IgA production, Signalling proteins and Toll-like receptors involved in the pathogenesis of canine Steroid-responsive Meningitis-Arteritis

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To Felix

Da bambino volevo guarire i ciliegi
quando rossi di frutti li credevo feriti
la salute per me li aveva lasciati
coi fiori di neve che avevano perduti.

Un sogno, fu un sogno ma non durò poco
per questo giurai che avrei fatto il dottore
e non per un dio ma nemmeno per gioco:
perché i ciliegi tornassero in fiore,
perché i ciliegi tornassero in fiore.

Fabrizio De André, Un Medico
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BM</td>
<td>bacterial meningitis</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>CNS-Mix</td>
<td>miscellaneous non-inflammatory diseases of the CNS</td>
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<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>DAMPs</td>
<td>damage associated molecular pattern molecules</td>
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<tr>
<td>dL</td>
<td>deciliter</td>
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<td>e.g.</td>
<td>exempli gratia</td>
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<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>Fig.</td>
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<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<td>HSP</td>
<td>Heat shock proteins</td>
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<td>ICH</td>
<td>immunohistochemistry</td>
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<td>IE</td>
<td>idiopathic epilepsy</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>KD</td>
<td>Kawasaki Disease</td>
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<td>kg</td>
<td>kilogram</td>
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<td>M</td>
<td>molar</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
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<td>ME</td>
<td>meningoencephalomyelitides</td>
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<td>mg</td>
<td>milligram</td>
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<td>mL</td>
<td>milliliter</td>
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<td>mM</td>
<td>millimolar</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MUE</td>
<td>meningoencephalitis of unknown aetiology</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<td>µM</td>
<td>micromolar</td>
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ng  nanogram
PAMPs  pathogen-associated molecular patterns
PB  peripheral blood
PBS  phosphate-buffered saline
pg  picogram
PMNs  polymorphonuclear cells
rSpear  Spearman's rank correlation coefficient
SRMA  Steroid-responsive Meningitis-Arteritis
Syst. Infl.  systemic inflammatory diseases
Tab.  table
TGF-β₁  transforming growth factor beta 1
Th1  T helper 1
Th17  T helper 17
Th2  T helper 2
TLR  Toll-like receptor
Treg  regulatory T cells
VEGF  vascular endothelial growth factor
WBCC  white blood cell count
%  percent
°C  degree Celsius
Chapter 1: General Introduction and Literature Review

Inflammatory and infectious diseases are among the most important disorders of the central nervous system (CNS) of companion animals (TIPOLD 1995). Depending on the anatomical structure affected by the inflammatory process encephalitis, myelitis and/or meningitis may occur, affecting either the brain parenchyma, the spinal cord or the meninges (SORJONEN 1992; MUÑANA 1996). Due to the close anatomic contiguity, inflammatory lesions are commonly widespread. For this reason terms such as encephalomyelitis and meningoencephalomyelitis are often more appropriate (LUTTGEN 1988; TIPOLD and STEIN 2011). However, some diseases affect predominantly the meninges and thus are clinically distinct from encephalomyelitis.

Dogs of any age and breed may be affected, however the disease most commonly occurs in young adults of medium- to large-breeds (RUSSO et al. 1983; MERIC 1988; TIPOLD and JAGGY 1994). A breed predisposition is recognized for Beagles (RUBEN et al. 1989), Bernese Mountain Dogs (PRESTHUS, 1991, CIZINAUSKAS et al., 2000), Boxers (BEHR and CAUZINILLE 2006), Nova Scotia Duck-Tolling Retriever (CIZINAUSKAS et al., 2000, ANFINSEN et al., 2008) and Petit Basset Griffon Vendéen (VOSS et al. 2011). In the last two breeds a familial (ANFINSEN et al. 2008; VOSS et al. 2011) and genetic (WILBE et al. 2009) predisposition is recognized. The disease is equally described in male and female, either neutered or not (MERIC 1988; TIPOLD and JAGGY 1994; CIZINAUSKAS et al. 2000; BEHR and CAUZINILLE 2006).

Typically, affected dogs show a dramatic acute onset of cervical pain, stiffness, pyrexia and lethargy, followed by a waxing and waning course of improvement and relapse (TIPOLD and STEIN 2011). If not treated in this acute stage, the disease has a tendency to develop a more protracted course (CIZINAUSKAS et al., 2000). The neurological examination during the acute form is unremarkable, except for signs referring to pain manifestation. Additional neurological deficits, mainly reflecting the involvement of the spinal cord parenchyma, are observed during the protracted form (IRVING and CHRISMAN 1990; TIPOLD and JAGGY 1994). In rare cases an involvement of the cranial nerves, which may result in central vestibular syndrome (IRVING and CHRISMAN 1990; TIPOLD and JAGGY 1994; HESS and SELLON 1997; BEHR and CAUZINILLE 2006) and involvement of brain parenchyma resulting in seizures and coma (WRZOSEK et al. 2009), are described. Additional occasional findings are myclonus.
(TIPOLD and JAGGY 1994) and generalized tremors (GANDINI et al. 2003), possibly due to the extremely painful condition.

The most important extraneural sign is the elevation of the body temperature up to 42°C (TIPOLD and JAGGY 1994), making SRMA a disease of relevance even beyond neurology. Indeed, according to one retrospective study, SRMA was the most common cause of fever initially classified as of unknown origin (BATTERSBY et al. 2006). Isolated cases may show signs related to cardiac involvement, in two case reports one dog showed cardiac arrhythmias (SNYDER et al. 2010) and the other pericardial effusion (NAVARRO-CUBAS et al. 2011).

The laboratory findings reflect the systemic inflammatory nature of SRMA in most of the patients revealing a marked leukocytosis with a left shift and increased erythrocyte sedimentation (TIPOLD and JAGGY 1994; CIZINAUSKAS et al. 2000). However, the most representative abnormalities are found in the cerebrospinal fluid (CSF). A moderate to severe neutrophilic pleocytosis, coupled with elevated protein content, characterize the CSF of dogs affected by the acute form, as long as no glucocorticoid steroids are administered (MERIC et al. 1985; TIPOLD and JAGGY 1994; TIPOLD 1995). In this latter case and in patients with the protracted form, the CSF analysis may either show normal results or a slight increased protein content with or without a mild to moderate, mononuclear or mixed pleocytosis (CIZINAUSKAS et al. 2000; TIPOLD 2000; LOWRIE et al. 2009b).

Macroscopic alterations of the CNS are not constantly found at necropsy. In some cases, in which the vascular lesions led to rupture of major blood vessels, subarachnoidal haemorrhages may be seen extending over the entire spinal cord and brainstem (VANDEVELDE and FANKHAUSER 1972). Hydrocephalus may
occur, due to the obstruction of the flow of CSF after meningeal fibrosis (TIPOLD et al. 1995).

The histopathological examination of dogs during the acute stage of the disease shows an extensive suppurative leptomenigitis involving the meninges of the entire CNS, most severely in the cervical region, with invasion of macrophages, plasma cells, lymphocytes and polymorphonuclear cells (MERIC, 1988, TIPOLD et al., 1995). Immunohistochemistry reveals the presence of immunoglobulin (Ig) G-, IgM- and IgA-producing plasma cells in the meninges (TIPOLD et al. 1995).

In addition, alterations are found in the vascular system, these lesions range from periarteritis to necrotizing arteritis, with invasion of a mixture of inflammatory cells, and vascular thrombosis (TIPOLD et al. 1995). The most frequently and consistently affected vessels were the small- to medium-sized muscular arteries of the heart, cranial mediastinum, and cervical spinal meninges (SCOTT-MONCRIEFF et al., 1992, SNYDER et al., 1995). In protracted stages of the disease the manifestation of the meningitis is milder, consisting of fibrous thickening and focal mineralisation of the leptomeninges (TIPOLD et al. 1995). The vascular lesions also reflect chronic progression of previous episodes of vasculitis, characterized by a combination of intimal and medial fibrosis, thickened intima, ruptured elastic laminae, mineralisation, and mild perivasculitis (TIPOLD et al., 1995, SNYDER et al., 1995). Some vessels show organization of thrombi and some others recanalisation (TIPOLD et al. 1995).

Acute and chronic vascular lesions may be found in the same section, reflecting the relapsing nature of the disease (TIPOLD and JAGGY, 1994, SNYDER et al., 1995).

Diagnosis of SRMA in young dogs referred with acute onset of cervical pain, fever and leukocytosis is easily confirmed by CSF analysis and exclusion of the diseases that might mimic these signs. In protracted cases, age and clinical
manifestation overlap several other multifocal diseases, making the definitive diagnosis more challenging (TIPOLD and SCHATZBERG 2010).

CSF analysis remains the most reliable antemortem diagnostic test, allowing a diagnosis in most acute cases. For those cases in which the CSF analysis is inconclusive, the detection of increased levels of acute phase proteins can help finding the diagnosis (BATHEN-NOETHEN et al. 2008; LOWRIE et al. 2009a).

Treatment strategies are focused to suppress the overreaction of the immune system and reduce the inflammation (TIPOLD and SCHATZBERG 2010). Non-steroidal anti-inflammatory drugs can be used in dogs with mild initial signs and mild pleocytosis, while in case of relapse, worsening of the symptoms or severe pleocytosis, long-term therapy with glucocorticosteroids is mandatory (TIPOLD and SCHATZBERG 2010). An immunosuppressive dose of prednisolone is generally recommended for the first days, while a gradual decreasing anti-inflammatory dosage is given for a period of at least six months. In both steroidal and non-steroidal therapies gastro-intestinal protectors are recommended (TIPOLD and SCHATZBERG 2010). The prognosis is good to excellent in dogs promptly and adequately treated in the acute stage of the disease. The prognosis is guarded if the disease progresses to chronic stage (TIPOLD and JAGGY, 1994, CIZINAUSKAS et al., 2000).

Despite almost 20 years of research, the aetiology of SRMA is still unknown. Pathologic and laboratory findings, together with the marked response to steroids, suggest that immunopathologic mechanisms are involved in SRMA (TIPOLD 2000). Infectious agents, environmental factors or self-antigens might be involved in
triggering an inadequate immune response, because activated T cells have been found (TIPOLD et al. 1996).

However, any attempt to identify infectious agents has been inconclusive (HARCOURT 1978; SCOTT-MONCRIEFF et al. 1992; PONCELET and BALLIGAND 1993; TIPOLD et al. 1995).

Previous studies have described abnormalities such as antinuclear antibodies (TIPOLD and JAGGY 1994), lupus erythematosus cells (RUSSO et al. 1983; MERIC et al. 1985; TIPOLD and JAGGY 1994), circulating immune complexes (HAYES et al. 1989; TIPOLD et al. 1995) and IgM rheuma factors (HAYES et al. 1989; TIPOLD and JAGGY 1994), thus suggesting immune-mediated mechanisms. Some morphological features of the vascular changes in the affected animals share some similarities to immune complex-mediated vascular injury. However, unequivocal deposits of Ig in blood vessels have not been reported (HARCOURT 1978; SPENCER and GREAVES 1987; HAYES et al. 1989; SCOTT-MONCRIEFF et al. 1992). In one study (TIPOLD et al. 1995) Ig-deposits were not found in any acute vascular lesions, but some chronic cases showed diffuse positive staining for IgG and IgM in the thickened adventitia of a few stenosed arteries. Immune complexes may be involved at some point, but it remains doubtful, however, if they were related to the initial lesion or not (TIPOLD et al. 1995). Additional studies focused on characterisation of leukocytes in SRMA. Schwartz et al. (2008b) demonstrated an increase of T helper to cytotoxic T cells ratio in blood lymphocytes and a selective recruitment (or massive proliferation) of B lymphocytes into the subarachnoidal space. The same research group found that SRMA patients expressed low levels of Th1-response-related cytokines and high levels Th2 cytokines, suggesting that in SRMA an overreacting Th2-mediated immune response takes place (SCHWARTZ et al. 2011). An overexpression of
adhesion integrin CD11a on leukocytes and production of matrix metalloproteinases 2 and 9 in dogs affected with SRMA are believed to substantially contribute to the massive infiltration of neutrophils in the meninges, hallmark of the disease (SCHWARTZ et al. 2008a; SCHWARTZ et al. 2010).

Vascular Endothelial Growth Factor (VEGF) is a potent, multifunctional factor, which regulates angiogenesis and blood vessel permeability (DVORAK et al. 1995; NAGY et al. 2008). In human medicine, the role of VEGF has been widely investigated in systemic vasculitides (TERAI et al. 1999; YASUKAWA et al. 2002; MONACH et al. 2011). SRMA represents a naturally occurring animal model for several human vasculitides of unknown origin, in particular Kawasaki Disease (KD). Fever, systemic arteritis, increased numbers of peripheral blood B lymphocytes, increased serum levels of several cytokines and IgA characterize both diseases (BURNS et al., 1991, SNYDER et al., 1995). We hypothesize that enhanced VEGF production is responsible for the vascular lesion in SRMA dogs. In order to investigate the role of VEGF in SRMA we measured VEGF concentrations in CSF and serum, by means of an enzyme-linked immunosorbent assay (ELISA).

