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Pathogenetical factors contributing to high IgA levels and marked neutrophilic pleocytosis in canine Steroid-responsive Meningitis-Arteritis

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List of abbreviations

bp	base pairs
°C	degree celcius
CBC	complete blood count
CD	Cluster of Differentiation
cDNA	complementary DNA
CNS	central nervous system
CO_2	carbon dioxide
ConA	concanavalin A
COX	cyclooxygenase
CSF	cerebrospinal fluid
CSF WBC	cerebrospinal fluid white blood cell
CT	computed tomography
DNA	desoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EF	elongation factor
ELISA	enzyme-linked immunosorbent assay
Fig.	figure
g	gravity
GAPDH	glyceraldehyd-3-phosphate dehydrogenase
GRO-α	growth-related gene product alpha
h	hour
HUVEC	human umbilical vascular endothelial cell

i.e.	that is
Ig	immunoglobulin
IFN	interferon
IL	interleukin
kg	kilogram
KS	Kawasaki Syndrome
mAb	monoclonal antibody
mg	milligram
µg	microgram
µl	microliter
min	minutes
ml	milliliter
MNC	mononuclear cell
mM	millimolar
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
ng	nanogram
nM	nanomolar
NTC	no template control
OD	optical density
PB	peripheral blood
PBMNC	peripheral blood mononuclear cell
PBPMN	peripheral blood polymorphonuclear cell

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear cell
PNS	peripheral nervous system
r_{Spear}	Spearman´s rank correlation coefficient
RNA	ribonucleic acid
RPE	R-phycoerythrine
SID	once per day
SRMA	Steroid-responsive Meningitis-Arteritis
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TIMP	tissue inhibitor of metalloproteinase
U	enzymatic activity
WBC	white blood cell

Chapter 1: General Introduction

Canine *Steroid-responsive Meningitis-Arteritis* (SRMA) is known under various terms, all reflecting certain characteristics of the disorder. These names include *Beagle Pain Syndrome* (Hayes et al., 1989), *Canine Pain Syndrome* (Burns et al., 1991), *Necrotizing Vasculitis* (Brooks, 1984), *Polyarteritis* (Harcourt, 1978), *Canine Juvenile Polyarteritis Syndrome* (Felsburg et al., 1992), *Aseptic Suppurative Meningitis* (Presthus, 1991) and *Corticosteroid-responsive Meningomyelitis* (Irving and Chrisman, 1990). Results of post mortem examinations strongly suggest that these descriptions all refer to the identical disease entity of SRMA.

Steroid-responsive Meningitis-Arteritis is one of the most frequently diagnosed inflammations of the central nervous system (CNS) of dogs and was reported to account for 15 % of these cases (Tipold, 1995). In this species SRMA is known to be the most common type of meningitis (Meric, 1988). In another study SRMA was found to be the cause for fever in 11 % of dogs that had been referred for diagnostic work-up of this clinical sign (Battersby et al., 2006).

Juvenile to young adult dogs are most frequently affected with ages at the time of diagnosis ranging from 2 months (Russo et al., 1983) to 9 years (Cizinauskas et al., 2000).

Individuals from all breeds and cross-breed dogs seem to be susceptible, however, breed predispositions have been identified for Boxers (Behr and Cauzinille, 2006; Tipold and Jaggy, 1994), Bernese Mountain Dogs (Cizinauskas et al., 2000; Presthus, 1991; Tipold and Jaggy, 1994) and Beagles (Joshua and Ishmael, 1968), of which many are kept as laboratory animals

(Brooks, 1984; Felsburg et al., 1992; Harcourt, 1978; Hayes et al., 1989; Scott-Moncrieff et al., 1992). No sex predilection was found in any of these studies.

The natural course of SRMA is relapsing with a tendency to progressively deteriorate (Brooks, 1984). The classical acute and a chronic protracted form of SRMA have been described (Tipold and Jaggy, 1994). The latter may develop with inadequate treatment resulting in relapses (Tipold, 2000).

The acute form is characterized by fever and pain upon palpation and manipulation of the cervical region. No neurological deficits, suggesting involvement of the CNS parenchyma, are evident on a neurological examination. The chronic form, however, may be accompanied by deficits indicative for a spinal cord or multi-focal CNS lesion (Tipold and Jaggy, 1994). These lesions most frequently become evident as gait abnormalities (Tipold and Jaggy, 1994) that rarely progress to a state of plegia (Hoff and Vandevelde, 1981; Joshua and Ishmael, 1968). Additional neurological symptoms may include central vestibular signs (Behr and Cauzinille, 2006; Tipold and Jaggy, 1994), optic nerve lesions (Meric et al., 1985; Russo et al., 1983) and myoclonus (Tipold and Jaggy, 1994) or generalized tremor (Gandini et al., 2003).

A hematological examination shows a neutrophilic leukocytosis in the majority of patients (Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994). In some patients, especially in those that are presented for a relapse (Cizinauskas et al., 2000), the complete blood count (CBC) reveals a lymphopenia (Burns et al., 1991), in other patients, however, a lymphocytosis may be evident (Meric et al., 1986). Further hematological abnormalities may include monocytosis (Cizinauskas et al., 2000; Scott-Moncrieff et al., 1992), mild normocytic, normochromic anemia (Burns et al., 1991; Harcourt, 1978; Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994) and thrombocytosis (Hayes et al., 1989). The albumin concentration is decreased in

some dogs (Bathen-Noethen et al., 2008; Burns et al., 1991; Hayes et al., 1989; Lowrie et al., 2008; Scott-Moncrieff et al., 1992) and globulin-/α_2-globulin levels may be elevated (Behr and Cauzinille, 2006; Brooks, 1984; Cizinauskas et al., 2000; Hayes et al., 1989; Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994).

Findings in cerebrospinal fluid (CSF) of affected dogs depend on whether animals suffer from the acute or chronic form of SRMA. Changes in the acute form are characterized by a moderate to marked pleocytosis with predominance of non-degenerated neutrophils (Behr and Cauzinille, 2006; Harcourt, 1978; Tipold and Jaggy, 1994). The content in total protein is usually moderately to markedly elevated (Behr and Cauzinille, 2006; Tipold and Jaggy, 1994). Frank blood (Behr and Cauzinille, 2006; Irving and Chrisman, 1990) or xanthochromia (Bathen-Noethen et al., 2008; Meric et al., 1986) may be found in CSF of some dogs reflecting hemorrhage into the subarachnoidal space due to vasculitis-induced destruction of pial blood vessels. In dogs that have entered the chronic stage of SRMA (Tipold and Jaggy, 1994) or have received glucocorticosteroid treatment prior to presentation (Behr and Cauzinille, 2006; Poncelet and Balligand, 1993) a mild to moderate mononuclear to mixed pleocytosis is usually present. In most of these cases the protein content is normal to slightly elevated (Tipold and Jaggy, 1994).

A gross pathological examination reveals changes only in some individuals. These consist of varying degrees of hemorrhage, ranging from petechial (Snyder et al., 1995) to massive bleedings (Joshua and Ishmael, 1968; Vandevelde and Fankhauser, 1972). In addition, amyloid depositions in various organs (Snyder et al., 1995), enlarged lymph nodes (Scott-Moncrieff et al., 1992) and meningeal fibrosis resulting in obstructive hydrocephalus internus (Gerhardt et al., 1998; Tipold and Jaggy, 1994) may be evident.

Multi-focal mainly extra-parenchymal lesions are found in histopathological examinations (Burns et al., 1991). These consist of a peri- and panarteritis of small to medium-sized vessels (Brooks, 1984), characterized by progressive fibrinoid necrosis of the vessel (Meric et al., 1986) with infiltrations of macrophages, plasma cells, lymphocytes and variable numbers of neutrophils (Brooks, 1984; Tipold et al., 1995). These findings occur mainly within the spinal meninges, in which concurrent meningitis is present (Tipold and Jaggy, 1994). In some dogs identical vascular changes may also be detected in extra-mural coronary arteries, mediastinum, thyroid gland, thymus and different segments of the gastro-intestinal and uro-genital tract (Hayes et al., 1989; Scott-Moncrieff et al., 1992). In the chronic phase of SRMA fibrinoid enlargement of the vessel walls may cause compression of the surrounding nervous tissue, resulting in neurological deficits (Tipold and Jaggy, 1994; Tipold et al., 1995). These acute and chronic changes frequently occur concurrently (Snyder et al., 1995).
Similar vascular changes, mainly within the extra-mural coronary arteries, were found in many asymptomatic Beagles that had been included in various toxicological studies, either as control dogs or as recipients of tested agents (Hartman, 1987, 1989; Kemi et al., 1990; Ruben et al., 1989; Spencer and Greaves, 1987; Stejskal et al., 1982).

Diagnosis of the acute form of SRMA is straightforward in most cases whereas recognition of the chronic form may be difficult. Identification of SRMA is based on the combination of the following findings: pain upon cervical palpation, fever, marked neutrophilic leuko- and pleocytosis, immediate and sustained response to glucocorticosteroid treatment, exclusion of other disease processes that may cause similar symptoms. Concomitant elevation of immunoglobulin (Ig) A concentration in serum and CSF supports a diagnosis of SRMA (Cizinauskas et al., 2000; Tipold and Jaggy, 1994). Detection of increased serum levels of

acute phase proteins is helpful in supporting the diagnosis of SRMA (Bathen-Noethen et al., 2008; Lowrie et al., 2008).

Steroid-responsive Meningitis-Arteritis is treated with a long-term course of glucocorticosteroids (≥ 6 months), starting with 4 mg/kg body mass once daily, decreasing to 0.5 mg/kg every second day. While in mild cases treatment with non-steroidal anti-inflammatory drugs may be sufficient to achieve resolution of clinical signs, refractory courses of the disease may require additional administration of cytostatic agents. Decreasing the drug dosage should be attempted only when hematological and CSF analyses have yielded results within reference values. Concurrent administration of protective drugs to prevent damage of gastro-intestinal mucosal membranes is recommended (Tipold, 2000). Treatment is usually well tolerated (Cizinauskas et al., 2000; Tipold and Jaggy, 1994) and prognosis is favorable if appropriate therapy is initiated early in the course of SRMA (Behr and Cauzinille, 2006; Cizinauskas et al., 2000; Gandini et al., 2003; Meric et al., 1985; Poncelet and Balligand, 1993). Prognosis is considered guarded in older individuals, in which transition into the chronic form has occurred (Cizinauskas et al., 2000; Tipold and Jaggy, 1994).

The etiology and pathogenesis of SRMA are largely unknown. A dysregulation resulting in an overshooting immune reaction is thought to occur, the triggering event, however, is yet obscure. All attempts to identify an infectious agent have failed until now (Cizinauskas et al., 2000; Harcourt, 1978; Meric et al., 1985; Poncelet and Balligand, 1993; Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994) and no genetic factor promoting development of SRMA could be identified to date (Hayes et al., 1989; Meric et al., 1986; Poncelet and Balligand, 1993; Ruben et al., 1989; Scott-Moncrieff et al., 1992).

In a previous study our group demonstrated that the inflammatory response in the acute form of SRMA is associated with high proportions of B cells among lymphocytes of the peripheral blood and CSF. In addition, the CD4+:CD8α+ lymphocyte ratio is increased in comparison to controls, suggesting low numbers of circulating cytotoxic T cells in SRMA (Schwartz et al., 2008b). These findings in combination with an increased systemic and intrathecal IgA production (Cizinauskas et al., 2000; Tipold and Jaggy, 1994) indicate that a type 2 immune response with predominant involvement of Th2 cells occurs in SRMA.
We thus investigated mRNA expression of Th1 (interleukin (IL)-2, interferon (IFN)-γ) and Th2 signature cytokines (IL-4, -5, -10) in peripheral blood mononuclear cells (PBMNCs) and CSF white blood cells (CSF WBCs) by means of reverse-transcriptase real-time polymerase chain reaction (PCR) and compared values from dogs in the acute phase of SRMA with those under glucocorticosteroid treatment for SRMA and dogs suffering from other CNS inflammations and neoplasias.

Another intriguing finding in the classical form of SRMA is the invasion of enormous numbers of leukocytes, mainly neutrophils, into the subarachnoidal space (Behr and Cauzinille, 2006; Harcourt, 1978; Tipold and Jaggy, 1994). This attraction is at least partially due to an increased chemotactic activity for peripheral blood polymorphonuclear cells (PBPMNs) in the CSF of affected dogs (Burgener et al., 1998). Additional factors, however, are required to allow extravasation and migration of leukocytes into the CSF. These include β_2 integrin mediated leukocyte adhesion to and crawling along endothelial cells (Phillipson et al., 2006) and crossing of the basement membrane as one component of the blood-CSF-barrier (Ransohoff et al., 2003). Matrix metalloproteinases (MMPs)-2 and -9 were shown to

be potent substances that degrade this basement membrane in inflammations of the CNS (Mun-Bryce and Rosenberg, 1998; Paul et al., 1998).

We hypothesized that up-regulation of β_2 integrins occurs on PBPMNs in the acute form of SRMA and that this represents one mechanism that facilitates the development of marked neutrophilic pleocytosis. We therefore quantitated CD11a, b and c expression on these cells by immunophenotyping and subsequent flow cytometric measurement. In addition, we suspected that in the acute phase of SRMA leukocytic up-regulation of MMP-2 and -9 allows their migration into the CSF and that concomitant counter-regulation of their tissue inhibitors of metalloproteinases (TIMPs)-2 and -1 may occur. To investigate this assumption mRNA expression levels of the respective MMPs and TIMPs were determined in PBMNCs, PBPMNs and CSF WBCs by means of reverse-transcriptase real-time PCR.

In both studies results were compared to those from individuals under glucocorticosteroid treatment for SRMA and dogs with other inflammatory and neoplastic diseases of the CNS.

Chapter 2: Pathogenetical factors for excessive IgA production: Th2-dominated immune response in canine steroid-responsive Meningitis-Arteritis

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2.1 Abstract

Canine Steroid-responsive Meningitis-Arteritis (SRMA) is a systemic inflammatory disease with a predominant manifestation within the cervical meninges, increased immunoglobulin A (IgA) levels in serum and cerebrospinal fluid (CSF), and a shift of the B:T cell ratio towards a higher percentage of B cells. We therefore hypothesized that the inflammatory reaction in SRMA is associated with a Th2-dominated immune response.

Samples from dogs in the acute phase of SRMA (n = 16) and under glucocorticosteroid treatment for SRMA (n = 16) were investigated in comparison with other inflammatory (n = 9) and neoplastic disorders (n = 10) of the central nervous system. Pellets of peripheral blood mononuclear cells (PBMNCs) and CSF white blood cells (CSF WBCs) were studied for interleukin (IL)-2, interferon (IFN)-γ, IL-4, IL-5 and IL-10 mRNA expression by means of reverse-transcriptase real-time polymerase chain reaction (PCR). Values were normalized to the geometric mean of the internal reference genes glyceraldehyd-3-phosphate dehydrogenase (GAPDH), β-actin and elongation factor (EF)-1α.

PBMNCs of dogs in the acute phase of SRMA expressed low levels of Th1 response related cytokines (IL-2, IFN-γ) whereas IL-4, which is indicative for a Th2-dominated immune reaction, was up-regulated in comparison to the other diseases. Interleukin-5 and -10 levels were similar among all groups. The IL4:IL2 ratio was similar in PBMNCs and CSF WBCs in SRMA and IL-10 levels were increased in CSF WBCs when compared to PBMNCs.

These findings indicate that SRMA is associated with a Th2-dominated immune response with a pronounced production of IL-4 and the distribution of Th1 and Th2 subsets is similar in PBMNCs and CSF WBCs in this disease. The described type 2 immune reaction may be an

important pathogenetical factor for high systemic and intrathecal IgA production in the acute phase of SRMA and under glucocorticosteroid treatment.

Keywords: CNS immune response; Dog; humoral; IL-4; T helper 2 cells

Abbreviations: CBC = complete blood count; cDNA = complementary DNA; CSF = cerebrospinal fluid; CSF WBCs = cerebrospinal fluid white blood cells; CNS = central nervous system; EF = elongation factor; GAPDH = glyceraldehyd-3-phosphate dehydrogenase; IFN = interferon; IL = interleukin; PBMNCs = peripheral blood mononuclear cells; SRMA = Steroid-responsive Meningitis-Arteritis

2.2 Introduction

Steroid-responsive Meningitis-Arteritis (SRMA) is a systemic inflammatory disease with a predominant manifestation within the cervical meninges (Tipold and Jaggy, 1994). It is one of the most frequently diagnosed inflammatory disorders of the central nervous system (CNS) in dogs (Tipold, 1995) and accounts for the majority of canine meningitides (Meric, 1988). Typically, juvenile to young adult dogs are affected and the natural course of SRMA is relapsing with episodes of severe inflammation and symptom-free intervals in-between (Tipold and Jaggy, 1994).

Laboratory hallmarks consist of a marked neutrophilic leuko- and pleocytosis, which is, in the majority of patients, accompanied by a simultaneous elevation of immunoglobulin A (IgA) levels in serum and cerebrospinal fluid (CSF) (Tipold and Jaggy, 1994; Tipold et al., 1995).

To date, all attempts to identify an infectious agent have failed (Cizinauskas et al., 2000; Harcourt, 1978; Meric et al., 1985; Poncelet and Balligand, 1993; Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994) and the disease is suspected to result from a dysregulation of the immune system.

In a previous study we demonstrated that, in addition to increased IgA levels, a shift of the B:T cell ratio towards a high percentage of B cells is present in the peripheral blood of dogs with SRMA. This shift is even more pronounced in the CSF and a large proportion of B cells is positively correlated with high IgA levels. In addition, the CD4:CD8α ratio in the peripheral blood is increased in these dogs suggesting low numbers of circulating cytotoxic lymphocytes (Schwartz et al., 2008b).

Since the mid 1980´s it is known that the CD4+ T helper cell population is not homogenous regarding its cytokine profile and according to the Th1/Th2 paradigm may be classified as

either T helper 1 (Th1) or T helper 2 (Th2) cells. T helper 1 cells are mainly characterized by their signature cytokines interferon-γ (IFN-γ) and interleukin-2 (IL-2) whereas Th2 cells express IL-4, -5, and -10. T helper 2 cells support the humoral arm of the immune system by stimulation of B cells and their cytokines are crucial in the initiation of IgA production (Briere et al., 1994; Harriman et al., 1988; Murray et al., 1987). Both subsets promote the predominance of their own kind and suppress cells of the other sub-category. Therefore prolonged immune reactions may result in a polarized type 1 or type 2 response with a predominant involvement of the respective cytokines (Abbas et al., 1996; London et al., 1998; Mosmann and Sad, 1996; O'Garra, 1998; Romagnani, 1997).

We thus hypothesized that the inflammatory response in SRMA is associated with a type 2-skewed immune response and investigated the mRNA expression pattern of cytokines that are indicative for either a type 1 or type 2 immune response in peripheral blood mononuclear cells (PBMNCs) and cerebrospinal fluid white blood cells (CSF WBCs) of dogs in the acute phase of SRMA. These values were compared to those of dogs that were under glucocorticosteroid treatment for SRMA and dogs that suffered from other CNS inflammatory diseases and neoplasias.

2.3 Materials and Methods

2.3.1 Animals and Samples

Samples used for mRNA quantitation derived from dogs with SRMA and other CNS disorders (Table 1) and included pellets consisting of PBMNCs and CSF WBCs. Immunophenotyping of these lymphocytes had been carried out in a previous study (Schwartz et al., 2008b).

Table 1: Disease categories, number of dogs, inclusion criteria and age of included animals

Disease categories	Number of dogs	Inclusion criteria	Age[a] [years]
SRMA with symptoms	16	Dogs showing symptoms of SRMA at time of sampling	0.75 (0.5 – 1.5)
SRMA without symptoms	16	Dogs with SRMA that were under glucocorticosteroid treatment and did not show symptoms at time of sampling (≈ 1 mg/kg SID; follow-up examinations)	1.0 (0.5 – 5.0)
CNS inflammation	9[b]	Dogs with CNS inflammation[c] other than SRMA	7.75 (1.0 – 8.75)
CNS neoplasia	10	Dogs with CNS neoplasia[c] (no tumors of the hematopoetic system included)	9.0 (5.5 – 14.5)

SRMA = Steroid-responsive Meningitis-Arteritis; SID = once per day; [a] = given as mean and range; [b] = for one of these dogs no CSF WBC pellet was available; [c] = including the nerve roots; CNS = central nervous system

All dogs were client-owned and presented to the Department of Small Animal Medicine and Surgery, University of Veterinary Medicine, Hannover, Germany, for a diagnostic work-up of

neurological signs. Animals were ascribed to one of the following groups: “SRMA with symptoms”, “SRMA without symptoms”, “CNS inflammation”, and “CNS neoplasia”. Each patient was subject to a general and neurological examination, which was followed by a complete blood count (CBC), routine blood chemistry, and urinalysis. In addition, a suboccipital CSF tap and CSF analysis (WBC count, cytomorphological differentiation, and total protein) were performed in every dog. Samples containing erythrocytes were accepted only if xanthochromia, suggesting *in vivo* leakage of red blood cells into the subarachnoid space due to impaired vascular integrity, was present (Jamison and Lumsden, 1988). Immunoglobulin A (IgA) content in serum and CSF was determined in all animals using a previously described enzyme-linked immunosorbent assay (ELISA) to support the diagnosis of SRMA (Tipold et al., 1994). Ancillary tests performed to obtain a definitive diagnosis included radiographic studies, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), as well as electrodiagnostic testing. If available, results of tests for infectious agents and pathological examinations were considered to achieve the diagnosis. A possible history of glucocorticosteroid administration within one week prior to presentation (within 6 weeks if depot formulations were given) was noted for each patient.

