cells than *ctrl*s (ANOVA, p = 0.0079). For single comparisons, this was significant in treatment groups A (Aa, ANOVA, p = 0.025) and B (Bb, ANOVA, p = 0.013). Control samples of A had significantly fewer calprotectin-positive cells in *Dret* than of C (*, ANOVA, p = 0.0261) and D (*, ANOVA, p = 0.0065). *Treat* samples of B contained significantly more calprotectin-positive cells than those of A (* ANOVA, p = 0.0454). As a trend, treatment samples of C contained more MAC387-positive cells than those of A, too (ANOVA, p = 0.0654).

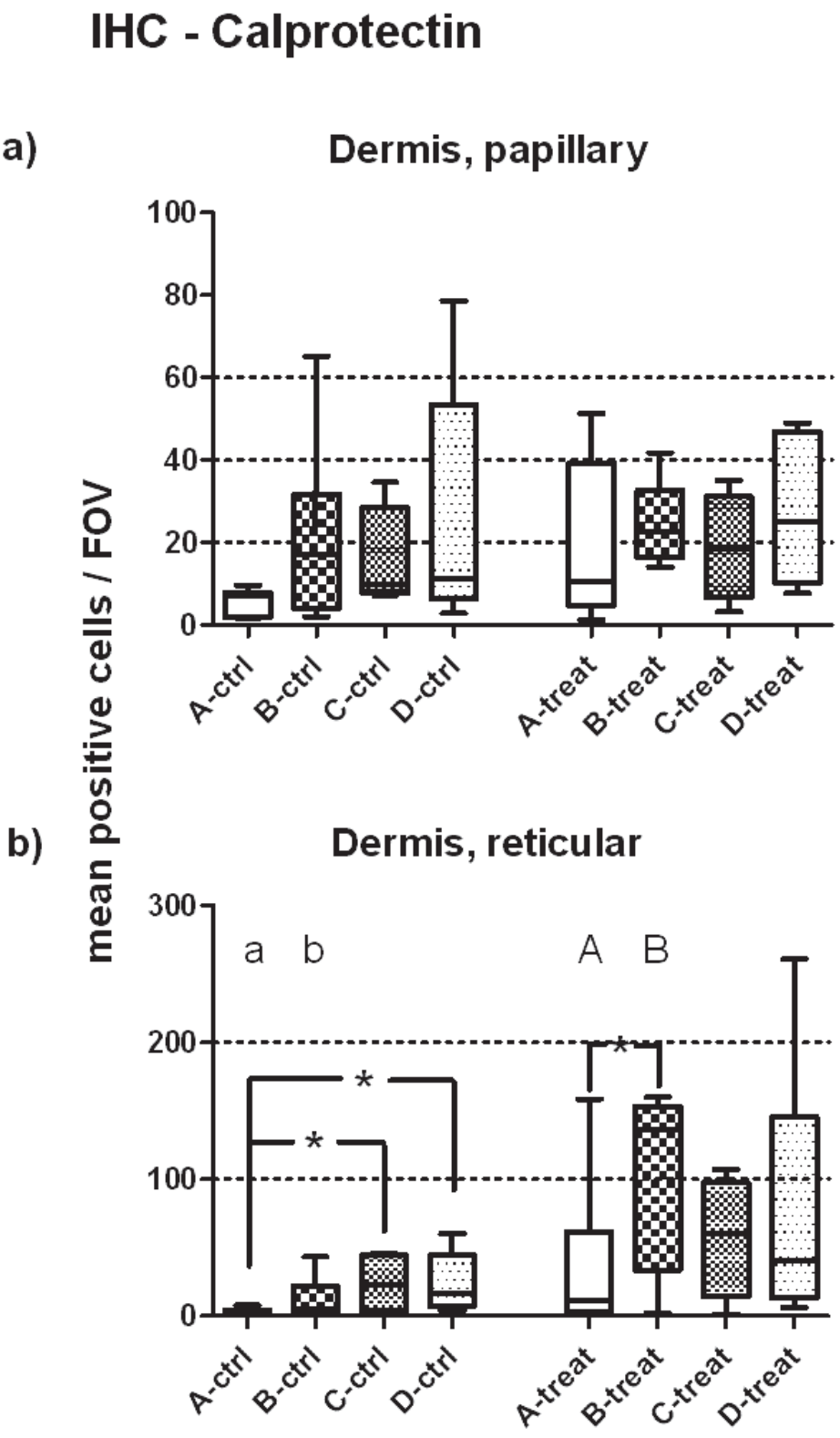


Figure 8 Calprotectin (MAC387) positive cells in immunohistochemistry

Local expression of IL-12 and IL-18 proteins in skin samples after treatment

The IL-12 and IL-18 proteins were stained by monoclonal antibodies in IHC for the investigation of transgene expression.

In general, IL-12 and IL-18 were present in the same tissues and cell types in skin samples of the sites treated and of healthy control animals: squamous epithelium, hairs, glands (sebaceous and apocrine), fibrocytes, endothelia (unstained in some bigger vessels), pericytes, muscle cells, nerves (in parts) and leukocytes (Supplements 5 – 10).

However, intensity varied, and some cell types (e.g. inflammatory cells) were more frequently present in samples after treatment, resulting in a theoretically higher amount of locally available IL-12 and IL-18.

The *treat* samples contained more IL-12 positive cells than the *ctrl*s. This was statistically significant for single comparisons in treatments A and B in both layers, papillary dermis (*Dpap*) and *Dret*. Furthermore, the *Dret ctrl*s contained significantly fewer IL-12-positive cells in A than the *Dret ctrl*s in D. The *treat* samples did not differ statistically significantly between different treatments in the absolute numbers of IL-12-positive cells in any layer (details given in Figure 9).

There was no statistically significant overall difference in the IL-18-positive cells in the *Dpap* between *treat* and *ctrl*. The *Dpap ctrl*s contained statistically significantly fewer IL-18-positive cells in A than in C. The *treat* samples in *Dret* contained overall more IL-18-positive cells than the *ctrl*s. This was statistically significant in A. The *treat* samples did not differ statistically significantly between different groups in the numbers of IL-18-positive cells (details given in Figure 10).

Figure 9 Interleukin 12-positive cells in immunohistochemistry

Interleukin-12-positive cells per FOV plotted for the *Dpap* (a) and *Dret* (b) for each treatment (A – D) and site (*Ctrl*: control; *treat*: local treatment). Median, quartiles, minimum and maximum plotted for each dataset. Asterisks (*) with brackets (⌜ ⌝) and same letters (Aa, Bb) represent significantly different comparisons. In *Dpap* (a), *treat* specimens overall contained more IL-12-positive cells than *ctrl*s (ANOVA, p = 0.0027). This was significant for single comparisons in treatments A (Aa, ANOVA, p = 0.0158) and B (Bb, ANOVA, p = 0.0366). In *Dret* (b), too, *treat* specimens contained significantly more IL-12-positive cells than *ctrl*s (ANOVA, p = 0.0004). For single comparisons, this was again significant in treatment groups A (Aa, ANOVA, p = 0.0005) and B (Bb, ANOVA, p = 0.0455). In *Dret*, *ctrl* samples of A contained significantly fewer IL-12-positive cells than those of D (*, ANOVA, p = 0.022) and, as a trend, than controls of B (ANOVA, p = 0.0602).

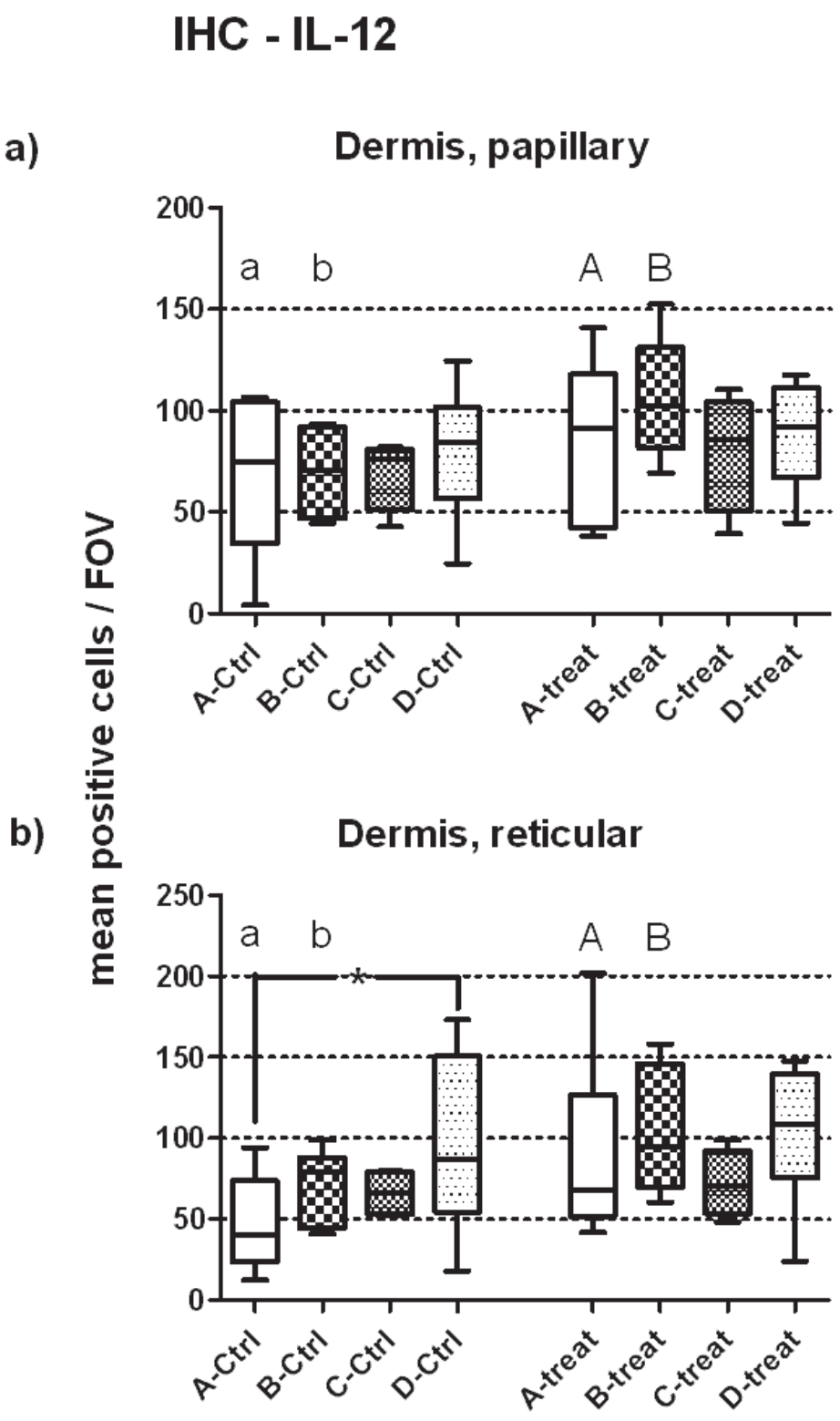


Figure 9 Interleukin 12-positive cells in immunohistochemistry

Figure 10 Interleukin 18-positive cells in immunohistochemistry

Interleukin-18-positive cells per FOV plotted for the *Dpap* (a) and *Dret* (b) for each treatment (A – D) and site (*Ctrl*: control; *treat*: local treatment). Median, quartiles, minimum and maximum plotted for each dataset. Asterisks (*) with brackets (┌ ┐) and same letters (Aa) represent significantly different comparisons. Numbers of IL-18-positive cells in the *Dpap*(a) in *ctrl* samples of A were significantly lower than those of C (ANOVA, p = 0.0414) and, as a trend, than those of B (ANOVA, p = 0.0815) and D (ANOVA, p = 0.086). In *Dret* (b), *treat* samples overall contained more IL-18-positive cells than *ctrl*s (ANOVA, p = 0.0726). For single comparisons, this was significant in treatment group A (ANOVA, p = 0.0397). The *Ctrl* samples of A contained fewer IL-18-positive cells in *Dret* than those of C (ANOVA, p = 0.052) and D (ANOVA, p = 0.0503) without reaching statistical significance.

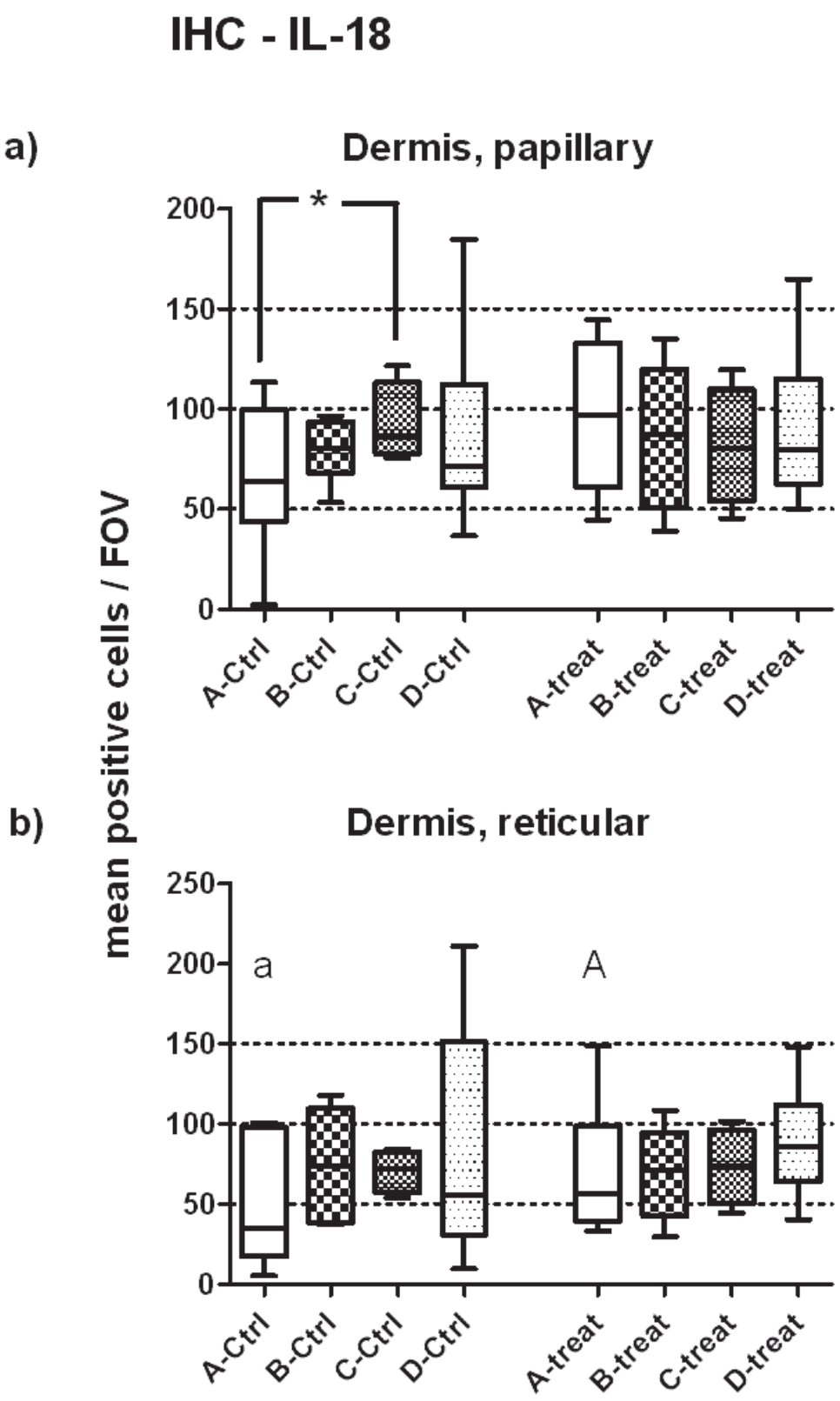


Figure 10 Interleukin 18-positive cells in immunohistochemistry

Cytokine and chemokine mRNA in skin biopsies

For further investigation of *in vivo* expression of recombinant *IL-12* and *IL-18*, these two cytokines were investigated on the mRNA-level in skin biopsies by SYBR green qPCR as well as the downstream mediators typically induced by these, IFNγ (Heinzerling et al., 2001) and CXCL-10 (Coughlin et al., 1998), (Table 1).

The detection of IL-12 expression by qPCR was hampered by the interference of MIDGE- Th1 DNA in the (*treat*) samples, as MIDGE-Th1 vectors present at the site of injection could not be digested completely prior to transfer of mRNA into cDNA (complementary DNA), and PCR primers could not be designed to discriminate between equine recombinant *IL-12* transcripts expressed by MIDGE-Th1 vectors and endogenous *IL-12* transcripts. Consequently, measurement of *IL-12* by qPCR always resulted in positive results and discrimination from false-positive results was not possible.

Copy number differences between *treat* and *ctrl* samples of *IL-18* were lowest in group A, which varied statistically significantly from B and D (details given in Figure 11a).

Copy number differences of *CXCL-10* between *treat* and *ctrl* of individual horses were statistically significantly lower in group A compared to the other groups. Group B, furthermore, showed statistically significantly higher differences of *CXCL-10* mRNA copy numbers between *ctrl* and *treat* than the other groups treated with complexed DNA (C or D) (details given in Figure 11b).

Similar to the blood samples, *IFNγ* mRNA/cDNA was not detected at all. Again, insufficient sensitivity of the assay cannot be excluded.

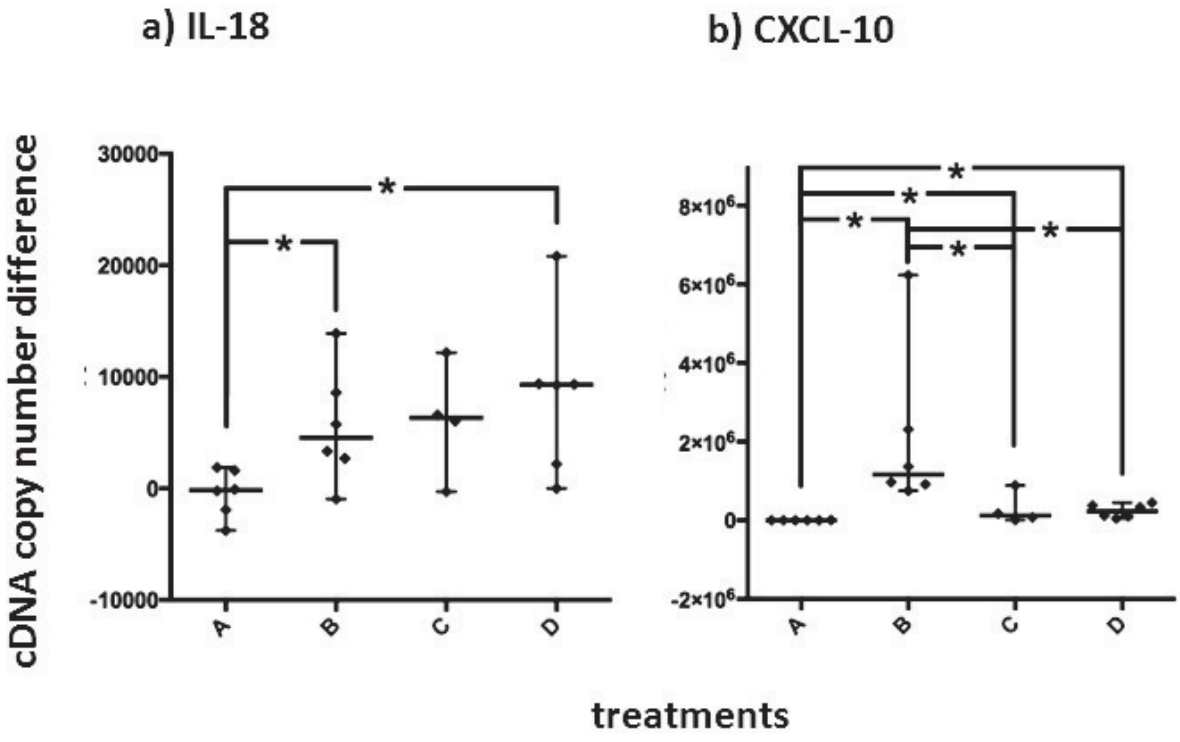


Figure 11 Treatment effects on mRNA expression in the skin

The *treat-ctrl* differences of copy numbers of IL-18 (a) and CXCL-10 (b) measured by qPCR in skin samples are shown in histograms. Horizontal bars represent medians and ranges. Asterisks (*) with brackets (┌ ┐) represent significantly different comparisons. The expressions of IL-18 increased by local treatment in comparison to *ctrl*s in groups B – D. The *treat-ctrl* difference of IL-18 mRNA (a) was significantly higher in B (*, Wilcoxon, p = 0.0306) and D (*, Wilcoxon, p = 0.0131) than in A. The expression of CXCL-10 in skin biopsies of the *treat* site was overall significantly higher than in the *ctrl* samples in all treatment groups (Wilcoxon, p = 0.0001). The *treat-ctrl* difference (b) of copy numbers examined was significantly lower in A than in the other treatment groups [*, Wilcoxon; B: p = 0.0051, C: p = 0.0142, D: p = 0.0051]. Group B, furthermore, displayed significantly higher *treat-ctrl* differences of CXCL-10 copy numbers than C (Wilcoxon, p = 0.0252) or D (Wilcoxon, p = 0.0051).