High IgA concentrations in both serum and CSF have been reported to be characteristic for SRMA (TIPOLD and JAGGY, 1994, CIZINAUSKAS et al., 2000), not only in the acute phase of the disease, but also in dogs under treatment and in protracted forms (CIZINAUSKAS et al. 2000; SCHULTE et al. 2006). Whereas the findings of Lowrie et al. (2009b) support these results in 20 cases, similar changes could not be detected by Behr and Cauzinille (2006) using a smaller number of cases.
We hypothesized that measurement of IgA concentrations in CSF and serum indeed represents a useful ancillary test in the diagnostic work-up of suspected SRMA cases and therefore determined sensitivity and specificity of a concurrent elevation of IgA concentrations in CSF and serum in a large number of dogs.

Interleukin 6 (IL-6) is a proinflammatory cytokine, which activates lymphocytes, increases antibody production, induces fever and acute-phase protein production (MURPHY et al. 2008b), while Transforming Growth Factor Beta 1 (TGF-β1) induces a class switching of B lymphocytes towards IgA production (MURPHY et al. 2008c). Hogenesch et al. (1995) investigated IL-6 in serum of dogs with juvenile polyarteritis syndrome and detected increased IL-6 serum values. In preliminary studies, measurement of IL-6 in CSF was considered to be a valuable biomarker for the diagnosis of SRMA (LOWRIE et al. 2008). Information about TGF-β1 and the bioactive form of IL-6 in CSF is still missing.

We hypothesize that IL-6 and TGF-β1 are causes for excessive IgA production and the systemic inflammatory response, especially for acute phase protein production and development of T-helper cell subtypes. We thus investigated concentrations of IL-6 and TGF-β1 in CSF and serum of dogs affected with SRMA, by means of an ELISA.

Toll-like receptors (TLRs) are pattern recognition receptors that identify a wide spectrum of ligands deriving from both invading pathogens (through pathogen-associated molecular patterns, PAMPs) and endogenous molecules produced by injured tissue (through damage-associated molecular patterns, DAMPs) (MONTERO VEGA and DE ANDRES MARTIN 2009). This recognition process regulates many
aspects of innate immunity, but participates also in the development of adaptive immune response (IMLER and HOFFMANN 2001; WONG et al. 2009). Additionally, they may be involved in the induction of chronic inflammation and autoimmune reactions (LIU et al. 2003; HURST and VON LANDENBERG 2008; WONG et al. 2009; GREEN and MARSHAK-ROTHSTEIN 2011).

Due to the multiplicity of functions of TLRs, they have become the subject of many immunological studies. Examples of systemic diseases in which an association with TLRs has been found are numerous (MARSHAK-ROTHSTEIN 2006) and include systemic lupus erythematosus (BARRAT et al. 2005), giant cell arteritis (MA-KRUPA et al. 2005; DENG et al. 2009), Sjögren’s syndrome (LOVGREN et al. 2006), autoimmune arthritis (ABDOLLAHI-ROODSAZ et al. 2008) and multiple sclerosis (PRINZ et al. 2006).

Several studies fuel the hypothesis that SRMA derives from a dysfunction of the immune system, in which a Th2-mediated immune response predominate (TIPOLD et al. 1995; SCHWARTZ et al. 2008b; SCHWARTZ et al. 2011). These studies mainly focused on the role of lymphocytes, but studies on the innate immune system in SRMA are still missing. In order to study their involvement in SRMA, we investigated the expression profile of cell surface TLRs (TLR2, TLR4 and TLR5) and intracellular TLRs (TLR3 and TLR9) of canine leukocytes, by means of immune phenotyping and flow cytometric analysis.
Chapter 2: Determination of IgA concentrations in serum and cerebrospinal fluid in dogs: an estimation of its diagnostic value for canine steroid-responsive meningitis-arteritis

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2.1 Abstract

Previous studies on steroid-responsive meningitis-arteritis (SRMA) in dogs suggested that concurrent elevation of immunoglobulin A (IgA) concentrations in both serum and cerebrospinal fluid (CSF) is specific for SRMA throughout the different stages of the disease. Other recent studies, however, have raised concerns about the value of this test.

The purpose of this study was to investigate the diagnostic value of IgA concentration testing in paired CSF and serum samples. IgA concentration of 525 paired canine CSF and serum samples was evaluated. Paired samples derived from dogs with SRMA (n = 311) and dogs affected by miscellaneous diseases (n = 214), such as other inflammatory central nervous system (CNS) diseases (n = 34), CNS tumours (n = 46), idiopathic epilepsy (n = 42), intervertebral disc disease (n = 46) and diseases not affecting the CNS (n = 46).

Serum IgA concentrations were significantly higher in dogs with the acute form of SRMA in comparison to dogs with other diseases. IgA concentrations in CSF were also significantly higher in dogs with SRMA comparing other disease categories, with the exception of inflammatory CNS diseases. The sensitivity for simultaneous elevation of IgA concentrations in serum and CSF was 91% with a specificity of 78%, respectively. Analysis of a large number of samples confirmed that IgA production was higher within the group of dogs with SRMA compared to the remaining disease categories examined. Calculation of the diagnostic value to determine IgA confirmed that this test is still an important tool and highly sensitive to confirm the diagnosis of SRMA. Testing paired CSF and serum samples for IgA is recommended for the diagnostic work-up in suspected cases of SRMA, particularly in those animals
receiving glucocorticosteroids prior to CSF puncture. However, since the specificity is low, other diseases causing neck pain have to be ruled out in the protracted form.

Keywords: Steroid-responsive meningitis-arteritis; Immunoglobulin A; Cerebrospinal fluid; Central nervous system; Dog.
Chapter 3: Signalling proteins involved in the pathogenesis of Steroid Responsive Meningitis-Arteritis

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Submitted to BMC Veterinary Research
Abstract

3.1.1 Background

Steroid responsive meningitis-arteritis (SRMA) is a common cause of inflammation of the canine central nervous system (CNS). To verify the hypothesis that transforming growth factor beta 1 (TGF-β₁), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) are involved in the production of excessive immunoglobulin A (IgA), the induction of acute phase proteins and in the development of a systemic necrotizing vasculitis, characteristic of SRMA, these three signalling proteins were evaluated.

3.1.2 Results

Cerebrospinal fluid (CSF) and serum samples of dogs during the acute phase of SRMA (SRMA) were tested for IL-6, VEGF and TGF-β₁. Results were compared to those of dogs affected with SRMA during treatment (SRMA Th) and during relapse (SRMA R), to dogs with other meningoencephalomyelitides (ME), with miscellaneous non-inflammatory diseases of the CNS (CNS-Mix), with idiopathic epilepsy (IE), with systemic inflammatory diseases (Syst. Infl.) and with healthy dogs (Healthy).

Concentrations of IL-6 and VEGF in CSF were significantly elevated in the SRMA group compared to the other disease categories (p< 0.05). The CSF concentrations of TGF-β₁ were increased in SRMA group, but statistically significant differences were found only in comparison with Healthy and CNS-Mix groups. No differences were detected in the serum concentrations of TGF-β₁ between the different groups.

A positive correlation (r_Spear = 0.3549; P = 0.0337) between concentrations of TGF-β₁ and IgA concentration was found in CSF, while concentrations of IL-6 and VEGF in
CSF correlated positively with the degree of pleocytosis \( (r_{\text{Spear}} = 0.8323; P < 0.0001 \) and \( r_{\text{Spear}} = 0.5711; P = 0.0166, \) respectively)

### 3.1.3 Conclusions

Our results suggest that these three signalling proteins play an important role in the pathogenesis of SRMA and the systemic development of an arteritis. Both TGF-\( \beta_1 \) and IL-6 are considered to be involved in the excessive IgA production. The detected combined intrathecal increase of TGF-\( \beta_1 \) and IL-6 in SRMA could possibly force CD4 progenitors to differentiate towards the newly described Th17 lymphocyte subset and enhance the autoimmune response.
3.2 Keywords
Steroid Responsive Meningitis-Arteritis (SRMA), interleukin-6 (IL-6), transforming growth factor beta 1 (TGF-\(\beta_1\)), Vascular endothelial growth factor (VEGF), cerebrospinal fluid (CSF), dog, central nervous system (CNS).

3.3 Background
Steroid Responsive Meningitis-Arteritis (SRMA) is a relatively common inflammatory disease of the canine central nervous system (CNS) (TIPOLD 1995), being the primary cause of meningitis (MERIC 1988; MUÑANA 1996) and one of the most common causes of fever in referred dogs (BATTERSBY et al. 2006). Typically affected dogs manifest severe neck pain in addition to signs of systemic illness, such as fever and lethargy. The meningeal and systemic inflammation is usually reflected by laboratory findings, such as pleocytosis and leukocytosis (CIZINAUSKAS et al. 2000) and the elevation of acute phase proteins (BATHEN-NOETHEN et al. 2008; LOWRIE et al. 2009a; LOWRIE et al. 2009b). A concurrent increased intrathecal and systemic production of immunoglobulin A (IgA) has been found in dogs affected with SRMA (TIPOLD et al. 1994), and its determination supports the diagnosis (MAIOLINI et al. 2011). Recent studies underlined the importance of a Th2-mediated immune response in SRMA patients (SCHWARTZ et al. 2008b; SCHWARTZ et al. 2011), but the exact etiopathogenesis of the disease is still unclear.

Signalling proteins are molecules, such as cytokines, growth factors, hormones and neurotransmitters, that interact with receptors. Vascular Endothelial Growth Factor (VEGF) is a potent, multifunctional factor, which regulates angiogenesis and blood vessel permeability (DVORAK et al. 1995; NAGY et al.
In the last two decades the role of VEGF has been widely investigated in systemic vasculitides (TERAI et al. 1999; YASUKAWA et al. 2002; MONACH et al. 2011) and malignant tumours (LIN and CHAO 2005; SAHARINEN et al. 2011). In veterinary medicine VEGF has been studied in some inflammatory diseases (SILVERSTEIN et al. 2009) and tumours (CLIFFORD et al. 2001; AL-DISSI et al. 2010), but its role in systemic vasculitis is still unknown. Interleukin 6 (IL-6) is a proinflammatory cytokine, which activates lymphocytes, increases antibody production, induces fever and acute-phase protein production (MURPHY et al. 2008b), while Transforming Growth Factor Beta 1 (TGF-β1) induces a class switching of B lymphocytes towards IgA production (MURPHY et al. 2008c).

To test the hypothesis that the signalling proteins VEGF, IL-6 and TGF-β1 are involved in the pathogenesis of SRMA, these proteins were determined in cerebrospinal fluid (CSF) and serum. We hypothesize that enhanced VEGF production is responsible for an increased vascular permeability (vasculitis) in SRMA dogs, while IL-6 and TGF-β1 are responsible for the excessive IgA production and the systemic inflammatory response, especially for acute phase protein production and development of T-helper cell subtypes.

### 3.4 Methods

#### 3.4.1 Serum and cerebrospinal fluid (CSF) samples

Serum and cerebrospinal fluid (CSF) samples were collected from client-owned dogs, presented to the Neurology Service of the Department of Small Animal Medicine and Surgery, University of Veterinary Medicine, Hannover, Germany.
All dogs underwent a standard neurological examination performed either by a veterinary neurology resident or a Board-certified veterinary neurologist. Depending on the diagnosis, animals were assigned to one of the following groups: ‘SRMA untreated’ (SRMA), ‘SRMA relapse’ (SRMA R), ‘SRMA therapy’ (SRMA Th), ‘idiopathic epilepsy’ (IE), ‘Meningoencephalomyelitides’ (ME), and ‘miscellaneous non-inflammatory CNS diseases’ (CNS-Mix).

Diagnoses were based on the results of general and neurological examinations, complete blood cell count (CBC), blood chemistry, CSF analysis and further specific diagnostic procedures if considered useful to achieve a definitive or ‘highly likely’ diagnosis (e.g., radiographic and ultrasound examination, electrodiagnostics, computed tomography, magnetic resonance imaging, surgery, histopathology).

Dogs not suffering from neurological condition, but otherwise affected with systemic inflammatory diseases were also included in the study (‘Syst. Inflam.’).

Healthy dogs with a normal general and neurological examination and laboratory values in the reference range served as negative control group (‘Healthy’).

CSF and serum samples were stored at -20°C until analyzed.

The study was conducted following the ethical guidelines of the University for Veterinary Medicine Hannover. The experimental protocols and procedures in healthy dogs were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the authorities in Lower Saxony (animal experiment number 33.42502/05-12.05).

### 3.4.2 Quantification of IL-6

Serum and CSF IL-6 concentrations were measured with a commercially available canine-specific Enzyme Linked Immunosorbent Assay (ELISA) following
manufacturer’s instructions (Quantikine Canine IL-6 Immunoassay; R&D Systems, Minneapolis, MN, USA). The mean minimum detectable value given by the manufacturer was 6.1 pg/mL. Values lower than 6.1 pg/mL were considered negative for IL-6. If the value ranged between 6.1 pg/mL and the highest dilution of the standard curves (31.25 pg/mL) a fixed value of 3 pg/mL was assigned for statistical analysis. If the value exceeded the highest value of the standard curve (2000 pg/mL), the sample was diluted and measured again.

All samples were analyzed in duplicates and mean values were calculated.

The IL-6 bioactivity in serum and CSF was verified testing its ability to stimulate proliferation of B9 cell line, a mouse B cell murine hybridoma cell line that requires IL-6 for survival and proliferation (DSMZ No ACC 211, German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany) (AARDEN et al. 1987; LEMAY et al. 1990; CARTER et al. 1999; RAU et al. 2007).