Diagnosis of SRMA was based on the combination of the following findings: pain upon cervical palpation, fever, marked neutrophilic leuko- and pleocytosis, immediate and sustained response to glucocorticosteroid treatment, and exclusion of other disease processes that may cause similar symptoms. Concomitant elevation of IgA in serum and CSF supported the diagnosis (Tipold and Jaggy, 1994).

The groups “CNS inflammation” and “CNS neoplasia” included diagnoses of meningoencephalitis of unknown etiology (n = 2), granulomatous meningoencephalitis (n = 1), CNS neosporosis (n = 2), acute polyradiculoneuritis (n = 2), bacterial meningomyelitis (n

= 2), and intracranial meningioma (n = 5), pituitary macroadenoma (n = 1), malignant blastoma of the spine/spinal cord (n = 2), and malignant nerve sheath neoplasia (n = 2).

Isolation of PBMNCs was achieved by means of density gradient centrifugation with two subsequent washing steps (Schwartz et al., 2008b). Cerebrospinal fluid WBCs were spun down (200 x g; 10 min; room temperature) and the supernatant was removed. Cerebrospinal fluid WBC pellets therefore contained a mixture of leukocyte populations and were immediately frozen and stored at - 80°C until RNA isolation was performed.

2.3.2 RNA purification and reverse transcription

Purification of total RNA from cell pellets was performed using RNeasy Mini Kit columns (Qiagen) according to the manufacturer's protocol for animal cells. To prevent contamination with genomic DNA an on-column DNase digestion step with RNase-free DNase (Qiagen) following the manufacturer's instructions was included. The yield of RNA was subsequently determined with a GeneQuant pro device (Biochrom Ltd.) by means of spectrophotometry at OD_{260}. The remaining eluate was immediately placed in liquid nitrogen and then stored at - 80°C until reverse transcription was undertaken.

Reverse transcription of RNA into complementary DNA (cDNA) was performed with Omniscript RT (Qiagen), Random Primers (Promega), and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen). Forty microliters of master mix containing ≤ 2 µg of total RNA was produced according to Qiagen's protocol for reverse transcription. Incubation for 60 min at 37°C took place in a thermocycler (PTC-200 Peltier Thermal Cycler; MJ Research).Complementary DNA was stored at - 20°C in aliquots until its use in real-time PCR experiments.

2.3.3 Primers

Primers used in this study (Table 2 and 3) were either taken from the literature (as cited) or designed with Primer3 software (Rozen and Skaletsky, 2000) or Beacon Designer version 2.1 software (Premier Biosoft International). Primers were produced by Eurofins MWG Operon. The specificity of each PCR product was confirmed by DNA sequencing (AGOWA genomics).

Table 2: Primers used for production of standard dilution series

Target gene	Primer sequence in 5´- 3´ (forward primers on top, reverse primers on bottom)	Size of amplicon [bp]	GenBank accession number
IL-2	ACCTCAACTCCTGCCACAAT GCACTTCCTCCAGGTTTTTG	289	D30710
IFN-γ[a]	GCAAGTAATCCAGATGTATCG TTATCGCCTTGCGCTGGACC	283	S41201
IL-4[b]	CTGATTCCAACTCTGGTCTGC TTGCCATGCTGCTGAGGTTC	283	AF239917
IL-5[c]	AGGCAAACACTGAACATTTC TCTCCAAAATCTTCCACTAC	468	AF331919
IL-10[a]	CCTGGGTTGCCAAGCCCTGTC ATGCGCTCTTCACCTGCTCC	212	U33843
GAPDH[d]	AAGGTCGGAGTCAACGGATT GCAGAAGAAGCAGAGATGATG	365	AB038240
β-actin	AACACCCCAGCCATGTATGT CTTCTCCAGGGAGGACGAG	333	NM_001003349
EF-1α[e]	AGCCCTTGCGCCTGCCTCTC CAGACACATTCTTGACATTGAAGC	219	X03558

bp = base pairs; IL = interleukin; IFN = interferon; GAPDH = glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor; [a] Markus et al. (2002); [b] Barnes et al. (2000); [c] Yang et al. (2001); [d] Puff et al. (2008); [e] von Smolinski et al. (2005)

Table 3: Primers used for real-time PCR

Target gene	Primer sequence in 5´- 3´(forward primers on top, reverse primers on bottom)	Size of amplicon [bp]	GenBank accession number
IL-2	CCAACTCTCCAGGATGCTCAC TCTGCTAGACATTGAAGGTGTGTA	81	D30710
IFN-γ	AGCATGGATACCATCAAGGAAGA AGATCGTTCACAGGAATTTGAATCA	104	S41201
IL-4	CTCCAAAGAACACAAGCGATAAGG TGTTGGAGCAGTTGTGTGTATAGA	84	AF239917
IL-5	CCTATGTTTCTGCCTTTGCTGTAG GCCTATCAGCCAAGTTCGATGA	95	AF331919
IL-10	GGTGGGAGCCAGCCGACACCAG AAGAAGATCTTCACCCACCCGAAGG	120	U33843
GAPDH[a]	GTCATCAACGGGAAGTCCATCTC AACATACTCAGCACCAGCATCAC	84	AB038240
β-actin	TCTACGAGGGGTACGCCTTG TTCCTTGATGTCACGCACGAT	149	NM_001003349
EF-1α[b]	AGCCCTTGCGCCTGCCTCTC CAGACACATTCTTGACATTGAAGC	219	X03558

bp = base pairs; IL = interleukin; IFN = interferon; GAPDH = glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor; [a] Puff et al. (2008); [b] von Smolinski et al. (2005)

2.3.4 Standard dilution series

In order to allow calculation of a standard curve tenfold dilution series (10^2 – 10^8 copies) were produced for each target gene. These dilution series also served as standards for inter-run calibration to account for run-to-run variations. Amplicons were produced by conventional end-point PCR (PTC-200 Peltier Thermal Cycler; MJ Research), agarose gel electrophoresis, and DNA extraction with NucleoSpin Extract II columns (Macherey-Nagel) according to the

manufacturer's protocol. Spectrophotometrical determination of DNA concentration was performed at OD_{260} (GeneQuant pro; Biochrom Ltd.) and copy numbers per volume were calculated according to the following formula: copies/µl = 6 x 10^{23} x DNA concentration (ng/µl) x 10^{-9}/[size of amplicon (base pairs) x 660].

Complementary DNA of canine PBMNC that were stimulated with concanavalin A (Con A) (5µg/ml) for 24 h served as template for IL-2, -4, -5, and IFN-γ. Template for IL-10, GAPDH, β-actin, and EF-1α was cDNA from DH-82 cells (Wellman et al., 1988). Reaction conditions were as follows: initial denaturation at 94°C for 1 minute; 40 cycles of denaturation at 94°C for 1 minute, annealing at 59°C (IL-2, IL-10, IFN-γ, GAPDH) / 60°C (β-actin, EF-1α) for 2 minutes and elongation at 72°C for 1 minute; final elongation at 72°C for 5 minutes. PCR conditions for IL-4 and IL-5 were set according to Barnes et al. (2000) and Yang et al. (2001), respectively. Amplification was achieved with recombinant *Taq* DNA Polymerase (Invitrogen) in 1x PCR Buffer (Invitrogen) with 1.25 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP mixture (Applied Biosystems), and 300 nM of each primer (Table 2).

2.3.5 Real-time PCR

Real-time PCRs were run on an Mx3005P QPCR system (Stratagene) with Brilliant SYBR Green QPCR Core Reagent Kit (Stratagene) in 8x Strip Tubes with Optical Cap (Stratagene). Due to the number of samples, experiments for each target gene were run on separate plates for PBMNC and CSF WBC samples. Measurements were conducted the same day using the identical standard dilution series. Standards were kept at + 4°C in-between runs. The plates included the standard dilution series as well as a no template control (NTC) in duplicate. Reaction volume was 25 µl including 1 µl template per reaction.

Reaction conditions were as follows: initial denaturation at 95°C for 10 minutes; 40 cycles with denaturation at 95°C for 30 seconds, annealing at 59°C (IL-2, IL-5, IFN-γ) / 60°C (β-actin, EF-1α) / 61°C (IL-10) / 64°C (IL-4, GAPDH) for 1 minute and elongation at 72°C for 30 seconds; final extension at 72°C for 1 minute; dissociation program beginning at 95°C for 1 minute followed by increasing the temperature by 0.5°C every 30 seconds from 55°C to 95°C. The master mix contained 0.025 U/µl SureStart *Taq* DNA Polymerase in 1x core PCR buffer with 2.5 mM (IL-10, IFN-γ, GAPDH) / 5.0 mM (IL-2, IL-4, IL-5, β-actin, EF-1α) $MgCl_2$, 800 µM dNTP mix, 150 nM of each primer (Table 3), 8 % glycerol, and 4 % dimethyl sulphoxide (DMSO) (3 % for IL-10, IFN-γ, GAPDH). Final SYBR Green concentration was 0.25x and Rox was used as a reference dye at 30 nM.

The software tool “Multiple Experiment Analysis” (MxPro QPCR Software v4.01; Stratagene) allowed run-to-run comparison between the results for PBMNC and CSF WBC samples.

To account for variations in sample input, extraction and reaction efficiencies, and presence of inhibitors, copy numbers of the genes of interest were normalized against the geometric mean of the internal reference genes GAPDH, β-actin and EF-1α: $copies_{normalized}$ (target gene) [%] = $copies_{non-normalized}$ (target gene) x 100/ [copies (GAPDH) x copies (β-actin) x copies (EF-1α)]^(1/3).

2.3.6 Statistical analysis

In addition to descriptive methods, the Wilcoxon rank sum test was applied for comparison of the results deriving from the different disease categories. For these comparisons results of the groups “CNS inflammation” and “CNS neoplasia” were combined. It was tested whether

results of dogs with a history of glucocorticosteroid pretreatment differed significantly from the non-pretreated ones. The Wilcoxon signed-rank test served as a tool to compare values deriving from PBMNCs and CSF WBCs of dogs in the acute phase of SRMA. The Spearman´s rank correlation coefficients (r_{Spear}) were calculated to detect overall correlations among the whole study population and correlations for “SRMA with symptoms”. The parameters included normalized copy numbers and results of CBC, CSF, IgA level, and flow cytometric lymphocyte subtype analysis (Schwartz et al., 2008b). Statistical significance was set at the 5% level ($p \leq 0.05$).

2.4 Results

Dissociation procedures at the end of real-time PCRs for IL-2, INF-γ, IL-4, IL-5, IL-10, GAPDH, β-actin, and EF-1α, produced clearly defined single melting temperatures at 77,5°C, 79,1°C, 78,5°C, 79,6°C, 83,8°C, 81,1°C, 85,4°C, and 82,3°C, respectively. PCR products for these genes were considered specific because temperatures corresponded with those of the standard dilution series. These standards contained a single gene product that had been sequenced and recognized as the respective gene of interest.

All reference genes could be measured in every PBMNC sample and normalization was therefore always possible. However, copy numbers for only 1 or 2 of the reference genes could be determined in 9 CSF WBC samples and these samples were excluded from further analysis since an accurate normalization was not possible. These included 3 samples belonging to the category “SRMA without symptoms”, 1 to “CNS inflammation”, and 5 samples to “CNS neoplasia”.

Results of IL-2 mRNA expression analysis are displayed in Figure 1. After normalization PBMNC samples of dogs in the acute phase of SRMA contained the least copy numbers followed by dogs treated for SRMA. Although the distribution of values was fairly homogenous within the group “SRMA with symptoms”, there was a large degree of variation within the remaining groups.

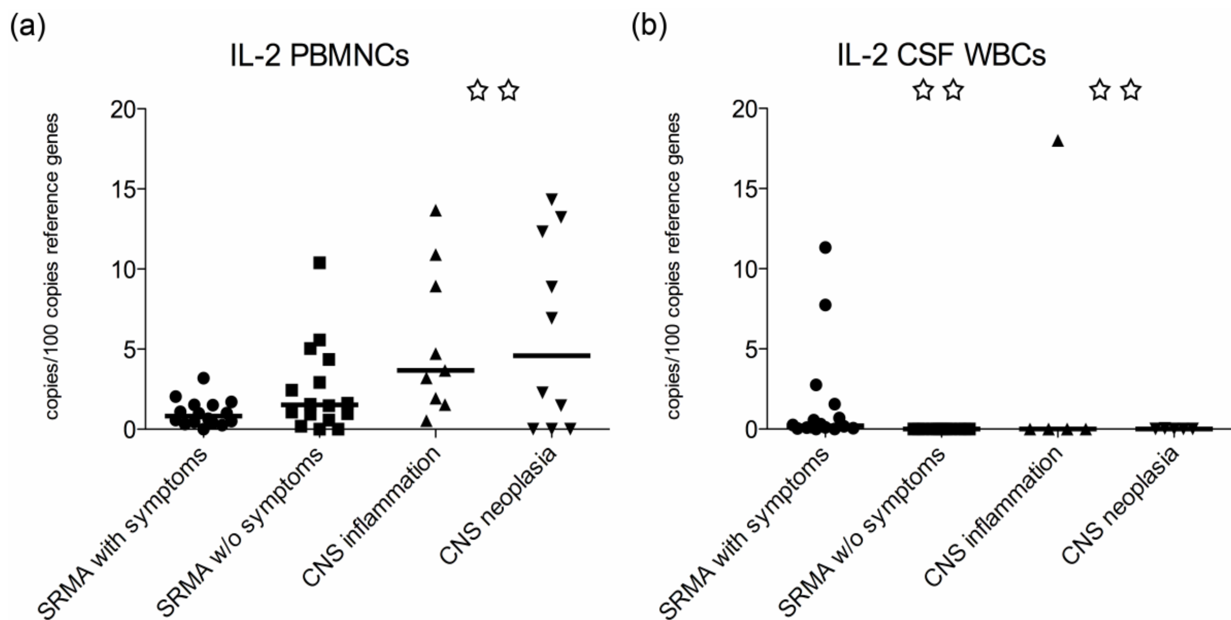

Figure 1: Results of real-time PCR for IL-2 mRNA expression of PBMNCs (a) and CSF WBCs (b). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-actin, and EF-1α and are given as the percentage of the respective geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category “SRMA with symptoms” ($p \leq 0.01$). For purpose of statistical analysis samples from “CNS inflammation” and “CNS neoplasia” were grouped as one disease category (IL = interleukin; PBMNC = peripheral blood mononuclear cells; CSF WBC = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor).**

As displayed in Figure 2, IFN-γ mRNA was expressed least in PBMNC samples that derived from individuals in the acute phase of SRMA and the level of expression was highest in "CNS neoplasia". Similarly to IL-2, IFN-γ expression was most homogenous in dogs with symptoms of SRMA.

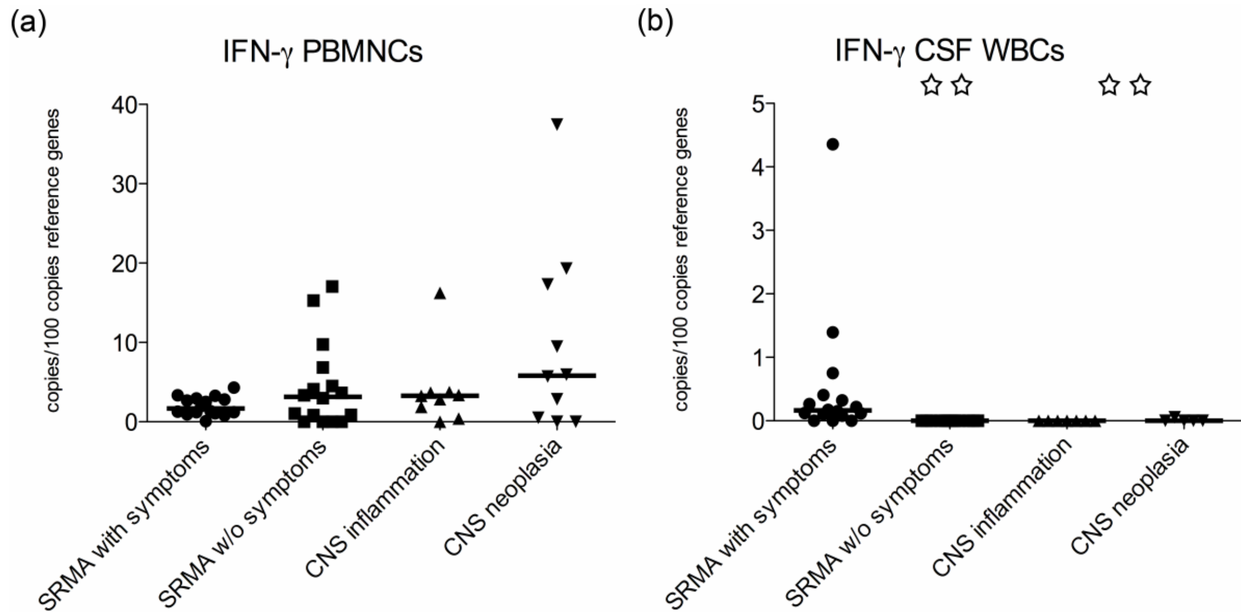

Figure 2: Results of real-time PCR for IFN-γ mRNA expression of PBMNCs (a) and CSF WBCs (b). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-actin, and EF-1α and are given as the percentage of the respective geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category "SRMA with symptoms" ($p \leq 0.01$). For purpose of statistical analysis samples from "CNS inflammation" and "CNS neoplasia" were grouped as one disease category. Please note the non-uniform scaling of the 2 diagrams (IFN = interferon; PBMNC = peripheral blood mononuclear cells; CSF WBC = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor).**

Values for IL-4 mRNA are visualized in Figure 3. The median value for IL-4 mRNA expression in PBMNCs was highest in the group "SRMA with symptoms" followed by "SRMA without symptoms". While no gene expression could be recorded in 50 % of dogs receiving glucocorticosteroids for treatment of SRMA, few individuals displayed a remarkably strong transcriptional activity. In addition, IL-4 mRNA transcripts were found in only few dogs with CNS inflammation and neoplasia.

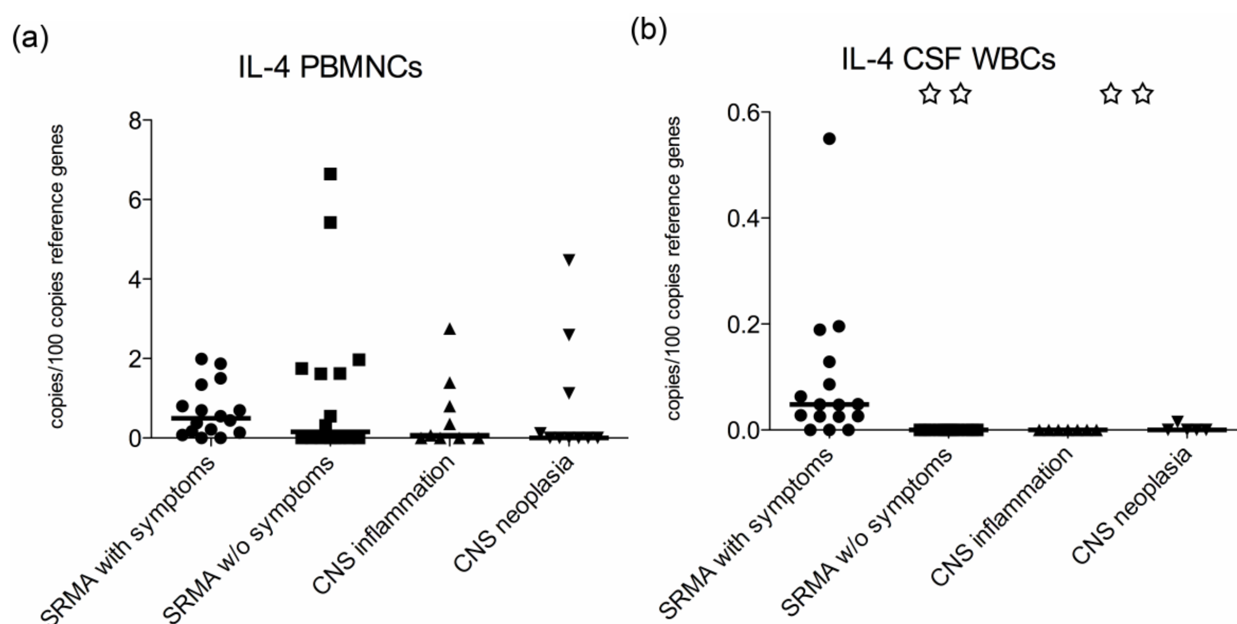

Figure 3: Results of real-time PCR for IL-4 mRNA expression of PBMNCs (a) and CSF WBCs (b). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-actin, and EF-1α and are given as the percentage of the respective geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category "SRMA with symptoms" ($p \leq 0.01$). For purpose of statistical analysis samples from "CNS inflammation" and "CNS neoplasia" were grouped as one disease category. Please note the non-uniform scaling of the 2 diagrams (IL = interleukin; PBMNC = peripheral blood mononuclear cells; CSF WBC = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor).**

Normalized values of IL-5 mRNA transcripts are given in Figure 4. Results for PBMNCs were comparable among the disease categories with slightly higher median values in “SRMA without symptoms” and “CNS neoplasia”.