Transgene expression

In vitro expression after transfection of mammalian cells with MIDGE-Th1 eqIL12 and eqIL18 was proven on the mRNA level and *in vivo* transfection of MIDGE-Th1 vectors was generally confirmed in other species, e.g. in rats, on the protein level as well (Machelska et al., 2009).

Although expression of recombinant IL-12 and IL-18 in group B could not be verified directly by detection of *IL-12* and *IL-18* transcripts, the mRNA of *CXCL-10*, a downstream mediator of IL-12 and IFNγ (Cassatella et al., 1997; Tannenbaum et al., 1996), was significantly elevated in skin samples treated with expressing MIDGE-Th1 (group B, *treat*). As it has been shown that effects on downstream mediators are amplified due to enhancing loops (Tannenbaum et al., 1996), *CXCL-10* mRNA could be detected even if recombinant IL-12 and IL-18, and IFNγ induced by these, are not detectably increased. As CXCL-10 has been shown to be produced after intratumoural injection of IL-12 plasmids in human melanoma (Heinzerling et al., 2005), subsequent to IFNγ after IL-12 treatment in mice (Tannenbaum et al., 1996) and after transfection of B16 melanoma with IL-18, also in mice (Nagai et al., 2002), it is likely that CXCL-10 is a relevant anti-tumour mediator in melanoma immunotherapy with IL-12/IL-18 DNA also in horses.

Furthermore, the general inflammatory responses after DNA treatment were most distinct in horses of group B, which had received expressing DNA vectors. Thus, the recombinant expression of IL-12 and IL-18 seems to intensify the systemic response described for DNA vaccines (Goncalves, 2000).

Treatment with DNA complexed with SAINT-18 did not lead to vast amounts of cytokines being released by PBMC or expressed by blood leukocytes. The *IL-12* and *IL-18* expression levels were not increased in blood cells after treatments and IL-12 protein could not be detected by a bead-based assay [validated in horses previously (Durán et al., 2013)] in PBMC supernatants, even after stimulation as evaluated in samples of eight horses of groups A – D, including four responders (data not shown). It can be concluded that circulating leukocytes and tissues influencing these were not transfected to a great extent by expressing DNA vectors, as recombinant *IL-12* and *IL-18* were not statistically significantly elevated in post-treatment blood samples of B and only a moderate increase in the production of IFNγ protein was detected as an induced down-

stream cytokine of IL-12 and IL-18 (Del Vecchio et al., 2007; Shizuo, 2000; Trinchieri, 1995a).

However, treatment locally with DNA expressing IL-12 and IL-18 (B) induced an increase in IL-12-positive cells in the dermis in comparison to PBS controls. This could be due to local transfection of cells or to induction of endogenous IL-12. This cannot be discriminated herein by means of IHC.

Influences of horse factors on treatment effects

The authors noted great interindividual variances in treatment effects on most parameters. The following possible influencing factors were evaluated to elucidate the origin of these variances: age, sex (mares vs. geldings; two stallions were excluded from analysis due to limited number), type (breed) and colour of coat.

As treatment differences were usually between treatment with (groups B, C and D) and without DNA (group A), horses of groups B, C and D were combined for the analysis of influences on treatment effects.

The SAA increases and lymphocyte decreases in response to treatments tended to be stronger in younger horses without reaching significance (Figure 12). Mares showed higher increases in WBC (t-test, $p = 0.0068$) and neutrophils (t-test, $p = 0.0282$), and higher TNFα responses (medium settings; t-test, $p = 0.0172$) than geldings (Figure 13). Thoroughbred type horses (ThB) showed higher TNFα responses (medium settings; t-test, $p = 0.0240$) than Warmblood type horses (WBl) (Figure 14).

Influences of age, sex and type are known to influence immunological parameters (Adams et al., 2008; Giraldo et al., 2013; Hansen et al., 2013; Katepalli et al., 2008; Wilmink et al., 2003) and the influences found herein were not unexpected. As the treatment groups of horses were balanced with respect to these horse parameters, bias of the interpretation of the results concerning treatment effects is not likely.

Figure 12 Influence of age on systemic parameters

The SAA levels in serum (a) of horses treated with DNA (groups B – D) measured at t24 and t48 (Y-axes) are plotted against age (X-axes). Two outliers were excluded (aged 2 years and 8 years). The SAA tends to decrease with age (uv regression, p = 0.0720).

Lymphocyte differences to individual time-of-day-matched baselines (b) of horses treated with DNA (groups B – D) calculated for at t12 and t24 are plotted against age. Two outliers were excluded (aged 2 years and 8 years). Lymphocytes tend to decrease less in older horses (uv regression, p = 0.0952).

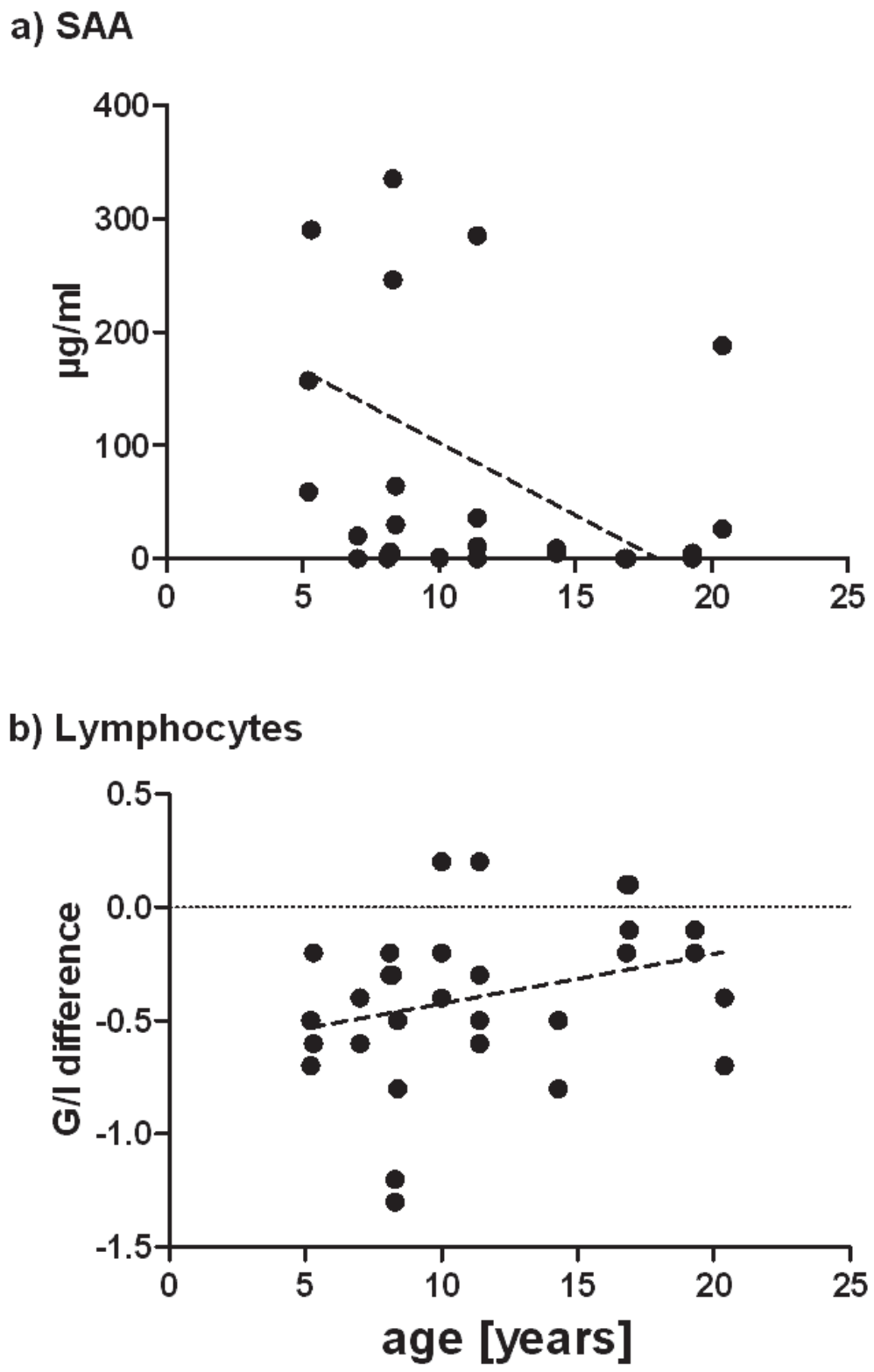


Figure 12 Influence of age on systemic parameters

Figure 13 Influence of sex on systemic parameters

Differences to individual baselines of WBC (a), neutrophils (b) and TNFα in medium settings (c) of horses treated with DNA (groups B – D) calculated at t12 and t24 are plotted in histograms for mares and geldings. Stallions could not be evaluated due to their small number. Horizontal bars represent mean and SEM. Asterisks (*) with brackets (⌐ ¬) represent significantly different comparisons. The WBC, neutrophil and TNFα increases to individual time-of-day-matched baselines were significantly higher in mares than in geldings (t-test).

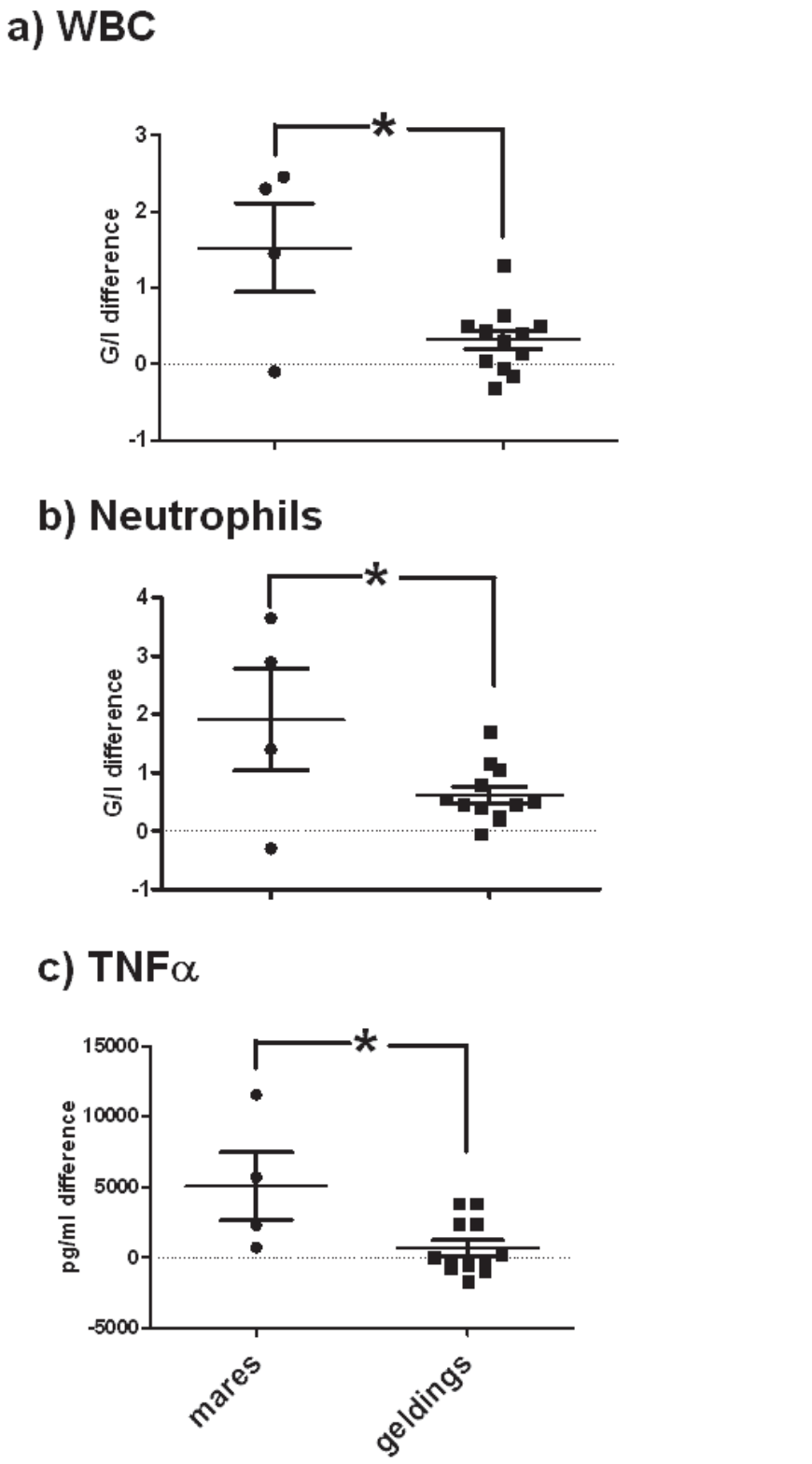


Figure 13 Influence of sex on systemic parameters

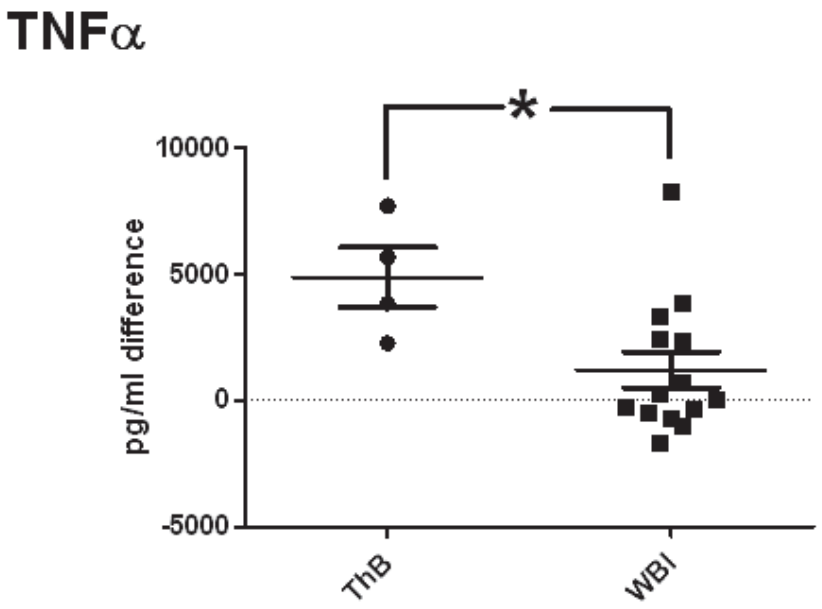


Figure 14 Influence of type on systemic parameters

The TNFα differences to individual baselines in PBMC supernatants (medium settings) of horses treated with DNA (groups B – D) calculated at t12 and t24 are plotted in histograms for ThB type breeds and WBl type breeds. Horizontal bars represent mean and SEM. Asterisks (*) with brackets (⌐ ¬) represent significantly different comparisons. Increases of TNFα were significantly higher in ThB than in WBl (t-test).

In addition to the factors mentioned above, the coat colour of the horses influenced their responses to treatment with DNA. Grey horses showed statistically significantly fewer lymphocyte decreases than horses of other colours (t-test, p = 0.0037) and fewer increases of *ex vivo* secreted TNFα in LPS settings (t-test, p = 0.0470) (Figure 15). Statistically significantly fewer myeloid (calprotectin-, MAC-387-positive) cells were found (t-test, p = 0.0413) (Figure 16) in the *Dret* of *treat* samples of grey horses than of non-grey horses, resulting in statistically significantly lower *treat-ctrl* differences (t-test, p = 0.0379).

Furthermore, none of the grey horses included was classified as a responder to DNA treatment with regards to systemic parameters. Therefore, the acute immune reaction of grey horses to DNA treatment seems to be milder than that of non-grey horses. Some previously reported differences between grey and non-grey horses in immunological parameters, such as different associations of microsatellites and single nucleotide polymorphisms (SNP) with immune responses to EHV vaccination (Rusek et al., 2013), match the findings of different immune reactivity herein.

The milder response of grey horses to DNA complexed with transfection reagent may require stronger stimulation in DNA vaccination, as inflammation enhances DNA uptake and antigen processing (Weide et al., 2008).

It might even be speculated whether this different acute immunological response contributes to melanoma development in grey horses, as genetic bases of grey coat colour are proposed to have an influence on other organ systems, including cell cycle regulation and immune functions (Comfort, 1958; Mayr et al., 1979; Pielberg et al., 2008), which are involved in tumour development and immune escape. Based on the present data of healthy horses, this is speculative and certainly requires further investigation.

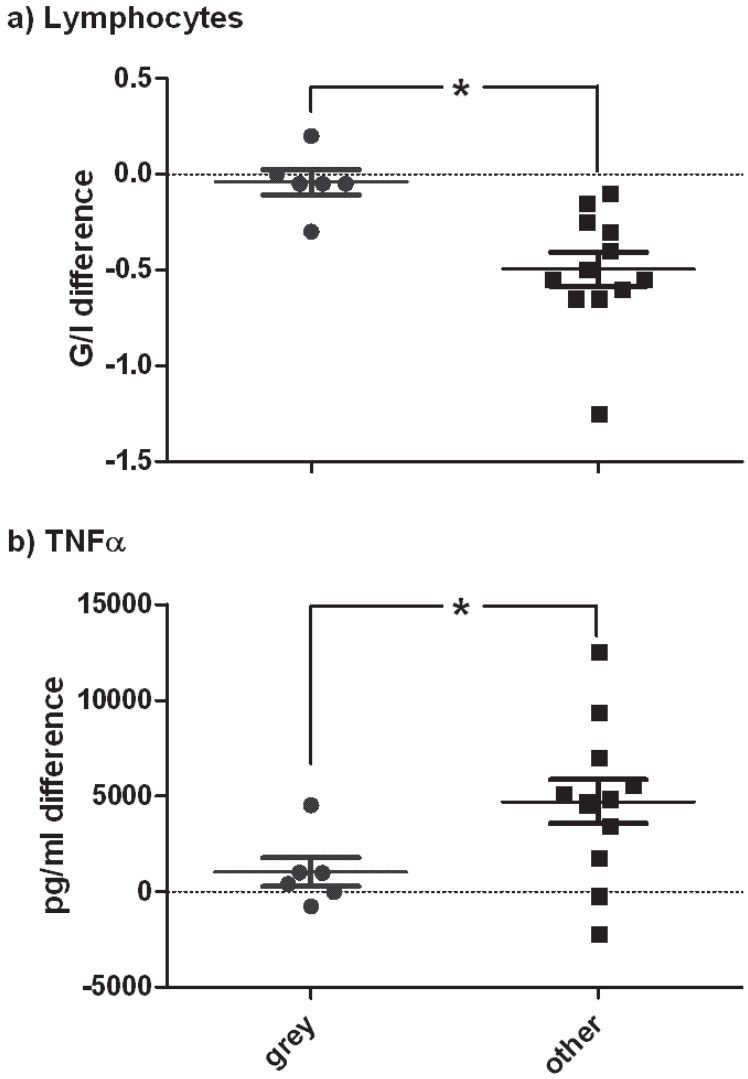


Figure 15 Influence of colour on systemic parameters

Differences of lymphocytes (a) and TNFα in LPS settings (b) to individual baselines of horses treated with DNA (groups B – D) calculated at t12 and t24 are plotted in histograms for grey horses (grey) and horses of other colours (other). Horizontal bars represent mean and SEM. Asterisks (*) with brackets (┌ ┐) represent significantly different comparisons.