Cells were cultured at 37°C and 5% CO₂ in Rosewell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Gibco® RPMI Media 1640) containing 10% heat-inactivated fetal bovine serum, 50 pg/mL recombinant canine IL-6 (rcIL-6) (Recombinant Canine IL-6; R&D Systems, Minneapolis, MN, USA) and 50µM 2-mercaptoethanol.

Cells for the assay were washed twice in the above described medium without IL-6. For each assay, a standard curve was prepared using rcIL-6 with serial threefold dilutions starting at 2000pg/mL.

Samples were tested in duplicates at dilutions ranging from 1:2 to 1: 240.

For the IL-6 bioactivity assay B9 cells (5000 cells/well) were incubated for 48 hours with rcIL-6 standard dilutions or and diluted canine CSF and serum samples in opaque 96-well microtiter plates (F96 MicroWell™ Plates, Nunc™, Denmark). Cell
proliferation and viability was quantified using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI), according to manufacturer’s recommendations. Luminescence in each well was measured 30 min after reagent addition using a scanning multiwell spectrophotometer equipped with the analysis software Gen 5 (Synergy2 HT multi-mode microplate reader, BioTek Instruments Inc., Bad Friedrichshall Germany).

3.4.3 Quantification of VEGF

Serum and CSF VEGF concentrations were measured with commercially available canine-specific ELISA following manufacturer’s instructions (Quantikine Canine VEGF Immunoassay, R&D Systems, Minneapolis, MN, USA). The minimum detectable values given by the manufacturer were < 9.8 pg/mL for CSF and < 19.5 pg/mL for serum. Similar to the described IL-6 ELISA, a content of 0 pg/mL was assigned, if the amount of VEGF detected was less than the minimum detectable dose.

If the amount ranged between 9.8 pg/mL for CSF or 19.5 pg/mL for serum and the highest dilution of the standard curves (19.5 pg/mL and 39.1 pg/mL, respectively) a fixed value of 2 pg/mL and 3.9 pg/mL instead of zero was assigned for statistical analysis.

All samples were analyzed in duplicates and mean values were calculated.

3.4.4 Quantification of TGF-β1

Serum and CSF TGF-β1 concentrations were measured with a commercially available ELISA following manufacturer’s instructions (Human TGF-beta 1 DuoSet,
R&D Systems, Minneapolis, MN, USA). This assay is designed to detect the biologically active natural and recombinant human TGF-β1. Canine TGF-β1 shares 94% nucleotide sequence identity to human TGF-β1 (MANNING et al. 1995). Therefore, we suspected a relative high cross-reactivity, that allows the measurement of canine TGF-β1 by using this human ELISA Development Kit. The final dilution was 1:15 for the CSF samples and 1:30 and 1:60 for the serum samples. All dilutions were tested in duplicates on 96-well microplates (Nunc-Immuno™-Plate, Nunc™, Denmark). The absorbance was recorded at a wavelength of 450 nm using a plate reader (Dynatech, Denkendorf, Germany).

Antibodies were tested by immunohistochemistry (IHC) detecting canine TGF-β1 on canine tissue. Different canine tissues (activated lymph nodes, brain tumour, brain and spinal cord from SRMA affected dogs) were stained with the capture antibody of the previously mentioned ELISA-Kit. All tissues were routinely formalin-fixed and paraffin-embedded and the sections were mounted on positively charged slides (SuperFrost Plus®, Menzel-Gläser, Wiesbaden, Germany). Tissue sections were deparaffinised and the endogenous peroxidase was blocked with 0.5 % H₂O₂ in 70 % ethanol over 30 minutes. After washing the slides with PBS three times for five minutes, citrate buffer (10 minutes, autoclave, 121 °C) was used for antigen retrieval. The capture antibody of the ELISA-Kit, a monoclonal mouse-anti-human TGF-β1-antibody was diluted 1:40 in PBS (pH 7.5) with 1% bovine serum albumin (BSA) and incubated overnight by 4 °C. As secondary antibody we used a biotinylated goat-anti-mouse-antibody (Vector Laboratories, Burlingame, USA) (diluted 1:200 in PBS, incubation for 45 minutes, room temperature). Staining was completed using an ABC Kit (Vector Laboratories, Burlingame, USA).
3.4.5 Data analysis

Data were analyzed for normal distribution using the Kolmogorov-Smirnov test, and non-parametric tests were used to analyze data not conforming to a Gaussian distribution.

In addition to descriptive methods, the Kruskal-Wallis test was used to evaluate the analysis of variance. Multiple comparisons between the SRMA groups and groups of other diseases were performed using the Wilcoxon rank-sum test.

The Spearman's rank correlation coefficients ($r_{\text{Spear}}$) were calculated in the “SRMA untreated” group to detect correlations between signalling proteins and different parameters, such as IgA concentrations, complete blood cell count and CSF cell count.

Data were analyzed by using the statistical software package (GraphPad Prism®, version 5; GraphPad Software, La Jolla, CA, USA) and values of $P < 0.05$ were considered significant.
3.5 Results

CSF and serum samples of 172 dogs were collected and analysed. The small amount of some of the samples did not allow performing the quantification of all three signalling proteins in each sample. The distribution of the samples for each ELISA is shown in Table 1.

Table 1 - Number of CSF and serum samples analysed for each signalling protein in each group

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-6</th>
<th>VEGF</th>
<th>TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSF</td>
<td>Serum</td>
<td>CSF</td>
</tr>
<tr>
<td>SRMA</td>
<td>26</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>SRMA R</td>
<td>9</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>SRMA Th</td>
<td>41</td>
<td>44</td>
<td>41</td>
</tr>
<tr>
<td>ME</td>
<td>13</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>CNS-Mix</td>
<td>21</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Syst. Inflam.</td>
<td>7</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>IE</td>
<td>22</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Healthy</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

IL-6: interleukin-6; VEGF: vascular endothelial grow factor; TGF-β1: Transforming growth factor beta; CSF: cerebrospinal fluid; CNS: central nervous system; SRMA: steroid-responsive meningitis-arteritis; Syst Inflam: systemic inflammatory diseases; ME: other inflammatory CNS diseases; CNS-Mix: miscellaneous non-inflammatory CNS diseases; IE: idiopathic epilepsy
Table 2 - Concentration of signalling proteins in different disease categories (given as median and 25-75% range)

<table>
<thead>
<tr>
<th></th>
<th>SRMA</th>
<th>SRMA R</th>
<th>SRMA Th</th>
<th>Syst Inflam</th>
<th>ME</th>
<th>CNS-Mix</th>
<th>IE</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>CSF</td>
<td>1582</td>
<td>637.7</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(pg/mL)</td>
<td>(163.1-3473)</td>
<td>(17.19-1507)</td>
<td>(0-1.5)</td>
<td>(0-31.25)</td>
<td>(0-175.6)</td>
<td>(0-3)</td>
<td>(0-0.75)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Serum</td>
<td>3</td>
<td>17.13</td>
<td>0</td>
<td>17.13</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>(pg/mL)</td>
<td>(3-124)</td>
<td>(3-36.24)</td>
<td>(0-0)</td>
<td>(0-77.1)</td>
<td>(0.3)</td>
<td>(0-0)</td>
<td>(0-0)</td>
</tr>
<tr>
<td>VEGF</td>
<td>CSF</td>
<td>36.31</td>
<td>20.80</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(pg/mL)</td>
<td>(1-175.2)</td>
<td>(0-69.96)</td>
<td>(0-0)</td>
<td>(0-0)</td>
<td>(0-65.71)</td>
<td>(0-0)</td>
<td>(0-0)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Serum</td>
<td>43.92</td>
<td>33.71</td>
<td>3.9</td>
<td>3.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(pg/mL)</td>
<td>(3.9-94.5)</td>
<td>(0.98-42.9)</td>
<td>(0-3.9)</td>
<td>(3.9-57.8)</td>
<td>(0-3.9)</td>
<td>(0-0)</td>
<td>(1.95-3.9)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>CSF</td>
<td>90</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>58</td>
<td>0</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>(pg/mL)</td>
<td>(0-429)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(0-214)</td>
<td>(0-95)</td>
<td>(0-0)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Serum</td>
<td>13.57</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>22.84</td>
<td>19.31</td>
<td>23.84</td>
</tr>
<tr>
<td></td>
<td>(ng/mL)</td>
<td>(9.93-19.27)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(11.86-27.96)</td>
<td>(14.77-23.25)</td>
<td>(18.19-29.16)</td>
</tr>
</tbody>
</table>

IL-6: interleukin-6; VEGF: vascular endothelial growth factor; TGF-β1: Transforming growth factor beta; CSF: cerebrospinal fluid; CNS: central nervous system; SRMA: steroid-responsive meningitis-arteritis; Syst Inflam: systemic inflammatory diseases; ME: other inflammatory CNS diseases; CNS-Mix: miscellaneous non-inflammatory CNS diseases; IE: idiopathic epilepsy; n.a.: not available
3.5.1 Quantification of IL-6

IL-6 concentrations in CSF and serum and results of the statistical analysis are illustrated in Figure 1A and 1B. The highest concentrations of IL-6 were detected in CSF of dogs with SRMA (median 1582 pg/mL; range 163.1-3473 pg/mL). This group differed significantly from the other disease categories, with the exception of dogs with relapsed SRMA (median 637.7 pg/mL; range 17.19-1507 pg/mL).

Similarly, high concentrations of IL-6 were detected in serum in the ‘SRMA’ group (median 3 pg/mL; range 3-124 pg/mL), ‘SRMA R’ group (median 17.13 pg/mL; range 3-36.24 pg/mL) and ‘Syst. Inflam.’ group (median 17.13 pg/mL; range 0-77.1 pg/mL). In the remaining groups the IL-6 concentration was significantly lower than in the ‘SRMA’ group.

Approximately 30% of the samples used for the ELISA were also tested with the bioassay.

The B9 cells could proliferate only when incubated with samples with high IL-6 values measured by the ELISA.

3.5.2 Quantification of VEGF

VEGF concentrations in CSF and serum and results of the statistical analysis are illustrated in Figure 1C and 1D. The highest concentrations of VEGF were detected in CSF (median 36.31 pg/mL; range 1-175.2 pg/mL) and serum (median 43.92 pg/mL; range 3.9-94.5 pg/mL) of dogs with SRMA. Similarly, high concentration of VEGF were found in CSF (median 20.80 pg/mL; range 0-69.96 pg/mL) and serum (median 33.71 pg/mL; range 0.98-42.9 pg/mL) of dogs with relapsed SRMA and in serum (median 3.9 pg/mL; range
3.9-57.8 pg/mL) of dogs with a systemic inflammatory disease. In the remaining groups the VEGF concentrations were significantly lower than the ones of the ‘SRMA’ group.
Figure 1 - Quantification of signalling proteins

Quantification of signalling proteins: IL-6 in CSF (A) and serum (B), VEGF in CSF (C) and serum (D), TGF-β1 in CSF (E) and serum (F). Boxes contain values from 1st to 3rd quartile, lines inside boxes indicate median values, endpoints of vertical lines display 5th–95th percentile and • represent the outliers. Asterisks indicate statistically significant differences from the disease category ‘SRMA untreated’ (* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.005 \)).

IL-6: interleukin-6; VEGF: vascular endothelial growth factor; TGF-β1: Transforming growth factor beta 1. CSF: cerebrospinal fluid; CNS: central nervous system; SRMA: steroid-responsive meningitis-arteritis; ME: other inflammatory CNS diseases; CNS-Mix: miscellaneous non-inflammatory CNS diseases; Syst Inflam: systemic inflammatory diseases; IE: idiopathic epilepsy
3.5.3 Quantification of TGF-β₁

TGF-β₁ concentrations in CSF and serum and results of the statistical analysis are illustrated in Figure 1E and 1F.

Compared with the ‘Healthy’ group, TGF-β₁ concentrations in CSF were significantly higher in ‘SRMA’ (median 90 pg/mL; range 0-429 pg/mL) and ‘ME’ (median 58 pg/mL; range 0-214 pg/mL) groups. In serum ‘SRMA’ group showed the lowest concentrations of TGF-β₁ (median 13.57 ng/mL, range 9.93-19.27 ng/mL). These concentrations were significantly lower than those found in the remaining groups.

The capture antibody of the commercial human ELISA-Kit succeeded in staining canine TGF-β₁ in the canine tissues used as positive controls, such as lymph nodes with suppurative lymphadenitis and brain and spinal cord with SRMA (Figure 2). In the negative control sections no TGF-β₁ was detected.

Figure 2 - Spinal cord and meninges of a dog with SRMA
Arrow indicates TGF-β₁ positive lymphocyte; immunohistochemistry, anti-TGF-β₁-ABC, x400.
SRMA: steroid-responsive meningitis-arteritis
TGF-β₁: Transforming growth factor beta 1
3.5.4 Correlation analysis

A summary of the statistically relevant correlations found in the ‘SRMA’ group is shown in Table 3. A positive correlation between signalling protein and IgA concentration was found between CSF TGF-β₁ and CSF IgA (\( r_{\text{Spear}} = 0.3549; \ P = 0.0337 \)).