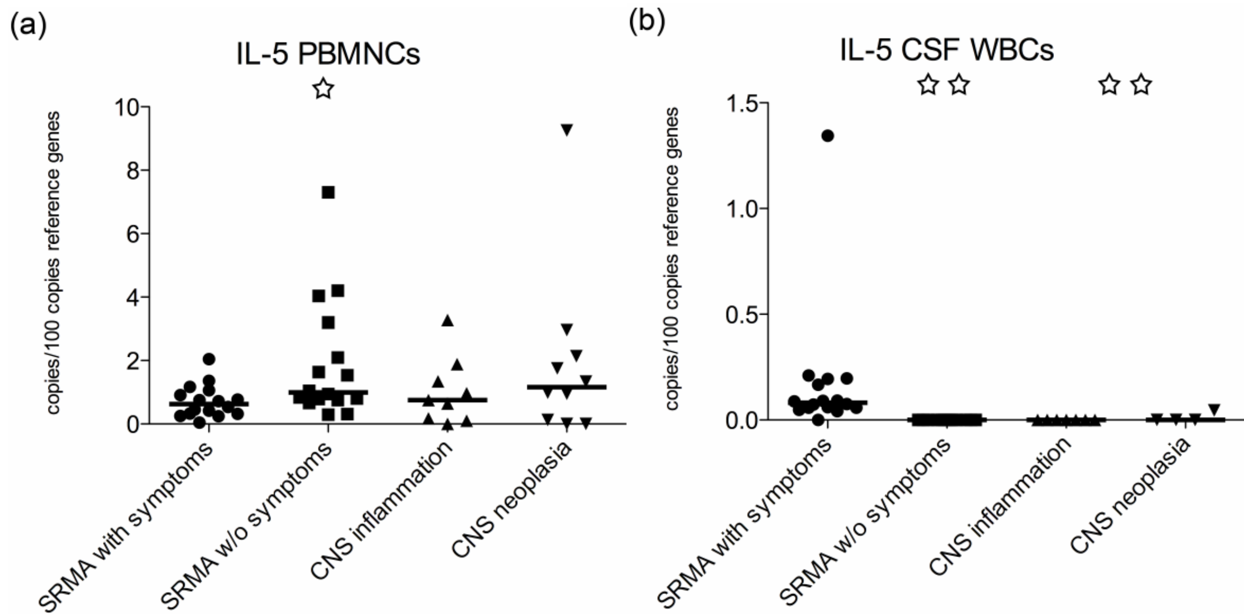

Figure 4: Results of real-time PCR for IL-5 mRNA expression of PBMNCs (a) and CSF WBCs (b). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-actin, and EF-1α and are given as the percentage of the respective geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category “SRMA with symptoms” (* $p \leq 0.05$; ** $p \leq 0.01$). For purpose of statistical analysis samples from “CNS inflammation” and “CNS neoplasia” were grouped as one disease category. Please note the non-uniform scaling of the 2 diagrams (IL = interleukin; PBMNC = peripheral blood mononuclear cells; CSF WBC = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor).

Figure 5 shows the quantity of mRNA transcripts for IL-10. Median values in PBMNC pellets did not differ significantly among the different disease categories, a greater degree of dispersion, however, was noticeable in dogs with neoplastic and non-SRMA inflammatory disorders.

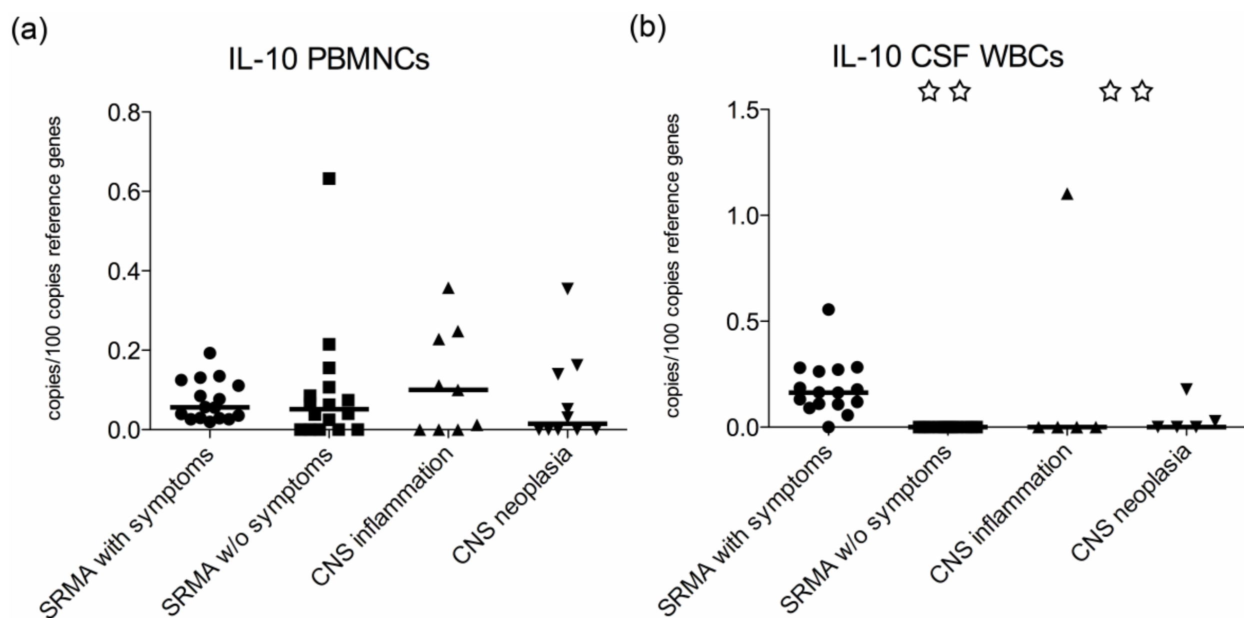

Figure 5: Results of real-time PCR for IL-10 mRNA expression of PBMNCs (a) and CSF WBCs (b). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-actin, and EF-1α and are given as the percentage of the respective geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category "SRMA with symptoms" ($p \leq 0.01$). For purpose of statistical analysis samples from "CNS inflammation" and "CNS neoplasia" were grouped as one disease category. Please note the non-uniform scaling of the 2 diagrams (IL = interleukin; PBMNC = peripheral blood mononuclear cells; CSF WBC = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor).**

Pretreatment with glucocorticosteroids, after exclusion of “SRMA without symptoms”, was recorded for 8 dogs (4/16 in “SRMA with symptoms”, 3/9 in “CNS inflammation”, 1/10 in “CNS neoplasia”) and did not cause significantly differing results in CBC, CSF analysis, flow cytometrically determined lymphocyte subsets, and mRNA expression of genes of interest, except for a more severe pleocytosis ($p < 0.05$), higher CSF IgA content ($p < 0.01$), and increased PBMNC IL-10 mRNA levels ($p < 0.05$) in pretreated dogs. Studying dogs in the acute phase of SRMA separately, the only significant difference that could be detected were lower IL-10 mRNA levels in CSF WBCs of pretreated individuals ($p < 0.05$).

The IL-4:IL-2 ratio was used as an indicator for the Th2:Th1 ratio and was highest in PBMNCs of “SRMA with symptoms” (median: 0.77; range: 0 – 8.84). Values were significantly higher when compared to “CNS inflammation” + “CNS neoplasia” ($p \leq 0.05$) (median: 0.03; range: 0 – 1.42), whereas differences to “SRMA without symptoms” did not reach the level of significance ($p = 0.338$) (median: 0.20; range: 0 – 8.84). The Th2:Th1 ratio in peripheral blood of dogs in the acute phase of SRMA did not differ significantly from that in the CSF ($p = 0.826$) (median: 0.22; range: 0 – 1.79).

In CSF WBC pellets belonging to “SRMA with symptoms” the expression of all cytokines could be recorded, whereas they were found only in single samples of the “CNS inflammation” and “CNS neoplasia” group. Moreover, none were detected in the “SRMA without symptoms” group. Normalized values for cytokine mRNA in CSF cell pellets were below those of PBMNCs. These differences were statistically significant for IL-4, IL-5, and IFN-γ ($p \leq 0.05$). An exception were IL-10 mRNA levels, which were higher in CSF WBCs when compared to PBMNC levels. Cerebrospinal fluid WBC pellets contained a considerable

proportion of neutrophils that also contributed to the normalization factors, calculated from reference gene mRNA expression levels, thus causing a certain "dilution effect".

For the entire study population no statistically significant correlation could be identified between cytokine mRNA expression and B:T cell or CD4:CD8α T cell ratios. Correlations that reached the level of significance were weak ($r_{Spear} < \pm 0.5$) and reflect that findings typically present in SRMA (marked neutrophilic leuko- and pleocytosis + increased IgA levels) are associated with low mRNA expression for IL-2 and IFN-γ and strong expression for IL-4 in PBMNCs (Table 4).

Table 4: Correlations between quantity of mRNA expression and other tested parameters (for the entire study population)

Cytokine	Cellular response	Spearman´s rank correlation coefficient	Level of significance
IL-2 in PBMNCs	Degree of neutrophilic leukocytosis	- 0.393**	p = 0.004
	Degree of pleocytosis	- 0.365**	p = 0.008
	IgA level in serum	- 0.403**	p = 0.002
IFN-γ in PBMNCs	IgA level in CSF	- 0.319*	p = 0.022
IL-4 in PBMNCs	Degree of neutrophilic leukocytosis	+ 0.365**	p = 0.008

IL = interleukin; PBMNCs = peripheral blood mononuclear cells; IgA = immunoglobulin A; IFN = interferon; CSF = cerebrospinal fluid

Within "SRMA with symptoms" high expression of IFN-γ in PBMNCs was associated with a low B:T cell ratio in the peripheral blood (r_{Spear} = - 0.487; $p \leq 0.05$) and strong IL-10 expression in CSF WBCs correlated with milder degrees of pleocytosis (r_{Spear} = - 0.500; $p \leq 0.05$).

2.5 Discussion

Steroid-responsive Meningitis-Arteritis is frequently diagnosed in dogs (Battersby et al., 2006; Meric, 1988; Tipold, 1995) and our knowledge about the pathogenesis of SRMA is still limited. The disease is suspected to arise from a dysregulation of the immune system and the presented data show that SRMA is associated with a type 2-skewed immune response. In a previous study we could demonstrate that the acute phase of SRMA is associated with a lymphocyte shift towards high proportions of B cells, in the peripheral blood and particularly obvious in the CSF. This observation correlates positively with high IgA levels (Schwartz et al., 2008b). Immunoglobulin A production is known to be positively influenced by IL-4, -5, -6, and -10 (Briere et al., 1994; Murray et al., 1987; Ramsay et al., 1994), all belonging to the classical Th2 cytokine spectrum (Abbas et al., 1996). We therefore hypothesized that T helper cells in SRMA predominantly belong to the Th2 subset and thus investigated the cytokine expression pattern in PBMNCs and CSF WBCs by means of reverse-transcriptase real-time PCR. Values from dogs in the acute phase of SRMA were compared to those from dogs under treatment and dogs with other inflammatory and neoplastic diseases of the CNS. In order to assure that regulation of reference genes does not influence the results, data were normalized to a set of three reference genes each belonging to a different functional class in cell

metabolism thus rendering a simultaneous up- or down-regulation unlikely (Vandesompele et al., 2002).

Indeed, PBMNCs of dogs with signs of SRMA expressed low levels of IL-2 and IFN-γ, whereas IL-4 expression was higher in these samples. These findings are compatible with a polarized type 2 immune response with a pronounced production of IL-4. In contrast, IL-5 and -10 mRNA levels were within the same range as those from dogs with other inflammatory and neoplastic CNS disorders. In general, there was a strong overlap of values for all cytokines among the different disease categories and variations of values were most pronounced in "CNS inflammation" and "CNS neoplasia" reflecting the heterogeneity of these groups.

Polarization of the immune response is a dynamic process that progresses and thus requires time to develop. Polarization is therefore most noticeable in chronic diseases while early states of inflammation are usually associated with mixed patterns of cytokine production (London et al., 1998; O'Garra, 1998). This explains our findings of a mixed cytokine pattern with a predominance of type 2 associated cytokines in dogs with SRMA. Due to the highly painful nature of the disease dogs are usually referred to specialty facilities within hours to few days after onset of clinical signs and this time span may be too short to develop full-blown polarization of the immune response. In addition, it was realized that the Th1/Th2 paradigm is an oversimplification for the description of *in vivo* inflammations in many cases. It is now suggested that Th1 and Th2 cells with their classical cytokine expression patterns rather represent extreme phenotypes and that there is a large variety of cytokine expression patterns in single CD4+ T cells. On a population level, however, the cytokine profile may certainly be skewed in either type 1 or type 2 direction (Kelso, 1995; London et al., 1998) as found in SRMA. Whether inflammatory reactions tend to polarize towards a type 1 or 2

immune response depends on multiple factors. Among the promoters for type 2 immune responses are a predominance of IL-4 in the microenvironment of naïve T helper cells and increasing dosages of antigen (Abbas et al., 1996; London et al., 1998; Mosmann and Sad, 1996; Romagnani, 1997). The latter and the finding that repeated T helper cell stimulation promotes type 2 immune responses make environmental antigens typical elicitors of inflammatory reactions with high IL-4 levels (Abbas et al., 1996). Involvement of such an environmental factor in the pathogenesis of SRMA has been suggested earlier (Tipold et al., 1999). There is also some evidence that antigen-presentation by B cells stimulates Th2 cell development (Abbas et al., 1996), which is of interest in light of the finding that high percentages of B cells circulate in peripheral blood and CSF of dogs with SRMA (Schwartz et al., 2008b). Another factor that participates in determination which route an inflammatory reaction takes is the genetic background of the host (London et al., 1998; O'Garra, 1998). Although no genetic factor could be identified to date, the fact that certain breeds are over-represented in the population of diseased dogs (Behr and Cauzinille, 2006; Cizinauskas et al., 2000; Harcourt, 1978; Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994) suggests that a certain genetic background could facilitate the development of SRMA, possibly because of a more pronounced tendency to develop a type 2 immune reaction.

Although IL-5 is one of the signature cytokines of Th2 cells, levels of expression were within a comparable range among the disease categories, which can be explained by the fact that each cytokine gene may be expressed independently (Kelso, 1995; London et al., 1998). Interleukin-5 was ascribed an important role in the differentiation of IgA B cells into IgA-secreting plasma cells (Harriman et al., 1988) and presence of IL-4 further boosts this secretion (Murray et al., 1987). Messenger RNA encoding for IL-5 could be detected in each PBMNC pellet of dogs with SRMA, both in the acute phase and under treatment, suggesting

that IL-5, with the aid of IL-4, contributes to increased IgA levels and is most probably responsible for persistently elevated levels in many dogs under glucocorticosteroid treatment (Cizinauskas et al., 2000).

The expression level of the immunomodulatory cytokine IL-10 did not show considerable differences in PBMNC samples of the different disease categories. Whereas IL-10 was originally considered a typical Th2 cytokine, it is now clear that also lymphocytes of the Th1 subset as well as B cells and monocytes, amongst others, are capable to produce IL-10. Regulatory T cells (T_{Reg}) are now considered a major source of IL-10 (Taylor et al., 2006). This cytokine acts suppressive on both Th1 and Th2 cells and given the strong, presumably overshooting, immune response in SRMA it is not surprising that no up-regulation of this cytokine could be recognized in PBMNCs. The finding of increased IL-10 mRNA levels in CSF WBCs when compared to PBMNCs may represent an endogenous response for CNS protection. In addition to its immunosuppressive function, IL-10 also has the potential to induce differentiation of B cells into IgA secreting plasma cells (Briere et al., 1994).

Comparison of mRNA expression levels in PBMNCs of dogs in the acute phase of SRMA and after 2 months of glucocorticosteroid treatment revealed a tendency towards a “depolarization” of the immune response with single individuals exhibiting an even more pronounced type 2 immune response. Glucocorticosteroids are known to inhibit the transcription of both Th1 and Th2 associated cytokines, long-term treatment, however, tends to result in a dominance of Th2 cells (Elenkov, 2004; Liberman et al., 2009). This may explain that IgA levels remain elevated in a number of individuals despite treatment with clinical improvement (Cizinauskas et al., 2000).

In the present study we compared results of dogs suffering from SRMA with inflammatory and neoplastic diseases of the CNS. It was shown that CNS neoplasias tend to be associated

with serum cytokine levels that are shifted towards a type 2 immune response (Kumar et al., 2006). The same applies for bacterial infections of the CNS (Raziuddin et al., 1995). Therefore, one can assume that the overall results of the groups “CNS inflammation” and “CNS neoplasia” are rather shifted towards a Th2-dominated than towards a Th1-dominated immune response, indicating that SRMA is associated with an even more pronounced shift towards an IL-4 dominated type 2 immune response.

2.6 Conclusion

Canine SRMA is associated with a lymphocyte shift towards high proportions of B cells and increased IgA levels. The presented data on mRNA expression levels suggest that the immune response in SRMA tends to polarize towards a type 2 immune reaction. The inflammation is characterized by comparatively high IL-4 and low IL-2 and IFN-γ levels in PBMNCs, whereas IL-5 and IL-10 expression was similar to those of dogs suffering from other inflammatory and neoplastic CNS disorders. Cytokine profiles in CSF WBCs showed a similar Th2:Th1 ratio to that in PBMNCs. We thus conclude that distribution of Th1 and Th2 subsets does not differ significantly between peripheral blood and CSF and that the type 2 immune reaction represents an important pathogenetical factor for high systemic and intrathecal IgA production in the acute phase of SRMA and under glucocorticosteroid treatment.

2.7 Acknowlegements

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2.8 Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Chapter 3: Selective CD11a upregulation on neutrophils in the acute phase of Steroid-responsive Meningitis-Arteritis in dogs

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3.1 Abstract

Steroid-responsive Meningitis-Arteritis (SRMA) is a systemic inflammatory disease of juvenile to young adult dogs with a relapsing course and most prominent manifestation in the cervical meninges. The most important laboratory finding is a marked neutrophilic pleocytosis.

Integrin (CD11a, b, c) expression on polymorphonuclear cells (PMNs) was quantified by immunophenotyping and subsequent flow cytometric measurements. Values were determined for peripheral blood in the acute phase of SRMA (n = 14) as well as during glucocorticosteroid treatment (n =16). Results were compared to those from dogs with other neurological diseases (n = 49) and healthy individuals (n = 7). Integrin expression was also investigated on PMNs deriving from cerebrospinal fluid (CSF) of dogs in the acute phase of SRMA (n = 14). In a second part of the study PMNs of healthy dogs were incubated with sera of dogs in the acute phase of SRMA (n = 12). The influence on integrin expression was studied and results were compared to those after incubation with pooled sera of dogs suffering from idiopathic epilepsy (n = 3).

PMNs in peripheral blood of dogs in the acute phase of SRMA showed higher values of CD11a expression when compared to dogs under treatment and to control groups, whereas CD11b and c expression was comparable among the different groups. In the acute phase of SRMA CD11b expression on PMNs in CSF was increased in comparison to that in peripheral blood. Incubation with SRMA sera caused a stronger upregulation of CD11a than did pooled epilepsy sera in 9/12 cases whereas an upregulation of CD11b and c was observed in single cases only.

High CD11a expression on PMNs in peripheral blood appears to be an important factor in the pathogenesis of SRMA. This integrin is known to be essential for adhesion of PMNs within the neutrophil recruitment cascade and therefore might mediate the enhanced invasion of neutrophils into the subarachnoidal space eventually leading to meningitis and clinical signs. Since sera of dogs suffering from SRMA selectively induce an upregulation of CD11a it can be suspected that this fluid contains one or multiple factors being responsible for this.

Key words: canine, CNS immune response, compartmentalization, flow cytometry, immunoglobulin A, integrin

Abbreviation list: CBC = complete blood cell count; CNS = central nervous system; CSF = cerebrospinal fluid; IgA = immunoglobuline A; IL = interleukin; KS = Kawasaki Syndrome; mAb = monoclonal antibody; PBS = phosphate buffered saline; PMN = polymorphonuclear cell; SRMA = Steroid-responsive Meningitis-Arteritis; WBC = white blood cell

3.2 Introduction

Steroid-responsive Meningitis-Arteritis (SRMA) is a systemic inflammatory disease with a relapsing course. Juvenile to young adult dogs are affected and inflammation is most prominent in the cervical meninges. In addition, extraneural signs may be detected elsewhere, e.g., in the extramural coronary arteries (Hayes et al., 1989; Scott-Moncrieff et al., 1992). There is a breed predisposition for Boxers, Bernese Mountain Dogs and Beagles, however, multiple breeds and crossbred dogs have also been reported to be affected (Meric et al., 1985; Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994). SRMA is a naturally occurring animal model for several human vasculitides of unknown origin, including Kawasaki Syndrome (KS) and Polyarteritis nodosa (Burns et al., 1991; Snyder et al., 1995). Furthermore, SRMA allows studies on the general phenomenon of compartmentalization of immune responses into the subarachnoid space.

Most prominent laboratory findings in SRMA are a marked neutrophilic leuko- and pleocytosis as well as an increase in immunoglobuline (Ig) A levels in serum and cerebrospinal fluid (CSF) (Tipold and Jaggy, 1994).

Neutrophilic pleocytosis is an uncommon finding in neurologic diseases of dogs. In addition to SRMA, it is also observed in a small percentage of dogs with intracranial meningiomas (Dickinson et al., 2006) and in dogs suffering from bacterial central nervous system (CNS) infection. These bacterial meningoencephalomyelitides, however, are exceptionally rare in dogs (Radaelli and Platt, 2002). Attraction of neutrophils into the CNS can, amongst others, be mediated by interleukin-8 (IL-8 = CXCL8) and growth-related gene product alpha (GRO-α = CXCL1), which both are significantly upregulated in CSF of human patients with bacterial meningitis (Spanaus et al., 1997). And in fact, Burgener et al. (1998) could show

that also in CSF of dogs with SRMA, chemotactic activity for neutrophils is increased and that this is, at least in part, caused by IL-8 or an IL-8-like factor. Since high chemotactic activity persists during successful glucocorticosteroid treatment with clearance of pleocytosis, Burgener and colleagues suspected that a decrease in endothelial adhesion might be responsible for the lack of neutrophil invasion into the CSF. This endothelial adhesion, which is followed by crawling of the migrating white blood cells (WBCs) along the vessel wall, is crucial for the extravasation of WBCs. Adhesion is mediated by CD11a whereas CD11b is responsible for crawling (Phillipson et al., 2006). Linked to CD18 these leukocytic surface molecules and CD11c form a heterodimer and are referred to as β_2 integrins (Abbas and Lichtman, 2003). Blockage of CD18 in rabbits with experimentally induced bacterial meningitis could prevent the development of pleocytosis, confirming the importance of these molecules in the pathogenesis of meningitis (Tuomanen et al., 1989).