Lymphocyte decreases (a) to individual time-of-day-matched baselines were significantly less in grey than in other horses (t-test). The TNFα increases to individual baselines in PBMC supernatants were significantly lower in grey than in other horses (t-test).

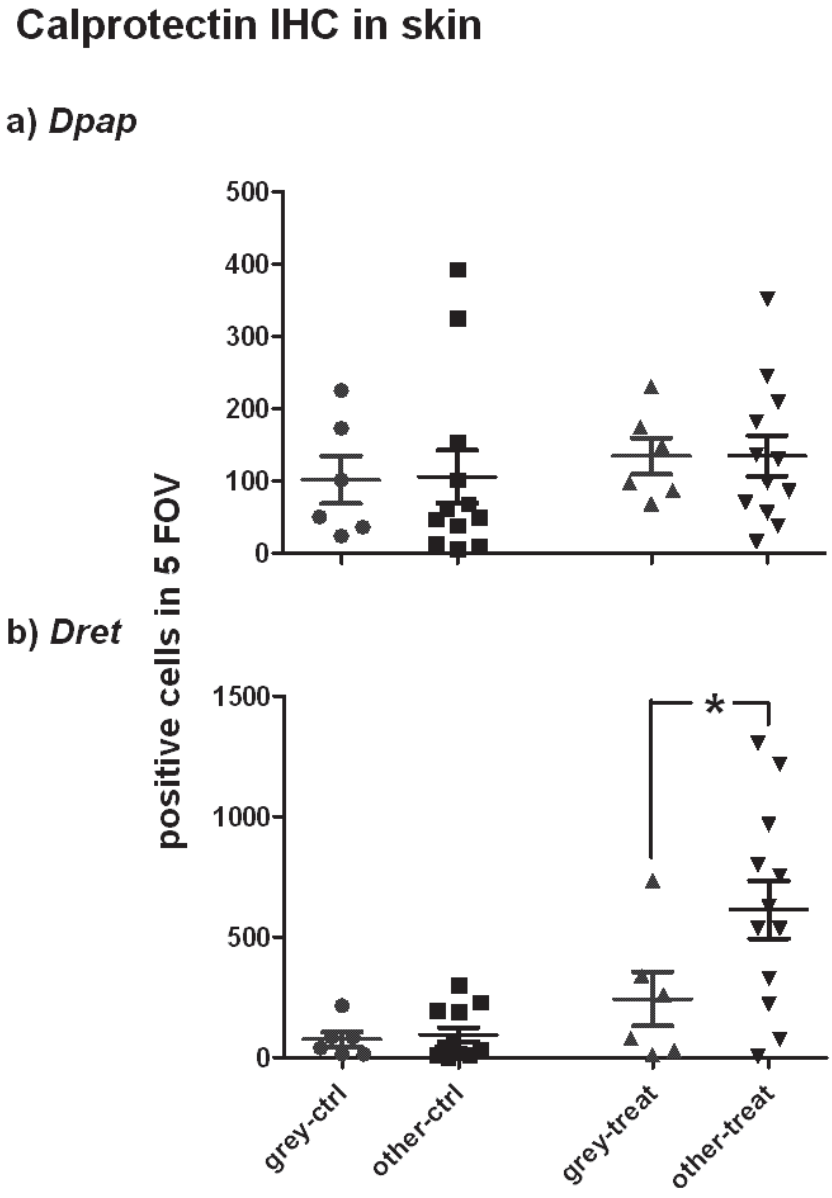


Figure 16 Influence of colour on immunohistochemistry results

Calprotectin-positive cells (Y-axes) in five fields of view (FOV) are plotted for the papillary dermis (*Dpap*, a) and the reticular dermis (*Dret*, b) in histograms for PBS *ctrl* and *treat* sites of grey and non-grey (other) horses treated with DNA (groups B – D). Colour-site combinations are announced on X-axes. Mean and STD plotted; asterisks (*) with brackets (⌜ ⌝) represent significantly different comparisons. Significantly fewer calprotectin-positive cells were found in the *Dret* (b) of *treat* sites of grey horses than in samples of non-grey (other) horses (t-test).

Conclusions

Treatment with DNA complexed with the transfection reagent SAINT-18 *in vivo* is immunostimulatory in healthy horses, independent of transgene expression and CG motif content, and might be used as a therapeutic adjuvant. The reactivity in grey horses to DNA treatment systemically and locally differs from that of horses of different colour, and grey horses seem to be non-responders with respect to unspecific systemic DNA effects. It cannot be excluded that this characteristic of grey horses may contribute to their melanoma development. Further research elucidating this issue is desirable.

Supplemental files

Supplement 1 Histology of epithelia and papillary dermis

Illustration of findings in representative skin samples (H&E staining). Evaluation was performed for single layers: epithelium (*Ep*), papillary dermis (*Dpap*), reticular dermis (*Dret*).

a) Overview of *Ep* with two ulcers (arrows) and areas of subepithelial infiltration

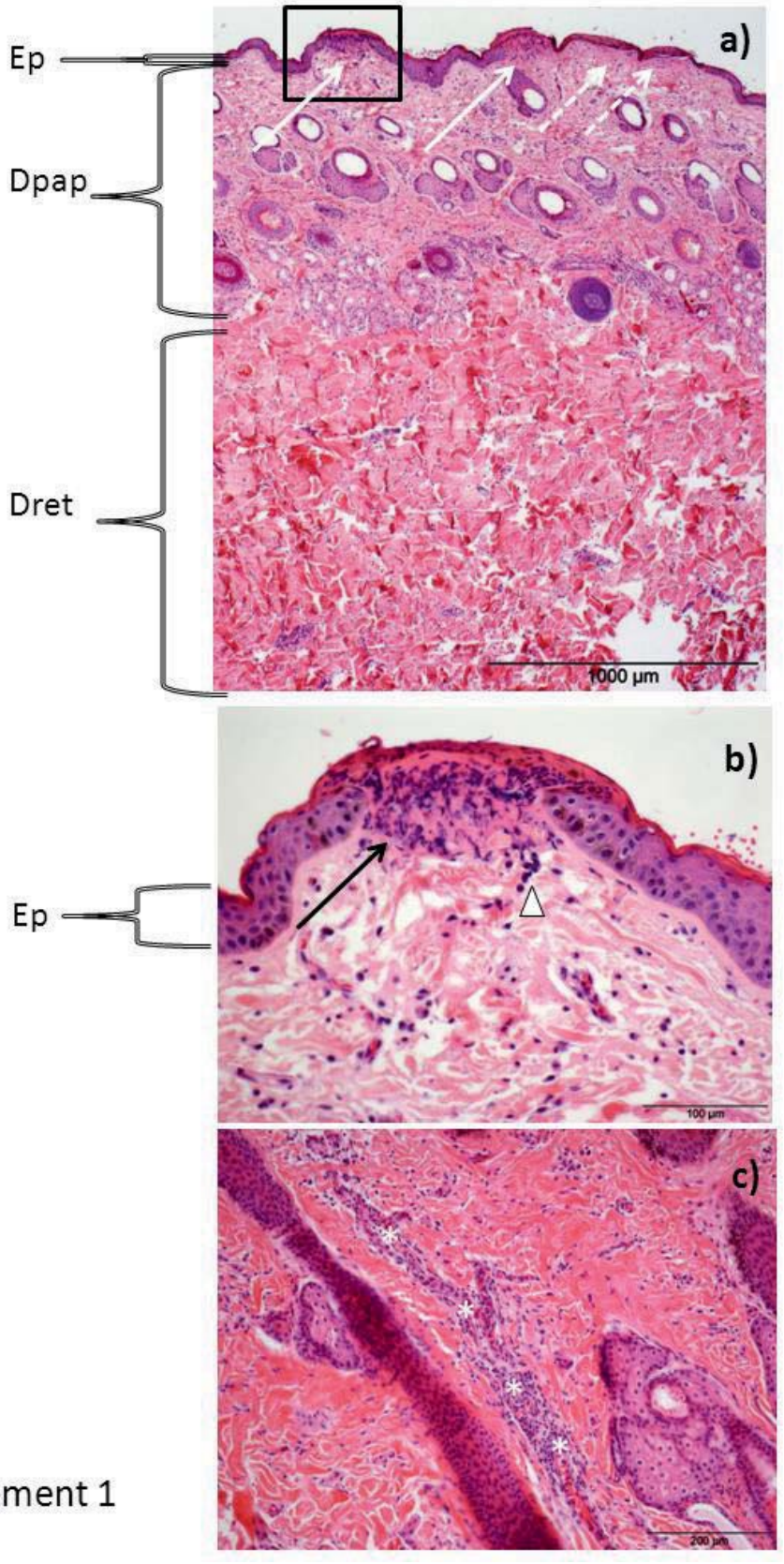
Note beginning of ulceration in two additional localisations (dashed arrows).

b) Detail of *Ep* with ulceration (magnification of frame in A)

Note cell debris in ulcer (arrow) and subepithelial infiltration of leukocytes (arrowhead) in *Dpap*.

c) Inflammation in *Dpap* with adnexa

Distinct infiltration of leukocytes in perivascular localisations (asterisks), while only a few leukocytes are present in a diffuse distribution pattern in other areas.



supplement 1

Supplement 2 Histology of the reticular dermis

Illustration of findings in representative skin samples (H&E staining) in *Dret*.

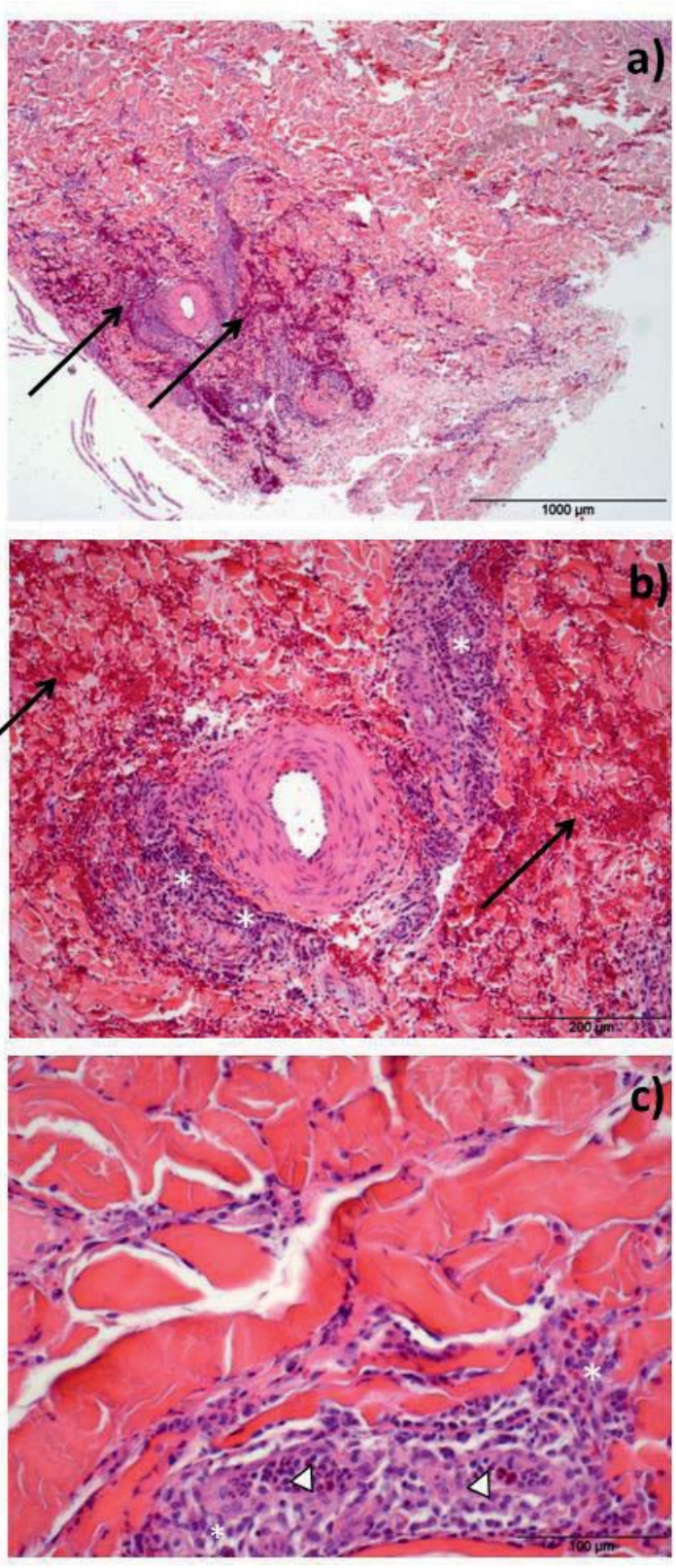
a) **Overview of *Dret* with haemorrhage (arrows)**

b) **Detail of *Dret* with haemorrhage (magnification of A)**

Note haemorrhage (arrows) and massive perivascular infiltration of leukocytes (asterisks).

c) **Detail of inflammation in *Dret***

Distinct infiltration of leukocytes in perivascular (asterisks) and intravascular (arrowheads) localisation, while only a few leukocytes are present in a diffuse distribution pattern in other areas.



supplement 2

Supplement 3 Immunohistochemistry for calprotectin

Immunohistochemical localisation of calprotectin (red) in representative skin samples (counterstained with haematoxylin).

a) Overview of *Ep* and Dermis with low grade inflammation

Calprotectin-positive cells are present in *Dpap* (arrows). In addition, *Ep* and hairs were immunopositive in parts.

b) Overview of *Dret* with haemorrhage (arrows)

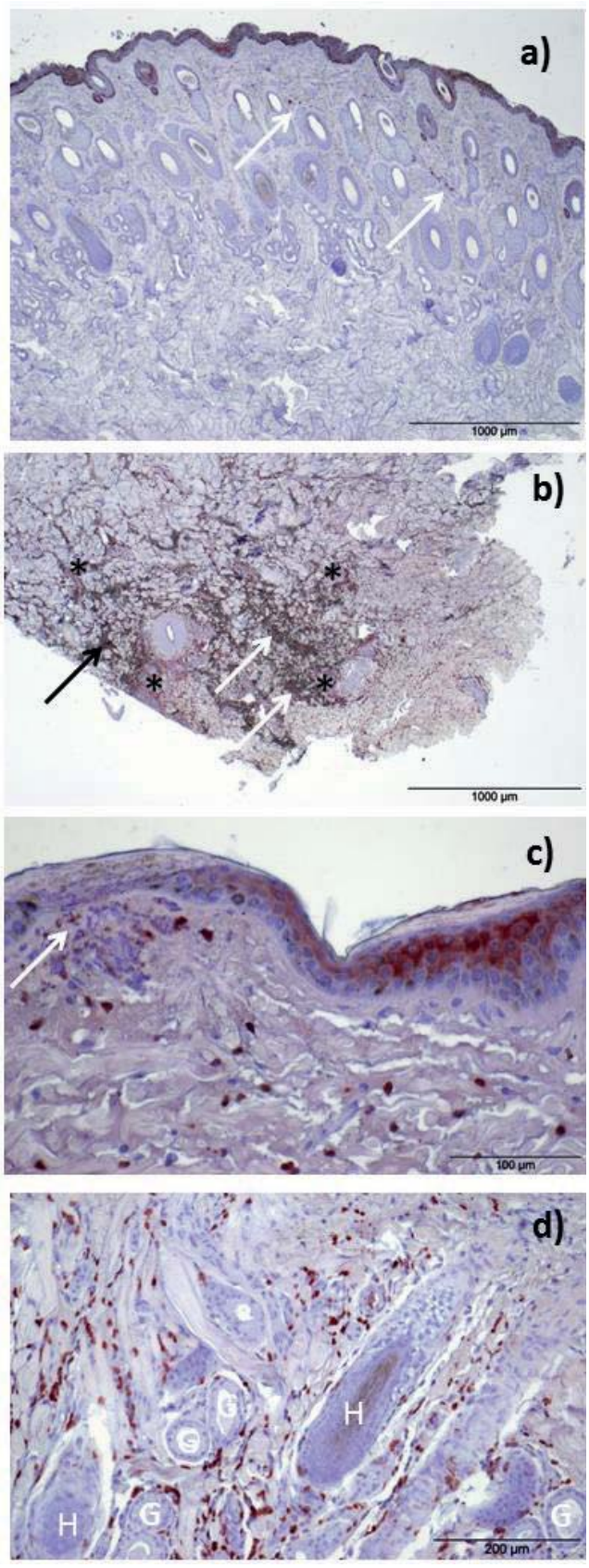
Some leukocytes (asterisks) near and within haemorrhage stained positive for calprotectin (red).

c) Epithelial Ulcer (arrow)

Subepithelial leukocytes frequently stained calprotectin-positive (red). In addition, parts of the *Ep* (*Str. spinosum and Str. granulosum*) are positive for calprotectin.

d) *Dpap* with adnexa

Infiltrating calprotectin-positive (red) leukocytes in perivascular localisation indicate a distinct inflammation. Hairs (H) and excretory ducts of apocrine glands (G) were negative.



supplement 3

Supplement 4 Detail of calprotectin immunohistochemistry (continued)

Immunohistochemical localisation of calprotectin (red) in representative skin samples (counterstained with haematoxylin).

a) *Dret* with perivascular inflammation around larger vessels

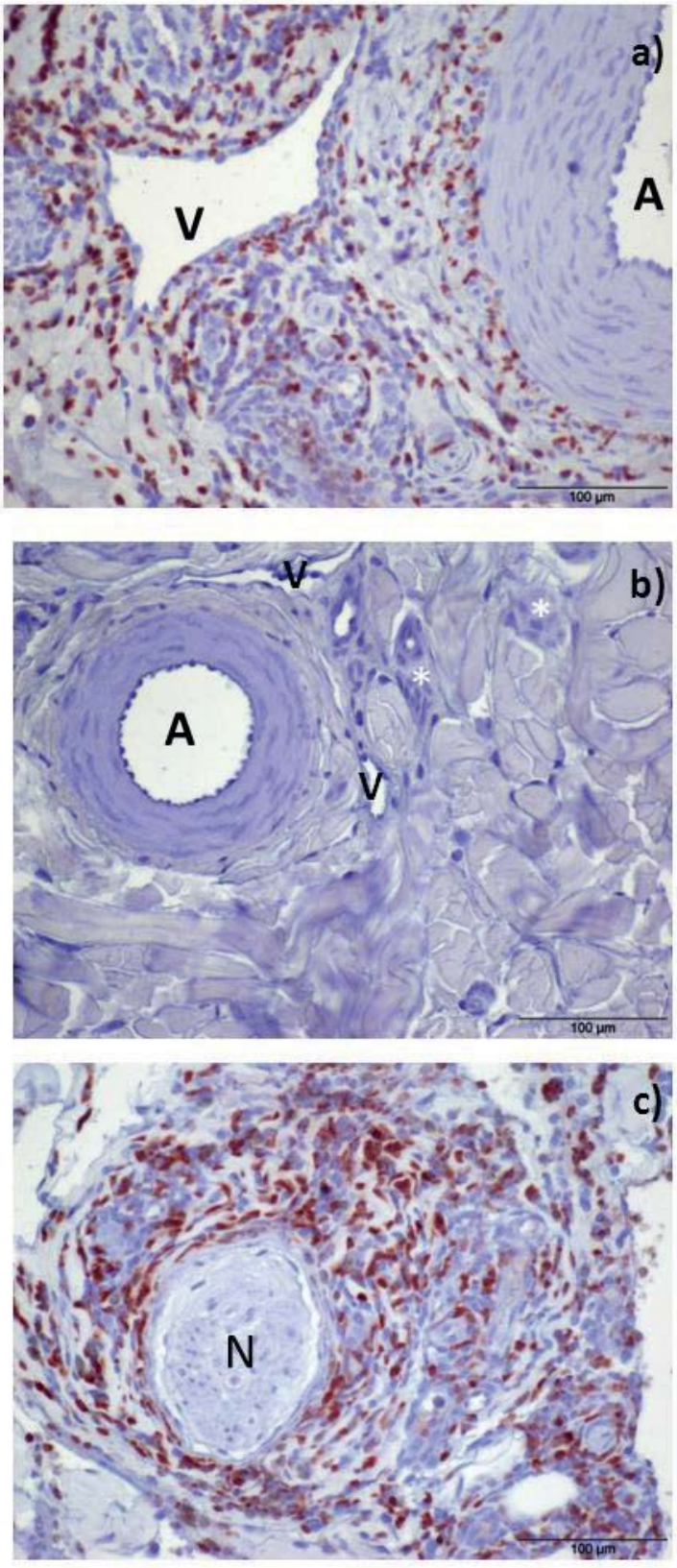
Detail of large artery (A) and vein (V), which are surrounded by multiple calprotectin-positive (red) leukocytes.