CSF concentrations of IL-6 and VEGF had a strong positive correlation with the degree of pleocytosis (\( r_{\text{Spear}} = 0.8323; \ P < 0.0001 \) and \( r_{\text{Spear}} = 0.5711; \ P = 0.0166 \), respectively). A positive correlation was also found between concentration of IL-6 and VEGF, both in CSF (\( r_{\text{Spear}} = 0.8246; \ P < 0.0001 \)) and serum (\( r_{\text{Spear}} = 0.5045; \ P = 0.0086 \)). A similar correlation was not found in serum of ‘Syst Inflam.’ group (\( r_{\text{Spear}} = 0.1340; \ P = 0.339 \)).

**Table 3 - Significant correlations for the group ‘SRMA’**

<table>
<thead>
<tr>
<th>Evaluated parameters</th>
<th>Spearman’s rank correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of IL-6 in CSF</td>
<td>Concentration of VEGF in CSF</td>
</tr>
<tr>
<td>Concentration of IL-6 in serum</td>
<td>Concentration of VEGF in serum</td>
</tr>
<tr>
<td>Concentration of IL-6 in CSF</td>
<td>Number of leukocytes in CSF</td>
</tr>
<tr>
<td>Concentration of VEGF in CSF</td>
<td>Number of leukocytes in CSF</td>
</tr>
<tr>
<td>Concentration of TGF-β₁ in CSF</td>
<td>Concentration of IgA in CSF</td>
</tr>
</tbody>
</table>

SRMA: steroid-responsive meningitis-arteritis  
IL-6: interleukin-6; VEGF: vascular endothelial grow factor; TGF-β₁: Transforming growth factor beta; IgA: immunoglobulin A; CSF: cerebrospinal fluid  
* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.005 \)
3.6 Discussion

The aim of the present study was to verify the hypothesis that the three signalling proteins, IL-6, VEGF and TGF-β\textsubscript{1} are involved in the pathogenesis of SRMA.

We chose to investigate VEGF concentrations, since this parameter has been widely studied in human patients affected with Kawasaki Disease (KD), an acute febrile systemic vasculitis of children. SRMA has been proposed as an animal model for KD (BURNS et al. 1991; BURNS and GLODE 2004). In KD patients, levels of VEGF were found significantly elevated (TERAI et al. 1999; YASUKAWA et al. 2002; KARIYAZONO et al. 2004). The histopathology of meningeal arteries of dogs euthanised during the acute stage of SRMA typically revealed endothelial and subendothelial oedema, hyaline degeneration and a mild to moderate periarteritis (TIPOLD et al. 1995). Therefore, we hypothesized that elevated VEGF might cause these early vascular changes. Indeed, our results showed that VEGF was increased both systemically and intrathecally in SRMA patients during the clinical phases of the disease (‘SRMA’ and ‘SRMA R’) supporting this theory.

In other different inflammatory processes within the CNS (‘ME’ group), VEGF was found to be increased only in single cases, but overall the VEGF concentrations in the CSF of this group was significantly lower than in the SRMA group. We conclude that VEGF does not play an important role in other meningoencephalomyelitides. However, due to the heterogeneity of the diseases included in the ME group (e.g. granulomatous meningoencephalomyelitis, necrotising encephalitides and meningoencephalomyelitis of unknown origin) we cannot exclude that VEGF is involved in the pathogenesis of some of these diseases.

The values of VEGF in cases with systemic inflammation were lower than in dogs with SRMA (median 3.9 pg/mL and 43.92 pg/mL, respectively). However, the
difference was not statistically significant. This is in accordance with a recent study in which VEGF values were elevated in 64% of dogs with systemic inflammatory response syndrome. However, a correlation with clinical signs or increased permeability could not be proved (SILVERSTEIN et al. 2009).

VEGF is involved in the proposed pathogenesis of vasculitis in KD: after the permeability of the vessels is increased under the influence of VEGF, platelets might adhere to the vascular wall and inflammatory cells cross the loose endothelium, accumulating in the intima and becoming a source of proinflammatory cytokines. As final consequence there is a thickening of the intima as found in both diseases, KD and SRMA. If coronary artery lesions occur, this might even lead to life-threatening complications (BURNS and GLODE 2004). In KD patients VEGF was correlated with the development of coronary artery lesions (KARIYAZONO et al. 2004).

Interestingly in our study VEGF was not only found increased in the acute stage of SRMA, but also during relapses of SRMA (median in CSF 20.80 pg/mL; median in serum 33.71 pg/mL). Therefore, VEGF in SRMA may enhance vascular wall destruction in the acute phase of the disease before immune complexes appear (TIPOLD et al. 1995). Consequently, VEGF may be also involved in the development of the arterial lesions found during the chronic phase, such as increased wall thickness, stenosis and fibrosis (TIPOLD et al. 1995). On the other hand, VEGF might indicate vascular damage. A limitation of this study is the lack of comparison to other pure vasculitides. Experimental in vivo studies are probably necessary to elucidate the long-term effect of VEGF on canine vessels.
The recruitment and activation of different lymphocytes subsets after alteration of the CNS tissue by an environmental factor are caused by multiple mechanisms (TIPOLD et al. 1999). These include chemotactic agents (BURGENER et al. 1998), probably additional mechanisms such as changes of the blood-brain barrier (LACY et al. 1995; SPRENGER et al. 1996), and altered expression of selectins and integrins (GRANGER and KUBES 1994; SCHWARTZ et al. 2008a). To add more information to these previous studies and to verify the hypothesis that IL-6 and TGF-β₁ are involved in the pathogenesis of fever, pleocytosis and increased IgA production in SRMA, these proteins were determined in CSF and serum samples. Previous studies on cytokine expression in SRMA patients, showed an up-regulation of IL-4 and IL-8, while IL-2 and IFN-γ were found in low levels (BURGENER et al. 1998; SCHWARTZ et al. 2011). Hogenesch et al (1995) investigated IL-6 in serum of dogs with juvenile polyarteritis syndrome and detected increased IL-6 serum values. In preliminary studies, measurement of IL-6 in CSF was considered to be a valuable biomarker for the diagnosis of SRMA (LOWRIE et al. 2008). Information about the bioactive form of IL-6 in CSF is still missing.

In the current study IL-6 values were increased intrathecally and systemically in SRMA patients, the highest concentrations were found in CSF samples (median 1582 pg/mL in ‘SRMA’ and median 637.7 pg/mL in ‘SRMA R’ groups). In case of other inflammatory diseases of the CNS (‘ME’ group) the concentrations of CSF IL-6 were significantly lower (median 3 pg/mL), leading to the conclusion that IL-6 in SRMA might have a crucial role in the pathogenesis of the disease. Moreover, IL-6 in SRMA strongly correlated with the degree of pleocytosis indicating, that IL-6 values might be the result of the severe pleocytosis because of its production by macrophages (MURPHY et al. 2008b). Lowrie et al. (2008) also detected elevated IL-
6 CSF values in samples of dogs with a putative relapse and a normal CSF cell count, making the latter hypothesis less likely. Nevertheless further studies should address causes and consequences of IL-6 CSF values.

IL-6 has long-range effects, indeed it is one of the most important endogenous pyrogens, induces hepatocytes to synthesize acute-phase proteins, stimulates neutrophil mobilization from bone marrow and stimulates terminal differentiation of B cells to secret immunoglobulins (NAKA et al. 2002; TIZARD 2004c; MURPHY et al. 2008b). Therefore it is very likely that an overproduction of IL-6 is a major mediator of the most peculiar findings, such as fever, increased acute-phase proteins, neutrophilic pleocytosis and leukocytosis and increased IgA production during the acute phase of SRMA. The extreme high values of IL-6 in CSF also during relapses suggest that IL-6 exert its major functions intrathecally and throughout the waxing and waning course of the disease. As previously mentioned, the cell population in CSF of dogs during the acute phase of SRMA is predominantly composed of neutrophils. During the chronic form macrophages tend to prevail. Upregulation of CD11a on neutrophils (SCHWARTZ et al. 2008a), increased IL-8 levels in CSF (BURGENER et al. 1998) and the destruction of the blood–brain barrier (SCHWARTZ et al. 2010) have all been shown to be involved in neutrophil migration into CSF. Factors involved in the accumulation of monocytes in CSF of SRMA patients during the protracted form have not been investigated. Interestingly, IL-6 has been recently proposed to be a regulator of the transition from a neutrophil-dominated to a macrophage-dominated process (KAPLANSKI et al. 2003). We therefore propose a leading role for IL-6 in both the acute and protracted forms of the disease.
As expected, serum IL-6 concentrations were similar to the group of systemic inflammatory diseases supporting other studies, where IL-6 has been used not only as a general marker of inflammation (LEMAY et al. 1990), but in particular as a prognostic factor in canine systemic inflammatory response syndrome and sepsis (RAU et al. 2007). Also in KD IL-6 was increased in serum, but contrary to VEGF, the increase was not correlated with the development of coronary artery aneurysms and dilatation (KIM 1992; LIN et al. 1992).

In SRMA dogs both VEGF and IL-6 values were much higher in CSF compared to serum levels. This might reflect a more severe inflammatory process affecting meninges and meningeal vessels compared to peripheral vessels. Another possible explanation would be an excessive intrathecal production of these signalling proteins, followed by a secondary leakage into the systemic circulation. Further studies including protein associated gene expression and immunohistochemistry of meningeal and peripheral vessels might be necessary to elucidate the site of production.

TGF-β₁ in SRMA patients was decreased in serum (median 13.57 ng/mL) and elevated in CSF (median 90 pg/mL). The increased concentration in CSF was not specific for SRMA, indeed similar values have been found in the other meningoencephalomyelitides (median 58 pg/mL), while the reduced concentration in serum statistically differed from the other groups. The serum levels of TGF-β₁ were found to be decreased also in patients with KD (MATSUBARA et al. 1997), but to the authors knowledge data concerning levels of TGF-β₁ in CSF of patients with KD are lacking. The hypothesis that TGF-β₁ might be the most important pathogenetic factor for the excessive IgA production in SRMA could be partially rejected in the current study. Our results support the suggestion
that TGF-β₁ has a minor role in systemic production of IgA, whereas it is highly likely that it might still play an important role in the intrathecal production of IgA. Indeed, TGF-β₁ was positively correlated with IgA concentrations in CSF ($r_{\text{spear}} = 0.3549; P = 0.0337$). The rather unspecific elevation of TGF-β₁ values in CSF samples also supports a more immunoregulatory function of this cytokine in inflammatory CNS diseases (BETTELLI et al. 2006; MURPHY et al. 2008d). Further experimental in vivo and in vitro studies are needed to confirm this hypothesis.

Our findings indicate that the CSF cytokine profile of SRMA dogs during the acute phase was characterized by increased values of IL-6 and TGF-β₁. Recent progress in immunology led to the discovery of Th17 cells, a new subset of T helper cells (HARRINGTON et al. 2005; PARK et al. 2005). According to one study, the combined influence of both IL-6 and TGF-β₁ is necessary for the Th17 lineage to develop, while TGF-β₁ alone shifts the development of naïve T-cells towards T regulatory cells, a T-cell subset that restrains inflammation and maintains tolerance (BETTELLI et al. 2007).

The detected combined high intrathecal production of TGF-β₁ and IL-6 in SRMA could possibly lead to an increase of Th17 lymphocyte subset and enhance the development of an autoimmune response. Th17 cells produce IL-17A, the founding member of the IL-17 family. IL-17 members play an active role in inflammatory response and in autoimmune diseases (KOLLS and LINDEN 2004). Indeed, IL-17 family members coordinate local tissue inflammation inducing proinflammatory cytokines and neutrophil-mobilizing cytokines, such as IL-6 and TNF-alpha (KOLLS and LINDEN 2004). Experimental studies showed that overexpression of IL-17A in the joint space of mice with collagen-induced arthritis leads to increased neutrophil recruitment (LUBBERTS et al. 2001). This mechanism might be an additional
explanation of the massive invasion of neutrophils into the subarachnoidal space, the hallmark of the clinical-pathological findings in dogs affected with SRMA.

To date, SRMA has been believed to be a Th2-mediated immune disorder (SCHWARTZ et al. 2011), our results produce evidence that in SRMA Th17 skewed immune response might have a major role, particularly in the development of the meningitis.

### 3.7 Conclusions

Analysis of the pattern of signalling proteins production in SRMA showed many similarities with results in KD supporting the usefulness of this animal model. In our study increased concentrations of VEGF and IL-6 in serum and CSF of dogs affected with SRMA were found. TGF-β\(_1\) was increased in CSF and decreased in serum. This study gives evidence that these three signalling proteins might be involved in the pathogenesis of vasculitis, especially in a pronounced permeability and vessel wall damage. Both TGF-β\(_1\) and IL-6 are considered to be involved in the excessive IgA production. However, the presented data indicate that additional proteins may influence IgA production. The pleocytosis, the continuous ongoing of the disease and the invasion of neutrophils are supported by extremely high intrathecal IL-6 production. The hypothesis that SRMA might be a Th17-mediated disorder should be further investigated.