Since neutrophil migration into CSF is a key feature of SRMA and CD11a, b and c might play a crucial role in this event, the purpose of the study was to characterize the pattern of integrin expression in blood and CSF of dogs with SRMA and to compare these findings to those of various control groups. Furthermore we investigated if sera of SRMA patients contain substances, which have the ability to induce changes in β_2 integrin expression on non-activated neutrophils *in vitro*.

3.3 Materials and Methods

3.3.1 Animals and Samples

Blood samples to determine *in vivo* integrin expression on polymorphonuclear cells (PMNs) in peripheral blood, derived from 71 dogs of various breeds and both sexes. All dogs were presented in the Department of Small Animal Medicine and Surgery, University of Veterinary Medicine, Hannover, Germany between July 2006 and March 2008 because of neurological disorders. The categorization into one of the groups "SRMA with symptoms", "SRMA without symptoms", "Idiopathic epilepsy", "Spinal cord traumata", "Inflammatory diseases", "Neoplastic diseases" and "Miscellaneous" (Table 1) was based on results of general and neurological examinations and laboratory testing (blood, CSF, urine) under consideration of electrodiagnostic studies (electromyography, nerve conduction velocity studies, electroencephalography) and imaging findings (X-ray, computed tomography, magnetic resonance imaging). In those cases where results of a pathological examination were available, these contributed to the diagnoses. Diagnosis of SRMA was based on the presence of the following findings: fever, cervical pain, neutrophilic leukocytosis, neutrophilic pleocytosis, increased IgA levels in serum and CSF, immediate and sustained response to glucocorticosteroid treatment and the absence of other causes for these signs (Tipold and Jaggy, 1994). Possible glucocorticosteroid treatment within seven days prior to sampling (in case of depot formulations within six weeks prior to sampling) was noted for all dogs. The integrin expression was also determined in seven clinically healthy Beagle dogs summarized as "Healthy controls". Health status in these animals was determined by general examination and complete blood cell count (CBC). In dogs with symptoms of SRMA (n = 14) integrin expression was also investigated on PMNs in CSF.

Table 1: Disease categories (age given as mean and range [years])

Disease categories	Number of dogs	Inclusion criteria	Age
SRMA with symptoms	14[a]	Dogs showing symptoms of SRMA at time of sampling	1.0 (0.5 – 1.5)
SRMA without symptoms	16	Dogs with SRMA that were under glucocorticosteroid treatment and did not show symptoms at time of sampling (control examinations)[b]	1.0 (0.5 – 5.0)
Idiopathic epilepsy	6	Dogs with idiopathic epilepsy	3.75 (1.75 – 8.5)
Spinal cord traumata	6	Dogs with intervertebral disk protrusion/herniation, vertebral subluxation	8.25 (6.0 – 11.25)
Inflammatory diseases	12	Dogs with inflammatory diseases of the CNS or PNS	7.5 (1.0 – 9.75)
Neoplastic diseases	10	Dogs with CNS neoplasia	9.0 (4.25 – 14.5)
Miscellaneous	15	Dogs with neurological symptoms due to other diseases	6.0 (1.0 – 10.5)
Healthy controls	7	Healthy dogs	5.0 (4.75 – 6.5)

SRMA = Steroid-responsive Meningitis-Arteritis; [a] = eight individuals are also included in "SRMA without symptoms" at a later date (follow up study); [b] = one dog did not receive medication and was in a subclinical phase of SRMA; CNS = central nervous system; PNS = peripheral nervous system

For flow cytometric quantification of granulocytic CD11a, b and c expression in peripheral blood samples 10 ml blood was collected from either the cephalic or saphenal vein in tubes containing ethylene diamine tetraacetic acid (EDTA). Mean WBC count of dogs with SRMA was 27 500 WBCs/µl (range: 16 200 – 48 000/µl). CSF samples were taken from the cerebellomedullary cistern in general anesthesia with dogs being in lateral recumbency. CSF analysis was performed immediately after sample collection and consisted of cell count, cytomorphological differentiation and total protein content determination. Samples containing erythrocytes were only included in this study if CSF was xanthochrome and blood contamination was therefore considered to be due to arteritis (Jamison and Lumsden, 1988). Mean WBC count in CSF of dogs with SRMA was 1 670 WBCs/µl (range: 18 – 8 534/µl). IgA levels in serum and CSF were determined at time of sampling using an enzyme linked immunosorbent assay (ELISA) system established by Tipold et al. (1994).
Whether an integrin upregulation could be induced by incubation of non-activated granulocytes with serum of dogs with SRMA was tested in a second part of the study. Three healthy large breed dogs served as PMN donors. To ensure that neutrophils of these dogs were in a physiological activation state a general examination and CBC were carried out in each dog. Twenty-five milliliter blood of each donor dog was collected in EDTA tubes as described above.

3.3.2 Monoclonal antibodies (mAbs)

Monoclonal antibodies (mAb) of murine origin and directed against canine surface antigens, and their dilutions used in this study are listed in Table 2. Primary antibodies belonged to the IgG1 isotype and were purchased from Serotec (Duesseldorf, Germany). An $F(ab')_2$-fragment

specific RPE-labeled goat-anti-mouse IgG antibody (Dianova, Hamburg, Germany) served as secondary antibody (1:200 dilution).

Table 2: Monoclonal antibodies

Target antigen	Clone	Dilution
CD11a	CA11.4D3	1:300
CD11b	CA16.3E10	1:6
CD11c	CA11.6A1	1:6
IgG1 negative control	W3/25	1:6

3.3.3 Sample processing for the *ex vivo* examination

The method of indirect membrane immunofluorescence with subsequent flow cytometric measurement was elected for determination of integrin expression.

Sample processing for the first part of the study, addressing the *in vivo* CD11a, b and c expression on peripheral blood PMNs, was initiated immediately after venipuncture. PMNs were obtained by means of density gradient centrifugation as described by Danilenko et al. (1992) with the exception that phosphate buffered saline (PBS) was used instead of 6 % dextran for dilution of blood and no autologous plasma was added to PBS for washing isolated PMNs. After the separation cell viability was determined by trypan blue exclusion and PMNs were counted using a hemocytometer. Cell suspension was then adjusted to 5 x 10^5 vital PMNs/50 µl by adding CellWASH solution (BD Biosciences, Erembodegem, Belgium). To prevent unspecific Fc-receptor binding of mAbs the cell suspension was incubated with heat-inactivated (56°C; 30 min) pooled goat serum for 10 min (1:11 dilution) (Schwartz, 2007). Negative controls consisted of an isotype-matched primary antibody and incubation with the secondary antibody only. For each mAb 50 µl of cell suspension was used. A 30-min

incubation period for the primary antibodies was followed by two washing steps at 200 x g for 6 min using CellWASH solution. An incubation period with the secondary antibody (30 min) and subsequent washing steps identical to the before mentioned ones followed. Immediately after the second washing step cell suspensions were diluted with BD FACSFlow™ solution (BD Biosciences, Erembodegem, Belgium) in a ratio of 2:5 and integrin expression was flow cytometrically quantified using a FACSCalibur™ device with BD CellQuest™ Pro Version 5.2.1 software (Becton Dickinson, Heidelberg, Germany).

Preparation of PMNs from CSF was initiated immediately after tapping. CSF was centrifuged at 200 x g for 10 min and subsequently the supernatant was removed. The absolute number of leukocytes was calculated from CSF cell count and cell concentration was then adjusted to 5 x 10^5 leukocytes/50 µl by adding CellWASH solution. Blocking of Fc-receptors, washing steps and flow cytometric measurements were equal to those used for peripheral blood PMNs, duration of incubation with antibodies, however, was reduced to 15 min to minimize cell loss. In one dog only CD11b staining was performed.

3.3.4 Sample processing for the *in vitro* study

In the second part of the study changes in integrin expression on non-activated PMNs following incubation with sera of dogs with SRMA in comparison to dogs with idiopathic epilepsy were investigated. Three experiments were performed. In each experiment granulocytes of one donor dog were incubated with sera of four dogs with SRMA and with pooled sera from three epileptic dogs, which all had a normal general examination and laboratory results (incl. IgA levels) within reference range. Incubation with PBS served as an additional negative control. The method for isolation of PMNs was identical to that described

above (2.3. Sample processing for the *ex vivo* examination). Twenty microliter of serum was added to 100 µl of cell suspension containing 3 x 10^5 PMNs (adjusted with PBS) and a 25-min incubation period (37°C; 5 % CO_2) followed. All samples were processed as duplicates. After this period PMNs were washed three times at 200 x g for 6 min using CellWASH. Immunostaining was performed as described in the first part of the study (2.3. Sample processing for the *ex vivo* examination) with the exception that pooled goat serum was not used as a blocking agent to prevent interference of different sera types. Negative controls as used in the first part of the study helped to rule out unspecific mAb binding. All sera were stored at – 20°C prior to usage.

3.3.5 Flow cytometric analysis

Ten thousand events per sample were flow cytometrically registered. Setting of the region containing granulocytes was based on their size and complexity characteristics as illustrated in Figure 1.

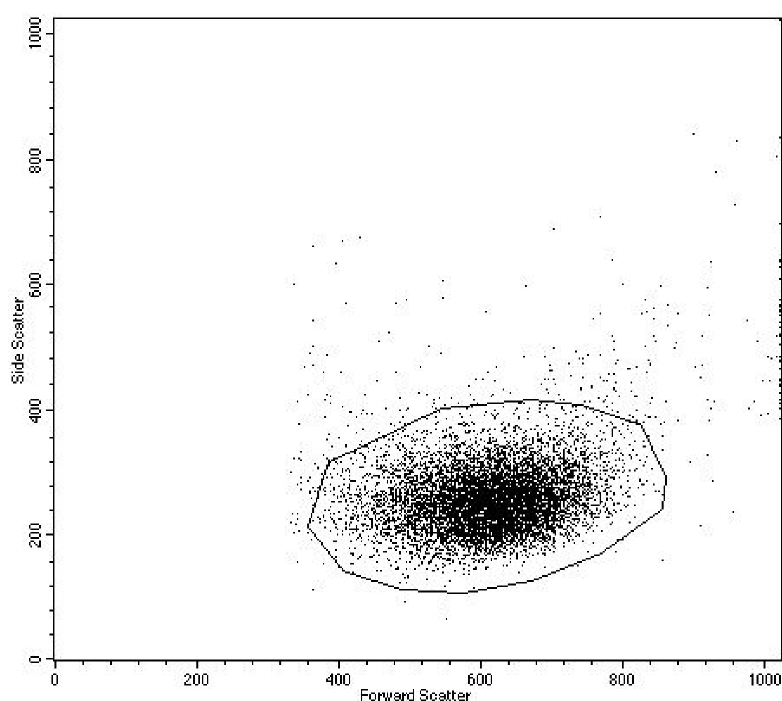


Figure 1: Result of flow cytometric measurement of polymorphonuclear cells (PMNs) from peripheral blood displayed as dot plot. PMNs were isolated by means of density gradient centrifugation. Setting of PMN containing region was based on size and complexity characteristics of cells. Note the absence of any lymphocytic contamination.

Mean fluorescence values were used for statistical analysis. In the second part of the study the arithmetic mean was calculated from values deriving from duplicate samples. For comparison of these values among the three experiments, fluorescence following incubation with pooled "Idiopathic epilepsy" sera (F_{IE}) was given a value of 100. Values resulting from incubation with PBS and with sera of dogs with SRMA (F_A) were put in relation to this given value resulting in a relative value (F_R). The following formula was used for this purpose: $F_R = F_A$ x $100/F_{IE}$.

3.3.6 Preliminary experiments

Preliminary experiments were performed to determine PMN viability at the time of flow cytometric measurement. Staining with To-Pro-3 (Molecular Probes™, Leiden, The Netherlands) resulted in > 99 % vital cells within the PMN region.

3.3.7 Statistical analysis

In addition to descriptive methods the Wilcoxon rank sum test was applied for comparison of the results deriving from the groups "SRMA with symptoms" and "SRMA without symptoms". Results from these two groups were also compared to those of the remaining disease categories. The Wilcoxon signed-rank test served as a tool to compare values deriving from blood and CSF of the same individual. The Spearman´s rank correlation coefficients were calculated to detect correlations of parameters within the two groups of SRMA. These parameters included neutrophil count in peripheral blood and CSF, IgA levels in serum and

CSF and values of *in vivo* integrin expression. Statistical significance was set at the 5 % level ($P < 0.05$).

3.4 Results

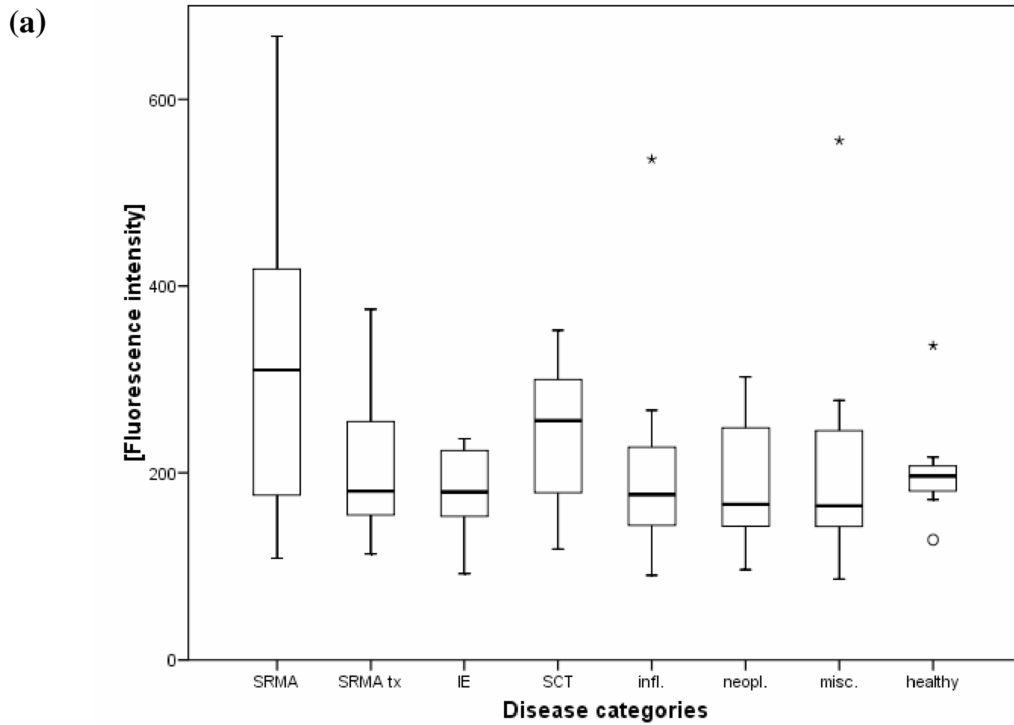
Three out of 14 dogs with symptoms of SRMA received a glucocorticosteroid treatment prior to sampling. In the group of animals with SRMA not showing any symptoms all but one dog where under glucocorticosteroid therapy with daily doses ranging from 0.8 – 1.0 mg/kg prednisolone. This one dog was in an asymptomatic phase of the disease at the time of sampling.

3.4.1 Results of the *ex vivo* examination

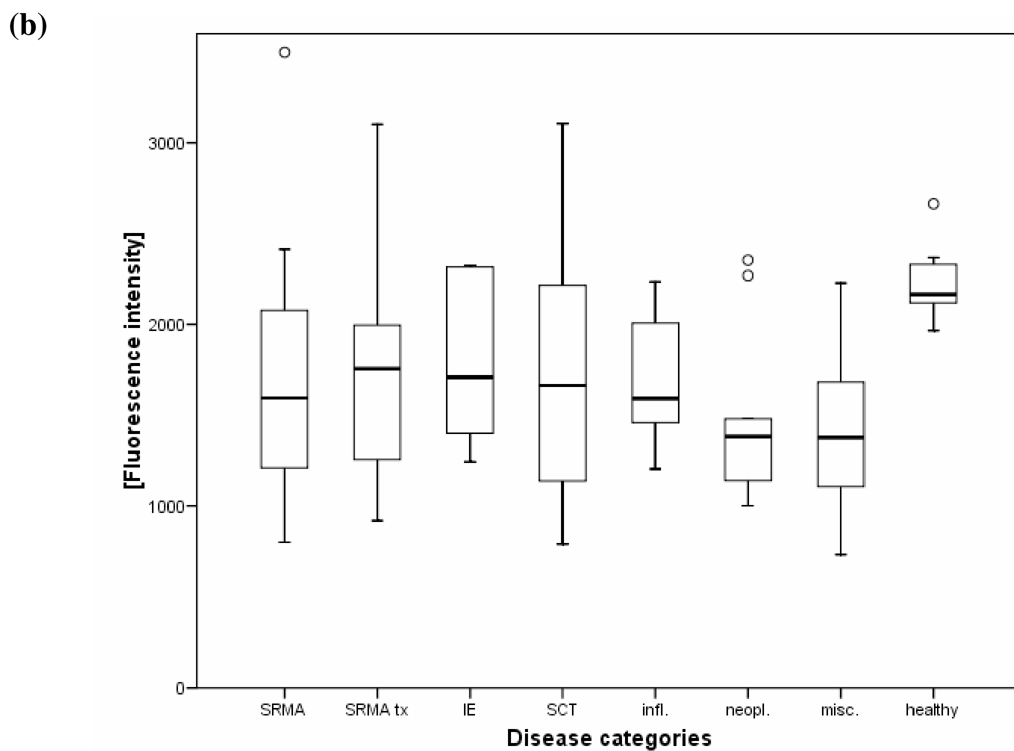
The results of the first part of the study addressing the quantification of integrin expression on PMNs in peripheral blood are illustrated as Box-and-Whisker Plots in Figure 2 a-c. The median value for fluorescence intensity after staining of CD11a was highest in the group of dogs suffering from symptoms of SRMA. Values within this group were significantly higher ($P < 0.05$) compared to the remaining disease categories with the exception of “Spinal cord traumata” and “Healthy controls”. Distribution of values showed a wide range within “SRMA with symptoms” with some individuals expressing as little CD11a as dogs in the other groups. However, only five dogs not displaying clinical signs of SRMA at time of sampling (i.e. < 7 %) showed higher CD11a expression than the median value of “SRMA with symptoms”. There was no more than one of these individuals per group. Determination of CD11b expression revealed a comparatively wide range of results throughout all groups except for “Healthy controls”. CD11b expression in dogs with signs of SRMA and under therapy was

significantly lower (P < 0.05) than that of healthy Beagle dogs, which was highest among all groups. Also median expression of CD11c was highest in "Healthy controls". CD11c expression in dogs displaying symptoms of SRMA was significantly higher (P < 0.05) than that of dogs with CNS neoplasia

(a)



(b)



(c)

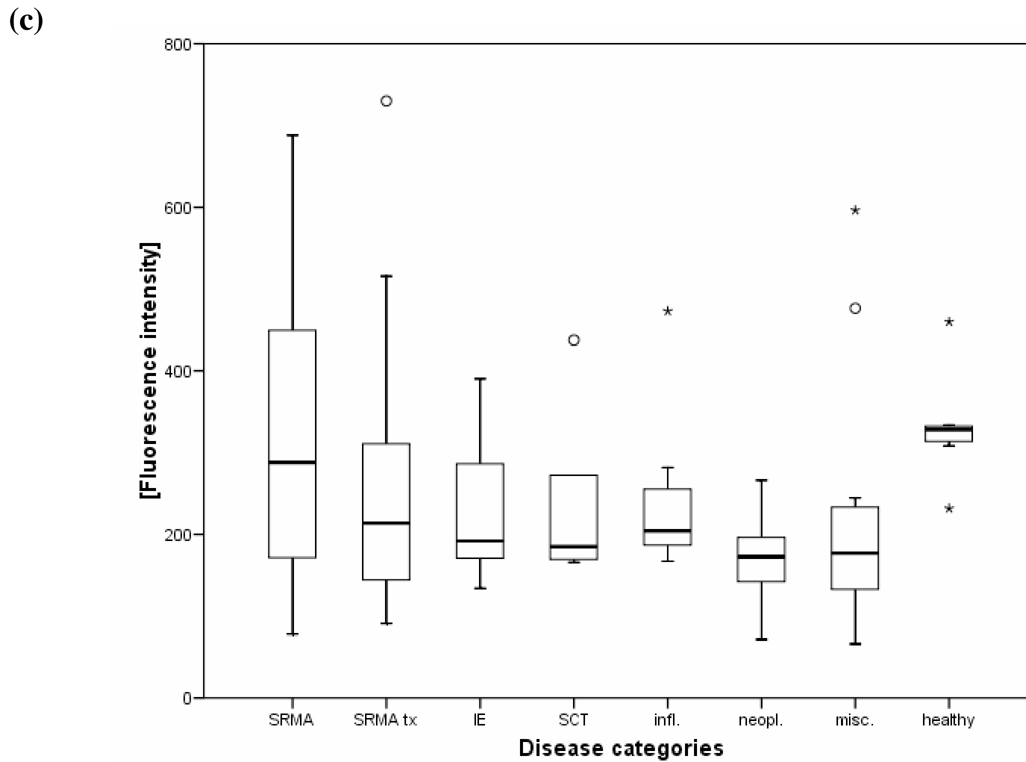


Figure 2: Integrin expression on polymorphonuclear cells in peripheral blood: CD11a (a), CD11b (b) and CD11c (c) (SRMA = SRMA with symptoms; SRMA tx = SRMA without symptoms; IE = Idiopathic epilepsy; SCT = Spinal cord traumata; infl. = Inflammatory diseases; neopl. = Neoplastic diseases; misc. = Miscellaneous; healthy = Healthy controls; SRMA = Steroid-responsive Meningitis-Arteritis; box contains values from 1st – 3rd quartile, line inside box indicates median value, endpoints of vertical line display minimum and maximum value, ○ = outlier (1.5 – 3 x box length outside of box), ★= extreme value (> 3 x box length outside of box))

Comparison of integrin expression in peripheral blood and CSF revealed a significant difference for CD11b only ($P < 0.05$). CD11b expression was greater in CSF. Figure 3 demonstrates the relation of integrin expression on PMNs in blood and CSF of dogs suffering from SRMA.