b) *Dret* – Isotype control

Similar localisation as a) with large artery (A) and smaller veins (V), where a perivascular infiltration (asterisks) is visible, but red staining is absent (negative).

c) Perinerval leukocyte infiltration in *Dret*

Detail of nerve (N) and vessels; note the distinct perivascular and perinerval infiltration with calprotectin-positive leukocytes.



supplement 4

Supplement 5 Immunohistochemistry for Interleukin 12

Immunostaining for IL-12 (red) illustrated in representative skin samples (counterstaining with haematoxylin).

a) Overview of *Ep* and dermis in inflamed skin

Ep, hairs (H) and sebaceous glands (G) stained immunopositive for IL-12 (red).

b) Detail of *Ep* and *Dpap*

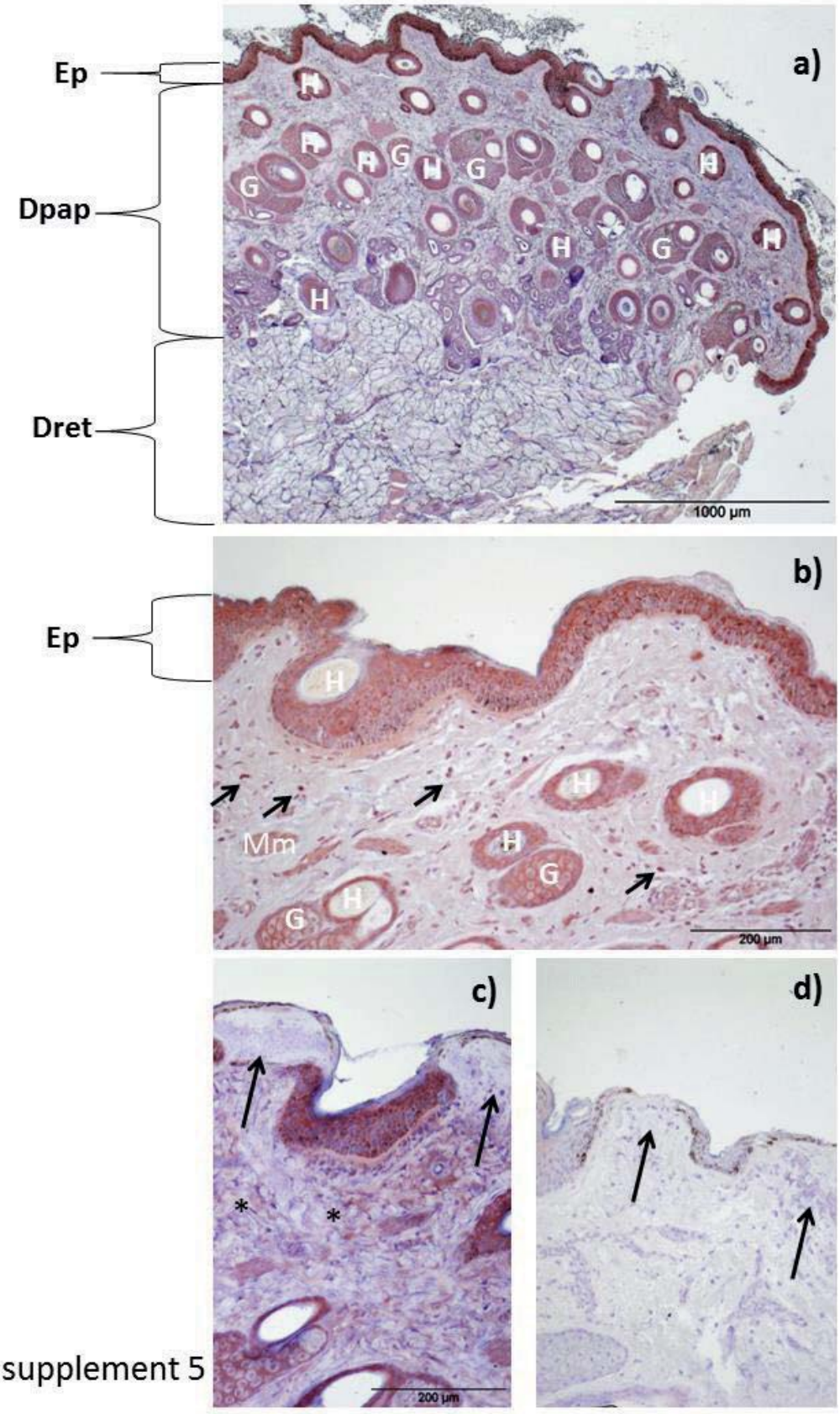
The *strata granulosum, spinosum* and *basale* of the stratified squamous epithelium (Ep) showed a cytoplasmic red staining for IL-12. Sebaceous glands (G) and hairs (H) usually stained positive for IL-12. *Musculi arrectores pilorum* (Mm) were positive for IL-12, as well. In addition, infiltrating leukocytes were frequently positive for IL-12 (arrows).

c) Epithelial ulcers

Ulcers (arrows) were unstained in otherwise IL-12-positive stained squamous epithelium. Parts of the fibrocytes (asterisks) in *Dpap* also stained positive for IL-12.

d) Isotype control

No immunopositive staining is visible in a similar localisation as in c).



supplement 5

Supplement 6 Immunohistochemistry for Interleukin 12 in papillary dermis

Red immunoreactions for IL-12 are shown in representative skin samples with haematoxylin counterstaining.

a) Detail of *Dpap* with adnexa

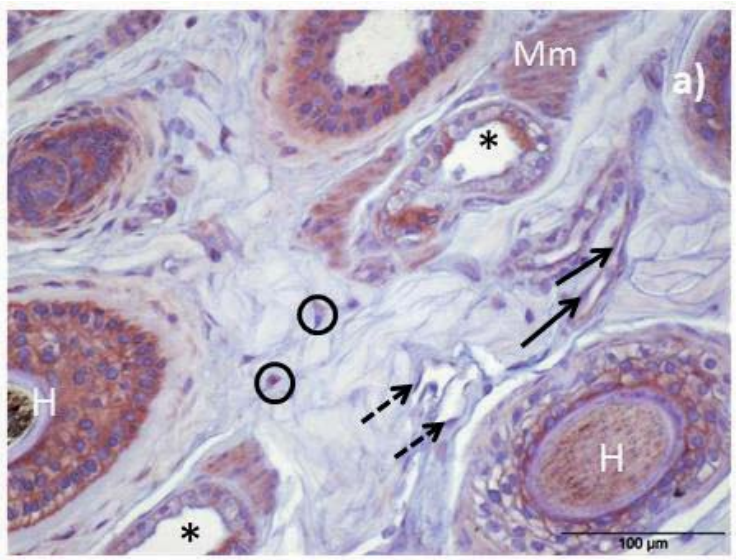
Epithelia of various kinds were usually positive for IL-12, e.g. cortex and epithelial root sheath of hairs (H), and glands and their excretory ducts (asterisks). Please note the patchy staining pattern of the excretory ducts. Endothelia stained positive (arrows) or negative (dashed arrows) for IL-12. Additionally, *Mm. arrectores pilorum* (Mm) stained positive for IL-12. Fibrocytes usually appeared negative for IL-12, but some perinuclear immunopositive staining of fusiform cells in the connective tissue could be noted (circles).

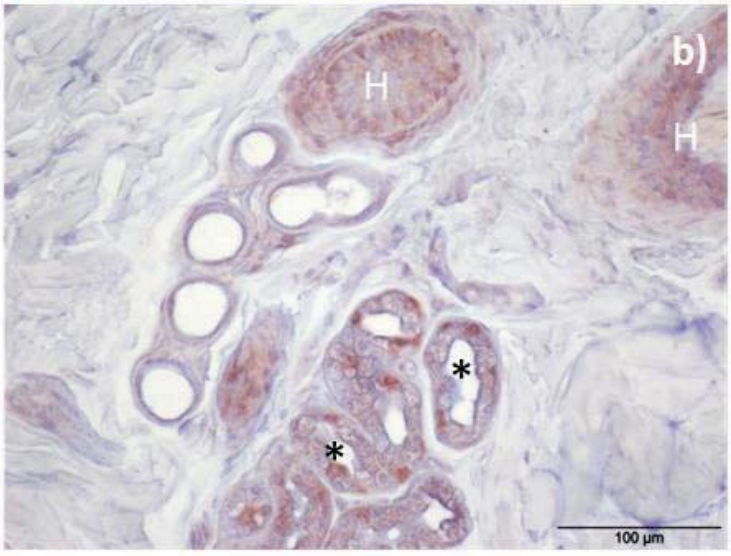
b) Detail of *Dpap* illustrating appearance of apocrine glands

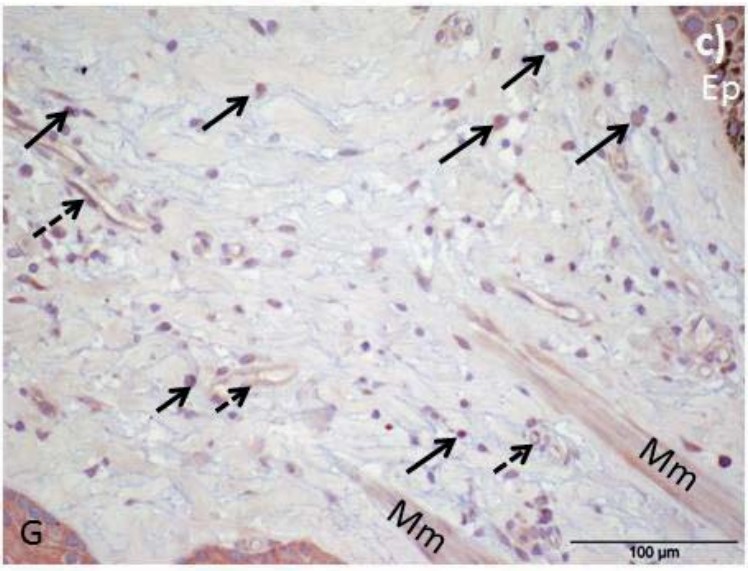
Apocrine glands were positive for IL-12 (asterisks) but showed a patchy staining. Epithelial components of hairs (H) were immunopositive, while fibrocytes in this detail were negative for IL-12.

c) *Dpap* – detail of subepithelial inflammation

Infiltrating leukocytes can be found perivascularly and are dispersed diffusely in the connective tissue. They frequently show a red perinuclear and cytoplasmic immunostaining for IL-12 (arrows). *Mm. arrectores pilorum* (Mm) and endothelia (dashed arrows) stained positive for IL-12, while fibrocytes were usually negative for IL-12. Epithelium (Ep) and a gland (G) are indicated at the edges of the image (top right and bottom left, respectively).







supplement 6 G

Supplement 7 Immunohistochemistry for Interleukin 12 in reticular dermis

Immunostaining for IL-12 (red) in representative skin samples (counterstaining with haematoxylin).

a) Large artery and nerve in *Dret*

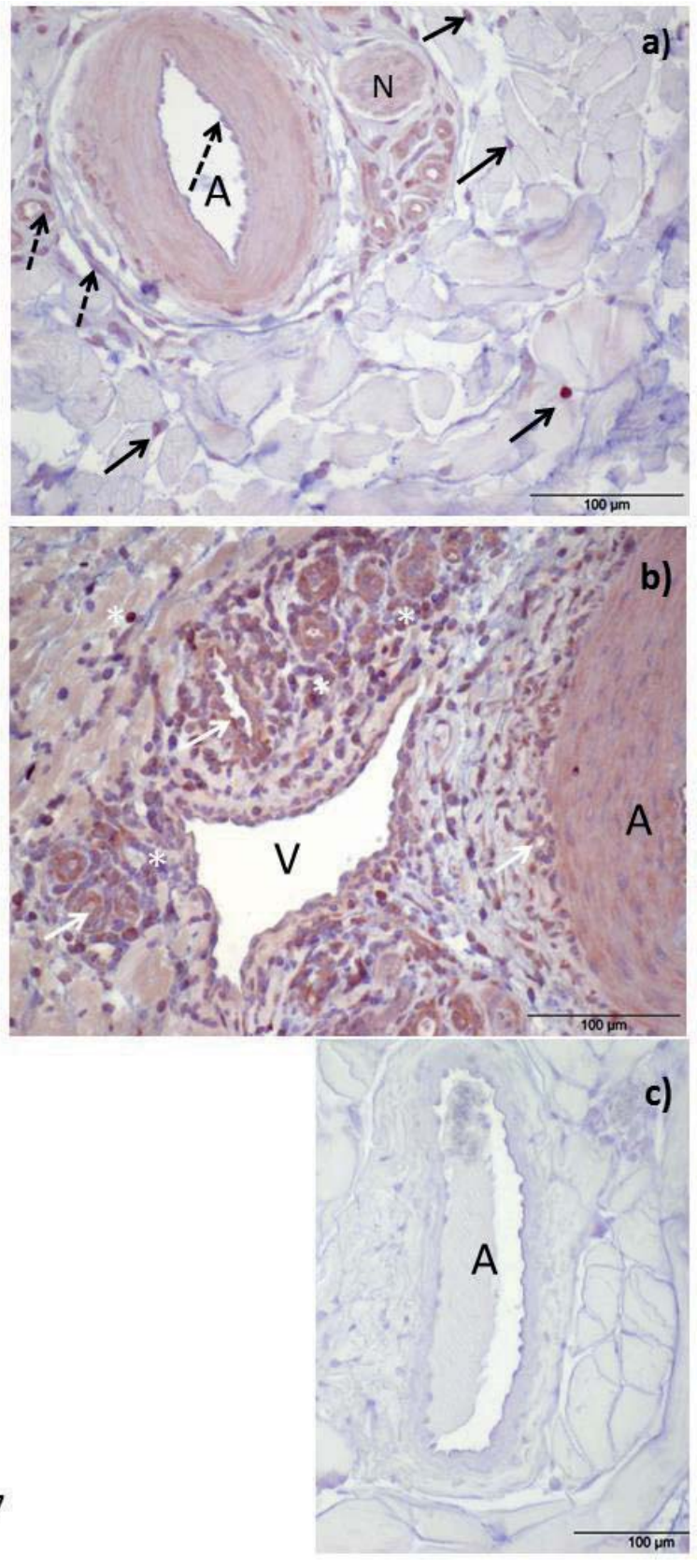
Walls and endothelia (dashed arrows) of a large artery (A) and small blood vessels stained positive for IL-12, as did the nerve (N). In addition, infiltrated leukocytes (arrows) were positive for IL-12. Fibrocytes and extracellular matrix were usually negative.

b) *Dret* – detail with perivascular inflammation

Leukocytic perivascular infiltration of high degree proximate to artery (A) and vein (V). In addition, vessel walls and endothelia (arrows) infiltrated cells (asterisks) are positive for IL-12. Fibrocytes and extracellular matrix are negative apart from a weak background staining.

c) Isotype control *Dret* with artery (A)

All tissue components are negative in detail of *Dret*.



supplement 7

Supplement 8 Immunohistochemistry for Interleukin 18

Illustration of red IL-18 in representative skin samples (haematoxylin counterstained).

a) Overview of *Ep* and dermis in mildly inflamed skin

Ep (except for ulcers, arrows), hairs (H) and glands (G) stained positive for IL-18, while the surrounding connective tissue is negative.

b) Isotype control – overview of *Ep* and dermis

No immunopositive staining is visible in a similar localisation as in a) [including squamous epithelium with ulcers (arrows) and dermis with hairs (H) and glands (G)] in the papillary layer.

c) Detail of *Ep* with ulcer (arrow) and *Dpap* with medium grade leukocyte infiltration

All epithelial tissues [*stratum granulosum, spinosum* and *basale* of the stratified squamous epithelium (Ep), cortex and epithelial root sheath of hairs (arrowheads), sebaceous glands (G)] showed a cytoplasmic immunoreaction for IL-18, while ulcers (arrow) were negative for IL-18. Infiltrating leukocytes (arrowheads) were frequently positive for IL-18. The IL-18 staining of fibrocytes and endothelia (dashed arrows) varied from positive to negative. In addition, hairs (H) and sebaceous glands (G) stained IL-18positive.

d) Detail of *Ep* and *Dpap*, isotype control

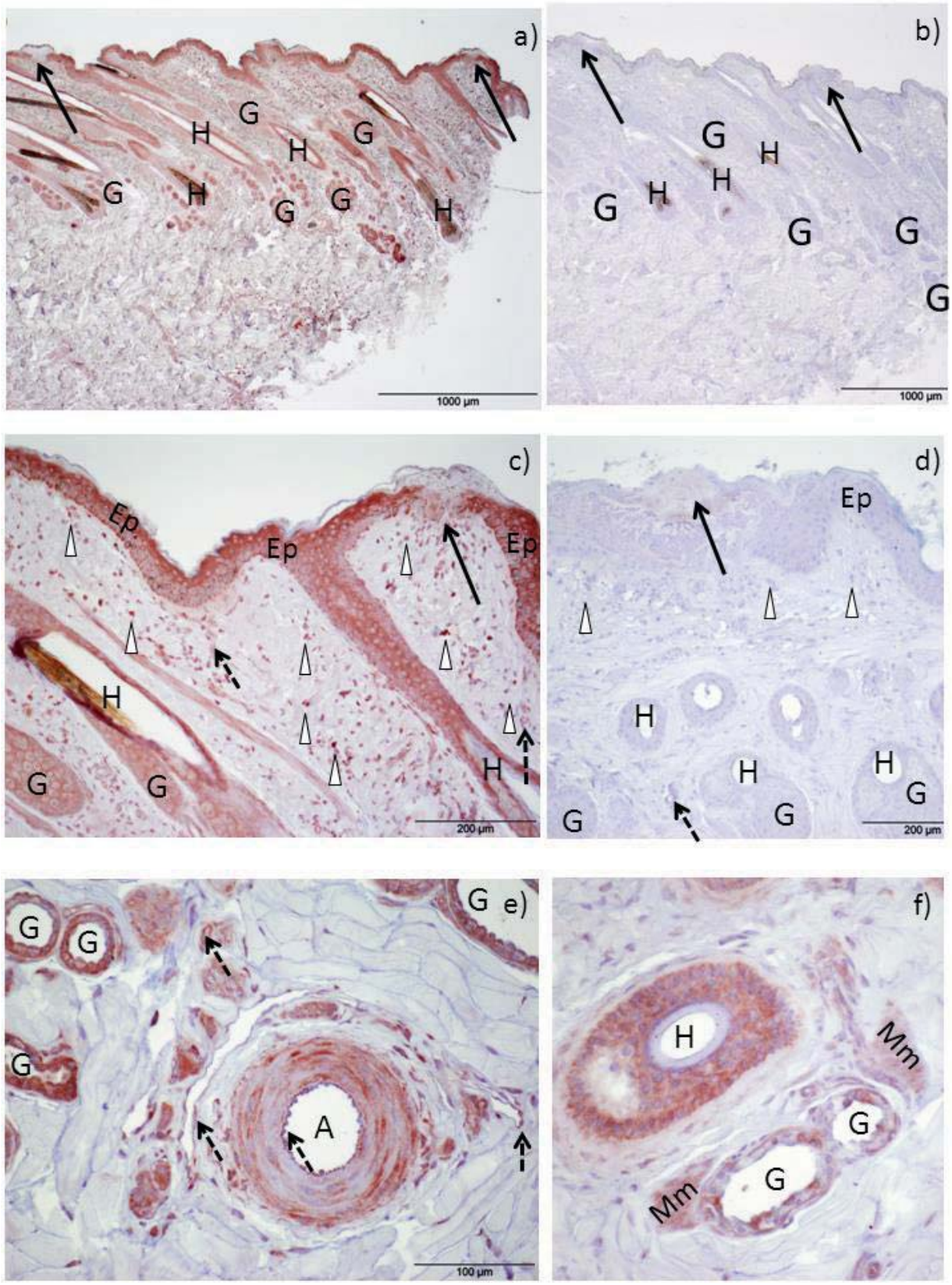
A corresponding section to c) did not show any reaction for IL-18. Symbols: stratified squamous epithelium (Ep) with an ulcer (arrow), hairs (cross section, H), sebaceous glands (G), endothelia (dashed arrows) and leukocyte infiltration (arrowheads);

e) Detail of *Dpap* with vessels and excretory ducts of apocrine glands (G)