### 3.8 List of abbreviations

CBC complete blood cell count
CNS | central nervous system
---|---
CNS-Mix | miscellaneous non-inflammatory diseases of the CNS
CSF | cerebrospinal fluid
ELISA | enzyme-linked immunosorbent assays
IE | idiopathic epilepsy
IgA | immunoglobulin A
IL | interleukin
KD | Kawasaki Disease
ME | meningoencephalomyelitides
rcIL-6 | recombinant canine IL-6
$r_{\text{Spear}}$ | Spearman’s rank correlation coefficient
SRMA | steroid responsive meningitis-arteritis
SRMA R | SRMA relapse
SRMA Th | SRMA therapy
Syst. Infl. | systemic inflammatory diseases
TGF-β₁ | transforming growth factor beta 1
Th | T helper cells
TNF | Tumor necrosis factor
VEGF | vascular endothelial growth factor

### 3.9 Competing interests

The authors declare that they have no competing interests.
3.10 Authors' contributions

AT designed and supervised the study. AM performed the experiments and analysed the data concerning the part of VEGF and IL-6. OT performed the experiments and analysed the data concerning the part of TGF-β₁. RC gave substantial contributions to acquisition, analysis and interpretation of the data in all the experiments. MHT provided the laboratory, materials, supervision and substantial contribution to acquisition of the data for the immunohistochemical part of the experiment on TGF-β₁. AM drafted the manuscript and all authors contributed to the critical revision of the manuscript for important intellectual content and have read and approved the final version.

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Chapter 4: Toll-like receptors 4 and 9 responsible for the maintenance of the inflammatory reaction in canine Steroid-responsive Meningitis-arteritis, a large animal model for neutrophilic meningitis

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4.1 Abstract

Steroid-responsive Meningitis-arteritis (SRMA) is a systemic inflammatory disease affecting young adult dogs and a large animal model for neutrophilic meningitis. Similarities between SRMA and infectious central nervous system (CNS) diseases in lymphocytes subsets suggest an infectious origin.

Toll-like receptors (TLRs) are pattern recognition receptors playing an important role in innate immunity. Due to their ability to recognize both self and non-self antigens, we hypothesize that TLRs are among the key factors for the induction of the inflammatory process in SRMA and provide an indirect hint on the aetiology of the disease.

Therefore, the expression profile of cell surface TLRs (TLR2, TLR4 and TLR5) and intracellular TLRs (TLR3 and TLR9) of canine leukocytes was analysed by immunophenotyping and subsequent flow cytometric measurements. Experiments were performed on cerebrospinal fluid (CSF) and peripheral blood (PB) samples of dogs affected with SRMA during the acute phase (n = 14) as well as during treatment (n = 23) and compared with those of dogs with bacterial meningitis (n = 3), meningoencephalitis of unknown aetiology (n = 6), neoplasia of the central nervous system (n = 6) and a group of dogs with miscellaneous neurological diseases (n = 9). Two additional control groups consisted of dogs with pyogenic infections (n = 13) and of healthy dogs (n = 6).

All examined groups showed high percentage of TLR 2, TLR 4 and TLR 5 positive PB polymorphonuclear cells (PMNs) in comparison to healthy dogs. Very high values of TLR9 positive PB PMNs were detected in acute SRMA. Only few similarities were found between SRMA patients and dogs with pyogenic infections, both groups were characterized by high expression of TLR4 positive PB monocytes.
Glucocorticosteroid therapy reduced TLR2, TLR4 and TLR 9 expression in PB monocytes. A relatively high expression of TLR4 and TLR9 in acute SRMA let suggest that these two receptors might be involved in the inflammatory process in SRMA, enhancing the autoimmune reaction. Systematic CSF cells analysis for TLRs can be performed in future treatment studies in larger animals such as dogs.
4.2 Introduction

Steroid-responsive Meningitis-arteritis (SRMA) is a systemic inflammatory disease affecting young adult dogs. It is the most common cause of meningitis (MERIC 1988) and the most common cause of fever of unknown origin in dogs (BATTERSBY et al. 2006). In the last years SRMA has become well-recognised in veterinary practice, although a deep understanding of the disease is still lacking. Similarities between SRMA and infectious central nervous system (CNS) diseases in lymphocytes subsets suggested that the immune response in SRMA might be triggered by an antigen (TIPOLD et al. 1999), but such infectious agents were not directly detected (TIPOLD et al. 1996). SRMA has been proposed as a large animal model for Kawasaki Disease (BURNS et al. 1991), systematic flow cytometric (FACS) analysis of CSF is feasible in larger animals such as dogs (TIPOLD et al. 1998).

Toll-like receptors (TLRs) are pattern recognition receptors which recognize both invading pathogens (through pathogen-associated molecular patterns, PAMPs) and endogenous molecules produced by injured tissue (through damage-associated molecular patterns, DAMPs) (MONTERO VEGA and DE ANDRES MARTIN 2008). This recognition process plays a role in innate immunity, but participates also in the development of the adaptive immune response (IMLER and HOFFMANN 2001; WONG et al. 2009). Additionally, TLRs may be involved in the induction of chronic inflammation and autoimmune reactions (LIU et al. 2003; HURST and VON LANDENBERG 2008; WONG et al. 2009; GREEN and MARSHAK-ROTHSTEIN 2011). There are many examples of systemic human diseases in which an association with TLRs has been found (MARSHAK-ROTHSTEIN 2006) and include systemic lupus erythematosus (BARRAT et al. 2005), giant cell arteritis (MA-KRUPA...
et al. 2005; DENG et al. 2009), Sjögren’s syndrome (LOVGREN et al. 2006), autoimmune arthritis (ABDOLLAHI-ROODSAZ et al. 2008) and multiple sclerosis (PRINZ et al. 2006). In the dog TLRs have been found upregulated in inflammatory bowel disease (BURGENER et al. 2008). The TLR expression on CSF leukocytes was not yet widely studied.

To date SRMA is believed to be characterized by a Th2-mediated immune response (SCHWARTZ et al. 2011), but it is still unclear if this reaction is triggered by environmental factors or self antigen (hit-and-run principle).

Recognising both self (DAMPs) and non-self (PAMPS) molecules, TLRs are most probably involved in the inflammatory process in SRMA. To confirm the hypothesis that SRMA is triggered by an environmental factor changing the TLR pattern, the expression profile of cell surface TLRs (TLR2, TLR4 and TLR5) and intracellular TLRs (TLR3 and TLR9) should be examined on canine leukocytes. An indirect hint on the aetiology of SRMA was expected.
4.3 Materials and methods

4.3.1 Dog population and samples

The study population consisted of eighty dogs referred to the Department of Small Animal Medicine and Surgery, University of Veterinary Medicine, Hannover, Germany between May 2009 and April 2011. The studies were conducted according to the ethical guidelines of the University for Veterinary Medicine Hannover. Depending on the clinical diagnosis, the dogs were assigned to one of the following groups (see table 1).

SRMA Acute (“SRMA A“): The diagnosis of SRMA was supported by the detection of typical findings during physical and neurological examinations, complete blood and CSF examinations, cervical radiographs, elevated IgA concentrations in CSF and serum and the absence of other conditions causing cervical pain (TIPOLD and SCHATZBERG 2010). Dogs with the acute form of SRMA, but pretreated with glucocorticosteroids prior to CSF puncture were excluded from the study.

SRMA Therapy (“SRMA Th“): dogs from the former group under glucocorticosteroid treatment that did not show clinical signs at the time of sampling. Dogs under treatment for SRMA received prednisolone, the dosage ranged from 1mg/kg/24h to 0.5 mg/kg/48h.

Other groups were: Bacterial Meningitis or meningoencephalitis (“BM“); Meningoencephalitis of unknown aetiology (“MUE“); CNS Neoplasia (“Neopl“) and a group of dogs with miscellaneous neurological diseases (“Mix“) (see table 1). In dogs with BM, MUE, CNS neoplasia and dogs with miscellaneous neurological diseases, in addition to the diagnostic procedures described for SRMA, magnetic resonance
imaging (MRI), electrophysiological studies, surgery and histopathology contributed to the diagnosis.

Since SRMA is considered to be a systemic inflammatory disorder (BATHEN-NOETHEM et al. 2008), leukocytes of dogs with pyogenic infections not affecting the nervous system (“Pyo”) were evaluated as a further control group. Another control group (“Healthy”) consisted of private owned blood donors from the hospital and were considered to be healthy because history, complete physical examination, blood examination and clinical follow-up examinations did not reveal any abnormalities. The owners approved the blood examinations.

From each dog five mL of blood was collected via venipuncture of cephalic or saphenous veins into tubes containing ethylene diamine tetraacetic acid (EDTA) for collection of peripheral blood (PB) leukocytes.

Cisternal cerebrospinal fluid (CSF) collection was part of the work-up in all dogs for collection of CSF leukocytes with the exception of healthy animals and patients with pyogenic infections.
Table 1: Distribution of disease categories

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Findings</th>
<th>Number of dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRMA Acute (SRMA A)</td>
<td>Dogs with fever, cervical pain, neutrophilic leukocytosis and pleocytosis, no pre-treatment with glucocorticosteroid</td>
<td>14</td>
</tr>
<tr>
<td>SRMA Therapy (SRMA Th)</td>
<td>Dogs from “SRMA A” group, asymptomatic under long-term glucocorticosteroid treatment</td>
<td>23</td>
</tr>
<tr>
<td>Bacterial Meningitis (BM)</td>
<td>Dogs with meningitis/meningoencephalitis caused by bacterial infections</td>
<td>3</td>
</tr>
<tr>
<td>Meningoencephalitis of unknown aetiology (MUE)</td>
<td>Dogs with clinical, CSF, MRI and/or pathological findings consistent with meningoencephalitis, in which no causative agent has been identified.</td>
<td>6</td>
</tr>
<tr>
<td>Neoplasia (Neopl.)</td>
<td>Dogs with clinical, CSF, MRI and/or pathological findings consistent with neoplasia of the CNS</td>
<td>6</td>
</tr>
<tr>
<td>Miscellaneous (Mix)</td>
<td>Dogs with miscellaneous non-inflammatory neurological diseases including intervertebral disc disease, peripheral nervous system diseases and idiopathic epilepsy</td>
<td>9</td>
</tr>
<tr>
<td>Pyogenic infection (Pyo)</td>
<td>Dogs suffering from diseases caused by pyogenic infections, such as pyometra, pyothorax and bacterial peritonitis</td>
<td>13</td>
</tr>
<tr>
<td>Healthy</td>
<td>Healthy dogs</td>
<td>6</td>
</tr>
</tbody>
</table>

SRMA: steroid-responsive meningitis-arteritis; CSF: cerebrospinal fluid; CNS: central nervous system; MRI: Magnetic resonance imaging

4.3.2 Isolation, permeabilisation and fixation of peripheral blood leukocytes

After collection, 1 mL of EDTA PB was used for staining of cell surface TLRs (TLR2, TLR4 and TLR5) and 0.5 mL for intracellular staining (TLR3 and TLR9). Leukocytes were fixed, in order to preserve their marker expression.

For the staining of cell surface TLRs, the cells were fixed using a previously described method of preparing blood leukocytes for flow cytometric analysis.
(HAMBLIN et al. 1992). The method has been previously validated from Burgener and Jungi (2008) for detection of TLRs on canine leukocytes. Briefly, the blood was mixed with the same volume of preheated 0.4% formaldehyde (diluted in phosphate buffered saline (PBS; containing 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium hydrogen phosphate, 1.5 mM monopotassium phosphate, pH 7.4) and incubated for 4 minutes at 37°C. Successively, 40 mL of warmed lysing buffer (0.83% ammonium chloride/0.01 M Tris chloride, pH 7.4) was added and the mixture was incubated at 37°C until red-cell lysis was observed (about one to two minutes). After centrifugation at 160 x g for 10 minutes the supernatant was discarded and the pellet was washed twice with PBS.

For intracellular staining the blood was mixed with BD FACS™ Lysing Solution, twice its volume, diluted 1:10 (BD Biosciences, Erembodegem, Belgium), and incubated for 10 minutes. After a washing step at 500 x g for 5 minutes using PBS containing 1.25% pooled dog serum, BD FACS™ Permeabilizing Solution 2 was added for 10 minutes according to the description of the manufacturer (BD Biosciences, Erembodegem, Belgium). Ultimately, an additional washing step was performed.

The number of leukocytes was determined in both procedures using a hemocytometer and the cell suspension was adjusted to $2.5 \times 10^5$ leukocytes/50 µL using PBS containing 1.25% pooled dog serum.

### 4.3.3 Isolation, permeabilisation and fixation of cerebrospinal fluid leukocytes

Immediately after tapping, CSF was aliquoted in two tubes and centrifuged at 200 x g for 10 minutes, as described by Schwartz et al. (2008a).
As described above for blood leukocytes, one CSF aliquot underwent fixation (cell surface TLRs) and the other CSF aliquot underwent permeabilisation (intracellular TLRs). Both procedures were performed as described in Section 4.3.2 with the exception of the lysing steps.

4.3.4 Monoclonal antibodies (mAbs) and immunostaining

A study from Burgener et al. (2008) demonstrated that commercial antibodies against human TLRs cross-react with canine TLRs. According to this study, human antibodies were chosen and listed in Table 2.

In addition, antibodies against cell surface antigens were used to identify leukocyte subclasses, such as lymphocytes (CD3⁺ or CD21⁺), polymorphonuclear cells (CD11a⁺/CD11b⁺) and monocytes (CD14⁺), (see table 2). The secondary antibody was an F(ab’)₂-fragment specific RPE-labeled goat-anti-mouse IgG antibody (Dianova, Hamburg, Germany) (1:200 dilution).