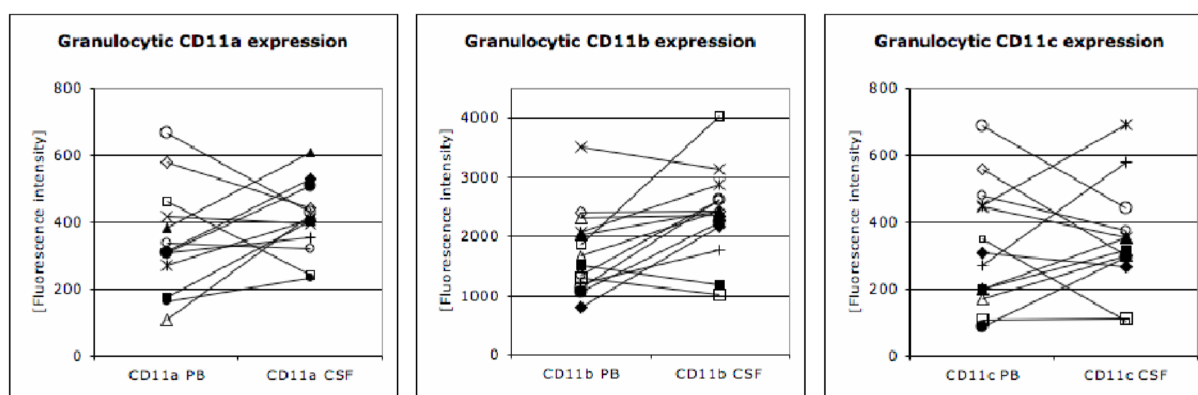


Figure 3: Integrin expression on granulocytes in peripheral blood (PB) and cerebrospinal fluid (CSF) is displayed for each individual with symptoms of Steroid-responsive Meningitis-Arteritis. In the majority of dogs integrin expression is increased in CSF (CD11a: 62 % of dogs; CD11b: 79 % of dogs; CD11c: 54 % of dogs).

3.4.2 Correlation of parameters

Beta$_2$ integrin expressions on PMNs in peripheral blood and the degree of neutrophilic leukocytosis correlated positively ($P < 0.05$) within the category "SRMA with symptoms", as did the expressions of CD11a, b and c in the blood with one another (Table 3). Also in "SRMA without symptoms" β_2 integrin expressions on peripheral blood PMNs correlated positively ($P < 0.05$) with one another. No correlation could be established between integrin expression, neither in peripheral blood nor in CSF, and severity of neutrophilic pleocytosis, as well as between integrin expressions and IgA levels.

Table 3: Significant correlations for the group “SRMA with symptoms” (P < 0.05)

Parameter	Parameter	Spearman´s rank correlation coefficient
CD11a in PB	Number of neutrophils in PB	+ 0.824**
CD11b in PB	Number of neutrophils in PB	+ 0.662**
CD11c in PB	Number of neutrophils in PB	+ 0.688**
CD11a in PB	CD11b in PB	+ 0.727**
CD11a in PB	CD11c in PB	+ 0.727**
CD11b in PB	CD11c in PB	+ 0.657

SRMA = Steroid-responsive Meningitis-Arteritis; PB = peripheral blood; ** = P < 0.01

3.4.3 Results of the *in vitro* study

In the second part of the study changes in integrin expression following incubation with sera of dogs with symptoms of SRMA were investigated. Figure 4 summarizes the results of the three single experiments. In general, incubation of neutrophils with sera of either group (SRMA and epilepsy) led to higher integrin expressions in comparison to incubation with PBS. Nine out of 12 sera from dogs with SRMA (75 %) induced a stronger CD11a upregulation in comparison to incubation with epilepsy sera. The histogram plot in Figure 5 shows an example for this comparatively strong upregulation of CD11a. A PMN subpopulation expressing particularly large amounts of CD11a is visible (marked with arrow). The remaining three dogs, whose sera resulted in $F_R < 100$, showed either very low or very high *in vivo* CD11a expression. In contrast to this finding, a stronger upregulation of CD11b and c was detected in only two and one out of 12 tested SRMA sera, respectively, when compared to pooled sera from epileptic dogs. All other sera caused lower CD11b and c expressions in comparison to epilepsy sera.

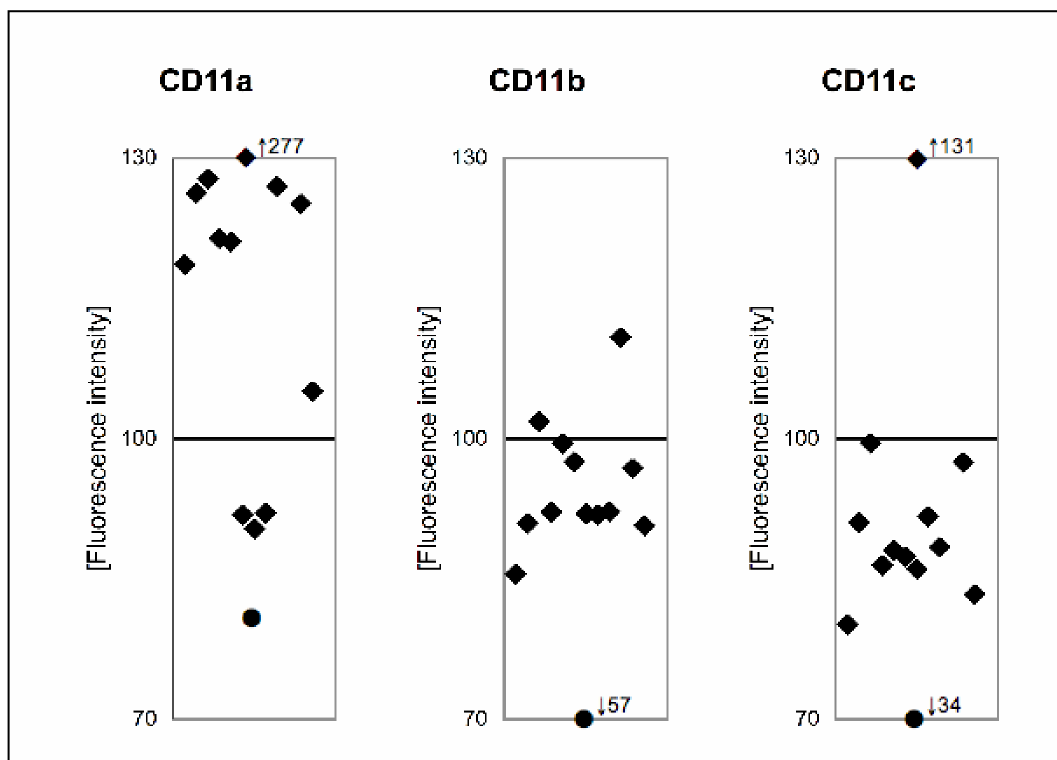


Figure 4: Integrin expression on polymorphonuclear cells after incubation of non-activated granulocytes from a donor dog with sera of dogs (n = 12) belonging to disease category "SRMA with symptoms". Each square depicts the fluorescence intensity after incubation with serum from one dog. Circles represent mean fluorescence intensity after incubation with PBS. Values were put in relation to those from incubation with pooled sera from dogs belonging to disease category "Idiopathic epilepsy" (fluorescence intensity resulting from incubation with pooled epilepsy sera (F_{IE}) was given a value of 100).

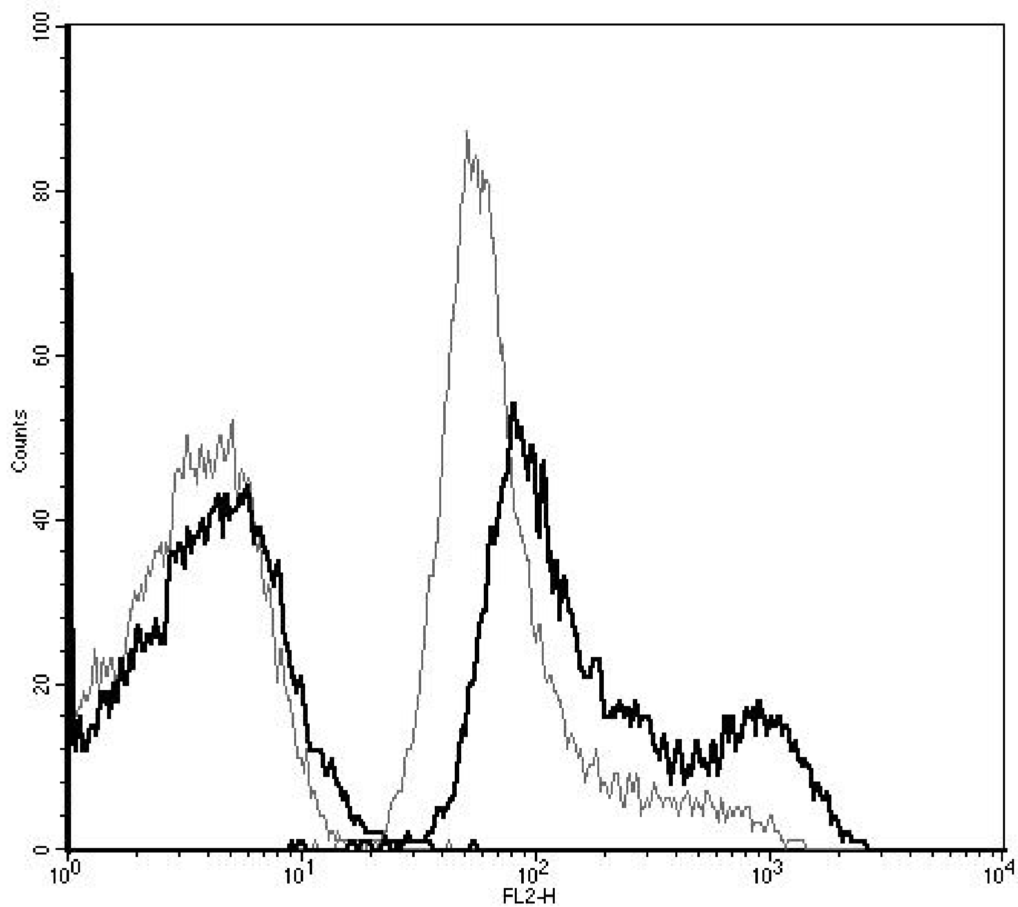


Figure 5: Histogram plot displaying CD11a expression after incubation with sera (negative controls for immunostaining procedure included (dashed arrow)). Grey graphs result from incubation with pooled serum from dogs belonging to disease category "Idiopathic epilepsy", bold black graphs from incubation with serum from a dog belonging to "SRMA with symptoms". Graphs show a comparatively strong upregulation of CD11a after incubation with SRMA serum and a separation of a cell subpopulation with a particularly marked upregulation (solid arrow) (x-axis displays fluorescence intensity).

3.5 Discussion

SRMA is a naturally occurring animal model for various systemic vasculitides of unknown origin in humans. Especially Kawasaki Syndrome (KS) and SRMA bear many similarities (Burns et al., 1991). In both diseases an involvement of the meninges occurs (in ≈ 25 % of KS patients) and in each of the disorders increased IgA levels are detected without their deposition being responsible for initiating vascular lesions (Rowley and Shulman, 1998; Tipold et al., 1995). As an animal model SRMA is of a great value due to its comparatively high incidence. As one of the most frequently occurring canine CNS inflammations, it accounts for approximately 15 % of these cases (Tipold, 1995). Since the systemic disease SRMA shows a strong tendency to manifest in the cervical meninges, findings in this animal model might as well be applied to other medical conditions, in which a compartmentalization of the immune response occurs. Extensive studies on this canine disease will help to elucidate pathogenetical principles of the development of immune mediated meningitides and immune response compartmentalization in general. This in long-term might give rise to the development of new treatment strategies for both, humans and dogs. The aim of this study was to quantify integrin expression on PMNs in SRMA, as one striking feature of the disease is the migration of enormous numbers of neutrophils into CSF (Tipold and Jaggy, 1994) and integrins are crucial for this extravasation (Tuomanen et al., 1989). It was shown that this attraction of neutrophils in SRMA is, at least partially, due to the presence of IL-8 or an IL-8-like factor in the CSF (Burgener et al., 1998).

Our study showed that neutrophils of dogs with symptoms of SRMA exhibit stronger CD11a expression than those of the remaining disease categories, whereas expression of CD11b and c was comparable among all groups. The wide range of integrin expression is likely to reflect

the situation that patients with SRMA are presented in different periods of the symptomatic phase. In some dogs, a compensatory endogenous anti-inflammatory response might already have been initiated, as it was shown to occur in canines with multiple organ dysfunction syndrome secondary to sepsis (Weiss et al., 2004). In the cited study examinations were performed for the β_2 integrin CD11b/CD18 only. It seems reasonable, however, to assume that such an anti-inflammatory reaction also affects CD11a/CD18 and CD11c/CD18. Our finding that the expression of investigated integrins correlated positively with one another supports this assumption. The natural course of SRMA, with its clinically silent periods in between periods with fever and cervical pain, might be attributable to such compensatory mechanisms. A reduced migration due to decreased CD11a-mediated endothelial adhesion would explain that no or only few neutrophils are present in the subarachnoid space in these asymptomatic periods (Phillipson et al., 2006; Tuomanen et al., 1989) while chemotactic activity of the CSF remains high (Burgener et al., 1998). Since glucocorticosteroids are known to reduce β_2 integrin expression on activated human neutrophils *in vitro*, assumedly via induction of downregulating proteins (Filep et al., 1997), and > 90 % of our patients with SRMA without symptoms were under prednisolone therapy, it would be extremely interesting in this regard to monitor integrin expressions in the natural course of disease with its waxing and waning pattern. Due to the highly painful nature of SRMA, however, ethical considerations prohibit such an approach. The expression of all integrins in peripheral blood correlated positively with the severity of neutrophilic leukocytosis in the state of inflammation. Thus, a strong systemic immune response was accompanied by high β_2 integrin expression. This was most obvious for CD11a and led to the suspicion that this particular surface molecule plays a major role in the pathogenesis of the suppurative meningitis as the

cardinal manifestation of SRMA. This molecule was shown to be essential for neutrophil adhesion to endothelial cells, which in turn is a crucial step within the leukocyte recruitment cascade (Phillipson et al., 2006). The selective upregulation of CD11a is in accordance with results of Sahin et al. (1996) who investigated neutrophil adhesion properties in Behçet´s disease, a human vasculitis with recurrent episodes of remission and possible affection of the CNS (Hirohata and Kikuchi, 2003). As in SRMA elevated IgA levels are detected in serum (Sunakawa and Ohshio, 1989) and, if the CNS is affected, in CSF (Jongen et al., 1992). Their *in vitro* experiments led to the insight that patients´ sera cause an upregulation of CD11a on neutrophils deriving from healthy controls whereas CD11b expression is unaffected. This coincides with increased adhesive properties. Furthermore, blockage with anti-CD11a mAbs causes a more pronounced inhibition of neutrophil adhesion to cultured human umbilical vascular endothelial cells (HUVECs) than does blockage with anti-CD11b mAbs.

The second part of our experiment supports our *in vivo* findings in peripheral blood. Initially non-activated neutrophils expressed CD11a to a greater extent after incubation with 75 % of SRMA patients´ sera when compared to a serum pool of dogs with idiopathic epilepsy. In contrast to this, CD11b and c expression was higher in only 17 % and 8 %, respectively, meaning that the vast majority of sera caused a decreased CD11b and c upregulation in comparison to the epilepsy pool. The three dogs that had relative fluorescence values < 100, i.e. their sera induced less CD11a than did sera form epileptic dogs, exhibited either extremely high or low *in vivo* CD11a expressions. This discrepancy of high CD11a expression *in vivo* and poor stimulatory properties *in vitro* could be explained with an anti-inflammatory response that was already initiated *in vivo*, without having affected integrin levels on neutrophils yet.

We suppose that sera of diseased dogs contain one or multiple factors that exhibit a selective boosting effect on CD11a whereas CD11b and c expressions remain unaffected or are even depressed. One can suspect that high CD11a expression in SRMA reflects high expression of the functional CD11a/CD18 molecule. In the past, a considerable redundancy between the function of β_2 integrins was suspected. A recent study using an *in vivo* approach with CD11a and CD11b knock-out mice, however, could demonstrate fundamentally different roles within the neutrophil recruitment cascade. While initial adhesion is mediated by CD11a, subsequent crawling along endothelium was shown to be largely dependent on CD11b (Phillipson et al., 2006). High CD11a expression in SRMA therefore is most likely associated with increased neutrophil adhesion properties to endothelial cells. This eventually would facilitate neutrophil migration to CSF. The depression of the factor causing the CD11a upregulation in SRMA and changes on endothelial cells in the natural course of disease as well as under treatment might be the cause for the absence of neutrophils in CSF of dogs in subclinical periods despite the persistence of increased chemotactic activity in CSF (Burgener et al., 1998).

Comparison of integrin expression on neutrophils in peripheral blood and CSF led to variable results. In a marked number of dogs (39 %) lower CD11a expression on cells isolated from CSF was measured. Since it is known that CD11a is an important molecule for neutrophils´ crossing of the blood-CSF barrier, it is conceivable, that in these patients a previously present upregulation of CD11a had already been reversed, either by a distinct milieu of stimulating factors in CSF or due to changes that occur in perishing neutrophils. It is known from *ex vivo* examinations that especially neutrophils have only a short survival time in CSF (Steele et al., 1986).

Taking into account the results of Weiss et al. (2004) who showed that in dogs with non-septic and septic inflammatory diseases an increase in CD11b and decrease in CD18 on

peripheral blood neutrophils occurs, integrin expression pattern in SRMA, with its selective increase in CD11a, seems to differ from that in other inflammatory conditions.

3.6 Conclusion

Our results demonstrate a disproportionally strong upregulation of CD11a on neutrophils in peripheral blood of dogs with SRMA, whereas CD11b and c expressions are comparable to those in control groups. Since incubation of sera from dogs in a clinically apparent phase of SRMA with initially non-activated neutrophils results in a comparatively strong CD11a expression, whereas CD11b and c expressions remain lower than after incubation with control sera, we hypothesize that SRMA serum contains one or multiple factors that promote this selective increase of CD11a. CD11a is highly suspicious to account for the massive invasion of neutrophils into the CSF and therefore might play a major role in the pathogenesis of the disease.

3.7 Acknowledgments

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Chapter 4: Marked MMP-2 transcriptional up-regulation in mononuclear leukocytes invading the subarachnoidal space in aseptic suppurative Steroid-responsive Meningitis-Arteritis in dogs

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4.1 Abstract

Canine Steroid-responsive Meningitis-Arteritis (SRMA) is a suitable animal model for studies on the development of neutrophilic pleocytosis in aseptic meningitis.

Samples of dogs in the acute phase of SRMA (n = 16) were examined for gene expression of matrix metalloproteinases (MMP) -2 and -9 and tissue inhibitors of metalloproteinases (TIMP) -1 and -2. Results were compared to those of dogs under glucocorticosteroid treatment for SRMA (n = 16) and dogs with other inflammatory and neoplastic diseases of the central nervous system (CNS) (n = 19). Samples included mononuclear (PBMNCs) and polymorphonuclear cells (PBPMNs) of peripheral blood and cerebrospinal fluid white blood cells (CSF WBCs).

In the acute phase of SRMA CSF WBCs showed mRNA expression for MMP-2 and -9 and TIMP-1 and -2, highlighting a contribution of these cells to the overall content of MMPs and TIMPs in CSF. MMP-2 mRNA levels in CSF WBCs were significantly up-regulated in comparison to PBMNC expression levels, suggesting that MMP-2 is relevant for PBMNC invasion into the subarachnoidal space and that the expression is influenced by migratory activity through the blood-CSF-barrier.

Keywords: Canine, Blood-brain-barrier, Leukocyte extravasation, Matrix metalloproteinases

Abbreviations: CBC = complete blood count; cDNA = complementary DNA; CSF = cerebrospinal fluid; CSF WBCs = cerebrospinal fluid white blood cells; CNS = central nervous system; EF = elongation factor; GAPDH = Glyceraldehyd-3-phosphate dehydrogenase; MMP = matrix metalloproteinase; PBMNCs = peripheral blood mononuclear cells; PBPMNs = peripheral blood polymorphonuclear cells; SRMA = Steroid-responsive Meningitis-Arteritis; TIMP = tissue inhibitors of metalloproteinases

4.2 Introduction

Aseptic meningitis with predominance of polymorphonuclear cells (PMNs) is diagnosed in the human (Amir et al., 1991; Negrini et al., 2000) and in the canine patient (Tipold, 1995). However, understanding of principles that lead to the development of these suppurative meningitides is still incomplete.