Blood vessel walls and endothelia (dashed arrows) of a larger artery (A) and smaller vessels stained positive for IL-18 (dashed arrows) usually appeared with immunopositive *Lamina media* and endothelia (dashed arrows). Excretory ducts of apocrine glands (G) were positive for IL-18. Fibrocytes and extracellular matrix were usually negative.

f) Detail of adnexa in *Dpap*

The epithelial root sheath of a hair (H) and *Mm. arrectores pilorum* (Mm) always stained immunopositive for IL-18, while apocrine glands (G) stained positive, but with some patchy appearance. Fibrocytes and extracellular matrix were usually negative.



supplement 8

Supplement 9 Immunohistochemistry for Interleukin 18 (continued)

Immunostaining for IL-18 (red) in representative skin samples (counterstaining with haematoxylin).

a) *Dret* – detail with perivascular inflammation

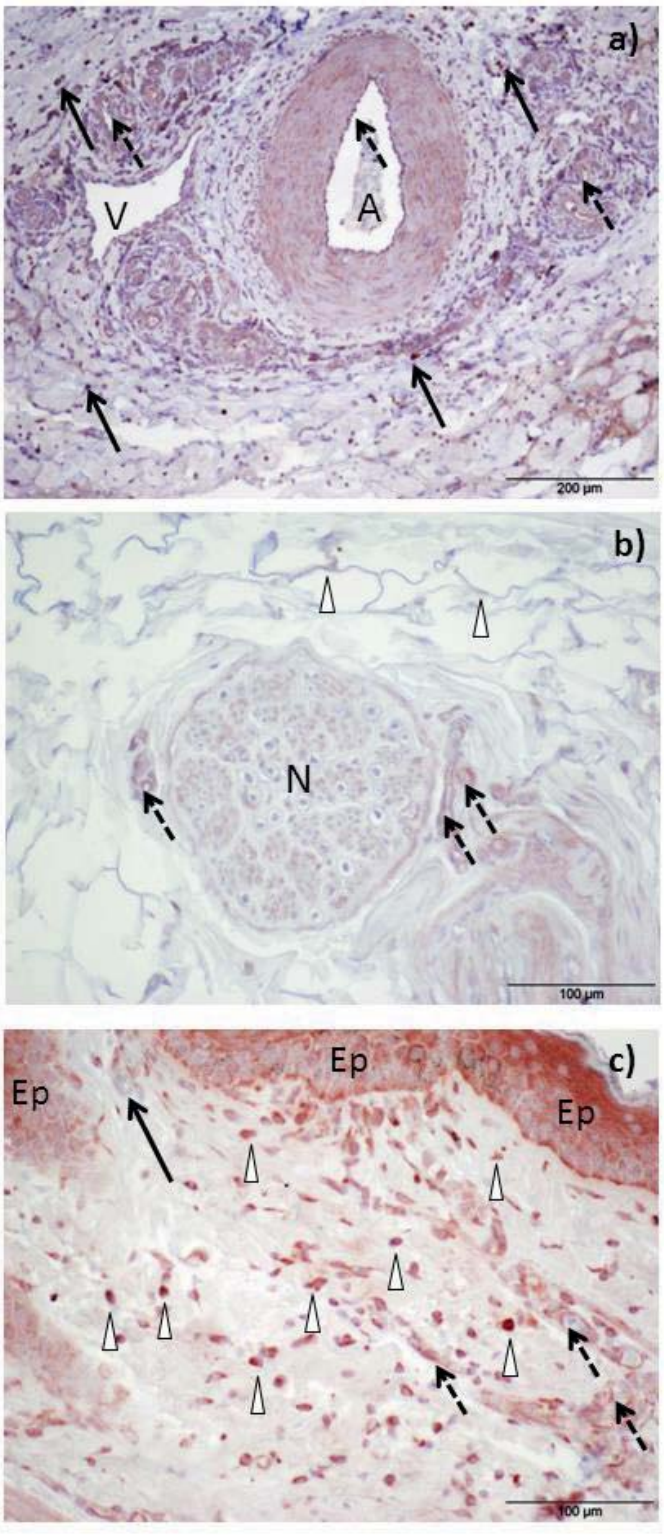
Leukocytic perivascular infiltration is visible proximate to artery (A) and vein (V). Vessel walls and endothelia (dashed arrows), as well as infiltrated cells (arrows) are positive for IL-18. Fibrocytes and extracellular matrix are negative apart from a weak background staining.

b) Cross-sectioned nerve (N) in subcutis

Most parts of the nerve (N) stained immunopositive. In addition, adjacent capillary endothelia were positive for IL-18 (dashed arrows).

c) Detail of infiltrated cells in *Dpap*

Infiltrating subepithelial immune cells were usually positive for IL-18 (arrowheads), as well as capillary endothelia (dashed arrows). Positive stratified squamous epithelium (Ep) and negative basis of an ulcer (arrow) are visible at the top edge of the detail.



supplement 9

Supplement 10 Staining characteristics of IL-12 and IL-18

As the results of IHC stainings for these two cytokines were very similar, the description of these will be presented together.

Stained tissues and cell types

The *strata granulosum, spinosum* and *basale* of the stratified squamous epithelium always stained cytoplasmically positive for IL-12 and IL-18. Melanocytes were difficult to evaluate due to the intensive dark brown colour of the melanin granules contained. Hairs were positive for IL-12 and IL-18 in the cortex and epithelial root sheath. Sebaceous glands were also positive for both cytokines, except for necrotic areas. Apocrine glands and their excretory ducts usually stained positive for IL-12 and IL-18 with some patchy appearances and a few exceptions of unstained glands. Myoepithelial cells around sweat glands always stained positive. Muscles (skeletal and smooth) were also always weakly immunostained (see Supplements 5a-c; 6a, b; 8a, c, e, f; 9c).

Fibrocytes in the connective tissue layers of the skin were either stained or unstained for IL-12 and IL-18; however, when stained, the immunoreaction was found in the cytoplasm. More positive fibrocytes were found after treatments than in healthy non-treated skin. Nevertheless, fibres and extracellular matrix were usually negative, but the appearance varied due to some background staining in more severely inflamed tissues. Plasma in vessels and intercellular fluid (oedema or exudate) was often found to be weakly stained (see Supplements 5a-c; 7a; 8a, c, e, f; 9a, c).

Adipocytes were found to be negative or positive for IL-12 and IL-18. Positive cells were usually associated with adjacent inflammation and there was often some uncertainty about the differentiation of adipocytes from inflammatory cells (data not shown).

Endothelia were usually positive for IL-12 and IL-18 with a few exceptions. Negative endothelia were found more often in control tissues of healthy non-treated equine skin, but hardly at all in any capillaries. Pericytes of capillaries were usually positive for IL-12 and IL-18. Vessel walls were generally positive for IL-12 and IL-18 in the *Tunica media* (see Supplements 6c; 7a, b; 8e, f; 9a, c). Nerves were positive for IL-12 and IL-18 in parts (see Supplements 7b; 9b).

Infiltrating immune cells were frequently positive for IL-12 and IL-18. A specifically strong immunoreaction was observed in macrophages and some lymphocytes (see Supplements 5b; 6a, c; 7c, d; 8c, e, f; 9a, c;).

Staining intensities varied between and within individual samples, especially in immune cells.

Isotype controls showed no staining at all, except for very pale background staining in one necrotic area of an epithelial ulcer (see Supplements 5d; 7c; 8b, d).

Supplement 11 Evaluation of shaving and scrubbing effects on skin irritation

Materials and methods:

Six additional clinically healthy horses (one mare, three geldings and two stallions) aged three to 24 years (mean 13.2 years) with a body weight of 520 kg to 663 kg (mean 579 kg) were included in the study. There was one ThB (English ThB) and five WBls (two Oldenburg WBls, two German WBls and one Standardbred). One horse was grey, four were brown and one was piebald.

These horses were euthanized in the course of an unrelated study on diagnostic imaging (animal experiment No. 33. 14-42502-04-13/1219) and were clipped and shaved 60 h and scrubbed 24 h before euthanasia, as described for preparation of i.d. injections (groups A – D), at one site on the neck. Immediately postmortem, skin biopsy specimens were taken from this site (treatment E, shaving samples) and from another site which was only shaved and briefly scrubbed immediately before sampling (control, healthy skin).

Biopsy specimens were treated and analysed as described for the samples of groups A – D.

Results

Macroscopically shaved and scrubbed skin was unchanged at the time of sampling.

Differences between treatment and control samples from the *Ep* were significantly different between treatments (Kruskal-Wallis test, $p = 0.0049$). Shaving and scrubbing resulted in ulceration of the *Ep*. Significantly higher grades (scores) of ulceration could be found in comparison to untreated controls (Wilcoxon signed-rank test, $p = 0.0313$), which showed no ulcers at all.

An increase of macrophages in the *Dpap* in a diffuse pattern was noticed after shaving only in comparison to untreated samples ($p = 0.0625$).

Higher scores in the *Dpap* and *Dret* were found for treatment and control sites in groups A – D than in shaved horses, without reaching statistical significance. There were more

neutrophils in the dermis (*Dpap* and *Dret*) of samples of A – D (independent of *ctrl* or *treat* sites) than in the samples of shaved horses (E).

Discussion and conclusion

Shaving and scrubbing alone induce ulcerative dermatitis. Effects in the dermis are usually limited to superficial layers. Treatment effects of i.d. injections in A – D are not likely to be hidden by shaving effects.

Shaving, if necessary, should be performed immediately prior to sampling to prevent iatrogenic ulcerative dermatitis and artefacts in samples.

Methods

Study Design

A prospective, randomised, double-blind study in 24 horses with internal controls was performed. Horses were allocated to four groups of six horses each (A – D) paired randomised by the criteria of colour, age and breed (Tables 3 and 4).

Animals

Twenty-four clinically healthy horses (six mares, 15 geldings and three stallions) aged between two and 21 years (mean 10.6 years) with a body weight of 425 – 680 kg (mean 572 kg) were included in the study. There were six ThB type horses (two Arabians, two Arabian-mix and two English ThBs) and 18 WBl type horses (nine Hanoverian WBls, one Hessian WBl, one Polish WBl, two Pura Raza Españolas (PRE), one PRE-mix, one Standardbred, one Oldenburg WBl, and one Westfalian WBl) (Table 4). No systemic and potentially immunomodifying treatments were administered in the two weeks preceding the experiments.

The animals were kept in stables of the Clinic for Horses, University of Veterinary Medicine, Hannover, Foundation, housed in standard single boxes on straw or wood shavings under a natural light-dark cycle of German summer (15 – 16.3 h of light, sunrise between 05:12 and 05:44, light from large windows or adjacent paddocks). The horses were fed hay and concentrates twice a day (at 07:00 and 18:00), according to their body weight, and had access to water ad libitum. Horses had access to an outdoor 20 x 60 m sand area once a day, in the late afternoon or early evening, with a minimum of 2 h rest prior to examination and sampling.

The horses were allowed to acclimatise for at least two days before starting the experiment and were trained by classical and operant conditioning for blood sampling to minimise stress during blood drawings. Horses stayed in the clinic for eight days (t-96 – t72). Examinations were performed and samples were acquired at the horses' home stables at day 11 (t264) after treatment (Figure 1).

Table 4 Treatment groups

Treatment group	Treatment	Mean age (years)	Mean weight (kg)	Mares (n)	Geldings (n)	Stallions (n)	WBI (n)	ThB (n)	Grey (n)	Non-grey (n)
A	SAINT-18	11.1	561	2	3	1	4	2	2	4
B	SAINT-18+ eqIL12 + eq IL18	10.8	589	2	3	1	5	1	2	4
C	SAINT-18+ eqIL12-ATG + eq IL18-ATG	10.1	548	0	5	1	4	2	2	4
D	SAINT-18+ eqIL12-ATG-CG + eq IL18-ATG-CG	11.3	591	2	4	0	5	1	2	4

Treatment

Four horses at a time were kept in the clinic and treated with the four different blinded treatments, A – D, in neutral tubes in a volume of 1 ml buffered with PBS.
Group A received the transfection reagent (SAINT-18, Synvolux Therapeutics B.V., Groningen, Netherlands; containing 6 nmol/ml 1-methyl-4-(cis-9-dioleyl) methyl-pyridinium-chloride) only.
Group B received SAINT-18 and linear DNA vectors (MIDGE-Th1, eqIL12 and IL-1beta receptor antagonist protein (ILRAP)-eqIL18) encoding equine IL-12 [NM_001082511.1; NM_001082516.1] and IL-18 [NM_001082512.1] fused to the coding sequence of the IL-1beta receptor antagonist protein (ILRAP) signal peptide.
Group C received MIDGE-Th1 vector of equine IL-18 with point mutations in all ATG contained (as potential start codons) (nonsense DNA; eqIL18-ATG). These vectors had 1.02 times the number of total CG sequences, thus, potential CG motifs, as those given in the dose of group B.
Group D received SAINT-18 and nonsense DNA, similar to group C, but with inverted CG motifs (eqIL18-ATG-CG).

Ethical statement

All procedures were carried out according to the ethical guidelines of the law on animal welfare (Tierschutzgesetz) approved by the “Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit” (LAVES) in the animal experiment No. 33.9-42502-04-11/0399. Informed consent was obtained from all animal owners.

Construction of DNA-vectors

The construction and synthesis of MIDGE was performed by MOLOGEN AG (Berlin, Germany) analogous to the method described by Schirmbeck et al. (2001). Briefly, the expression cassettes containing the cytomegalie virus (CMV) immediate-early enhancer/promoter, a chimeric intron, the gene sequence (eqIL12; eqIL18; eqIL18-ATG or eqIL18-ATG-CG) and the SV40 late protein polyadenylation site were inserted into the plasmid pMCV1.4. The coding sequence for the ILRAP signal peptide was fused to the eqIL18 coding sequence in all eqIL18 MIDGE vectors. 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 on each free end. One of them was, analogous to Schirmbeck et al. (2001), linked to a peptide nuclear localisation sequence (a peptide with the amino acid sequence PKKKRKVEDPYC), (Schirmbeck et al., 2001). Unbound DNA fragments were digested with T7 DNA polymerase (MBI Fermentas, Vilnius, Lithuania) followed by purification.

Preparation of SAINT-18 complexes with DNA or PBS

The transfection reagent SAINT-18 dissolved in water was vortexed for 1 min. SAINT-18 (0.5 ml) was mixed manually with 2x PBS (Biochrom AG, Berlin, Germany) or the DNA dissolved in 2x PBS and incubated at room temperature (rt) for approximately 10 min prior to injection, achieving a mixture of 1 ml in 1x PBS. MIDGE-Th1/SAINT-18 complexes in groups B, C and D were formed at a ratio of 1 mg DNA to 0.75 µmol SAINT-18 and the DNA concentration was 0.4 mg/ml.

Applications

Half the volume (0.5 ml) was injected into the pectoralis muscle of the horses and the other half (0.5 ml) i.d. into the skin of the middle third of the neck under local anaesthesia with 1 ml of lidocaine (Lidocainhydrochlorid 2 %, Bela-Pharm, Vechta, Germany) after aseptical preparation of the injection site with chlorhexidine (Hibiscrub, tk pharma trade, Hasbergen, Germany). The i.d. injection site (*treat*) was marked. These injections were set as time point 0 (t0). All examination and sampling times are announced in hours before or after treatment (t-48 – t264) (Figure 1).

At the time of treatment (t 0), the contralateral side of the neck was treated intradermally with 0.5 ml of PBS as an internal control of local effects (*ctrl*).

Clipping and shaving of i.d. injection sites was performed at t-36 to allow local irritation to resolve until injections (t0) and biopsy sampling (t24).

Twenty-four hours prior to treatment (t-24), the horses were injected with PBS in the same manner, but contralaterally (0.5 ml i.m. and 1 ml i.d. split into two injections of 0.5 ml each, injected at least 5 cm distant from each other and 20 cm from the injection sites of t0) (Figure 1).

Examinations

The horses underwent a general clinical examination four times a day between t-48 and t48 and twice a day during the rest of their stay.
The skin at the injection sites was also examined clinically at these time points and digital photographs were taken. Changes were described in the categories oedema, pain, heat, depigmentation and exudate by a non-linear scale (0 – 3 representing absent – high grade, respectively). Scores were summarised as the sums of all categories in a single clinical skin score.
Blood samples were drawn at 09:00, 15:00 and 21:00 from t-24 – t24 and at 09:00 and 21:00 from t-48 – -24 and from t24 – t72. One general examination and blood sampling was performed on each horse at t264.

Blood Sampling

Blood samples were drawn from the jugular vein after disinfection on the surface area with ethanol, by use of a vacutainer system (Vacuette, greiner bio-one, Frickenhausen, Germany) with a 20 G single-use cannula. Blood was collected in ethylenediaminetetraacetate (EDTA)-coated tubes for routine clinical laboratory measurements, in clot-activator-coated tubes for serum, in heparinised (sodium-heparin) tubes for the determination of SAA, and in PAXgene blood tubes (BD, Heidelberg, Germany) for the isolation of PBMC and for the conservation of total mRNA.

Serum Preparation

Blood was allowed to clot at rt for 2 h and was then centrifuged at 1430 x g for 6 min. Serum without clots was stored in aliquots at 4 °C for a maximum of 48 h until further analysis.

Routine clinical laboratory measurements

Analysis by routine clinical laboratory measurements included total plasma protein (TPP), haematocrit (Hct), WBC and differential haemograms measured by a manual refractometer (TPP), a Sysmex kx-21N (Sysmex Deutschland GmbH, Norderstedt, Germany) (Hct, WBC) and an ADVIA 120 (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) (haemograms), respectively. Due to the availability of laboratory devices, differential haemograms were obtained after storage of EDTA-blood at rt for a maximum of 36 h. Absolute leukocyte subset (neutrophils, lymphocytes, monocytes) counts were calculated from percentages of the ADVIA measurement and total WBC measured immediately after sampling by Sysmex kx-21N.

Serum Amyloid A determination

Serum amyloid A was determined by an immunoturbimetric latex agglutination test (LZ Test Eiken SAA, Eiken Chemical Co. Ltd., Tokyo, Japan). Limits of detection are 5 – 300 µg/ml. This assay for human SAA had been evaluated for horses (Jacobsen et al., 2006).

Isolation of peripheral blood mononuclear cells

The PBMC were isolated from heparinised blood by density gradient separation. Briefly, after sedimentation at rt for 1 h, leukocyte-rich plasma was layered onto LSM 1077 (density gradient solution, PAA, Pasching, Austria) and centrifuged (1000 x g, rt, for 30 min). Interphase PBMC were harvested and washed three times in PBS (500/250/150 x g, rt, 10 min). Cells were counted using the Cellometer Auto T4 cell counting system (Nexcelom Bioscience, Massachusetts, USA).