Negative controls consisted of isotype-matched primary antibodies (see table 2) and cell suspensions stained with the secondary antibody alone.

Incubation was performed for 30 minutes at 4°C under light protection. The washing steps were performed with PBS with 1.25% of canine pooled serum in order to prevent unspecific Fc-receptor binding of mAbs.
### Table 2: Monoclonal antibodies

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Name</th>
<th>Clone</th>
<th>Provider</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD282/TLR2*</td>
<td>mouse anti human CD282</td>
<td>TL2.1</td>
<td>Serotec</td>
<td>1:26</td>
</tr>
<tr>
<td>CD283/TLR3*</td>
<td>mouse anti human CD283</td>
<td>TLR3.7</td>
<td>Serotec</td>
<td>1:26</td>
</tr>
<tr>
<td>CD284/TLR4*</td>
<td>mouse anti human CD284</td>
<td>HTA125</td>
<td>Serotec</td>
<td>1:26</td>
</tr>
<tr>
<td>CD289/TLR5*</td>
<td>mouse anti human CD289</td>
<td>85B152.5</td>
<td>Acris</td>
<td>1:26</td>
</tr>
<tr>
<td>CD289/TLR9*</td>
<td>mouse anti human CD289</td>
<td>5G5</td>
<td>Serotec</td>
<td>1:26</td>
</tr>
<tr>
<td>CD3</td>
<td>mouse anti dog CD3</td>
<td>CA17.2A12</td>
<td>Serotec</td>
<td>1:300</td>
</tr>
<tr>
<td>CD11a</td>
<td>mouse anti dog CD11a</td>
<td>CA11.4D3</td>
<td>Serotec</td>
<td>1:300</td>
</tr>
<tr>
<td>CD11b</td>
<td>mouse anti dog CD11b</td>
<td>CA16.3E10</td>
<td>Serotec</td>
<td>1:6</td>
</tr>
<tr>
<td>CD14*</td>
<td>mouse anti human CD14β</td>
<td>TÜK4</td>
<td>Dako</td>
<td>1:15</td>
</tr>
<tr>
<td>CD21</td>
<td>mouse anti canine CD21β</td>
<td>CA2.1D6</td>
<td>Serotec</td>
<td>1:6</td>
</tr>
<tr>
<td>IgG1</td>
<td>mouse IgG1 negative control</td>
<td>W3/25</td>
<td>Serotec</td>
<td>1:6</td>
</tr>
<tr>
<td>IgG2a</td>
<td>mouse IgG2a controlβ</td>
<td>PPV-04</td>
<td>ImmunoTools</td>
<td>1:6</td>
</tr>
</tbody>
</table>

*Reacts with canine species; §RPE-Conjugated

### 4.3.5 Flow cytometry

Samples were analyzed using a standard FACSCalibur™ flow cytometer and the BD CellQuest™ Pro Version 5.2.1 software (Becton Dickinson, Heidelberg, Germany).

Leukocyte populations (Fig. 1) were gated according to light scatter properties and CD expression into lymphocytes, monocytes and polymorphonuclear cells (PMNs), as previously described (DUQUE et al. 2002).

All events in CSF samples and a minimum of 10000 events in blood samples were collected.
Figure 1 Population of CSF leukocytes: granulocytes (R1, green), lymphocytes (R2, pink) and monocytes (R3, red).

4.3.6 Statistical analysis

The percentage of positive cells and the Mean Fluorescence Intensity (MFI) of each group were used for statistical analysis using a commercial statistical program (GraphPrism). The Wilcoxon rank sum test and the Kruskal–Wallis one-way analysis of variance were applied for comparison of the results deriving from the different groups. Statistical significance was set at the 5% level ($P < 0.05$).
4.4 Results

4.4.1 Expression of TLRs on CSF and PB leukocytes

The expression of intracellular and surface TLRs on CSF and PB leukocytes of untreated dogs affected with SRMA are summarised in table 3, results are given as % of positive cells. Statistically relevant results among the different leukocyte subsets and disease categories are shown in Figures 2, 3 and 4.

4.4.1.1 Expression of TLRs on PMNs

Generally, all groups with diseased dogs displayed a high percentage of TLR2, TLR4 and TLR5 positive PB PMNs in comparison to healthy dogs.

In SRMA dogs under treatment a tendency ($P = 0.0669$) was noted towards a decreased expression of TLR2 on PMNs (median 97.5; range 97.1-98.7 %) in comparison to dogs with the acute form of SRMA (median 98.7 %; range 98.1-99.3 %) (see Fig. 2-C). In CSF there were no significant differences among the groups, however the highest values of TLR2 positive PMNs were detected in dogs in the acute form of SRMA (“SRMA A”, median 83.8 %; range 79.6-90.9 %) (see Fig 2-D).

SRMA dogs showed higher expression ($P = 0.0023$) of TLR4 positive PB PMNs (“SRMA A”, median 98.6 %; range 98.1-99.7 %) in comparison to healthy controls (“Healthy”, median 89.8 %; range 82.5-93.7 %) and to dogs affected with miscellaneous diseases of the nervous system (“Mix”, median 95-6 %; range 94-97.5 %) ($P = 0.0095$) (see Fig 2-E). In CSF the highest values of TLR4 positive PMNs were found in dogs in the acute stage of SRMA (median 93.1 %; range 81.7-95.3 %)
with significant differences \((P < 0.05)\) to cases with encephalitides of unknown aetiology (“MUE”, median 67 %; range 23.1-77.9 %) and with CNS neoplasia (“Neopl”, median 58.1 %; range 24-70.7 %) (see Fig 2-F).

All groups of diseased animals had significantly higher percentages of TLR5 positive PB PMNs \((P < 0.05)\) in comparison to healthy animals (“Healthy”, median 79.5; range 45.7-90.6 %) (see Fig 2-G). In CSF higher percentage of TLR5 positive PMNs \((P < 0.05)\) was found in “SRMA A” (median 91.6 %; range 70.9-95.3 %) in comparison with “MUE” (median 52.1 %; range 16-6-72.6 %) and “Neopl” (median 35.8 %; range 18.7-59.3 %) (see Fig 2-H).

Dogs from “SRMA A” (median 7.5 %; range 5.2-11 %), “SRMA Th” (median 6.3%; range 3.9-10.4 %), “Neopl.” (median 26.1; range 8.9-35.8 %) and “Mix” (median 7.5; range 8.9-35.8 %) groups showed a higher percentage of PMNs positive for TLR3 \((P < 0.05)\) in comparison to dogs with pyogenic infections (“Pyo”, median 2.4; range 1.3 - 4.7 %). “SRMA Th” and “Pyo” dogs had a significant lower \((P < 0.05)\) percentage of TLR3 positive PMNs in comparison to healthy dogs (see Fig 2-A). No significant differences were found between the groups examined for expression of TLR3 positive PMNs in CSF. However, a lower percentage of TLR3 positive PMNs was observed in “SRMA A” (median 2.4 %; range 1.5-3.7 %) in comparison to “Neopl” (median 10.5 5; range 4.3-14.8 %).

Healthy dogs showed lowest values of TLR9 positive PB PMNs (“Healthy”, median 20.82 %; range 10.1-41.2 %) in comparison to all other groups \((P < 0.05)\), with the exception of “MUE” and “Pyo” (see Fig 2-B). The highest values of TLR9 positive PMNs were detected in “SRMA A” (median 96.3; range 60.8-97.7). The groups did not differ statistically displaying TLR9 positive PMNs in CSF.
In general, the fluorescence expression intensity did not differ significantly among the groups examined. An exception was the expression intensity of TLR3 on PB PMNs, “SRMA A” had a lower TLR3 MFI in comparison to “Pyo” ($P = 0.0149$) and “Healthy” ($P = 0.0077$).
Figure 2 Percentage of TLR positive PMNs in different disease categories

TLR: Toll-Like Receptor; PMNs: polymorphonuclear cells; PB: peripheral blood; CSF: cerebrospinal fluid; SRMA: Steroid-responsive Meningitis-Arteritis; SRMA A: SRMA Acute; SRMA Th: SRMA under therapy; BM: Bacterial Meningitis; MUE: Meningoencephalitis of unknown aetiology; Mix: Miscellaneous diseases of the nervous system; Pyo: Pyogenic infection; Healthy: healthy dogs. Boxes contain values from 1st to 3rd quartile, lines inside boxes indicate median values, endpoints of vertical lines display 5th–95th percentile and • represent the outliers. Asterisks indicate statistically significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$)
4.4.1.2 Expression of TLRs on monocytes

Dogs with SRMA, similar to dogs with pyogenic infections, were characterized by high values of TLR4 positive PB monocytes. In SRMA dogs the percentage of TLR2, TLR4 and TLR9 positive PB monocytes decreased after therapy.

The highest percentages of TLR2 positive monocytes were found in dogs with pyogenic infections (“Pyo”, median 96.6%; range 95.4-97.9%) and untreated SRMA dogs (“SRMA A”, median 96.3%; range 94-97.9%), being significantly higher than in “SRMA Th” (median 90.7%; range 88.4-95.3%; P < 0.01), “Neopl.” (median 90.5%; range 87.7-96.4%; P < 0.05) and “Mix” (median 90.3%; range 76.3-94.9%; P < 0.01) (see Fig 3-C). The expression of TLR2 positive monocytes in CSF did not statistically differ among the groups, however, a tendency in “SRMA A” to have a higher percentage of TLR2 positive monocytes (median 87%; range 78.9-94.3%) in comparison with “MUE” (median 26.2%; range 15.7-62.4%) was observed.

Higher percentages of TLR4 positive PB monocytes were found in untreated SRMA dogs (“SRMA A”, median 95.6%; range 93.3-96.9%; P = 0.0032) and in dogs with pyogenic infections (“Pyo”, median 97.2%; range 94.8-97.9%; P = 0.0015) in comparison to control group (“Healthy”, median 84.8%; range 77.3-88.0%). SRMA dogs under therapy showed a decrease in TLR4 positive PB monocytes (“SRMA Th”, median 91.2%; range 86.6-95.5%; P = 0.019) in comparison to the untreated dogs (see Fig 3-D). The expression of TLR4 positive monocytes in CSF did not statistically differ among the groups.

Similar to “SRMA Th”, “MUE” and “Pyo”, untreated SRMA dogs showed higher percentage of TLR5 positive PB monocytes (“SRMA A”, median 95.3%; range 92.3-97.4%; P = 0.0233) in comparison to healthy dogs (“Healthy”, median 77.6%; range
27.4-91.6 %). The expression of TLR5 positive monocytes in CSF did not statistically differ among the groups.

Dogs affected with SRMA showed higher percentage of TLR3 positive PB monocytes (“SRMA A”, median 8.6 %, range 4.5.-15.21 %; \( P = 0.0073 \)) in comparison to dogs affected with pyogenic infections (“Pyo”, median 2.1%; range 1.4-4 %). Similar percentages have been found in the remaining groups, with the exception of the healthy controls (see Fig 3-A). The expression of TLR3 positive monocytes in CSF did not statistically differ among the groups.

Untreated dogs with SRMA showed higher (\( P = 0.003 \)) percentage of TLR9 positive PB monocytes (“SRMA A”, median 92.13 %; range 67.2-96.9 %) in comparison to healthy dogs (“Healthy”, median 38.8 %; range 18.7-54 %). Similar values were found also in the remaining groups. In dogs with SRMA the expression of TLR9 positive PB monocytes statistically decreased (\( P = 0.0499 \)) under therapy (“SRMA Th” median 77.1 %; range 47.1- 88.4 %) (see Fig 3-B). The expression of TLR9 positive monocytes in CSF did not statistically differ among the groups.

The fluorescence expression intensity did not differ significantly among the examined groups, however a tendency of “SRMA A” in expressing higher TLR9 MFI in PB PMNs in comparison to “Pyo” and “Healthy” was observed.
Figure 3 Percentage of TLR positive monocytes in different disease categories

TLR: Toll-Like Receptor; PB: peripheral blood; CSF: cerebrospinal fluid; SRMA: Steroid-responsive Meningitis-Arteritis; SRMA A: SRMA Acute; SRMA Th: SRMA under therapy; BM: Bacterial Meningitis; MUE: Meningoencephalitis of unknown aetiology; Mix: Miscellaneous diseases of the nervous system; Pyo: Pyogenic infection; Healthy: healthy dogs. Boxes contain values from 1st to 3rd quartile, lines inside boxes indicate median values, endpoints of vertical lines display 5th–95th percentile and ⋄ represent the outliers. Asterisks indicate statistically significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$)
4.4.1.3 Expression of TLRs on lymphocytes

Generally, lymphocytes of SRMA dogs were characterized by decreased percentage of TLR2 and TLR4 positive cells in PB and decreased TLR4 expression in CSF in comparison to other diseases, whereas TLR9 was highly expressed in PB.