Since standards for diagnostic work-up and treatment of companion animals, in particular the dog, are similar to that in human patients, investigation of analogous veterinary diseases offers a great opportunity to increase the knowledge about pathomechanisms in both species. While bacterially induced inflammation of the meninges is rare in dogs (Radaelli and Platt, 2002), by far the most frequently occurring canine disease associated with a polymorphonuclear pleocytosis is Steroid-responsive Meningitis-Arteritis (SRMA) (Meric, 1988; Tipold, 1995). This meningitis is associated with inflammation of small to medium-sized arteries, predominantely within the spinal meninges (Brooks, 1984; Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994). To date every attempt to identify a causative infectious agent has failed and the inflammation is believed to result from a dysregulation of the immune system (Cizinauskas et al., 2000; Harcourt, 1978; Meric et al., 1986; Meric et al., 1985; Poncelet and Balligand, 1993; Scott-Moncrieff et al., 1992; Tipold and Jaggy, 1994). Steroid-responsive Meningitis-Arteritis is associated with cerebrospinal fluid (CSF) neutrophil counts of several thousands per microliter and thus accounts for an ideal animal model to study neutrophil invasion into the central nervous system (CNS), especially since follow-up examinations with repeated CSF taps are part of the routine treatment protocol.

Entry of cells and molecules from the peripheral blood directly into the subarachnoidal space is limited and controlled by the special architecture of the choroid plexus and the pial vessels

(Allt and Lawrenson, 1997; Ransohoff et al., 2003). Opening of this protective shield with its tight junctions and underlying basement membrane is required to allow extravasation of leukocytes into the CSF.

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases that are involved in remodelling of the extracellular matrix and also function as activators of latent forms of MMPs and cytokines. These enzymes may thus exhibit both, beneficial and detrimental effects. To counteract the potentially harmful actions, the MMP production and activation of their proforms is tightly regulated (Candelario-Jalil et al., 2009; Yong et al., 2001). Important in this respect are the tissue inhibitors of metalloproteinases (TIMPs) of which TIMP-1 forms a specific complex with proMMP-9 and TIMP-2 with proMMP-2 (Brew et al., 2000).

Experimental studies have shown that MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are potent substances to interfere with the integrity of the barrier between blood circulation and CNS mainly by degradation of type IV collagen, the main component of the basement membrane (Mun-Bryce et al., 2002; Mun-Bryce and Rosenberg, 1998; Paul et al., 1998; Rosenberg et al., 1992; Timpl, 1989). These enzymes have also been implicated in the development of systemic vasculitis (Gavin et al., 2003). We therefore hypothesized that leukocytes migrating from peripheral blood into the cerebrospinal fluid might increase their transcriptional activity for MMPs as one important mechanism to allow crossing-over from the vascular bed into the subarchnoidal space in SRMA. and that counter-regulation of their respective tissue inhibitors may take place.

4.3 Materials and Methods

4.3.1 Animals and Samples

Samples used for mRNA quantitation included pellets containing three different kinds of cells: peripheral blood mononuclear cells (PBMNC), peripheral blood polymorphonuclear cells (PBPMN) and white blood cells in cerebrospinal fluid (CSF WBC).

These samples derived from client-owned dogs that were presented to the Department of Small Animal Medicine and Surgery, University of Veterinary Medicine, Hannover, Germany, for the diagnostic work-up of neurological signs. According to the diagnoses, animals were ascribed to one of the following groups: "SRMA with symptoms", "SRMA without symptoms", "CNS inflammation" and "CNS neoplasia" (Table 1).

Table 1: Disease categories (age given as median and range)

Disease categories	Number of dogs	Inclusion criteria	Age [years]
SRMA with symptoms	16	Dogs showing symptoms of SRMA at time of sampling	0.75 (0.5 – 1.5)
SRMA without symptoms	16	Dogs with SRMA that were under glucocorticosteroid treatment and did not show symptoms at time of sampling (≈ 1 mg/kg SID; follow-up examinations)	1.0 (0.5 – 5.0)
CNS inflammation	9	Dogs with CNS inflammation[a] other than SRMA	7.75 (1.0 – 8.75)
CNS neoplasia	10	Dogs with CNS neoplasia[a] (no tumors of the hematopoetic system included)	9.0 (5.5 – 14.5)

SRMA = Steroid-responsive Meningitis-Arteritis; SID = once per day; [a] = including the nerve roots; CNS = central nervous system

Each patient was subject to a general and neurological examination, which was followed by a complete blood count (CBC), routine blood chemistry and urinalysis. In addition, a suboccipital CSF tap and CSF analysis (WBC count, cytomorphological differentiation and total protein) was performed in every dog. Samples containing erythrocytes were included only if xanthochromia, suggesting *in vivo* leakage of red blood cells into the subarachnoid space, was present (Jamison and Lumsden, 1988). Immunoglobulin A (IgA) content in serum and CSF was determined in all animals using a previously described enzyme-linked immunosorbent assay (ELISA) system to support diagnosis of SRMA (Tipold et al., 1994). Ancillary tests performed to obtain a definitive diagnosis included radiographic studies, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI) as well as electrodiagnostic testing. If available, results of tests for infectious agents and of pathological examinations contributed to the diagnoses. A history of glucocorticosteroid administration within one week prior to presentation (within 6 weeks if depot formulations were given) was recorded for each patient.

Diagnosis of SRMA was based on the combination of the following findings: pain upon cervical palpation, fever, marked neutrophilic leuko- and pleocytosis, immediate and sustained response to glucocorticosteroid treatment, exclusion of other disease processes that may cause similar symptoms. Concomitant elevation of IgA levels in serum and CSF supported this diagnosis (Tipold and Jaggy, 1994).

Separation of WBC populations deriving from peripheral blood was achieved by means of density gradient centrifugation with two subsequent washing steps (Schwartz et al., 2008a; Schwartz et al., 2008b). Cerebrospinal fluid WBCs were spun down (200 x g; 10 min; room temperature) and supernatant was removed, therefore pellets contained a mixture of WBC

populations. Cell pellets were immediately frozen and stored at - 80°C until RNA isolation was undertaken.

In a few cases not all of the three kinds of cell pellets were available for investigation of mRNA expression. Two of the 16 dogs in “SRMA with symptoms” were lacking a PBPMN pellet, in 1/9 dogs in “CNS inflammation” a PBMNC pellet was available only and in 2/10 dogs in “CNS neoplasia” a PBPMN pellet was missing.

4.3.2 RNA purification and reverse transcription

Purification of total RNA from the cell pellets was performed using RNeasy Mini Kit columns (Qiagen) according to the manufacturer’s protocol for animal cells. To prevent contamination with genomic DNA an on-column DNase digestion step with RNase-free DNase (Qiagen) following the manufacturer’s instructions was included. The yield of RNA was subsequently determined with a GeneQuant pro device (Biochrom Ltd.) by means of spectrophotometry at OD_{260}. The remaining eluate was immediately placed in liquid nitrogen and then stored at - 80°C until reverse transcription.

Reverse transcription of RNA into complementary DNA (cDNA) was performed with Omniscript RT (Qiagen), Random Primers (Promega) and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen). Forty microliters of master mix containing ≤ 2 µg of total RNA was produced according to Qiagen’s protocol for reverse transcription. Incubation for 60 min at 37°C took place in a thermocycler (PTC-200 Peltier Thermal Cycler; MJ Research). Complementary DNA was stored at - 20°C in aliquots until its use in real-time PCR experiments.

4.3.3 Primers

Primers used in this study (Table 2 and 3) were either taken from the literature (as cited) or designed with Primer3 software (Rozen and Skaletsky, 2000) or Beacon Designer version 2.1 software (Premier Biosoft International). The probe for MMP-9 was designed with the latter program. Primers and probe were purchased from Eurofins MWG Operon. The specificity of each PCR product was confirmed by DNA sequencing (AGOWA genomics).

Table 2: Primers for production of standard dilution series

Target gene	Primer sequence in 5´- 3´ (forward primers on top, reverse primers on bottom)	Size of amplicon [bp]	GenBank accession number
MMP-2[a]	CCAAGAACTTCCGTCTGTCC AGCTATGACCACTGCCTTGC	606	AF177217
MMP-9[a]	GAGGTTCGACGTGAAGGCGCAGAT AGGTCACGTAGCCCACTTCGTCCAC	200	J05070
TIMP-1[b]	GCGTTATGAGATCAAGATGAC CTGGTCCGTCCACAAGCA	345	AF077817
TIMP-2[c,a]	GCAGAYGTAGTGATCAGRGR GTTYAGGCTCTTCTTCTGGG	315	AF095638
GAPDH[a]	AAGGTCGGAGTCAACGGATT GCAGAAGAAGCAGAGATGATG	365	AB038240
β-Actin	AACACCCCAGCCATGTATGT CTTCTCCAGGGAGGACGAG	333	NM_001003349
EF-1α[d]	AGCCCTTGCGCCTGCCTCTC CAGACACATTCTTGACATTGAAGC	219	X03558

bp = base pairs; TIMP = tissue inhibitor of matrix metalloproteinases; MMP = matrix metalloproteinase; GAPDH = Glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor; [a] Puff et al. (2008); [b] Barnes et al. (2000); [c] Clegg et al. (1999); [d] von Smolinski et al. (2005)

Table 3: Primers for real-time PCR

Target gene	Primer sequence in 5´- 3´(forward primers on top, reverse primers on bottom)	Size of amplicon [bp]	GenBank accession number
MMP-2[a]	GGAGATCTTCTTCTTCAAGGACCG AGAATGTGGCTACCAGCAGGG	89	AF177217
MMP-9[a]	CATGACATCTTCCAGTACCAAG GGTTCACCTCATTCCGAGAA Probe: FAM-CTACTTCTGCCAGGACCGCTTCTACT-TAMRA	85	AB006421
TIMP-1[a]	ACGGACACTTGCAGATCAAC GCAGCATAGGTCTTGGTGAA	94	AF077817
TIMP-2[a]	CCATCAAGCGGATTCAGT GGAAGGAGCCGTGTAGATAA	89	AF095638
GAPDH[a]	GTCATCAACGGGAAGTCCATCTC AACATACTCAGCACCAGCATCAC	84	AB038240
β-Actin	TCTACGAGGGGTACGCCTTG TTCCTTGATGTCACGCACGAT	149	NM_001003349
EF-1α[b]	AGCCCTTGCGCCTGCCTCTC CAGACACATTCTTGACATTGAAGC	219	X03558

bp = base pairs; TIMP = tissue inhibitor of matrix metalloproteinases; MMP = matrix metalloproteinase; GAPDH = Glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor; [a] Puff et al. (2008); [b] von Smolinski et al. (2005)

4.3.4 Standard dilution series

In order to allow calculation of standard curves tenfold dilution series (10^2 – 10^8 copies) were produced for each target gene. These dilution series also served as standards for inter-run calibration to account for run-to-run variation. Amplicons were produced by conventional end-point PCR (PTC-200 Peltier Thermal Cycler; MJ Research), agarose gel electrophoresis and DNA extraction with NucleoSpin Extract II columns (Macherey-Nagel) according to the manufacturer's protocol. Spectrophotometrical determination of DNA concentration was performed at OD_{260} (GeneQuant pro; Biochrom Ltd.) and copy numbers per volume were calculated according to the following formula: copies/µl = 6 x 10^{23} x DNA concentration (ng/µl) x 10^{-9}/[size of amplicon (base pairs) x 660].

Template was cDNA from DH-82 cells (Wellman et al., 1988). Reaction conditions were as follows: initial denaturation at 94°C for 1 minute; 40 cycles of denaturation at 94°C for 1 minute, annealing at 59°C (TIMP-1, TIMP-2, MMP-2, MMP-9, GAPDH) / 60°C (β-Actin, EF-1α) for 2 minutes and elongation at 72°C for 1 minute; final elongation at 72°C for 5 minutes. Amplification was achieved with recombinant *Taq* DNA Polymerase (Invitrogen) in 1x PCR Buffer (Invitrogen) with 1.25 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP mixture (Applied Biosystems) and 300 nM of each primer (Table 2).

4.3.5 Real-time PCR

Real-time PCRs were run on an Mx3005P QPCR system (Stratagene) with Brilliant SYBR Green QPCR Core Reagent Kit (Stratagene) in 8x Strip Tubes with Optical Cap (Stratagene). Due to the number of samples, experiments for each target gene were run on separate plates for PBMNC, PBPMN and CSF WBC samples. Measurements were conducted the same day

using the identical standard dilution series. Standards were kept at + 4°C in-between runs. The plates included the standard dilution series as well as a no template control (NTC) in duplicate. Reaction volume was 25 µl including 1 µl template per reaction.

Reaction conditions were as follows: initial denaturation at 95°C for 10 minutes; 40 cycles with denaturation at 95°C for 30 seconds, annealing at 57°C (TIMP-1, TIMP-2) / 60°C (β-Actin, EF-1α) / 64°C (MMP-2, GAPDH) for 1 minute and elongation at 72°C for 30 seconds; final extension at 72°C for 1 minute; dissociation program beginning at 95°C for 1 minute followed by increasing temperature by 0.5°C every 30 seconds from 55°C to 95°C. The master mix contained 0.025 U/µl SureStart *Taq* DNA Polymerase in 1x core PCR buffer with 2.5 mM (MMP-2, GAPDH) / 4.0 mM (TIMP-1, TIMP-2) / 5.0 mM (β-Actin, EF-1α) $MgCl_2$, 800 µM dNTP mix, 150 nM of each primer (Table 3), 8 % glycerol and 4 % dimethyl sulphoxide (DMSO) (3 % for MMP-2, GAPDH). Final SYBR Green concentration was 0.25x and Rox was used as a reference dye at 30 nM.

The constitution of the master mix for MMP-9 mRNA quantification differed because a sequence specific probe was used (Table 3). It contained 0.025 U/µl SureStart *Taq* DNA Polymerase in 1 x core PCR buffer with 5.0 mM $MgCl_2$, 800 µM dNTP mix, 300 nM of each primer, 200 nM of TaqMan probe and 76 nM Rox. The thermal profile was: initial denaturation at 95°C for 10 minutes; 40 cycles with 95°C for 15 seconds and 60°C for 1 minute.

The software tool “Multiple Experiment Analysis” (MxPro QPCR Software v4.01; Stratagene) allowed run-to-run comparison between the results for PBMNC, PBPMN and CSF WBC samples.

To account for variations in sample input, extraction and reaction efficiencies and presence of inhibitors, copy numbers of the genes of interest were normalized against the geometric mean of the internal reference genes GAPDH, β-Actin and EF-1α: $copies_{normalized}$ (target gene) [%] = $copies_{non\text{-}normalized}$ (target gene) x 100/ [copies (GAPDH) x copies (β-Actin) x copies (EF-1α)]^(1/3).

4.3.6 Statistical analysis

In addition to descriptive methods the Wilcoxon rank sum test was applied for comparison of the results deriving from the different disease categories. For these comparisons results of the groups "CNS inflammation" and "CNS neoplasia" were combined. It was also tested whether results for dogs with a history of glucocorticosteroid pretreatment differed significantly from those of the non-pretreated ones. The Wilcoxon signed-rank test served as a tool to compare values deriving from PB and CSF of dogs in the acute phase of SRMA. The Spearman´s rank correlation coefficients were calculated to detect correlations for "SRMA with symptoms". The parameters included normalized copy numbers and results of CBC and CSF analysis. Statistical significance was set at the 5% level ($p \leq 0.05$).

4.4 Results

Dissociation procedures at the end of real-time PCRs for MMP-2, TIMP-1, TIMP-2, GAPDH, β-Actin and EF-1α produced clearly defined single melting temperatures at 82,9°C, 84,4°C, 76,9°C, 81,1°C, 85,4°C and 82,3°C respectively. PCR products for these genes were considered specific because temperatures corresponded with those of the standard dilution

series. These standards contained a single gene product only that had been sequenced and recognized as the respective gene of interest.

Copy numbers for the three reference genes could be measured in every PBMNC and PBPMN sample and normalization was therefore always possible. In 9 CSF WBC samples, however, copy numbers for only 1 or 2 of the reference genes could be determined and these samples were excluded from further analysis since accurate normalization was not possible. In these cases a mild degree of pleocytosis or a normal CSF cell count reduced the size of the cell pellets, containing considerably less leukocytes than those of the other dogs.

Pretreatment with glucocorticosteroids, after exclusion of “SRMA without symptoms”, was recorded for 8 dogs (4/16 in “SRMA with symptoms”, 3/9 in “CNS inflammation”, 1/10 in “CNS neoplasia”) and did not cause significantly differing results neither in CBC and CSF analysis nor in mRNA expression, except for a more severe pleocytosis and higher CSF IgA levels in pretreated dogs ($p < 0.05$ and $p < 0.01$, respectively). Considering dogs in the acute phase of SRMA only, no differences were detected in pretreated and not pretreated individuals. Pretreated patients were therefore not excluded from further analysis.

Results of MMP-2 mRNA expression analysis are displayed in Figure 1. In most dogs no mRNA expression could be recorded in PBMNC samples. Exceptions were 1/16 dogs from the group “SRMA with symptoms” and 3/16 dogs from “SRMA without symptoms”. No or only slight expression was detected in PBPMN pellets. In contrast, 15/16 CSF WBCs samples from dogs in the acute phase of SRMA showed clear evidence for mRNA expression. Normalized copy numbers were significantly higher than those in samples from PBMNCs and PBPMNs ($p \leq 0.001$). The 4 positive CSF samples not belonging to dogs in the acute phase of SRMA all derived from dogs that also had marked pleocytoses with considerable

participation of neutrophils (50 – 89 %). Two of these dogs suffered from bacterial meningomyelitis, in the other 2 individuals an intracranial meningioma was diagnosed.

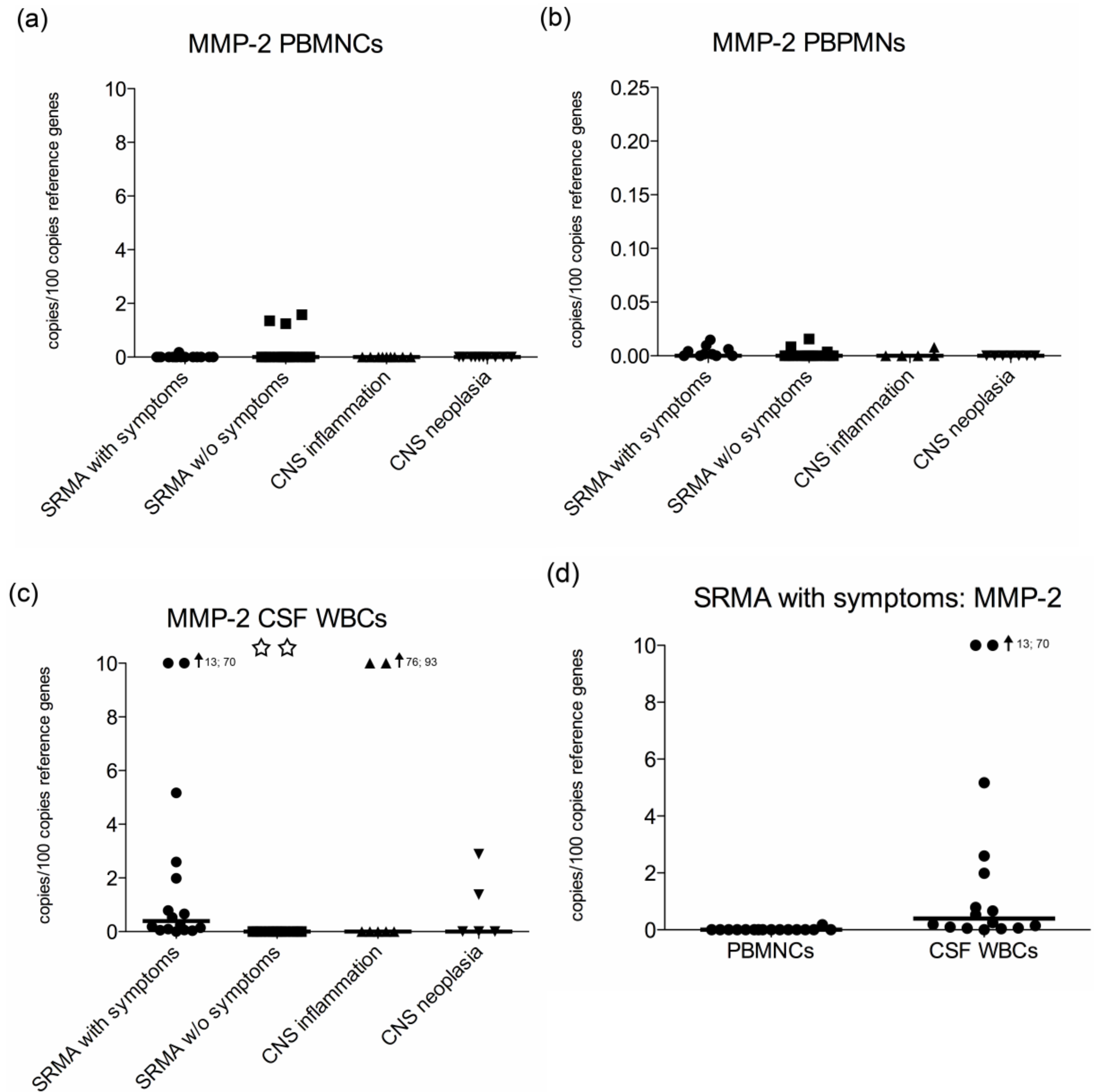

Figure 1: Results of real-time PCR for MMP-2 mRNA expression of PBMNCs (a), PBPMNs (b) and CSF WBCs (c) in all disease categories and PBMNCs + CSF WBCs in the acute phase of SRMA (d). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-Actin and EF-1α and are given as the percentage of the respective

geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category "SRMA with symptoms" ($p \leq 0.01$). For statistical analysis samples from "CNS inflammation" and "CNS neoplasia" were grouped as one disease category. Please note the non-uniform scaling of the diagrams (MMP = matrix metalloproteinase; PBMNCs = peripheral blood mononuclear cells; PBPMNs = peripheral blood polymorphonuclear cells; CSF WBCs = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = Glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor)**

Messenger RNA expression for MMP-9 is visualized in Figure 2. Expression in PBMNCs was lowest in "SRMA with symptoms" and the overall expression in this cell type was markedly below that of PBPMNs ($p \leq 0.001$). In these, values of dogs in acute stage of SRMA were comparable to those of dogs suffering from other CNS inflammations than SRMA ($p = 0.815$) and showed higher MMP-9 mRNA content than individuals from "SRMA without symptoms" ($p \leq 0.05$) and "CNS neoplasia" ($p = 0.07$). In 15/16 CSF WBC pellets from dogs with clinical signs due to SRMA mRNA expression of this gene could be noted. The remaining positive CSF samples derived from the two dogs with meningiomas that also showed significant mRNA expression for MMP-2. Levels of MMP-9 mRNA expression in "SRMA with symptoms" were lowest in PBMNCs, intemediate in CSF WBCs and highest in PBPMNs ($p \leq 0.01$).