Cell culture

A total of 2×10^6 PBMC per well were seeded in sterile cell culture 12-well plates and incubated for 12 h in a humidified atmosphere at 37 °C (5 % CO_2 in air) in 1 ml culture medium [(Roswell Park Memorial Institute: RPMI, Biochrom AG, Berlin, Germany) supplemented with 10 % heat-inactivated FCS (PAA Laboratories GmbH, Pasching, Austria) and penicillin 100 U/ml, streptomycin 0.1 mg/ml (PAA)] (medium setting) supplemented with 1 µg/ml LPS (#L2755, Sigma-Aldrich, Munich, Germany) (LPS setting), or

in medium supplemented with PMA (50 ng/ml) and ionomycin (1.34 µM) (Cell stimulation cocktail 500x, eBioscience, Frankfurt, Germany) (PMA/ionomycin setting). Rather high concentrations of PMA/ionomycin were chosen after preliminary experiments (data not shown) to obtain positive controls of cytokine secretion for each individual.
After incubation, cell-free supernatants were obtained and stored at -80 °C until further analysis. Thawing was performed at 37 °C immediately prior to the cytokine determinations.
Medium settings were used to evaluate spontaneous cytokine release. Lipopolysaccharide settings were used to evaluate TLR4-response (Ahmad-Nejad et al., 2002) and PMA/ionomycin stimulation to assess cytokine induction by non-specific stimulation (Barten et al., 2001; He et al., 2010; Li et al., 2008) as positive controls.

Determination of equine cytokines in cell culture supernatants

Tumour necrosis factor alpha was measured in duplicates by a sandwich ELISA for equine TNFα (Duo Set DY 1814, RnD, Wiesbaden, Germany), performed according to the manufacturer's protocol with the exception that coating was carried out at 4 °C overnight, as described previously (Lavoie-Lamoureux et al., 2010). Absorption was measured using a Synergy 2 instrument (BioTek, Bad Friedrichshall, Germany) and data was analysed by Gene 5 1.11 software (BioTek). The lower and upper limits of detection of the assay were 31.2 – 2,000 pg/ml. Dilutions of standards and supernatant samples (if necessary due to exceeding upper detection limit) were made with PBS containing 1 % bovine serum albumin (BSA; # P3688, Sigma-Aldrich).
Two samples were excluded from further analysis as the differences measured between TNFα duplicates were excessive.
Interferon alpha, IFNγ, IL-4, IL-10 and IL-17 were measured using a bead-based multiplex assay based on equine-specific monoclonal antibodies on a Luminex 100 System (Luminex, Austin, TX, USA), as validated previously (Wagner and Freer, 2009). Limits of detection were as follows: IFNα (12 – 30,000 pg/ml), IFNγ (10 – 5,000 U/ml), IL-4 (40 – 80,000 pg/ml), IL-10 (15 – 35,000 pg/ml) and IL-17 (10 – 10,000 U/ml).

Examination of messenger ribonucleic acid (mRNA)

Preparation of mRNA from blood samples

Ribonucleic acid was gained from whole blood in PAXgene blood RNA tubes with the PAXgene blood RNA Kit (Qiagen GmbH, Hilden, Germany). Isolation was performed according to the manufacturer's instruction. The RNA concentration of the resulting solution was measured by automated electrophoresis (Experion System, Bio-Rad Laboratories GmbH, Munich, Germany) using the Experion RNA StdSens Starter Kit (Bio-Rad Laboratories GmbH), according to the manufacturer's instructions.

Preparation of messenger ribonucleic acid of skin samples

Punch biopsies of 4 mm diameter were transferred into 300 µl RLT buffer (Qiagen GmbH) and homogenised with TissueLyser II (30 Hz, four times for 5 min each, Qiagen GmbH). Subsequently, the RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen GmbH), according to the manufacturer's instructions. The resulting RNA solution was used for analysis by qPCR.

Transcription into cDNA

In order to perform the qPCR, 100 ng RNA diluted to a final volume of 10 µl with DNase/RNase-free water was transcribed into cDNA. SuperScriptII RT (Invitrogen, Karlsruhe, Germany) was used in combination with RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), according to the manufacturer's instructions.
The resulting RNA solutions were stored at -80 °C and the cDNA solutions at -20 °C.

Standard series for qPCR

Target specific primers were designed for conventional and qPCR using the National Centre for Biotechnology Information (NCBI) primer blast in observance of the Equus caballus genome, and were produced by Eurofins MWG Operon, Ebersberg (Table 5).
In order to perform the real-time PCR based on SYBR green, standard series were produced for absolute quantification of the cDNA copy numbers.
Ribonucleic acid was eluated from equine liver, uterus and PBMC by standard procedures using the RNeasy Plus Mini Kit (Qiagen GmbH). The RNA was transferred into cDNA, as described previously.

A conventional PCR was performed with target-specific primer and Taq DNA Polymerase (5 U/ µl; Invitrogen). The reaction mixture had a final volume of 20 µl, consisting of 2 µl of 10× reaction mix, 0.4 µl of ROTI-MIX PCR 3 (pH 7; Carl Roth GmbH, Karlsruhe, Germany), 0.6 µl of magnesium chloride (50 mM; Invitrogen), 0.2 µl Taq DNA Polymerase (5 U/µl; Invitrogen), 1.5 µl of forward and 1.5 µl of reverse primer (5 pmol/µl), 12.8 µl DNase/RNase-free water (Sigma-Aldrich, Steinheim, Germany), and 1 µl of the cDNA. All samples were incubated at 95 °C for 10 min followed by 40 cycles of 30 sec at 95 °C, 30 sec at 56 °C, and 45 sec at 72 °C. Finally, the mixture was incubated at 72 °C for 10 min and stored at -20 °C.

All samples were analysed on 2.5 % agarose gel with Gel Loading Dye Blue (0.08 µl/ml; New England Biolabs GmbH, Frankfurt am Main, Germany) by electrophoresis. The rather high agarose concentration was chosen due to best separation, as determined in preliminary experiments (data not shown). When the expected size was reached, the product was isolated from the gel band by the QIAEX II Gel Extraction Kit (Qiagen GmbH), according to the manufacturer's instructions.

One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) was transformed with the target sequences isolated from the gel bands using the pCR 2.1 TOPO TA Cloning Kit (Invitrogen). The *E. coli* solution was transferred to LB medium (32 g LB agar powder (Lennox; Carl Roth GmbH) in 1 l *Aqua tridest*) breeding plates containing ampicillin (Sigma-Aldrich), 5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid (Invitrogen) and Isopropyl-β-D-thiogalactopyranosid (Invitrogen). The *E. coli* were cultured for at least 24 h at 37 °C. Afterwards, colonies were transferred into 5 ml liquid LB medium (20 g LB agar (Lennox; Carl Roth GmbH) in 1 l A. tridest.) containing 50 µl ampicillin, and cultured for 16 h at 37 °C and 370 rpm. Subsequently, the plasmids were isolated from transformed *E. coli* by the PureLink Quick Plasmid Miniprep Kit (Invitrogen). The plasmids were sequenced by Sequence Laboratories Göttingen GmbH (Göttingen, Germany) to monitor the success of the transformation. Properly transformed plasmids were linearised using the restriction enzyme ScaI (Thermo-Scientific, Schwerte, Germany). The concentrations of the resulting solutions were measured by BioPhotometer (Eppendorf, Hamburg, Germany) and the copy number per µl was calculated. Finally, solutions with a known concentration of copy numbers were diluted and used as standard series in the qPCR.

qPCR

Real-time SYBR Green PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with MicroAmp Fast Optical 96-Well Reaction Plates, 0.1 ml (Applied Biosystems) and MicroAmp Optical Adhesive Film (Applied Biosystems). A real-time PCR reaction mixture was used with 12.5 µl SYBR green (Applied Biosystems), forward and reverse primers (Table 5) and the addition of DNase/RNase-free water to achieve a final volume of 25 µl. One µl of a sample's cDNA solution was added. In order to quantify the amount of copy numbers, the standard series produced were used for comparison (10^2 – 10^6 copies/µl). All measurements were performed in duplicate. The samples were denatured for 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C. A DNA melting curve analysis was carried out as a control of the production of a single PCR product.

Table 5 qPCR settings

Cytokine	Accession number	Amplicon size (bp)	Forward primer (5'->3')	Forward primer volume (µl)	Reverse primer (5'->3')	Reverse primer volume (µl)	Concentration of primers (pmol/µl)
IL12p35	NM_001082511.1	76	GCTGACAGCCATTGACAAGCT	1.5	TTCAAGGGAGGGCTTTTGTG	4.5	0.5
IL12p40	NM_001082516.1	76	TGCTGTTCACAAGCTCAAGTATGA	1.5	GGGTGGGTCTGGTTTGATGA	1.5	0.5
IL18	NM_001082512.1	124	TGCTGGACCAGTAGAAGACA	1.5	AGGTTCAAGCCTGCCAAAGT	1.5	5
IFNγ	NM_001081949.1	417	GCTGTGTGCGATTTTGGGTT	1.5	CTCAGGTTAGCTTTGGGCGA	4.5	5
CXCL10	NM_001114940.1	153	GACTCTGAGTGGAACTCAAGGAAT	1.5	GTGGCAATGATCTCAACACG	4.5	5

Skin sampling

Four skin samples were taken 24 h after treatment at a distance of 1 cm from each injection site under local anaesthesia after aseptic preparation of the skin. Skin biopsy samples were obtained using 8 mm (for histology) and 4 mm (for qPCR) diameter biopsy punches (Stiefel, Munich, Germany). Biopsy sites were closed aseptically by routine surgical closure.

Punch biopsies for qPCR were snap frozen in 1.5 ml RNAlater (Ambion, Carlsbad, USA) in liquid nitrogen and stored at -80 °C until further analysis.

Biopsies for histological examinations were fixed in 4 % neutral buffered formalin (Lillie and Fullmer, 1976) for 24 h and then cut into two halves perpendicular to the surface.

Subsequently, formalin-fixed specimens were rinsed in slowly running tap-water for 6 h. After this, specimens were blinded again and embedded in paraffin for storage at rt until further analysis.

Histology

Three-micrometre sections of the paraffin-embedded tissues were cut, mounted on glass slides and dried at 60 °C overnight.

H&E stainings were prepared of each specimen for a general histopathological examination. One half of each specimen was chosen by the criteria of lowest artefacts, presence of all layers of the skin and best plane of cutting (exact sagittal slices preferred) for further evaluation.

Evaluation

Histopathological alterations were assessed according to a semi-quantitative score of inflammation (Table 6) separately in each layer of the skin [epidermis (Ep); dermis, papillary layer (*Dpap*) including adnexa; dermis, reticular layer (*Dret*); subcutis]. Inflammatory cell patterns were documented and individual cell types were ranked (0 – 3) according to their proportion of the cell infiltrate.

Table 6 Histological scores

Score	Epithelium	Dermis
0	no alterations (physiological)	no leukocytes
1	one small ulcer / few suspect areas with subepithelial infliltration of leukocytes	few leukocytes, usually perivascular (physiological)
2	few ulcers	some leukocytes, usually perivascular mononuclear cells (physiological)
3	some ulcers	many leukocytes, countable
4	frequent ulcers, intact epithelium between ulcers	many leukocytes, not countable
5	many ulcers, no epithelium without alterations	many leukocytes, not countable, tissue structure masked

Descriptions of histological scores for evaluations of epithelium and dermal layers (papillary and reticular) in skin samples stained with H&E.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on slices of the chosen half of the formalin-fixed paraffin-embedded skin samples. Macrophages and neutrophilic granulocytes were detected by staining of calprotectin, which is expressed by these cells (Düvel et al., 2012). Equine IL-12 and IL-18 were detected by cross-reactive monoclonal antibodies (mAbs), as described previously (Schnabel et al., 2013).

Slices of 3 µm were cut and dried on salined glass slides (Histobond, Marienfeld, Lauda-Königshofen, Germany) at 60 °C overnight. The sections were deparaffinised in xylene and rehydrated in a series of alcohols of descending grades. Endogenous peroxidase was blocked in 0.6 % hydrogen peroxide in 80 % ethanol at rt for 30 min. Sections were rinsed three times in PBS at rt for 5 min each.

All the following incubation steps were performed in a moist chamber. Pretreatment was performed with MAC 387 for optimum results (Table 7). The sections were incubated with proteinase K (#P2308, 7.0 – 14.0 U/mg, Sigma Aldrich) diluted in PBS at rt for 30 min. Then, the sections were rinsed in distilled water twice at rt for 2 min and once in PBS at rt for 5 min. Sections were incubated with heat-inactivated normal goat serum diluted 1:5 in PBS (NGS), for 20 min at rt to block unspecific protein binding.

The NGS was decanted and the sections were covered with primary antibodies (Table 7) diluted in PBS with 1 % BSA and incubated at 4 °C overnight. Negative controls were incubated with PBS/BSA only. On the following day, sections were rinsed three times in PBS for 5 min at rt (negative controls were handled separately).

The sections were then incubated with biotinylated secondary antibodies diluted in PBS at rt for 45 min, followed by rinsing in PBS and signal amplification with avidin-biotin complex (vector laboratories, Burlingame, Canada), according to the manufacturer's instructions for interleukins, or a ready-to-use kit (IHC Kit, DCS Immunoline, Hamburg, Germany) was employed, according to the manufacturer's instructions, for calprotectin as a secondary antibody and signal amplification (Table 7).

After rinsing the sections three times in PBS for 5 min, visualisation was performed with the chromogen, 3-amino-9-ethylcarbazole (AEC; Peroxidase-Substrat-Kit AEC, Biologo, Kronshagen, Germany), which was applied according to the manufacturer's instructions. After incubation for 10 min at rt, the slides were rinsed in PBS for 5 min and in slowly running tap-water for 10 min.

Sections were counterstained in Delafield's haematoxylin for 2 sec and rinsed in running tap-water for 10 min to facilitate identification of specific tissue components.
Slides were mounted with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany) and coverslips and left to dry at rt.
Sections were viewed with a Zeiss Axioskop (Carl Zeiss Jena GmbH, Jena, Germany) and images were digitally captured using an Olympus DP Soft Camera (Olympus Deutschland GmbH, Hamburg, Germany).
Distinct red staining of cells was interpreted as a positive reaction of the primary antibodies given that negative controls showed no such staining.

Table 7 Protocols in immunohistochemistry

Target	Antibody	Clone, immunoglobulin subclass	Source	Catalogue no.	Pretreatment	Dilution Final concentration	Secondary antibody, dilution	Signal amplification	Tissue for positive control	Isotype control
Calprotectin	Macrophage / MAC 387	MAC 387, mouse IgG1	DCS Immunoline, Hamburg, Germany	MI657C01	proteinase K 5 µg/ml, in PBS, 30 min	1:400 0.1 µg/ml	IHC Kit MouseLink, HRP Label (DCS Immunoline) enhancer	IHC Kit MouseLink, HRP Label (DCS Immunoline) polymer	tonsil	mouse IgG1 (Dako, Hamburg, Germany)
IL-12	Mouse anti-bovine IL-12	CC301, mouse IgG2a	AbD Serotec, Puchheim, Germany	MCA1782	none	1:100 10 µg/ml	anti-mouse-biotin (vector laboratories, Burlingame, Canada) 1:200	vectastain Elite ABC Kit (vector laboratories)	colon	mouse IgG2a (AbD Serotec)
IL-18	Mouse anti-pig IL-18	5-C-5, mouse IgG1	AbD Serotec	MCA2094	none	1:1000 1 µg/ml	anti-mouse-biotin (vector laboratories) 1:200	vectastain Elite ABC Kit (vector laboratories)	colon	mouse IgG1 (Dako)

Evaluation of IHC-stained specimens

Cells stained positive for the respective targets in IHC were separately evaluated in the different layers of the skin (*Ep*, *Dpap*, *Dret*). Since only a few specimens contained sufficient amounts of subcutis, this layer was not assessed further.
Five fields of view (FOV, 0.1435 mm2) with a 20,000 x magnification were chosen randomly in *Dpap* and *Dret*, and positively stained cells were counted in images of these by means of the manual tag function of the Image Pro programme (Media Cybernetics, Inc., Rockville, MD, USA). Epithelia were evaluated descriptively.

Statistical analysis

Statistical analysis was performed with SAS Analytics Pro (SAS Institute Inc., Cary, NC, USA) version 9.3 or higher. P-values < 0.05 were considered significant.
Cytokine data were log-transformed due to log-normal distribution of the values for statistical comparisons. Stimulation ratios (times release) were calculated for each cytokine as cytokine $_{LPS}$/cytokine $_{medium}$ and cytokine $_{PMA/ionomycin}$/cytokine $_{medium}$.

Determination of baselines for systemic parameters

ANOVA was employed with time-of-day as a fixed factor and horses as random factors considering the interaction of time-of-day and horses for the analysis of the influence of sampling times on all systemic parameters measured and calculated before treatment.
For parameters influenced by sampling time, baselines were calculated separately for each sampling time (09:00; 15:00; 21:00; 03:00) as means of all values measured at the respective time before treatment. For parameters independent of sampling time (cytokines in PBMC supernatants), baselines were calculated as means over all measurements before treatment.
All measurements were evaluated in relation to individual baselines. This decision was confirmed by an analysis of the influence of baseline values on measurements after treatment in an ANOVA with respect to interactions of treatment and baseline, which showed significant influences of the baseline measurements.

Influence of treatment on systemic parameters

The influence of the treatments on systemic parameters in relation to calculated baseline measurements was analysed by uv and mv ANOVA over all measurements post-treatment. If influences were significant in both models, they were considered relevant. Questionable parameters of treatment effect, judged by graphic evaluation and ANOVA results, were analysed in detail for measurements 12 h and 24 h after treatment, SAA as a long-term indicator, for all sampling times. The influences of single treatments were analysed by paired t-tests of time of day matched baseline values compared to values 12 h and 24 h after treatments. Comparison of treatment effects of A – D for 12 h and 24 h post-treatment was performed by estimated pairwise group differences with corresponding p-values and 95 % confidence intervals derived by ANCOVA methods with baseline as covariate (t-test, baseline adjusted), not adjusted for multiplicity.

Influence of treatment on local parameters

The Wilcoxon signed-rank test was employed for clinical and histological scores to compare treatment and control sample for each treatment and parameter.
The Kruskal-Wallis test was used to detect overall differences between treatments employing differences between *treat* and *ctrl* site for individual horses and Wilcoxon signed-rank-test to analyse differences from 0 or comparisons of single treatments was employed.
Data were log-transformed for immunohistochemistry parameters, except for IL-12/IL-18 ratios, and ANOVA was performed with a Tukey post-hoc test to determine differences between treatments and controls and between different treatments.

Determination of responders

Parameters statistically significantly affected by treatments (differences to baseline, t-tests) were used to determine responders herein. Thresholds and response periods discriminating responders from non-responders were defined by graphic evaluation of courses of the measurements in each parameter. Horses were regarded as responders if their values exceeded the thresholds in at least three parameters. Responders and parameters with responses exceeding thresholds were counted for each group.

Influence of treatment on mRNA expression

The difference of copy numbers of mRNA in *treat* (in blood t12) and *ctrl* samples (in blood t0) was calculated for the analysis of mRNA expression in blood and skin samples. These differences were analysed by Wilcoxon signed-rank test.

Influences of the horse factors, age, sex, type and color

Horses of groups which had shown no difference by treatment on respective parameters were analysed together for these parameters. The influences of age were analysed by uv ANOVA. Sex, type and colour influences were analysed by unpaired t-tests.

Abbreviations

*The references of this manuscript are included in the references of the doctoral thesis (**Abbreviations**, pp. viii).*

Competing interests

DO and CJ are employees of Mologen AG. MOLOGEN AG owns a patent for the MIDGE-Th1 vector (PCT/DE02/03798P74). These affiliations do not alter the authors' adherence to the policies of Biomed Central Veterinary Research on sharing data and materials. BWi consulted and received funding from Mologen AG.

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Authors' contributions

CLS, HJS, KF and JMVC designed the concept of the study and created the manuscript. BWi, CJ and DO designed the treatment constructs, determined the application details and contributed to quantitative PCR assays. CLS, PS, MK, HME, SW, BWa, CP and AM contributed essentially to the development of analysis methods used herein. CLS and PS conducted the experiments of the present study and acquired the laboratory data. PJ performed the statistical analysis. All authors contributed to the critical revision and finalisation of the manuscript.