Dogs affected with SRMA showed lower percentages ($P = 0.0094$) of TLR2 positive PB lymphocytes (“SRMA A”, median 6.2%; range 1.5-13.6%) in comparison to healthy dogs (“Healthy”, median 26.8%; range 18.1-38.9%). Similar findings were seen in the other groups, with the exception of “MUE” and “Neopl”. The lowest values were found in dogs with bacterial meningoencephalitis (“BM”, median 0.2%; range 1.8-0.1%), being statistically lower than those found in “SRMA A” ($P = 0.0197$) (see Fig. 4-C). “SRMA A” and “SRMA Th” dogs showed also lower fluorescence intensity values than “Pyo” and “Healthy”, being statistically relevant only for “SRMA Th” ($P < 0.01$). “SRMA A” had higher percentage of TLR2 positive lymphocytes in CSF (median 13%; range 8.1-18%) than “BM” (median 0.2%; range 0.1-0.7%) (see Fig. 4-D). Similar differences were observed between these two groups in MFI values.

“SRMA A” ($P = 0.0076$) and the remaining groups (except “MUE” and “Pyo”) had a lower percentage of TLR4 positive PB lymphocytes than “Healthy” (median 30.6; range 21.1-31.9%) (see Fig. 4-E). “SRMA Th” had lower MFI values than “Pyo” ($P = 0.0002$) and “Healthy” ($P = 0.004$). Dogs with untreated SRMA displayed statistically lower ($P < 0.01$) percentage of TLR4 positive lymphocytes in CSF (“SRMA A”, median 13%; range 3-23.9%) compared to SRMA dogs under therapy (“SRMA Th”, median 43.8%; range 19.2-70.7%) and dogs with miscellaneous diseases of the nervous system (“Mix”, median 45.3%; range 33.4-56.2%) (see Fig.
4-F). In “SRMA A” also the MFI values were statistically lower ($P = 0.0004$) in comparison to “SRMA Th”.

The percentage of TLR5 positive lymphocytes in PB and CSF did not differ among the groups. However, significant differences among the groups were found in the fluorescence expression intensity of TLR 5 in PB lymphocytes. Dogs from both “SRMA A” and “SRMA Th” groups showed lower MFI values in comparison to dogs affected with pyogenic diseases ($P = 0.02$ and $P = 0.003$, respectively). Additionally, there was a tendency for “SRMA A” towards lower MFI values in comparison to healthy dogs, but this difference was statistically relevant only for “SRMA Th” ($P = 0.0056$).

The percentage of TLR3 positive lymphocytes in PB in dogs affected with SRMA did not differ from the other groups (see Fig. 4-A). Also statistically relevant differences in TLR3 expression on CSF lymphocytes were not found. Nevertheless, a tendency of lower percentage of TLR3 positive CSF lymphocytes in “SRMA A” (median 2.9 %; range 1-11.6 %) comparing to “SRMA Th” (median 19.35 %; range 4.4-38.4 %) was detected.

Higher percentage ($P < 0.05$) of TLR9 positive PB lymphocytes in untreated SRMA dogs (“SRMA A”, median 85.24 %; range 71.2-92.8 %) and under treatment (“SRMA Th”, median 81.9 %, range 33-89 %) were found in comparison to dogs with pyogenic diseases (“Pyo”, median 31.63 %; range 6.6-51.4 %) and healthy dogs (“Healthy”, median 35.8; range 24.2-49.6 %) (see Fig. 4-B). The percentage of TLR9 positive lymphocytes in CSF did not statistically differ among the investigated groups.
Figure 4 Percentage of TLR positive lymphocytes in different disease categories

TLR: Toll-Like Receptor; PB: peripheral blood; CSF: cerebrospinal fluid; SRMA: Steroid-responsive Meningitis-Arteritis; SRMA A: SRMA Acute; SRMA Th: SRMA under therapy; BM: Bacterial Meningitis; MUE: Meningoencephalitis of unknown aetiology; Mix: Miscellaneous diseases of the nervous system; Pyo: Pyogenic infection; Healthy: healthy dogs. Boxes contain values from 1st to 3rd quartile, lines inside boxes indicate median values, endpoints of vertical lines display 5th–95th percentile and • represent the outliers. Asterisks indicate statistically significant differences (* P < 0.05; ** P < 0.01; *** P < 0.005)
Table 3: Expression of TLRs in/on leukocytes in SRMA (percentage of positive cells, median and 25%–75% range)

<table>
<thead>
<tr>
<th>TLRs</th>
<th>SRMA A</th>
<th>significant differences</th>
<th>tendencies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CSF PMNs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>2.4 (1.5-3.7)</td>
<td>none</td>
<td>Neopl†</td>
</tr>
<tr>
<td>TLR9</td>
<td>94.1 (53.8-99.0)</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>83.8 (79.6-90.9)</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>93.1 (81.7-95.3)</td>
<td>MUE↓</td>
<td></td>
</tr>
<tr>
<td>TLR5</td>
<td>91.6 (70.9-95.3)</td>
<td>MUE↓</td>
<td></td>
</tr>
<tr>
<td><strong>CSF monocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>14.4 (3.7-30.5)</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>TLR9</td>
<td>85.0 (48.0-95.0)</td>
<td>none</td>
<td>MUE↓</td>
</tr>
<tr>
<td>TLR2</td>
<td>87.0 (78.9-94.3)</td>
<td>none</td>
<td>MUE↓</td>
</tr>
<tr>
<td>TLR4</td>
<td>82.6 (74.5-93.2)</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>TLR5</td>
<td>83.3 (62.8-94.1)</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td><strong>CSF lymphocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>2.9 (1.0-11.6)</td>
<td>none</td>
<td>SRMA Th↑</td>
</tr>
<tr>
<td>TLR9</td>
<td>2.9 (18.8-1.5)</td>
<td>none</td>
<td>mix↓</td>
</tr>
<tr>
<td>TLR2</td>
<td>13.0 (8.1-18.0)</td>
<td>BM↓</td>
<td>MUE↓</td>
</tr>
<tr>
<td>TLR4</td>
<td>13.0 (3.0-23.9)</td>
<td>SRMA Th↑</td>
<td>BM↓</td>
</tr>
<tr>
<td>TLR5</td>
<td>11.1 (3.7-18.8)</td>
<td>none</td>
<td>BM↓</td>
</tr>
<tr>
<td><strong>PB PMNs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>7.5 (5.3-11)</td>
<td>Pyo↓</td>
<td>Neopl↑</td>
</tr>
<tr>
<td>TLR9</td>
<td>96.3 (60.8-97.7)</td>
<td>Healthy↓</td>
<td>Healthy↑</td>
</tr>
<tr>
<td>TLR2</td>
<td>98.7 (98.1-99.3)</td>
<td>Healthy↓</td>
<td>Mix↓</td>
</tr>
<tr>
<td>TLR4</td>
<td>98.6 (98.1-99.7)</td>
<td>Healthy↓</td>
<td>Mix↓</td>
</tr>
<tr>
<td>TLR5</td>
<td>99.0 (97.4-99.5)</td>
<td>Healthy↓</td>
<td></td>
</tr>
<tr>
<td><strong>PB monocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>8.6 (4.5-15.21)</td>
<td>Pyo↓</td>
<td>Neopl↑</td>
</tr>
<tr>
<td>TLR9</td>
<td>92.1 (67.2-96.9)</td>
<td>SRMA Th↓</td>
<td>Pyo↓</td>
</tr>
<tr>
<td>TLR2</td>
<td>96.3 (94.0-97.9)</td>
<td>Healthy↓</td>
<td>SRMA Th↓</td>
</tr>
<tr>
<td>TLR4</td>
<td>95.6 (93.3-96.9)</td>
<td>SRMA Th↓</td>
<td>Neopl↓</td>
</tr>
<tr>
<td>TLR5</td>
<td>95.3 (92.3-97.4)</td>
<td>Mix↓</td>
<td></td>
</tr>
<tr>
<td><strong>PB lymphocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>4.0 (3.1-5.8)</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>TLR9</td>
<td>85.2 (71.2-92.8)</td>
<td>none</td>
<td>Pyo↓</td>
</tr>
<tr>
<td>TLR2</td>
<td>6.4 (4.5-13.6)</td>
<td>BM↓</td>
<td>MUE↑</td>
</tr>
<tr>
<td>TLR4</td>
<td>8.2 (3.8-13.5)</td>
<td>Healthy↑</td>
<td>Neopl↑</td>
</tr>
<tr>
<td>TLR5</td>
<td>6.125 (4.4-18.7)</td>
<td>none</td>
<td>BM↓</td>
</tr>
</tbody>
</table>

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(continue from Table 3) TLR: Toll-Like Receptor; PMNs: polymorphonuclear cells; CSF: cerebrospinal fluid; SRMA: Steroid-responsive Meningitis-Arteritis; SRMA A: SRMA Acute; SRMA Th: SRMA under therapy; BM: Bacterial Meningitis; MUE: Meningoencephalitis of unknown aetiology; Mix: Miscellaneous diseases of the nervous system; Pyo: Pyogenic infection; Healthy: healthy dogs. 
↓ lower or ↑ higher values in comparison to “SRMA A”;

4.5 Discussion

In the last decades studies on the aetiopathogenesis of SRMA mostly focused on the role of lymphocytes (TIPOLD et al. 1995; TIPOLD et al. 1999; SCHWARTZ et al. 2008b; SCHWARTZ et al. 2011). Indeed, for many years the adaptive immune system has been believed to play the most important role triggering an inappropriate immune response. However, more recently, the innate immune system aroused much interest for its ability to modify the adaptive immune response, particularly in autoimmunity and immune-mediated diseases (PISETSKY 2008; MONTERO VEGA and DE ANDRES MARTIN 2009). TLRs are important components of the innate immune system: their ability to initiate and propagate inflammation protects the organism from infectious diseases (TAKEDA et al. 2003). On the other hand, an excessive activation of these receptors may lead to immune disorders (AKASHI-TAKAMURA and MIYAKE 2006; FISCHER and EHLERS 2008; HURST and VON LANDENBERG 2008; MONTERO VEGA and DE ANDRES MARTIN 2009). The ambivalent role of these receptors makes them interesting candidates for immune pathological studies in SRMA patients, especially because CSF cells can be studied by systematic flow cytometric studies in this large animal model. The activation profile of TLRs in SRMA was tested to support the hypothesis that these receptors are stimulated by infectious antigens or endogenous proteins (self-antigens). It was hypothesized, that they are key factors for the initiation of the inflammatory process
and provide an indirect hint on the aetiology of the disease. Therefore the expression of TLRs in dogs affected with SRMA was measured and compared to infectious diseases or other neurological conditions.

The current study did not show clear similarities between TLRs expression profiles of SRMA dogs and dogs affected with bacterial/pyogenic infections. We therefore concluded that SRMA is not maintained by a continuous bacterial infection. This hypothesis is also supported by the clear response to long term treatment with glucocorticosteroids (CIZINAUSKAS et al. 2000; TIPOLD 2000; LOWRIE et al. 2009). However, triggering an autoimmune reaction by bacteria cannot be ruled out completely.

Indeed, an exception was detected concerning TLR4, which was statistically more frequently expressed on monocytes of dogs with untreated SRMA and dogs with pyogenic infections. TLR4 recognizes not only lipopolysaccharides, but also some endogenous ligands such as heat shock proteins (HSP60, HSP70), fibronectin, hyaluronic acid, fibrinogen and heparan sulfate (TAKEDA et al. 2003). In the current study the triggering factor for increased TLR4 expression, a self or non-self antigen, was not examined. However, a recent study showed that HSP 70 is elevated in SRMA (MOORE et al. 2011). Therefore it seems to be very likely that the triggering protein in SRMA might derive from a self antigen such as the HSP 70.

The role of TLR4 in human patients with sepsis, but also in non infectious diseases such as inflammatory bowel disease and rheumatoid arthritis were studied (O'NEILL et al. 2009). In the canine patient the role of TLR4 during sepsis and the related systemic inflammatory response syndrome is still not investigated. However, some studies have already proven the role of TLR4 in dogs with osteoarthritis (KUROKI et al. 2010) and chronic enteropathies (BURGENER et al. 2008).
The role of TLR4 for neutrophil recruitment into the CNS was demonstrated in a murine model of systemic inflammation (ZHOU et al. 2009). Similar mechanisms might lead to the invasion of neutrophils into the subarachnoidal space in SRMA and explain the exorbitant neutrophilic pleocytosis in acute cases. A recent study on human large vessel vasculitides suggested that TLR4 is causing transmural panarteritis (DENG et al. 2009). Clinical and histopathological findings in dogs affected with SRMA include neutrophilic leukocytosis, neutrophilic pleocytosis and systemic vasculitis. Treatment with glucocorticosteroids reduce these pathological processes and the expression of TLR4 on monocytes declines significantly contemporarily ($P = 0.019$). These findings strongly suggest that TLR4 plays an important role in triggering the described pathological findings in SRMA. Additionally, it opens the discussion for new treatment modalities such as anti-TLR4 antibodies and TLR4 antagonist, some compounds from the latter class are already under clinical trials for treatment of sepsis in human patients (KANZLER et al. 2007; O’NEILL et al. 2009).