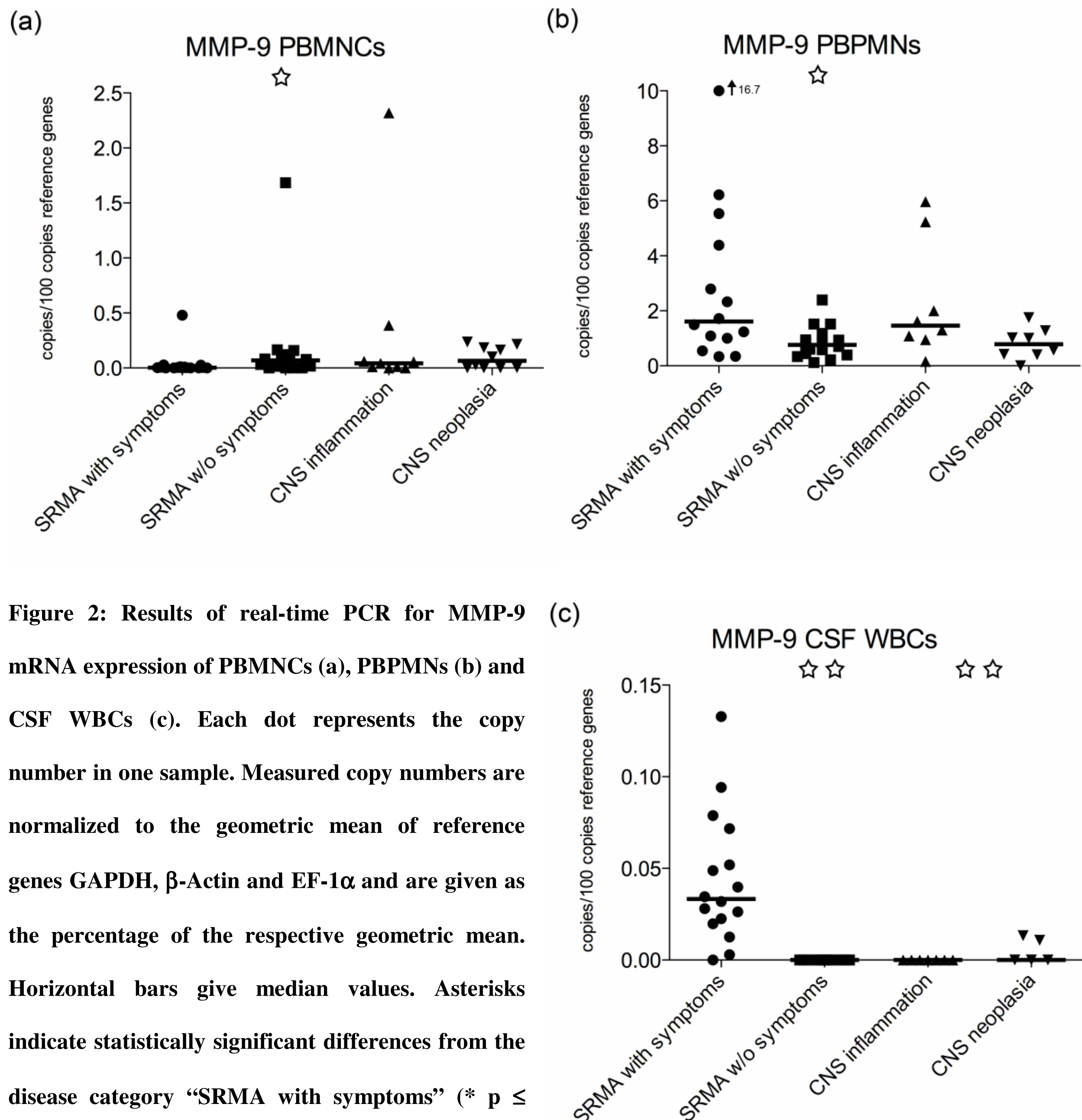

Figure 2: Results of real-time PCR for MMP-9 mRNA expression of PBMNCs (a), PBPMNs (b) and CSF WBCs (c). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-Actin and EF-1α and are given as the percentage of the respective geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category "SRMA with symptoms" (* $p \leq 0.05$; ** $p \leq 0.01$). For statistical analysis samples from "CNS inflammation" and "CNS neoplasia" were grouped as one disease category. Please note the non-uniform scaling of the diagrams (MMP = matrix metalloproteinase; PBMNCs = peripheral blood mononuclear cells; PBPMNs = peripheral blood polymorphonuclear cells; CSF WBCs = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = Glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor)

Figure 3 displays the quantity of TIMP-1 mRNA expression after normalization. Peripheral blood MNCs and CSF WBCs of dogs in the acute phase of SRMA showed the highest expression among the disease categories whereas copy numbers of PBPMN samples are similar within the 4 groups. Messenger RNA expression in PBMNCs and CSF WBCs was within the same magnitude while that of PBPMNs was markedly lower. The difference of CSF WBCs and PBPMNs in the acute phase of SRMA was statistically significant ($p \leq 0.01$).

Normalized values for TIMP-2 mRNA expression are given in Figure 4. Overall, samples from “SRMA with symptoms” showed the lowest level of expression. An exception were those deriving from CSF. In the acute phase of SRMA the amount of mRNA encoding for TIMP-2 per cell was lowest in PBMNCs, intermediate in CSF WBCs and highest in PBPMNs. These differences were statistically significant ($p \leq 0.01$).

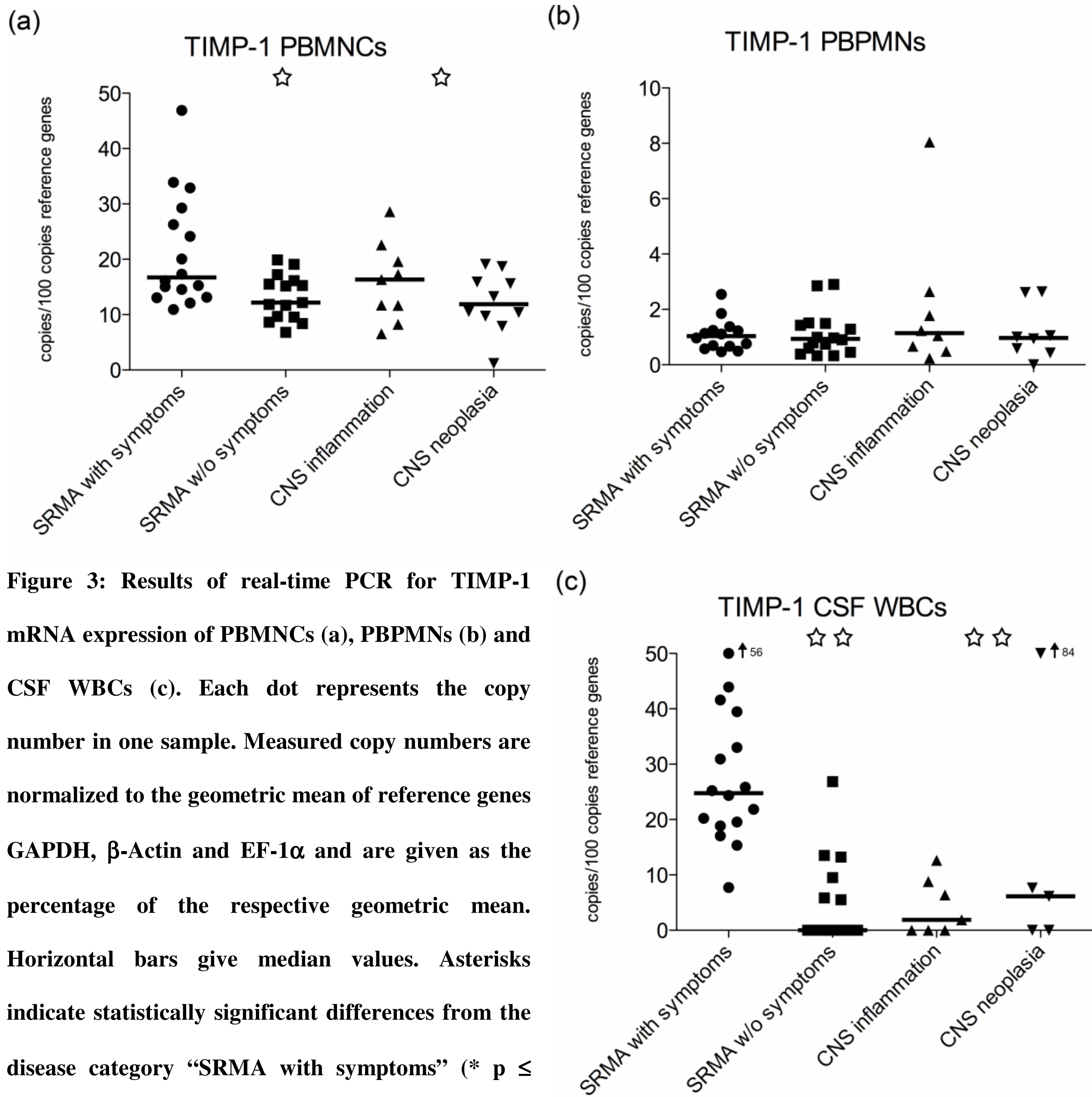

Figure 3: Results of real-time PCR for TIMP-1 mRNA expression of PBMNCs (a), PBPMNs (b) and CSF WBCs (c). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-Actin and EF-1α and are given as the percentage of the respective geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category "SRMA with symptoms" (* $p \leq 0.05$; ** $p \leq 0.01$). For statistical analysis samples from "CNS inflammation" and "CNS neoplasia" were grouped as one disease category. Please note the non-uniform scaling of the diagrams (MMP = matrix metalloproteinase; PBMNCs = peripheral blood mononuclear cells; PBPMNs = peripheral blood polymorphonuclear cells; CSF WBCs = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = Glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor)

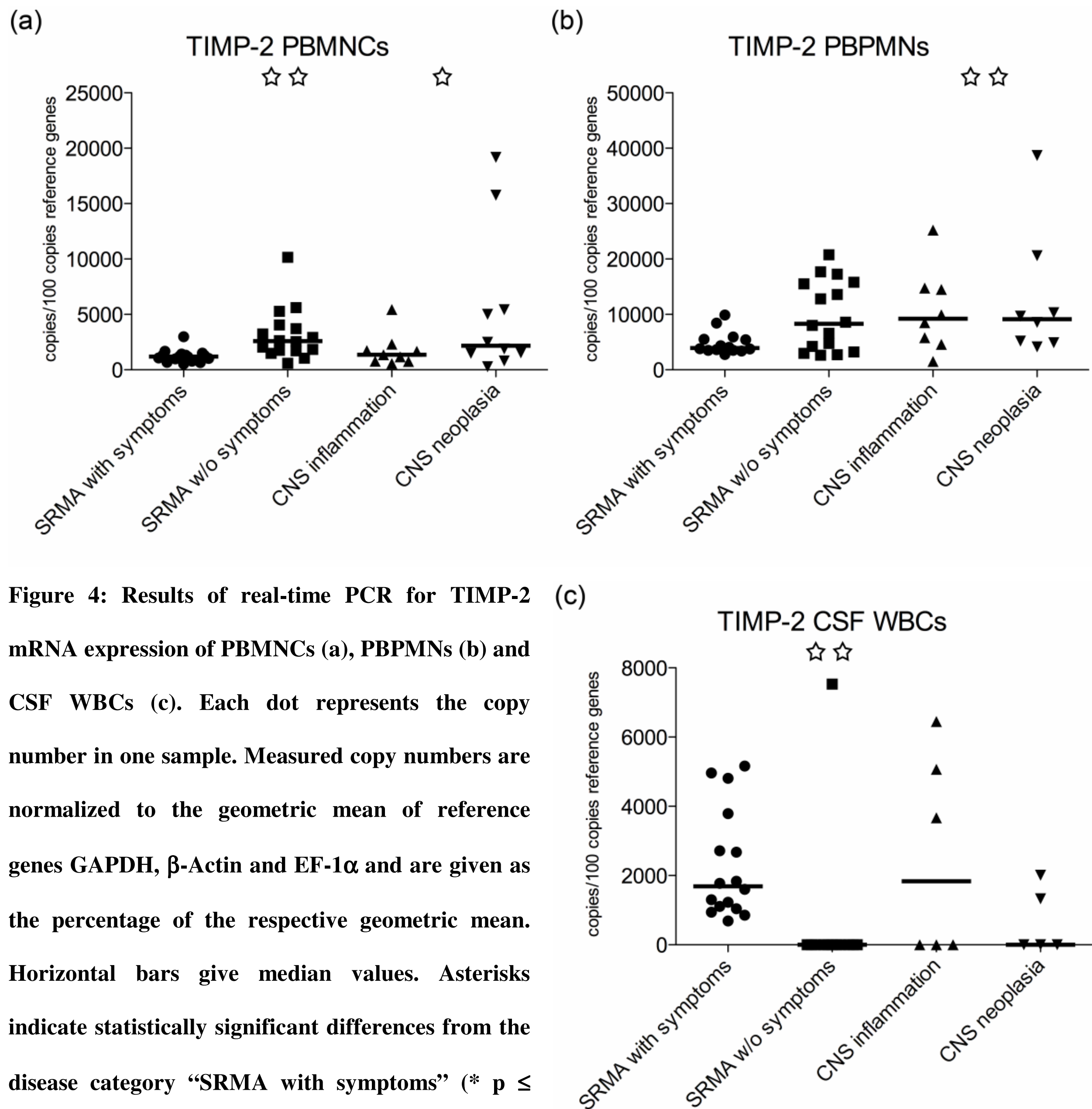

Figure 4: Results of real-time PCR for TIMP-2 mRNA expression of PBMNCs (a), PBPMNs (b) and CSF WBCs (c). Each dot represents the copy number in one sample. Measured copy numbers are normalized to the geometric mean of reference genes GAPDH, β-Actin and EF-1α and are given as the percentage of the respective geometric mean. Horizontal bars give median values. Asterisks indicate statistically significant differences from the disease category "SRMA with symptoms" (* $p \leq 0.05$; ** $p \leq 0.01$). For statistical analysis samples from "CNS inflammation" and "CNS neoplasia" were grouped as one disease category. Please note the non-uniform scaling of the diagrams (MMP = matrix metalloproteinase; PBMNCs = peripheral blood mononuclear cells; PBPMNs = peripheral blood polymorphonuclear cells; CSF WBCs = cerebrospinal fluid white blood cells; SRMA = Steroid-responsive Meningitis-Arteritis; GAPDH = Glyceraldehyd-3-phosphate dehydrogenase; EF = elongation factor)

No statistically significant differences were present for any of the genes between samples of dogs that were under treatment for SRMA and those that derived from individuals with other CNS inflammatory diseases and CNS neoplasias.

Statistically significant correlations and non-significant trends are given in Table 4.

Table 4: Significant correlations and non-significant trends between quantity of mRNA expression and other tested parameters (for the group "SRMA with symptoms")

Parameter	Parameter	Spearman´s rank correlation coefficient	Level of significance
MMP-2 in CSF WBCs	Percentage of lympho-cytes in CSF WBCs	+ 0.600*	p = 0.018
TIMP-1 in PBMNCs	TIMP-2 in PBMNCs	+ 0.744**	p = 0.001
	Degree of pleocytosis	- 0.591*	p = 0.016
TIMP-1 in PBPMNs	TIMP-1 in CSF WBCs	+ 0.556*	p = 0.039
	Degree of neutrophilia in peripheral blood	- 0.481	p = 0.081
TIMP-1 in CSF WBCs	TIMP-2 in CSF WBCs	- 0.632**	p = 0.009
TIMP-2 in PBMNCs	Degree of pleocytosis	- 0.612*	p = 0.012
TIMP-2 in CSF WBCs	Degree of pleocytosis	- 0.471	p = 0.066

SRMA = Steroid-responsive Meningitis-Arteritis; MMP = matrix metalloproteinase; CSF WBCs = white blood cells in cerebrospinal fluid; TIMP = tissue inhibitor of metalloproteinases; PBMNCs = peripheral blood mononuclear cells; PBPMNs = peripheral blood polymorphonuclear cells

4.5 Discussion

Meningitis with predominance of polymorphonuclear cells (PMNs) occurs in the human and canine patient. While, for a long time, these suppurative meningitides have been considered indicative for a bacterial etiology in human medicine, it is now recognized that a considerable

proportion of aseptic meningitis cases show a preponderance of neutrophils (Amir et al., 1991; Negrini et al., 2000) and that these cytomorphological characteristics may persist beyond 24 hours of initial symptoms (Negrini et al., 2000). The disease SRMA may provide a suitable model to increase the understanding of general pathogenetical principles that lead to the development of suppurative meningitis. A particular advantage of this naturally occurring canine model, in comparison to the established experimental rodent and rabbit models, is the possibility to perform repeated CSF taps with yield of large volumes.

Steroid-responsive Meningitis-Arteritis accounts for the majority of meningitis cases in dogs and is among the most frequently diagnosed CNS inflammations in this species (Meric, 1988; Tipold, 1995). It is the most likely cause for marked polymorphonuclear pleocytosis (Tipold, 1995), which is additionally seen in a small percentage of dogs suffering from intracranial meningiomas (Dickinson et al., 2006) and in the rare cases of bacterial CNS infection (Radaelli and Platt, 2002).

To allow development of pleocytosis leukocytes need to pass the tightly controlled barrier between vasculature and CNS. This may take place at different sites: 1. from parenchymal vessels into the perivascular space of brain and spinal cord tissue, 2. at the choroid plexus into the ventricles 3. from blood vessels within the meninges into the subarchnoidal space (Ransohoff et al., 2003). Since perivascular leukocytic infiltrates are lacking within CNS gray and white matter in SRMA (Brooks, 1984; Hayes et al., 1989; Scott-Moncrieff et al., 1992; Tipold et al., 1995), it seems rather unlikely that leukocyte trafficking takes place at this level. A number of leukocytes may certainly enter the CSF via the choroid plexus, the most plausible localization for the bulk of migration, however, are pial blood vessels, especially since their integrity is impaired by concurrent arteritis. And in fact, xanthochromia or non-iatrogenic hemorrhage is regularly seen in CSF samples (Behr and Cauzinille, 2006; Irving

and Chrisman, 1990; Meric et al., 1986), supporting the idea that this site of the blood-CSF-barrier is the one that is easiest to cross. In addition, leakage of albumin into the CSF of diseased dogs was demonstrated, which also indicates loss of integrity of the blood-CSF-barrier (Behr and Cauzinille, 2006).

Matrixmetalloproteinases play an important role in neuroinflammation. They contribute to its perpetuation by conversion of pro-forms of inflammatory molecules into mature forms and allow leukocyte recruitment into the CNS by permeabilizing the protective shield between blood stream and CSF (Candelario-Jalil et al., 2009; Yong et al., 2001). The action of these enzymes, with their potential to cause severe damage to surrounding substrates, is tightly regulated. Steps of regulation include the transcriptional and translational levels as well as the requirement for activation and finally the possibility of their subsequent inactivation (Rosenberg, 2002; Yong et al., 2001). Production of MMPs within the CNS occurs in a multitude of residential and migratory cells. These include endothelial cells, pericytes, astrocytes, oligodendrocytes, microglia, neurons and invading leukocytes (Rosenberg, 2002; Yong et al., 2001).

MMP-2 and -9 were shown to mediate opening of the blood-brain-barrier (Mun-Bryce and Rosenberg, 1998; Rosenberg et al., 1992), which made them good candidates to be investigated in our canine meningitis model. In contrast to most previous studies that have focused on the enzymatic activity in CSF (Bergman et al., 2002; Kolb et al., 1998; Leppert et al., 2000; Levine et al., 2006; Paul et al., 1998; Turba et al., 2007) or transcriptional activity in CNS parenchyma (Kieseier et al., 1999; Leib et al., 2000), we investigated the contribution of invading leukocytes themselves to this phenomenon. In addition, mRNA expression of tissue inhibitors of metalloproteinases, TIMP-2 and TIMP-1, that interact predominantly with MMP-2 and MMP-9 (Brew et al., 2000) was quantitated.