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References

*The references of this manuscript are included in the reference list of the doctoral thesis (**References**, pp. 193).*

5 General discussion

5.1 Finding suitable biomarkers

Studies in horses are often carried out with rather small numbers of individuals due to costs and requirements of husbandry. In particular when employing small numbers of such outbed animals, natural variances must be regarded carefully and distinguished from other effects. In this study, it was shown that high variances in immunological parameters and responses to treatments are often present in healthy horses. This kind of variation is paralleled by observations of variability of leukocyte properties in humans, rats and cattle (Böhmer et al., 2011; Schuberth et al., 2001; Webster et al., 1995) and of cytokines in humans and horses (Lembcke et al., 2012; Tarrant, 2010; Wong et al., 2008). A high individual variance among horses and variances between different laboratory assays hamper the establishment of fixed reference values and strongly argue for individual baselines, especially when using small numbers of individuals. Consequently individual baselines were used in the present study and enabled the author to notice responses by post-treatment differences to these baselines. Many effects would have been masked by variance when using absolute measurements only (manuscript III). This is in line with findings reviewed for establishment of reference values in human medicine (Geffré et al., 2009) and has been suggested for horses, wherever reliable reference intervals are missing (Ricketts, 1981).

5.1.1 Influencing factors

In addition to the overall high inter-individual variance, influencing factors could be identified for baselines (manuscript II) and for responses to treatments (manuscript III). Some influences corresponded before (baselines, manuscript II) and after (treatment effects, manuscript III) treatments (detail given in Annex, table 1). The same factor (e.g. sex) often influenced both, the baseline level of measurements and the extent of response within the same parameter (e.g. WBC).

In comparison to young individuals the older horses included showed lower baseline lymphocyte numbers, but larger cell sizes and responded to treatment with complexed

DNA with a less marked decrease in circulating lymphocytes. Granulocytes, on the other hand, were found in a higher baseline proportion of peripheral leukocytes in the older horses and in response to treatments they displayed the trend of lower amplitudes of increases without reaching statistical significance in comparison to younger horses. However, influences of age on cytokine release, found in baseline measurements for TNFα, IL-4 and IL-10, were not reflected in cytokine responses to the *in vivo* stimulus of application of complexed DNA.

Influence of sex on cytokines was demonstrated for spontaneous secretion of IL-10 before treatment, being lower in mares than in geldings without influencing the effect of *in vivo* DNA application on *ex vivo* IL-10 secretion of PBMC. However, after DNA treatment the TNFα response was higher in mares than in geldings. This matches the author's observation that TNFα secretion of (*in vivo*) untreated horses tends to be higher in mares than in geldings when their PBMC are stimulated with LPS *ex vivo* (data not shown). Thus, the more regulatory state of the immune system in geldings, indicated by the basic difference in IL-10 levels between mares and geldings, may become apparent in differences of pro-inflammatory cytokine release after *in vivo* or *ex vivo* stimulation of PBMC, represented here by TNFα.

Morphometric properties of leukocytes, identified as potential biomarkers in healthy horses (manuscript II) displayed no statistically significant effects of DNA treatment *in vivo*, although morphology of leukocytes was correlated with cytokine secretion in untreated horses (manuscript II). Cytokine secretion proved to be a biomarker which was indeed influenced by DNA treatment *in vivo*. The present results showed that morphology of leukocytes is correlated with cytokine release in general, but subtle immunological effects of *in vivo* manipulations may still be only detectable in either parameter.

The type of the horse (WBl / ThB) influenced cytokine stimulation ratios before treatments (baselines, manuscript II). IL-4 and IL-17 were more inducible in WBl than in ThB and the latter displayed lower IFNγ/IL-4 ratios. However, the increase in TNFα after treatments was the only parameter statistically significantly influenced by the type of horses, with higher increases in ThB than in WBl. This suggests that the extrapolation of findings in untreated horses of one type of horses onto that type's reaction to treatment should be seen with some reservation. Furthermore, the allocation of single breeds or horses to WBl or ThB is a limitation of this study as differences between breeds within

one type may be significant, while differences between the individuals allocated to either type may or may not. This allocation to two types might after all not be a criterion representing biological dichotomy.

Influences of colour were not statistically significant in untreated horses (data not shown), while responses to treatment by lymphocyte decrease and TNFα increase were attenuated in grey horses compared to non-grey horses. This impaired reactivity to treatment with complexed DNA in healthy horses is of particular interest for immunotherapy of equine melanoma, as discussed later.

5.2 Immunostimulation with DNA *in vivo*

5.2.1 General inflammatory response

In vivo application of DNA complexed with SAINT-18 resulted in immunostimulation in horses. The treated individuals displayed local and systemic signs of mild inflammation and a bias towards pro-inflammatory cytokines, as described in detail in manuscript III. Responses were usually mild with clinical examination results and haemograms staying within physiological ranges. Still, differences could be noticed with statistical significance, when the measured values were related to individual baselines again stressing their relevance. The low amplitude of alterations and the short duration of deviations from individual baselines indicate the quick compensation of immunostimulation by regulatory mechanisms in healthy horses. The indicators of the inflammatory response employed here (WBC, neutrophil and lymphocyte counts, SAA) are known to be short-time sensitive and return to baselines quickly (Jacobsen and Andersen, 2007; Reed et al., 2004). Returning to baselines can therefore be taken as a reliable sign of the resolution of systemic inflammatory states.

As analysed in locally treated skin samples, complexed DNA caused an immigration of mixed leukocyte subpopulations to the treated site. The leukocyte influx was also present in placebo-treated sites and may be in parts due to mechanical stimulation and the induced damage by high pressure within the tissue resulting from i.d. injections. The inflammatory response, estimated by the number of myeloid immune cells and cytokine expression, tended to be stronger in DNA-containing treatments compared to transfection reagent alone or PBS controls. This reveals immunostimulatory effects of com-

plexed DNA, observed locally in equine skin. Furthermore, the stronger local inflammation in response to systemic DNA effects (compared to treatment with SAINT-18 only) was also reflected in skin samples of PBS-treated control sites (at lower levels than in locally treated sites) of different treatment groups. These differences show a general inflammatory response to the treatment with or without DNA observable in tissue samples. Systemically applied complexed DNA caused higher reactivity to the local stimulus of trauma and injection, resulting in higher numbers of infiltrating immune cells and a higher expression of cytokines in control samples of horses systemically treated with complexed DNA. This matches reports of systemic and local inflammation [e.g. in the lungs of mice after *in vivo* administration of complexed DNA (Audouy et al., 2002; Dow et al., 1999b).

For skin samples the sampling time of 24h post-treatment was chosen to allow local transfection, expression and translation of transgene Interleukins (in group B) and primary immune reactions to the treatment. Furthermore, the author meant to avoid the trauma of biopsy sampling interfering with the short-term systemic observations. As distinct increases in lymphocytes and macrophages were noted in skin samples, secondary effects may have already occurred before biopsy sampling. First effectors of inflammation are neutrophilic granulocytes migrating quickly to tissues in response to local chemotactic substances produced by various resident cells, such as endothelia, fibrocytes, keratinocytes and dendritic cells (DC). DC process antigens and migrate to lymph nodes to present antigens to lymphoid cells (Tizard, 2013). Mononuclear cells, as frequently observed in skin samples in this study, are commonly thought to migrate to the site of stimulation in a secondary step (Tizard, 2013). Therefore it is possible that the local immune reaction to treatment in the present study (manuscript III) is quicker than expected or does not follow the classic cascade of immune cell migration as described. These two possibilities would explain the early presence of mononuclear cells at the injection sites.

In the study by Mählmann et al. (2015) local depigmentation was observed some weeks after (repeated) application. This effect and tumour remission in response to the applied complexed DNA was noticed after three weeks at the earliest. Although not completely comparable with the effects observed in healthy horses, this suggests that in addition to short-term responses local and systemic long-term effects in horses are likely induced by the applied complexed DNA-vectors.

The temporal course of local reactions to complexed DNA was not in the scope of the present study and has not been evaluated any further. As proven in experiments with rats (Endmann et al., 2014), MIDGE-Th1 vectors are detectable in skin long-term. The present results indicate that the identification of primary effects alone may be neither possible nor useful for elucidating the full mechanism of action. For these reasons, it seems desirable to further examine local effects of complexed DNA in future studies including the local temporal course of DNA effects.

5.2.2 Mechanism of immunostimulation by complexed DNA

Gene therapy with DNA vectors is usually believed to act via the expressed gene products of the introduced genes (Hunt et al., 2007). Beyond this, DNA has been identified to be immunostimulatory itself (Pisetsky, 1996). DNA dependent non-specific effects have here been shown to be induced by *in vivo* applied MIDGE-Th-1 vector DNA complexed with cationic transfection reagent (SAINT-18). Immunostimulatory effects were distinct in all treatment groups receiving complexed DNA (manuscript III).
Double stranded (ds) DNA is thought to stimulate three main pathways of the mammalian innate immune system, which are (1) CG-motif-dependent interaction with TLR-9 in endosomes, (2) stimulation of cyclic GMP-AMP synthase (cGAS) resulting in activation of stimulator of interferon genes (STING) and (3) inflammasome stimulation via AIM2 (Frese and Diamond, 2011; Unterholzner, 2013; Wu and Chen, 2014).

(1) Interaction of CpG-rich DNA containing stimulatory CG motifs with TLR-9 (usually after endocytosis to endosomes) leads to activation of nuclear factor kappa B ($NF_{\kappa}B$) and interferon regulatory factor (IRF) 7. This pathway is mediated through myeloid differentiation primary response gene (88) (MyD88), Interleukin-1 receptor-associated kinases (IRAK), TNF receptor-associated factor (TRAF) 3 and TRAF6 (Frese and Diamond, 2011; Hacker et al., 2000; Mutwiri et al., 2003).
(2) Independent of the DNA sequence, DNA molecules reaching the cytosol interact with several cytosolic proteins. Most of their downstream pathways have not been fully characterized yet. Interaction of dsDNA with cGAS leads to an increase in cyclic GMP-AMP (cGAMP) activating STING located at the endoplasmic reticulum (ER) (Mansur et al., 2014). STING activation leads to IRF3 and

IRF7 phosphorylation and $NF_{\kappa}B$ activation via TANK-binding kinase 1 (TBK1) (Wu and Chen, 2014).

(3) Cytosolic dsDNA further activates AIM2 via the adaptor protein ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD) and AIM2 promotes Caspase1 activation. Caspase1 is necessary for proteolytic cleavage of IL-1-family cytokines precursors into their (pro-inflammatory) active forms, e.g. IL-1β and IL-18 (Hornung et al., 2009; Wu and Chen, 2014).

The first two pathways via TLR-9 or cGAS and STING lead to expression of pro-inflammatory cytokines via $NF_{\kappa}B$ and of Type I IFNs via IRFs. The third pathway via AIM2 leads to inflammasome activation and pro-inflammatory cytokine secretion. In summary, all three pathways may lead to an inflammatory effect, but with a different spectrum of involved cytokines.

In horses TLR-9 and its effects have been described (Figueiredo et al., 2009; Leise et al., 2010; Waldschmidt et al., 2013; Zhang et al., 2008). Pathways for sensing of intracellular DNA and subsequent signalling have not been demonstrated so far. Since such innate immune mechanisms are highly conserved among mammalian species (Wu and Chen, 2014) it may be assumed that sufficiently homologous mechanisms also exist in horses.

In the present study the immunostimulation after *in vivo* application of complexed DNA appeared to be independent of recombinant expression or DNA CG content (manuscript III). DNA complexed with transfection reagents (as SAINT-18) more easily crosses cell membranes by the amphiphilic nature of the transfection reagent and should thus easily access the cytosolic compartment of target cells (Audouy et al., 2002; Tros de Ilarduya et al., 2010). Hence, it can reasonably be assumed that the complexed DNA applied here reached the cytosol and activated cytosolic receptors leading to inflammatory reactions via the pathways described above. Signs of inflammation could be noticed at the systemic and local level a short time after application. As different pathways lead to similar cytokine responses, especially beyond the single-cell level, the exact molecular mechanism(s) induced by the applied complexed MIDGE-Th1-DNA cannot be identified on the basis of the generated data. Further research elucidating this and determining the contribution of these immunostimulatory effects of DNA to the desired therapeutic effects is required.

5.2.3 Transgene expression

Transgene expression of MIDGE-Th1 vectors in general has been proven *in vitro* (Schirmbeck et al., 2001) and *in vivo* in equine skin with vectors encoding luciferase (Mählmann et al., 2015). For the vectors applied here encoding eqIL-12 and eqIL-18 (treatment group B, manuscript III), *in vitro* expression has been demonstrated in CHO cells (Mählmann et al., 2015). In the present study (manuscript III) indirect local signs of transgene expression were (1) the most distinct increase of myeloid cells in the *Dret*, (2) the highest number of IL-12 positive cells, and (3) the distinct increase of CXCL-10 expression in horses of group B treated with expressing vectors compared to other treatments. These findings indicate recombinant expression of (at least) bioactive IL-12. IL-12-induced IFNγ probably caused the induction of the downstream mediator CXCL-10 (Bukowski et al., 1999; Heinzerling et al., 2005; Tannenbaum et al., 1996) and of other chemotactic molecules promoting leukocyte migration (Tannenbaum et al., 1996).

Systemic signs of transgene expression were identified by the strongest inflammatory responses in horses treated with expressing DNA constructs. This included elevations of RT and rises of SAA (both statistically significant only in group B), highest mean increases of WBC and neutrophil numbers in group B and the most rapid decrease in lymphocyte numbers post-treatment. In addition to the general immunostimulatory effect of complexed DNA, as described above, the inflammatory responses may be this distinct in horses treated with expressing constructs due to pro-inflammatory pathways stimulated by transgene IL-12 and IL-18, as reviewed for different models and pathologies (Colombo and Trinchieri, 2002; Lebel-Binay et al., 2000; Trinchieri, 1995b).

In PBMC supernatants of horses receiving expressing vectors (group B), TNFα as the primary pro-inflammatory cytokine measured here, was not statistically significantly different from other treatment groups receiving non-expressing vectors. However, spontaneous IFNγ secretion was increased with statistical significance, which was not reached after the other treatments. IFNγ is known to be induced by IL-12 and IL-18 in horses (Heinzerling et al., 2005; McMonagle et al., 2001; O'Donovan et al., 2004; Tong et al., 2010) and indicates T_H1-biased immune responses (Aggarwal and Holmes, 1999; Wagner et al., 2010). It can reasonably be assumed that the expressing MIDGE-Th1-vectors induced this kind of bias in the horses treated.

5.2.4 Responder classification

Although statistical significance in some biomarkers indicated immune responses induced by complexed DNA, variances among data (internal controls and magnitudes) were usually high. For each parameter at least some horses were found not to respond to the treatments. Single parameters or combinations of these were not strictly predictive for values of other parameters, hampering simplification of the evaluation or reduction of the parameters measured. It is well known that individual immunological responses tend to vary widely (Marik and Zaloga, 2001; Pollmächer et al., 1996; Wray et al., 2013) and that responses of two similar parameters are not necessarily in line. This has for example been shown for the development of fever and increases in SAA, both parameters usually increased after surgery (Jacobsen et al., 2005).

To compare responses to DNA treatments, the author combined the identified biomarkers of systemic responses to develop a responder classification. Weighting of single parameters was not possible. This classification revealed individual responses to treatments more clearly for single horses than single parameters and was in agreement with these with respect to influencing factors. Such a classification may be useful in future studies, but must be validated in the very application for which it is to be used, especially if diseased horses are employed. Furthermore, it should be mentioned that thresholds and response periods were chosen after graphical evaluation. Should these be further used, it may be useful to re-examine the allocation into response or non-response in the intended setting.

In human medicine, individual responses have been known for a longer while and have led to the idea of individualizing therapies, especially immunotherapies (Koyanagi et al., 1997). This implies the adjustment of doses or application intervals to the intensity or duration of effects in an individual. To enable this, it must be evaluated which parameters are decisive for the effect and for the prognosis of treatment success in horses affected by the pathological condition in question.

5.2.5 Different response in grey and non-grey horses

Of the first examined influencing parameters (age, sex, type) none explained group differences, as the included horses had been allocated to the groups with respect to these parameters. Furthermore, none of these influencing factors explained the full variance of responses of any parameter. When influences on the responder classification within all DNA-treated horses were analysed, only the coat colour of the horses was predictive of classification. Grey horses were all classified as non-responders, i.e. showing responses exceeding the chosen thresholds in the chosen response periods in less than three out of six parameters. Differences of responses to DNA-containing treatments were statistically significant in TNFα secretion, lymphocyte decrease and myeloid cell infiltration of the *Dret*. Within these parameters grey horses showed fewer treatment effects, but overlapping ranges of response intensity of grey and non-grey horses were found. Summarizing these findings, grey horses may display an immunologic response to DNA treatment different from that in non-grey horses, which is most obvious, if different biomarkers are used in combination (see responder classification, manuscript III). Differences between grey and non-grey horses have been found in other parameters, including those related to immune functions (Comfort, 1958; Mayr et al., 1979; Pielberg et al., 2008). As development and progression of melanoma is thought to be associated with immune escape of tumour cells (Cavalleri et al., 2014; Heinzerling et al., 2001; Mählmann et al., 2015; Phillips and Lembcke, 2013), it is possible that impaired immune responses towards stimuli of the innate immune system (such as complexed DNA) may have underlying reasons, which also impair other mechanisms, e. g. involved in the immune reaction eliminating malignantly transformed cells. Thus, the milder immune response to DNA complexed with SAINT-18 found here in grey compared to non-grey horses and classification as non-responders may encourage further research elucidating the immunological details of melanoma development in grey horses, as well as treatment options.

Whether the immunologic response to complexed DNA applied *in vivo* is different in diseased horses cannot be answered based on the present data. This needs to be evaluated before employing time points and amplitudes of changes of healthy horses to design studies with diseased horses encountering immunostimulation by DNA application. Especially the adoption to immunotherapy of melanoma must be reviewed critically as melanoma patients display some dissimilarities to the horses examined in the pre-

sent study. Melanoma patients tend to be older than the present study population, as the incidence of melanoma is correlated with aging (Farcău et al., 2007; Fleury et al., 2000; M`Fadyean, 1933). Furthermore, grey horses displayed some immunological responses different from horses of other colours (manuscript III). Consequently, the latter cannot easily be used as *in vivo* models for grey horses. In addition, the presence of tumours in melanoma affected grey horses may alter the immunological response to immunostimulatory DNA as melanomas exhibit immunomodulating properties inhibiting curative immune responses towards tumour cells (Enk et al., 1997; Gajewski, 2007; Gajewski et al., 2006b). Both directions of variance in comparison to healthy horses are supposable (de Visser et al., 2006; Talmadge et al., 2007): On the one hand, it is possible that the immune response may be impaired by immune-escape mechanisms of tumours leading to local or general immunosuppression as reviewed for various malignancies (Gajewski et al., 2006a; Kiessling et al., 1999; von Bernstorff et al., 2001). On the other hand, it is probable that the notable immune response may be enhanced or prolonged by the presence of tumour antigens promoting acquired immune responses (Anichini et al., 2003; Halama et al., 2009; Heinzerling et al., 2005) after initial stimulation of innate immune mechanisms by the complexed DNA applied.

Comparing the present results with those of Mählmann and colleagues (2015) it can be noticed, that melanoma affected horses treated with complexed IL-12/-18 expressing DNA tended to develop stronger elevations of rectal temperatures than in the present study, while WBC stayed within physiological ranges, as in healthy horses here. As characterization of the innate immune response was not in the scope of Mählmann et al. , no more parameters can be compared to those measured in the present study.

Taken together and based on the present data, the resulting immunologic status after application of complexed DNA to melanoma-bearing grey horses cannot be predicted in detail. These considerations stress the need for evaluation of responses to immunostimulatory DNA and the influences on these responses in horses on the pathological condition in question, e. g. melanoma or hypersensitivities.

6 Conclusions

(1) DNA treatment with complexed MIDGE-Th1 vectors is short-term immunostimulatory in healthy horses independent of expression.

(2) The immunostimulatory effect was further independent of the CG motif content of the complexed DNA applied. It is thus likely to be exerted via pathways independent of TLR-9 and rather conducted via intracellular DNA receptors. To characterize the underlying immune mechanisms in detail further research is desirable, aiming at the improvement of the clinical application of DNA treatments e.g. for the therapy of equine melanoma.

