1 Introduction

1.1 Equine melanoma

1.1.1 Overview

Melanoma is a common, spontaneously occurring, usually progressive neoplastic skin disease with high prevalence in aging grey horses (Cavalleri et al., 2014; Cotchin, 1977; Jeglum, 1999; M`Fadyean, 1933; Valentine, 2006).
Genetic predisposition linked to the grey phenotype (genetically determined by a 4.6-kb duplication in intron 6 of syntaxin 17) evidently increases melanoma incidence, along with other mutations (e.g. a loss-of-function mutation in agouti signalling protein), (Pielberg et al., 2008). However, to date the underlying mechanisms of melanoma development in individual horses has not been completely understood (Cavalleri et al., 2014; Phillips et al., 2012; Pielberg et al., 2008).
Therapy of melanoma is usually not sufficient for complete remission of all tumours present in the affected horse. Most commonly, either no therapy is conducted or surgical excision is performed depending on localization and dimension of the tumour (Jeglum, 1999; Moore et al., 2013). Surgical therapy is usually limited to local treatment of early stage melanomas without eliminating metastases. Intra- and peritumoural chemotherapy with cisplatin is used experimentally with variable outcome and is usually limited to local treatment as well (Hewes and Sullins, 2006; Spugnini et al., 2011; Théon et al., 2007).

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1.1.2 Immunotherapy

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

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

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

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1.2 Immune effects of DNA

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

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

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

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

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

1.3 Immunological biomarkers in horses

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

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

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1.4 Aims

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

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2 Manuscript I

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

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Abstract

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

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

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

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No reactivity was shown for the remaining five antibodies in DB, although cross-reactivity of two of the antibodies was described previously.
The results obtained in this study can provide beneficial information for choosing of antibodies for immunological tests on equine cytokines.

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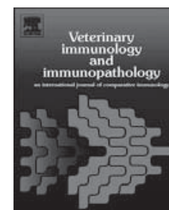

Research paper

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

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ABSTRACT

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

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

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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,

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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		

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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

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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).

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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

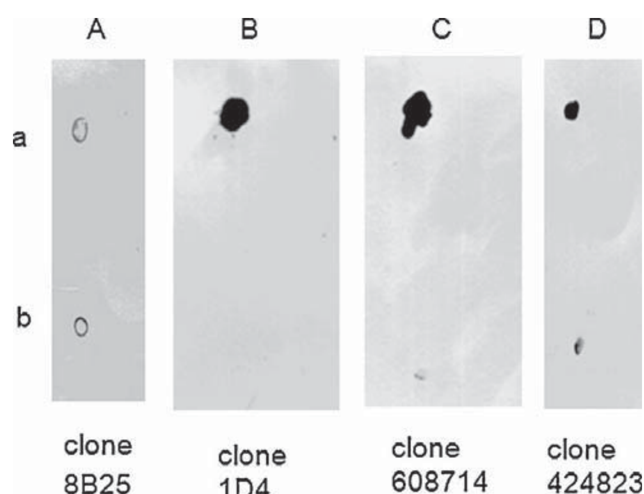

Fig. 1. Dot Blots prepared with mAbs detecting equine IL-1β: (a) primary Ab (pAb) applied as positive control; (b) tested equine rIL-1β produced in *E. coli*. Reactivity was detected for clones 8B25, 608714 and 424823.

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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

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