Albeit our measurements of mRNA encoding for MMP-2 in peripheral blood cells of dogs suffering from SRMA revealed only few samples with mRNA content above the detection limit, more than 90 % of CSF WBC specimens from the group “SRMA with symptoms” contained detectable amounts of MMP-2 mRNA with levels exceeding those found in PBMNCs. Also the CSF WBC sample of the dog, that had detectable mRNA in PBMNCs in the acute stage of SRMA, showed a > 25-fold increase of transcriptional activity for MMP-2 in comparison to the value in PBMNCs. The remaining 4 CSF samples, in which MMP-2 mRNA could be detected, all derived from dogs whose CNS pathology was also characterized by a predominantly polymorphonuclear pleocytosis. It was shown that MMP-2 is not produced by neutrophils (Opdenakker et al., 2001), therefore it is highly suspicious that the mononuclear leukocyte population accounts for the MMP-2 mRNA content in CSF samples. Detectable amounts of MMP-2 mRNA in some PBPMN pellets may arise from non-neutrophil PBPMNs. Since differential cell counts revealed predominance of polymorphonuclear cells in all CSF samples and these cells contribute to the quantity of reference genes, the actual increase in mRNA expression will even be stronger than indicated by our data.

Presence of MMP-2 in CSF of healthy individuals was confirmed by multiple studies and these levels remained unchanged in subjects with meningitis characterized by predominance of polymorphonuclear cells (Azeh et al., 1998; Kieseier et al., 1999; Leib et al., 2000; Paul et al., 1998). Messenger RNA expression was also unchanged in CSF WBCs in experimental pneumococcal meningitis in rats (Leib et al., 2001). In contrast to our experimental set-up, however, comparison was drawn to CSF WBC samples at the time of infection and not to expression levels in PBMNCs. In addition, copy numbers were normalized to the single reference gene GAPDH only and up-regulation might have remained undetected if it

coincided with that of GAPDH mRNA. Multiple studies have demonstrated that there is no universally applicable reference gene (Vandesompele et al., 2002) and *in vitro* GAPDH expression was shown to be increased by up to 70-fold in stimulated human lymphocytes (Bas et al., 2004). Vandesompele et al. (2002) therefore recommend normalization of reverse-transcriptase real-time PCR data to the geometric mean of at least 3 reference genes. It is conceivable that all PBMNCs that migrate into the subarachnoidal space will up-regulate their MMP-2 mRNA expression to facilitate crossing of the basement membrane, but that the amount of MMP-2 production is not sufficient to change the overall content of MMP-2 in CSF to be detectable by zymography or by ELISA. This assumption is supported by the finding that, conversely to the situation in the CSF, human T lymphocytes constitutively express MMP-9 and MMP-2 only upon stimulation. This MMP-2 expression is dose-dependant and activation is accompanied by increased migratory activity through a basement membrane model (Leppert et al., 1995). The fact that in our study MMP-2 mRNA expression levels in CSF WBCs were positively correlated with the proportion of lymphocytes in CSF supports the assumption that these cells largely account for the increase in mRNA encoding for MMP-2. Thus, SRMA seems to be a unique naturally occurring animal model to study MMP mRNA up-regulation in leukocytes that exhibit migratory activity through the blood-CSF-barrier.

Expression of TIMP-2 was present in each CSF WBC sample of dogs in the acute phase of SRMA. The role of TIMP-2 in the regulation of MMP-2 activity is not clear-cut. It was shown that TIMP-2 can either enhance activation of proMMP-2 or inhibit MMP-2 activity in a dose-dependent manner (Brew et al., 2000; Rosenberg et al., 1992). This inhibitor might therefore modulate the migration behavior of leukocytes into the CSF of dogs with SRMA.

We were able to show that leukocytes that have invaded the subarachnoidal space in the course of SRMA, exhibited transcriptional activity for MMP-9. This might be attributable to all participating cell populations as PBMNCs and PBPMNs both produce mRNA encoding for MMP-9. Given the fact that MMP-9 activity in CSF is a constant finding in bacterial meningitis (Azeh et al., 1998; Kieseier et al., 1999; Leib et al., 2000; Leppert et al., 2000; Lindberg et al., 2006; Paul et al., 1998) it seems plausible that leukocytes and their MMP-9 production also contribute to the pathogenesis of SRMA, amongst others by altering the integrity of the blood-CSF-barrier (Mun-Bryce and Rosenberg, 1998). Other studies have suggested that, beside leukocytes, also parenchymal cells up-regulate their production of MMP-9 and thus contribute to its activity in CSF (Leppert et al., 2000; Lindberg et al., 2006; Paul et al., 1998).

In addition, leukocytes that had entered the subarachnoidal space in the acute stage of SRMA also revealed transcriptional activity for TIMP-1, which inhibits MMP-9 activity by formation of a strong bond (Rosenberg, 2002). Detection of TIMP-1 in the CSF is a consistent finding in healthy controls and concentration is increased in meningitis patients (Kolb et al., 1998; Leppert et al., 2000; Paul et al., 1998). Our results support the idea that increase of TIMP-1 in CNS inflammation is at least partially due to invading leukocytes.

Steroid-responsive Meningitis-Arteritis is treated with long-term courses of glucocorticosteroids. One of the multiple modes of action by which glucocorticosteroids cause suppression of inflammation is the reduction of transcriptional activity for MMP-2 and -9 which was shown to follow drug application in the human (Aljada et al., 2001; Cundall et al., 2003). Our results with no detectable MMP-2 expression in PBMNCs of the majority of dogs under treatment and decreased MMP-9 expression in PBPMNs after initiation of treatment are in line with these findings.

4.6 Conclusion

Our data clearly show that transcriptional activity for MMP-2 increases markedly in mononuclear cells that have invaded the subarachnoidal space. This suggests that, although overall levels of concentration and activity are unchanged in meningitis, MMP-2 plays its role in migration of PBMNCs through the blood-CSF-barrier. In addition, we could demonstrate that leukocytes that have migrated into the CSF in meningitis express mRNA encoding for MMP-9 and TIMP-1 and -2. It is therefore highly likely that these cells themselves contribute to MMP and TIMP content in CSF.

4.7 Acknowlegements

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4.8 Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Chapter 5: General Discussion

Steroid-responsive Meningitis-Arteritis is a systemic inflammatory disease in dogs that predominantly manifests within the cervical meninges. It accounts for one of the most frequently diagnosed inflammatory disorders involving the CNS (Tipold, 1995) and is suspected to arise from a dysregulation of the immune system. Hallmarks of the disease are increased systemic and intrathecal IgA concentrations (Cizinauskas et al., 2000; Tipold and Jaggy, 1994) and a marked neutrophilic pleocytosis in the acute stage (Behr and Cauzinille, 2006; Harcourt, 1978; Tipold and Jaggy, 1994).

Canine SRMA is a recognized animal model for systemic vasculitides in humans (Burns et al., 1991; Felsburg et al., 1992; Snyder et al., 1995) and improvement in our knowledge of the involved pathogenetical principles will help both, the canine and human patient, and may eventually allow the development of more specific treatment strategies for these disorders.

In a previous study we demonstrated that, in addition to increased systemic and intrathecal IgA concentrations (Cizinauskas et al., 2000; Tipold and Jaggy, 1994), SRMA is associated with high proportions of circulating B cells and a high CD4+:CD8α+ lymphocyte ratio in peripheral blood (Schwartz et al., 2008b). In the first part of our study we therefore investigated whether, according to our hypothesis, the inflammation in the acute phase of SRMA is associated with a type 2 immune response with predominance of Th2 cells.

Our results reveal that the immune reaction in the acute form of SRMA is indeed characterized by strong IL-4 and weak IL-2 and IFN-γ expression with IL-5 and -10

expression levels being comparable to control groups. This finding is consistent with an incomplete type 2 skewed immune reaction (Abbas et al., 1996; London et al., 1998; Mosmann and Sad, 1996; O'Garra, 1998; Romagnani, 1997). The situation that affected dogs are usually presented soon after recognition of the first clinical signs may account for the fact that, in the most part, only tendencies could be detected. Complete immune response polarization, either towards Th1 or Th2 dominance, necessitates time to develop (London et al., 1998; O'Garra, 1998), thus explaining a lack of statistically significant differences for most cytokines. The production of IL-4 and -5 in both, PBMNCs and CSF WBCs, is highly likely to contribute to elevated IgA concentrations in SRMA since IL-5 was shown to induce B cell differentiation into IgA-secreting plasma cells (Harriman et al., 1988) and IL-4 to further enhance IgA secretion (Murray et al., 1987). In affected dogs increased IL-10 levels in CSF WBCs in comparison to values in PBMNCs may represent an endogenous counter-regulatory reaction that is initiated to protect the CNS.

At the time of the first recheck following initiation of glucocorticosteroid treatment, the cytokine profile reveals a “depolarization” of the immune response in most patients, with some individuals exhibiting an ongoing polarization towards Th2 dominance. This progressive polarization under the influence of glucocorticosteroids was described earlier (Elenkov, 2004) and may well explain why IgA levels remain elevated throughout treatment in some individuals (Cizinauskas et al., 2000).

Parts two and three of the presented study focused on mechanisms that facilitate the development of the marked neutrophilic pleocytosis in the acute stage of SRMA.

The β_2 integrin expression on PBPMNs was quantitated since these mediate the interaction between white blood cells and endothelial cells allowing the subsequent leukocyte extravasation (Tuomanen et al., 1989). Our results demonstrate that a selective up-regulation of CD11a occurs and that incubation of non-activated PBPMNs with sera of affected dogs also induces this selective up-regulation. CD11a is a surface molecule that is crucial for the neutrophil adhesion to the endothelium, which in turn is essential for the following cell migration towards extravascular sites of inflammation (Phillipson et al., 2006). Low CD11a expression levels under successful glucocorticosteroid treatment in SRMA may be one explanation why no or only few numbers of neutrophils are present in the CSF of treated dogs despite a persistently increased chemotactic activity on PBPMNs in CSF (Burgener et al., 1998). Glucocorticosteroids were shown to exhibit this down-regulatory effect on activated human neutrophils *in vitro* (Filep et al., 1997). The finding that sera of affected dogs induce such a selective CD11a up-regulation on non-activated PBPMNs *in vitro* suggests the presence of one or multiple hitherto unidentified factors that have stimulating properties on CD11a expression, leaving CD11b and c levels unaffected or even suppressing their expression.

We additionally investigated MMP-2 and -9 plus TIMP-1 and -2 expression in leukocytes deriving from peripheral blood and CSF. Matrix metalloproteinases may promote leukocyte invasion into the subarachnoidal space by opening of the blood-CSF-barrier (Mun-Bryce and Rosenberg, 1998; Rosenberg et al., 1992) and endogenous counter-regulation by TIMP production may take place to control potentially harmful effects of MMPs (Brew et al., 2000). Cerebrospinal fluid WBCs causing pleocytosis in the acute form of SRMA exhibit transcriptional activity for the studied MMPs suggesting their contributory role for leukocyte

entry into the subarachnoidal compartment. While previous studies on MMP content in the CSF mainly focused on demonstrating the sole presence of these gelatinases by zymography or ELISA (Bergman et al., 2002; Kolb et al., 1998; Leppert et al., 2000; Levine et al., 2006; Paul et al., 1998; Turba et al., 2007) our data strongly suggest that invading leukocytes represent one source of this MMP-2 and -9 content. Also TIMP expression is present in all CSF WBC samples of dogs in the acute stage of SRMA, which indicates that some degree of counter-regulation occurs in these cells. Expression of MMP-9 in PBPMNs of dogs in the acute phase of SRMA is markedly higher than in individuals under treatment. This down-regulation under therapy may represent, in addition to CD11a suppression, another mechanism by which glucocorticosteroids prevent neutrophil migration across the blood-CSF-barrier (Cundall et al., 2003) despite persistent neutrophil-attracting properties of the CSF (Burgener et al., 1998). Our results also demonstrate that migration of the mononuclear fraction of circulating leukocytes into the CNS is associated with marked MMP-2 up-regulation in diseased dogs.

Further studies should investigate the existance of Th17 lymphocytes in the canine species and, if existant, their role in the pathogenesis of SRMA. Strikingly many of the properties ascribed to this lymphocyte subtype are also characteristic features of the disease.

In addition, the determination of the actual MMP activity by zymography will further elucidate the enzymes’ role in leukocyte migration into the CNS of dogs with SRMA.

In conclusion, we were able to show that the inflammatory response in the acute form of SRMA is associated with a type 2 skewed immune reaction. This dominance of Th2 cells may explain previous findings of elevated systemic and intrathecal IgA levels and a high B:T cell

ratio in peripheral blood and CSF. Also the increase in CD4+:CD8α+ lymphocyte ratio in peripheral blood, suggesting reduced numbers of circulating cytotoxic T cells, is in line with a Th2-dominated immune reaction.

In addition, the presented data demonstrate that a selective up-regulation of CD11a occurs in the acute form of SRMA and that sera of affected dogs are capable to induce this selective augmentation on non-activated PBPMNs *in vitro*. These results most likely reflect increased adhesion properties of neutrophils to the endothelium *in vivo* and are likely to represent one mechanism that is involved in the development of the marked neutrophilic pleocytosis associated with the acute stage of SRMA. Matrix metalloproteinases-2 and -9, which are produced by leukocytes that migrate into the subarachnoidal space, seem to represent other factors that contribute to the pathogenesis of pleocytosis by permitting leukocyte migration across the blood-CSF-barrier.

Chapter 6: Summary

Pathogenetical factors contributing to high IgA levels and marked neutrophilic pleocytosis in canine Steroid-responsive Meningitis-Arteritis

Malte Schwartz

Canine *Steroid-responsive Meningitis-Arteritis* (SRMA) is a systemic inflammatory disease with predominant manifestation within the cervical meninges. Laboratory characteristics include simultaneous elevation of immunoglobulin A (IgA) concentrations in serum and cerebrospinal fluid (CSF) and marked neutrophilic pleocytosis. The first part of the presented study addressed the hypothesis that increased IgA levels are due to a Th2-dominated immune response. In a second and third part factors potentially facilitating neutrophil migration into the CSF were investigated. These included leukocytic matrix metalloproteinase (MMP) expression and β_2 integrin expression on peripheral blood polymorphonuclear cells (PBPMNs). Whereas MMPs have the potential to induce blood-CSF-barrier permeabilization, β_2 integrins are responsible for neutrophil-endothelium-interaction, which represents a crucial event for subsequent extravasation.

Throughout the study values deriving from dogs in the acute phase of SRMA were compared to those of dogs with SRMA under glucocorticosteroid treatment and individuals suffering from various neurological disorders. To detect Th2-dominance, pellets containing peripheral blood mononuclear cells (PBMNCs) and CSF white blood cells (CSF WBCs) were examined for their cytokine mRNA expression. Cytokines that were investigated included Th1-associated interleukin (IL)-2 and interferon (IFN)-γ as well as Th2 signature cytokines IL-4, -5, and -10. All results were normalized to the expression of 3 reference genes. A similar

approach allowed quantitation of MMP-2 and -9 expression in PBMNCs, PBPMNs and CSF WBCs. In addition, mRNA expression for tissue inhibitors of metalloproteinases (TIMP)-1 and -2 was investigated. $Beta_2$ integrin expression (CD11a, CD11b, CD11c) on PBPMNs was quantitated by means of indirect immunophenotyping and subsequent flow cytomentry.

The immune response occurring in SRMA is characterized by high levels of IL-4 and low IL-2 and IFN-γ expression, whereas IL-5 and -10 levels are similar among all disease categories. These results are consistent with a Th2 skewed immune reaction in the acute phase of SRMA, which may well explain previous findings of high IgA and B cell levels. In addition, increased IL-10 levels are found in CSF WBCs of affected dogs. This may reflect a counter-regulatory reaction that prevents involvement and damage of the central nervous system (CNS) parenchyma. Cerebrospinal fluid WBCs produce both, MMP-2 and -9, suggesting their contribution to leukocyte migration into the subarachnoidal space by disruption of the blood-CSF-barrier. These cells additionally produce TIMP-1 and -2, which may represent another mechanism that prevents destruction of the adjacent CNS parenchyma. Investigation of the β_2 integrin expression showed that selective CD11a expression occurs in SRMA, whereas CD11b and CD11c expression remains unchanged. The surface molecule CD11a is necessary for leukocyte adhesion to endothelial cells and up-regulation is likely to display another mechanism that contributes to high CSF neutrophil counts in the acute phase of SRMA.

In conclusion, our study reveals that SRMA is associated with a Th2-dominated immune response and that facilitation of leukocyte migration into the subarachnoidal space is mediated by MMP-2 and -9 production as well as CD11a up-regulation.

Chapter 7: Zusammenfassung

Pathogenetische Merkmale zur Entstehung hoher IgA-Spiegel und einer neutrophilen Pleozytose bei der steril-eitrigen Meningitis-Arteriitis des Hundes

Malte Schwartz

Die *steril-eitrige Meningitis-Arteriitis* (SRMA) des Hundes ist eine systemische Erkrankung mit vornehmlicher Manifestation der Entzündung im Bereich der zervikalen Meningen. Charakteristische Laborparameter sind erhöhte Immunglobulin A (IgA)-Spiegel in Serum und Liquor cerebrospinalis (CSF) sowie eine hochgradige neutrophile Pleozytose. Im ersten Teil dieser Studie wurde die Hypothese, daß erhöhte IgA-Konzentrationen auf eine Th2-dominierte Immunantwort zurückzuführen sind, bearbeitet. In einem zweiten und dritten Teil wurden Faktoren zur Entstehung der neutrophilen Pleozytose untersucht. In diesem Zusammenhang wurden leukozytäre Matrixmetalloproteinase (MMP)-Expression sowie $Beta_2$-Integrinexpression auf polymorphkernigen Zellen des peripheren Blutes (PBPMNs) untersucht. Während MMPs in der Lage sind, die Blut-Liquor-Schranke zu permeabilisieren, spielen $Beta_2$-Integrine bei der Extravasation von Leukozyten eine entscheidende Rolle, indem sie den Leukozyten-Endothel-Kontakt vermitteln.

Die Quantifizierung der $Beta_2$-Integrine (CD11a, b und c) auf polymorphkernigen Zellen des peripheren Blutes (PBPMNs) erfolgte mittels indirekter Immunphänotypisierung und anschießender durchflußzytometrischer Auswertung. Die Untersuchung der Zytokine sowie der MMPs und deren Inhibitoren (TIMP-1 und -2) wurde mittels mRNA-Quantifizierung in mononukleären Zellen des peripheren Blutes (PBMNCs) sowie in Leukozyten aus dem Liquor cerebrospinalis (CSF WBCs) durchgeführt. Die Expression von MMPs und TIMPs

wurde zudem in PBPMNs bestimmt. Interleukin (IL)-2 und Interferon (IFN)-γ wurden zum Nachweis einer Th1-Immunantwort untersucht, während die Zytokine IL-4, -5 und -10 der Identifikation einer Th2-Antwort dienten. Sämtliche Resultate wurden mithilfe der Expression von drei Referenzgenen normalisiert. Resultate von Hunden in der akuten Phase der SRMA wurden mit denen von Tieren verglichen, die sich zur Zeit der Probenentnahme wegen einer SRMA-Erkrankung unter Glukokortikosteroidtherapie befanden, sowie mit Ergebnissen von Hunden mit verschiedenen anderen neurologischen Erkrankungen.

Die bei SRMA ablaufende Immunreaktion ist durch eine hohe IL-4- und niedrige IL-2- und IFN-γ-Expression gekennzeichnet, wohingegen IL-5- und IL-10-Spiegel mit denen der übrigen Krankheitsgruppen vergleichbar sind. Diese Ergebnisse sprechen dafür, daß bei SRMA eine Verschiebung des Th1:Th2-Verhältnisses in Richtung einer verstärkten Beteiligung von IL-4-produzierenden Lymphozyten zu einer verstärkten IgA-Synthese und einem hohen Anteil zirkulierender und intrathekaler B-Lymphozyten führt. Eine gesteigerte IL-10-Expression von in den Subarachnoidalraum eingewanderten Leukozyten stellt einen möglichen Mechanismus dar, der eine Beteiligung und Schädigung des Neuroparenchyms im Zuge einer SRMA verhindert. Die CSF WBCs von Hunden in der akuten Phase der SRMA produzieren sowohl MMP-2- als auch -9-mRNA, was eine Beteiligung dieser Gelatinasen an der Störung der Blut-Liquor-Schranke sowie am Übertritt der Zellen in den Subarachnoidalraum nahelegt. Zusätzlich produzieren sie TIMP-1 und -2, die eine zusätzliche Schädigung des Neuroparenchyms bei SRMA verhindern können. Die PBPMNs der Hunde in der akuten Phase der SRMA zeigen eine selektive Aufregulation von CD11a. Diese konnte zudem durch Inkubation mit Sera von Hunden in der akuten Phase der SRMA induziert werden. Da CD11a die Adhäsion von Leukozyten an das Endothel vermittelt, erscheint es

wahrscheinlich, daß dessen Aufregulation bei SRMA zu der verstärkten Einwanderung der Neutrophilen in den Liquorraum beiträgt.

Zusammenfassend konnte in der vorliegenden Studie gezeigt werden, daß SRMA mit einer Th2-dominierten Immunreaktion einhergeht. Zudem wurden mit der CD11a-Aufregulation und MMP-Produktion zwei Mechanismen identifiziert, die das Einwandern von Neutrophilen in den Liquorraum begünstigen.

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DECLARATION

I herewith declare that I autonomously carried out the PhD-thesis entitled

Pathogenetical factors contributing to high IgA levels and marked neutrophilic pleocytosis in canine Steroid-responsive Meningitis-Arteritis.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institutions:

Department of Small Animal Medicine and Surgery, University of Veterinary Medicine Hannover, Hannover, Germany.

Department of Pathology, University of Veterinary Medicine Hannover, Hannover, Germany.

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

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Malte Schwartz