(3) The immune responses of horses to treatment with DNA complexed with SAINT-18 vary. Responders and non-responders seem to exist. Grey horses seem to display milder responses to immunostimulatory DNA treatment than non-grey horses. Still, validation of these data and identification of responsible mechanisms requires further investigations.

(4) Probably, recombinant expression of IL-12 and IL-18 by MIDGE-Th1 vectors tends to enhance immunostimulation and to promote T_H-1 bias.

(5) To identify immune effects evaluation of assays for immune parameters as biomarkers in horses is necessary in the very application and setting used.

(6) Beyond the evaluation of the detecting assay, validation of the choice of parameters as immunological biomarkers for the condition and setting of interest is essential.

(7) For the interpretation of alterations observed influencing factors must be taken into account in study designs and the choice of horses.

(8) To ensure the best validity of comparisons internal controls are important with regard to high interindividual variance in immune homeostasis.

7 Summary

Schnabel, Christiane

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

To investigate the mechanisms exerted by complexed minimalistic immunologically defined gene expression (MIDGE-Th1) vectors applied *in vivo* in horses, suitable biomarkers were evaluated, influencing factors were considered and treatment effects were identified at the systemic and local level in 24 healthy individuals.

Fifteen monoclonal antibodies specific for cytokines of different species were evaluated for reactivity with their corresponding equine cytokines by Dot Blot, Western Blot, immunohistochemistry and flow cytometry. Of these, ten reacted with four different equine cytokines in at least one of the applications.

Combining mAbs evaluated previously and in the present study, a panel of assays to detect candidate cytokines was chosen to evaluate responses to DNA treatments in combination with clinical, haematologic, flow cytometric and histologic evaluations.

To accurately employ the selected parameters as biomarkers for treatment effects, internal controls were included and evaluated for influencing physiological factors. Internal controls of systemic parameters were pre-treatment baseline measurements. The high inter-individual variances observed endorse the acquisition of individual baseline measurements to normalize treatment effects to these. The analyses conducted revealed that the age of the horses influenced leukocyte composition, cell morphology and cytokine release by peripheral blood mononuclear cells (PBMC) *ex vivo* in healthy individuals. Older horses had lower percentages of lymphocytes and higher percentages of (neutrophilic) granulocytes compared to younger horses. The size of blood lymphocytes and granulocytes increased with age. PBMC of older horses spontaneously secreted more tumour necrosis factor (TNF) α, Interleukin (IL-) 4 and IL-10, but revealed lower relative inducibility of these cytokines after *in vitro* stimulation of PBMC by mitogens. Geldings had smaller monocytes and their PBMC showed a higher spontaneous *ex vivo* production of IL-10 when compared to mares. The stimulation to spontaneous release ratios of TNFα, IL-4 and IL-17 as well as the basal Interferon (IFN)γ/IL-4 ratio in Warmblood type

horses exceeded those detected in Thoroughbred types. Sampling time influenced leukocyte composition and cell morphology only to a small extent.

These findings were considered for the evaluation of DNA treatment effects. The horses had been allocated to treatment groups with primary respect to colour and age. Secondarily, distributions of sex and breeds were regarded. Changes post-treatment were normalized to internal controls, usually matched for the respective sampling time.

Based on the allocations to four groups of six animals each, the horses were treated by simultaneous intradermal and intramuscular application of MIDGE-Th1 vectors encoding equine IL-12/-18 complexed with transfection reagent or comparative substances (transfection reagent only, complexed nonsense DNA, complexed nonsense DNA without CpG) in a prospective double-blind study. As an internal control for local effects, a different site was treated with phosphate buffered saline intradermally. The clinical and cellular responses of the horses were monitored over 72h following application.

Although no severe side-effects occurred, compared to individual baselines horses of all groups receiving DNA containing treatment showed systemic inflammatory responses. In these horses rectal temperatures were elevated after treatments and Serum Amyloid A (SAA) increased. Total blood leukocyte and neutrophil counts increased, while lymphocyte numbers decreased. In PBMC spontaneous *ex vivo* secretion of TNFα and IFNγ increased after treatments with DNA, while IL-10 secretion decreased. In skin samples of intradermal injection sites DNA-treated horses had significantly higher myeloid immune cell numbers and *CXCL-10* expression compared to horses treated with transfection reagent only. Summarizing these findings, an inflammatory response to complexed linear DNA vectors at the systemic and local level was induced *in vivo*. This general inflammatory response appeared independent of expression and CG content of the applied DNA and is likely to have been mediateded via intracellular DNA receptors integrated in mammalian innate immune systems.

In horses treated with IL-12/18 expressing DNA however, the strongest systemic inflammatory effects were observed. These animals showed the highest elevations of RT, highest mean increases of WBC and neutrophil numbers, earliest decreases in lymphocytes and statistically significant rises of SAA. Moreover, immunohistochemistry of local skin samples revealed most IL-12 positive cells when compared to the other treatment groups, and local *CXCL-10* expression was highest. As a downstream mediator of the T_H1 cytokines IL-12 and IFNγ, *CXCL-10,* in combination with the most marked local and

systemic effects, indirectly points towards an *in vivo* recombinant expression of IL-12 in horses treated with expressing MIDGE-Th1 vectors.

Influences on treatment effects of the age, sex and breed of the included horses were in partial agreement with those found in untreated horses and did not affect the comparison of treatment groups, as these had been balanced for the influencing horse factors.

The potential influence of the coat colour of the horses had been taken into account for group allocation as well, although this factor did not significantly influence any parameter pre-treatment. Interestingly, grey horses showed significantly fewer effects of DNA-treatments on blood lymphocyte counts, *ex vivo* TNFα secretion by PBMC and myeloid cell infiltration in the reticular dermis compared to non-grey horses. Based on a weaker systemic response post-treatment, the included healthy grey horses were all classified non-responders to immunostimulatory effects of complexed DNA, although in a previous study melanoma-bearing grey horses had responded to DNA treatment with complexed expressing vectors by partial tumour remission. Whether the different immunological reactivity of grey horses compared to horses of other colours found here may contribute to the susceptibility of grey horses to melanoma remains to be elucidated.

Summarizing the present results, the pro-inflammatory effect of complexed DNA independent of expression and CG content was characterized at the systemic and local level. This immunostimulatory property may contribute to previously observed antitumoural effects of the treatment. Moreover, expression of recombinant equine IL-12 and IL-18 enhances immunostimulation and induces T_H1-biased downstream mediators, known to be antitumoural in other mammalian species.

However, further research on suitable parameters and exact mechanisms of DNA treatment in melanoma-bearing grey horses is desirable, as the characterization of healthy grey horses as non-responders with respect to non-specific inflammatory effects of complexed DNA and dissimilarities in the amplitude of effects compared to melanoma bearing horses hampers a simple transfer of the present results to mechanisms in antitumoural treatments. Nevertheless, the detection methods, influencing factors and biomarkers identified in healthy horses established in this study form a solid basis for supplementary research on equine immune responses to complexed DNA in clinical settings.

8 Zusammenfassung

Schnabel, Christiane

Akute Immunantwort gesunder Pferde auf Interleukin 12/18 kodierende lineare DNA-Vektoren formuliert mit SAINT-18

Ziel der vorliegenden Arbeit war es, die Wirkmechanismen *in vivo* applizierter mit SAINT-18 formulierter minimalistischer immunologisch definierter Genexpressions (MIDGE-Th1)-Vektoren (SAINT-18/DNA) zu charakterisieren. Dazu wurden zunächst potentielle Biomarker untersucht und mögliche Einflussfaktoren geprüft. Unter Berücksichtigung dieser wurden die Behandlungseffekte von SAINT-18/DNA auf systemischer und lokaler Ebene an gesunden Pferden identifiziert.

Fünfzehn monoklonale Antikörper gegen Zytokine verschiedener Spezies wurden auf Reaktivität mit equinen Zytokinen im Dot Blot, im Western Blot, in der Immunhistochemie und in der Durchflusszytometrie überprüft. Zehn der Antikörper waren (kreuz-) reaktiv mit vier verschiedenen equinen Zytokinen.

Aus diesen und weiteren verfügbaren validierten Nachweisverfahren für das Pferd wurde eine Kombination geeigneter Biomarker für immunologische Effekte der Behandlung mit SAINT-18 formulierten DNA-Vektoren ausgewählt. Diese wurden mit klinischen, hämatologischen, durchflusszytometrischen und histologischen Methoden kombiniert, um ein Gesamtbild der systemischen und lokalen Effekte der Behandlung zu zeichnen.

Zur Sicherung korrekter Vergleiche wurden interne Kontrollen jedes Individuums in die Studie eingeschlossen und Einflüsse der Eigenschaften der Pferde und der Methodik auf die Messungen der Biomarker untersucht. Als interne Kontrollen der systemischen Parameter dienten Baseline-Messungen vor der Behandlung der Pferde. Hohe interindividuelle Unterschiede in diesen Messungen sprechen für die Normalisierung von Behandlungseffekten auf individuelle Kontrollen.

Anhand der Baseline-Messungen vor Behandlung wurde festgestellt, dass das Alter der Pferde die Zusammensetzung der Blutleukozyten, ihre Morphologie und die Zytokinsekretion von peripheren mononukleären Blutzellen (PBMC) gesunder Pferde beeinflusst. Ältere Pferde zeigten u. a. signifikant geringere Anteile von Lymphozyten zugunsten

von (neutrophilen) Granulozyten im peripheren Blut, sowie größere Lymphozyten und Granulozyten. Auch stieg die spontane Sekretion von Tumornekrosefaktor (TNF)α, Interleukin (IL-) 4 und IL-10 aus PBMC signifikant mit dem Alter der Pferde, die *in vitro* Stimulierbarkeit dieser Zytokine durch Mitogene nahm hingegen ab.
Weiteren Einfluss auf systemische Biomarker der Immunreaktion hatte das Geschlecht der Pferde. Wallache wiesen signifikant kleinere Monozyten und eine höhere spontane Produktion von IL-10 durch PBMC auf als Stuten. Die Stimulationsrate (im Verhältnis zur Spontansekretion) wurde weiterhin vom (Rasse-)Typ der Pferde beeinflusst. Die Sekretion von TNFα, IL-4 und IL-17 aus PBMC von Warmblutpferden war *in vitro* stärker induzierbar als die aus PBMC von Vollblutpferden. Auch das basale Interferon γ / IL-4-Verhältnis ergab signifikant höhere Werte für Warmblut- als für Vollblutpferde.
Die Tageszeit der Probennahme beeinflusste nur geringgradig die Zusammensetzung der Leukozyten im peripheren Blut sowie deren Morphologie.
Die hier festgestellten physiologischen und methodischen Einflüsse auf die gemessenen Parameter wurden bei der Beurteilung der Behandlungseffekte nach Applikation SAINT-18-formulierter DNA berücksichtigt. Die Pferde waren gepaart randomisiert nach ihrer Fellfarbe und ihrem Alter als primären Kriterien und bei (sekundär) möglichst ausgewogener Verteilung von Geschlechtern und Typen in die Behandlungsgruppen eingeteilt. Veränderungen der Messwerte nach Behandlung wurden für systemische Messparameter auf die individuellen Baselines unter Berücksichtigung der Tageszeit relativiert.
Nach der vorgenommenen Einteilung in vier Gruppen à sechs Tiere wurden die Pferde gleichzeitig intramuskulär und intradermal mit MIDGE-Th1-Vektoren kodierend für IL-12 und IL-18 formuliert mit SAINT-18 oder Vergleichsmischungen (nur SAINT-18, SAINT-18 mit nicht-exprimierender MIDGE-Th1-DNA, SAINT-18 mit nicht-exprimierender MIDGE-Th1-DNA ohne CpG) verblindet behandelt. Zum internen Vergleich auf der lokalen Ebene wurde eine kontralaterale Stelle intradermal mit Phosphat gepufferter Kochsalzlösung behandelt. Die klinischen und zellulären Effekte wurden über 72 Stunden nach der Behandlung in länger werdenden Intervallen von sechs bis 24 Stunden untersucht.
Während keine schwerwiegenden Nebenwirkungen auftraten, wurden in allen Gruppen, die Behandlungen mit DNA erhielten, Behandlungseffekte im Vergleich zu den internen Kontrollen gemessen. Die Rektaltemperaturen dieser mit SAINT-18/DNA behandelten Pferde und Serum Amyloid A (SAA) in ihrem Blutserum waren erhöht. Die Gesamtleu-

kozyten- und Neutrophilenzahl im Blut stiegen signifikant an, während die Lymphozytenzahlen abfielen. Die spontane *ex vivo* Sekretion von TNFα und Interferon (IFN) γ durch PBMC der Pferde war nach *in vivo* Behandlung höher als zu den Zeitpunkten der Baseline-Messungen, während die IL-10-Sekretion nach Behandlung mit SAINT-18/DNA sank. Mit SAINT-18/DNA behandelte Pferde wiesen signifikant höhere Anzahlen myeloider Zellen und eine höhere *CXCL-10*-Expression in Hautproben der intradermal behandelten Stellen auf, als Pferde, die nur SAINT-18 erhalten hatten.

Insgesamt zeigen diese Ergebnisse, dass SAINT-18 formulierte MIDGE-Th1-DNA *in vivo* eine Entzündungsreaktion auf systemischer und lokaler Ebene hervorrief. Diese allgemeine Entzündungsreaktion stellte sich von Expression und CG-Gehalt der Vektoren unabhängig dar und wird vermutlich über intrazelluläre DNA-Rezeptoren vermittelt, die hochkonservierte Bestandteile in Säugerimmunsystemen sind.

Pferde, die IL-12/18-exprimierende SAINT-18 formulierte MIDGE-Th1-Vektoren erhalten hatten, zeigten die stärksten systemischen Entzündungsreaktionen, die sich in den Anstiegen der Rektaltemperaturen, Gesamtleukozyten- und Neutrophilenzahlen und den frühesten Abfällen der Lymphozytenzahlen im Blut äußerten. Weiterhin waren die deutlichsten und nur in dieser Gruppe statistisch signifikanten Anstiege von SAA zu verzeichnen. Lokal behandelte Hautproben dieser Pferde enthielten mehr IL-12 positive Zellen als Proben anderer Behandlungsgruppen. Des Weiteren war die *CXCL-10*-Expression signifikant gegenüber allen anderen Gruppen erhöht. Als nachgeschaltetes Signalmolekül zu den T_H1-Zytokinen IL-12 und IFNγ deutet das lokal signifikant erhöhte *CXCL-10* in Kombination mit der Deutlichkeit der allgemeinen Entzündungsreaktion in dieser Behandlungsgruppe auf eine rekombinante Expression von IL-12 *in vivo* durch SAINT-18 formulierte MIDGE-Th1-Vektoren in der vorliegenden Studie hin.

Einflüsse von Alter, Geschlecht und Typ der verwendeten Pferde auf die Behandlungseffekte korrelierten überwiegend mit denen vor der Behandlung und beeinflussten die Vergleichbarkeit der Behandlungsgruppen nicht entscheidend, da diese nach dem Alter der Tiere und unter Berücksichtigung von Typ und Geschlecht randomisiert eingeteilt worden waren.

Der Einfluss der Fellfarbe war für die Gruppeneinteilung ebenfalls berücksichtigt worden, obwohl dieser Faktor vor der Behandlung keinen Messparameter signifikant beeinflusst hatte. Schimmel zeigten allerdings nach der Behandlung mit SAINT-18/DNA einen signifikant geringeren Abfall der Lymphozytenzahlen. Ebenso waren die *ex vivo*

TNFα-Sekretion von PBMC und die Infiltration myeloider Zellen in der tiefen Dermis im Vergleich zu Pferden anderer Farben vermindert. Aufgrund der deutlich schwächeren systemischen Immunantwort wurden diese Schimmel alle als Non-Responder auf SAINT-18/DNA eingestuft, obwohl Schimmel mit Melanomen in einer früheren Studie auf die Behandlung mit SAINT-18 formulierten MIDGE-Th1 kodierend für IL-12/18 mit partieller Tumorremission reagiert hatten. Ob die hier identifizierte unterschiedliche immunologische Reaktivität von Schimmeln im Vergleich zu Pferden anderer Farben zum erhöhten Risiko von Schimmeln beiträgt, Melanome zu entwickeln, sollte in weiteren Studien geklärt werden.

Zusammenfassend wurde in der vorliegenden Arbeit die pro-inflammatorische Wirkung SAINT-18 formulierter MIDGE-Th1-DNA gezeigt, die unabhängig von der Expression der Vektoren und von deren CG-Gehalt auf systemischer und lokaler Ebene dargestellt werden konnte. Diese immunstimulatorische Eigenschaft könnte zu der antitumoralen Wirkung beitragen, die in einer vorherigen Arbeit demonstriert wurde.

Des Weiteren wurde gezeigt, dass die Expression von rekombinantem IL-12 und IL-18 die Immunstimulation signifikant verstärkt und T_H1-Zytokine und Chemokine induziert, deren antitumorale Eigenschaften in anderen Säugern demonstriert wurden.

Weitere Forschung ist wünschenswert, die für an Melanomen erkrankte Schimmel adäquate Biomarker charakterisiert. Die hier festgestellten Unterschiede zwischen Schimmeln und Pferden anderer Farben, die Einstufung von Schimmeln als Non-Responder, sowie Anzeichen für eine schwächere Immunreaktion der gesunden Pferde in der vorliegenden Arbeit im Vergleich zur vorherigen Arbeit an Melanom tragenden Schimmeln verhindern die einfache Übertragung der Erkenntnisse. Die Ursachen der unterschiedlichen Reaktion gesunder Pferde auf SAINT-18/IL-12/18-DNA bedürfen einer genaueren Analyse, wie auch die Mechanismen, welche den antitumoralen Effekt der Behandlung gegen Melanome bei Schimmeln vermitteln.

Die hier entwickelten Nachweismöglichkeiten, Beeinflussungen der Biomarker und die identifizierten Wirkmechanismen in gesunden Pferden können hilfreiche Ansatzstellen für die Identifizierung des Wirkmechanismus in erkrankten Tieren und die Weiterentwicklung der Therapie bieten.

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10 Annex

Supplemental Table 1

influence parameter	**treatment effect**	colour BL	*colour treat*	age BL	*age treat*	sex BL	*sex treat*	type BL	*type treat*
SAA	**?**	n.d.		n.d.	+	n.d.		n.d.	
blood counts									
WBC	**+**			+		+	+		
neutrophils	**+**			*		+	+		
lymphocytes	**+**		+	*	+				
monocytes				*				*?	
Morphology (blood cells)									
granulocytes			*n.d.*	+	*n.d.*		*n.d.*		*n.d.*
lymphocytes			*n.d.*	+	*n.d.*		*n.d.*	+	*n.d.*
monocytes			*n.d.*	+	*n.d.*	+	*n.d.*		*n.d.*
PBMC supernatants									
TNFα spontaneous	**?**			+			+		+
TNFα LPS	**+**		+	+		+			
TNFα PMA/Iono				+					
TNFα stim ratio PMA/Iono				+				+	
IL-4 spontaneous				+					
IL-4 stim ratio PMA/Iono				+				+	
IFNγ spontaneous	**?**			?					
IFNγ LPS	**+**			+					
IFNγ PMA/Iono				?					
IL-10 spontaneous	**?**			+		+			
IL-10 LPS	**?**			+					
IL-10 PMA/Iono	**?**								
IL-17 stim ratio PMA/Iono				?				+	

Table 1 Presence of influences on systemic parameters and treatment effects

treatment effect: effect of treatment with DNA complexed with SAINT-18 *in vivo;*

+ influence observed with statistical significance; ? influence observed as a trend;

* influence observed in percentages, but not in absolute numbers; n.d. not determined;

BL: Prae-treatment, influences on levels / measured values within baselines;

treat: Post-treatment, influences on amplitudes of effects of complexed DNA;

Morphology summarizes FCS and SSC determined by flow cytometry for each cell subset.

Of the detected cytokines, only settings with relevant influences are presented in this table. spontaneous: medium setting; LPS: lipopolysaccharide setting; PMA/Iono: PMA and ionomycin setting;

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