TLR9 seems to be constantly increased on PB leukocytes in almost every disease examined in the current study. However, patients with SRMA and dogs affected with meningoencephalitides of unknown aetiology showed the highest expression of TLR9, suggesting a possible role of this TLR in inflammatory CNS diseases with a suggested autoimmune component. TLR9 is primarily involved in the recognition of bacterial DNA (TAKEDA et al. 2003). In human medicine TLR9 seems also to play an important role in class-switching to pathogenic autoantibody production in systemic autoimmunity (GREEN and MARSHAK-ROTHSTEIN 2011), leading again to the suggestion that an autoimmune reaction can maintain the inflammatory reaction in SRMA.
However, the role of TLR9 inducing autoimmunity is contrarily discussed. TLR9 can have a more regulatory function (MARTA et al. 2008) or enhance pathologic processes (PRINZ et al. 2006) in the same animal model of multiple sclerosis. Further studies are needed to elucidate the role of TLR9 in SRMA.

Dendritic cells, once activated by TLR4 and TLR9, produce interleukin-23 (IL-23), which subsequently activate CD4$^+$ T cells. The last are known to shift towards Th17-differentiation under the effect of IL-6 and transforming growth factor beta 1 (TGF$\beta_1$) (WEAVER et al. 2006; MONTERO VEGA and DE ANDRES MARTIN 2009). This relatively new class of T helper cells are believed to be involved in triggering aberrant immune responses and recruiting neutrophils (HARRINGTON et al. 2005; STOCKINGER and VELDHOEN 2007). A recent study of our research group showed indeed the concomitant increased intrathecal production of IL-6 and TGF beta-1 in dogs affected with SRMA (MAIOLINI et al. 2012), suggesting a new hypothesis, that the aberrant immune response in SRMA might be associated with Th17 cells maintaining the autoimmune reaction.

Although most TLR responses lead to inflammation, there are studies suggesting an important role of TLRs in homeostasis (EHLERS and RAVETCH 2007; MARTA et al. 2008).

The role of certain TLRs has been validated in different human diseases resulting in a wide research area focusing on possible new treatment strategies (KANZLER et al. 2007). In case of such multivalent receptors, the great challenge is to reduce the unnecessary inflammation without affecting regulatory functions of TLRs. For example, many efforts attempt to find partial TLR4 agonists, rather than antagonists, and some compounds are already currently available for human use (KANZLER et al. 2007; O'NEILL et al. 2009). So far in companion animals the interest has been
limited to TLR ligands for developing new vaccines (COFFEY and WERLING 2011), but considering the rapid progress in human medicine, a similar breakthrough is expected soon in veterinary medicine. SRMA would be an ideal model to study such treatment strategies.

In conclusion, TLRs are suggested to be involved in different aspects of the pathogenesis of SRMA. These findings support the hypothesis, that an infectious agent can only trigger the disease. SRMA itself seems to be maintained by multiple alterations of the immune system resulting in an autoimmune disease, TLRs such as TLR4 and TLR9 might be receptors maintaining the inflammation.

4.6 Conclusion

We suggest that TLRs are involved in different aspects of the pathogenesis of SRMA. This study supports the hypothesis that an infectious agent can only trigger the disease. SRMA itself seems to be maintained by multiple alterations of the immune system resulting in an autoimmune disease, TLRs such as TLR4 and TLR9 might act as receptors maintaining the inflammation.
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Chapter 5: General Discussion

Since the publication of the first report more than 40 years ago (JOSHUA and ISHMAEL 1968), SRMA became a well-recognised disease in canine practice, being the most common cause for meningitis and an important cause of fever in dogs (MERIC 1988; BATTERSBY et al. 2006; TIPOLD and STEIN 2011). Despite the assiduous years of research, the aetiology of SRMA is still unknown. SRMA shares many similarities with various systemic vasculitides of unknown origin in humans, enhancing the particular interest in understanding its aetiology and pathogenic mechanisms. Only when the pathogenesis and the origin of the disease will be well known, specific treatment strategies could be searched for. Furthermore SRMA is an ideal animal model for systemic vasculitides (BURNS et al. 1991; FELSBURG et al. 1992; SNYDER et al. 1995). Studying the disease might help to develop new treatment strategies for both humans and dogs.

The first part of this study focused on reviewing one of the first immunological abnormalities observed in SRMA, the increased systemic and intrathecal IgA production (TIPOLD and JAGGY 1994; TIPOLD et al. 1994; CIZINAUSKAS et al. 2000). High IgA values in paired CSF and serum samples of dogs are considered to support the diagnosis of SRMA. However, there is no general agreement on the reliability of this finding in SRMA (TIPOLD 1995; BEHR and CAUZINILLE 2006; LOWRIE et al. 2009b). Therefore, the aim of the first part of the study was to validate previous studies and to establish the diagnostic value of IgA determination. We investigated the IgA concentrations in a large population of dogs affected with SRMA and compared to dogs affected with other neurological diseases. In our study the
sensitivity of the test was 91% with an overall specificity of 78%. Our results showed that the test is a highly sensitive tool to confirm SRMA and should be part of the diagnostic work-up in suspected cases of SRMA. The moderate specificity of the test does not allow the clinician to rely only on this test, but other conditions, such as disc protrusion/extrusion have to be ruled out. However, we could show that IgA concentrations in serum and in CSF were higher in SRMA than in any other disease, confirming the hypothesis that this finding might play an important role in the pathogenesis of the disease.

SRMA is further characterized by a systemic inflammatory response and alteration of lymphocyte subsets. The second part of the study focused on possible mediators responsible for increased IgA and acute-phase protein production, shift of lymphocyte subsets, fever and systemic arteritis.

TGF-β₁ and IL-6 are the most important cytokines in controlling the antibody production. TGF-β₁ plays the main role inducing the IgA class switching of B cells, while IL-6 is crucial in the proliferation and differentiation of B cells into antibody-secreting plasma cells (MURPHY et al. 2008b, c).

Our results showed that in SRMA IL-6 and TGF-β₁ are indeed increased intrathecally, while systemically IL-6 is increased and TGF-β₁ decreased. Interestingly, high serum levels of IL-6 and low serum levels of TGF-β₁ were also found in patients with KD (KIM 1992; MATSUBARA et al. 1997) supporting the systemic nature of vascular changes in SRMA. To the authors’ knowledge, these signalling proteins have been not evaluated in CSF of patients affected with KD. A positive correlation was found between TGF-β₁ and IgA concentration in CSF, while IL-6 lacked to correlate with the IgA concentration. TGF-β₁ likely plays a direct
and important role in the intrathecal production of IgA. The two cytokines act on B cells at different time points. TGF-β₁ is necessary at the beginning of the IgA production, when the IgM- to IgA- switch takes place (TIZARD 2004c). IL-6 acts subsequently, inducing the terminal differentiation of IgA-producing plasma cells (TIZARD 2004c). The fact that in the latter phase also other cytokines are involved (e.g. IL-4, IL-5 and IL-10), might explain the absence of a correlation between IL-6 and IgA concentrations.

Our results suggest a primary role of IL-6 in SRMA, indeed the highest concentrations were found in CSF of SRMA patients, whereas other inflammatory diseases of the CNS showed only slight increase of IL-6 in CSF. In serum samples, the values of IL-6 were comparable to the group of systemic inflammatory diseases, serving in our study as positive control. We speculate that IL-6 is the major responsible factor triggering the systemic inflammatory response respectively acute phase protein production in both groups.

While previous studies already investigated concentrations of IL-6 in SRMA, the bioavailability of the cytokine was still unknown. In our study, CSF samples with high IL-6 values measured with an ELISA successfully stimulated IL-6 dependent cells, proving the high bioavailability of the protein and supporting the suspected functions in the pathogenesis of SRMA.

The analysis of different signalling proteins allowed us to determine a pattern of cytokine production in SRMA. Indeed, the increased concentration of TGF-β₁ in CSF was an unspecific finding, common also to other meningoencephalitides. However, a concomitant increase of IL-6 and TGF-β₁ was found only in SRMA.

IL-6 and TGF-β₁ influence the differentiation of CD4+ progenitors T cells (HARRINGTON et al. 2005; VELDHOEN et al. 2006; STOCKINGER and
VELDHOEN 2007). We propose that the synergistic effect associated with simultaneous presence of both cytokines intrathecally lead to a Th17-skewed immune response in SRMA, which should be further characterized in future studies.

VEGF, a signalling protein, has been widely studied in human patients affected with Kawasaki Disease (KD), one of the systemic vasculitides where SRMA is used as an animal model (BURNS et al. 1991; FELSBURG et al. 1992). We hypothesized that VEGF might be responsible for the vascular changes observed in the acute phase of SRMA, such as endothelial and subendothelial oedema, hyaline degeneration and periarteritis (TIPOLD et al. 1995). Our findings indicate that VEGF is increased both systemically and intrathecally during the clinical phases of SRMA. Systemic and intrathecal VEGF production reflect what is observed histopathologically. Indeed, even if vascular changes are mainly observed within the spinal meninges, a systemic vasculitis can affect heart, cranial mediastinum, thyroid gland, thymus and gastro-intestinal tract in dogs with SRMA (HAYES et al. 1989; SCOTT-MONCRIEFF et al. 1992; SNYDER et al. 1995). We can therefore propose that VEGF during the acute phase has a major role in increasing the permeability of the vessels, triggering a cascade of inflammatory reactions that lead to vascular wall destruction, even before immune complexes appear. The fact that VEGF is constantly increased also during relapses of SRMA suggests that VEGF maybe also involved in the development of the arterial lesions that appear in the chronic phase of the disease.

High concentrations of VEGF were found only sporadically in dogs affected with other inflammatory diseases, leading to the conclusion that VEGF does not play a principal role in this category of diseases.
The last part of our study investigated the role of the innate immune system in SRMA. The defence of the body is based on the innate and the adaptive immune system. Traditionally, the innate immune system has been considered to provide mainly an initial, rapid and unspecific defence against infections, while the adaptive immune system would later on respond with greater specificity and efficiency throughout the body (TIZARD 2004b; MURPHY et al. 2008a). However, in the last two decades the innate immunity has been shown to have additional roles in determining the response of the acquired immune system (MEDZHITOV 2001, 2009). The initiation of this process starts with the recognition of antigens, in which Toll-like receptors (TLRs) play the most important role. The activation profile of cell surface TLRs (TLR2, TLR4 and TLR5) and intracellular TLRs (TLR3 and TLR9) in SRMA was tested to support the hypothesis that these receptors are stimulated by infectious antigens or endogenous proteins (self-antigens).

Among the leukocyte subsets analysed, TLR2, TLR4 and TLR5 were highly expressed in polymorphonuclear cells (PMNs) of SRMA, but this was a common finding also in other disease categories. In general, dogs with SRMA had a different activation profile of TLRs than dogs with bacterial/pyogenic infections. We therefore concluded that SRMA is not maintained by a continuous bacterial infection. This hypothesis is also supported by the clear response to long term treatment with glucocorticosteroids. However, triggering an autoimmune reaction by bacteria cannot be ruled out completely.

Indeed, the most relevant results were found in the expression of TLR4 on peripheral blood monocytes. Dogs with SRMA and dogs with pyogenic infections had a high percentage of TLR4 positive monocytes. TLR4 recognizes lipopolysaccharides, but also some endogenous ligands, such as heat shock protein 70 (HSP 70). In the
current study the triggering factor for increased TLR4 expression, a self or non-self antigen, was not examined. However, a recent study showed that HSP 70 is elevated in SRMA (MOORE et al. 2011). Therefore, it seems to be very likely that the triggering protein might derive from a self antigen such as the HSP 70.

TLR4 is involved in neutrophil recruitment into the central nervous system in mice (ZHOU et al. 2009). Similar mechanisms might lead to the invasion of neutrophils into the subarachnoidal space in SRMA and explain the exorbitant neutrophilic pleocytosis in acute cases. In SRMA dogs during treatment with glucocorticosteroids and normal CSF cell count decreased percentages of TLR2, TLR4 and TLR9 positive PB monocytes occurred.

TLR9 seems to be constantly increased on PB leukocytes in almost every disease examined in the current study. However, patients with SRMA and dogs affected with meningoencephalitides of unknown aetiology showed the highest expression of TLR9, suggesting a possible role of this TLR in inflammatory CNS diseases with a suggested autoimmune component. TLR9 is primarily involved in the recognition of bacterial DNA (TAKEDA et al. 2003). In human medicine TLR9 seems also to play an important role in class-switching to pathogenic autoantibody production in systemic autoimmunity (GREEN and MARSHAK-ROTHSTEIN 2011), leading again to the suggestion that an autoimmune reaction can maintain the inflammatory reaction in SRMA.

TLR4 and TLR9 can also influence the differentiation of the above mentioned Th17 lymphocytes. Indeed, these two TLRs activate dendritic cells to produce factors that together with IL-6 and TGF-β1 drive CD4+ progenitors to differentiate into Th17 cells (WEAVER et al. 2006; MONTERO VEGA and DE ANDRES MARTIN 2009), further
supporting our new hypothesis, that SRMA is a disease with a Th17-skewed immune response.

In conclusion, IgA, IL-6, VEGF, TGF-β1 and TLRs are involved in different aspects of the pathogenesis of SRMA. All findings support the hypothesis, that an infectious agent can only trigger the disease. SRMA itself seems to be maintained by multiple alterations of the immune system resulting in an autoimmune disease. Two studies of this thesis provided evidence that a Th17-skewed immune response might dominate in SRMA and further investigations are recommended.
Chapter 6: Summary

IgA Production, Signalling proteins and Toll-like receptors involved in the pathogenesis of canine Steroid-responsive Meningitis-Arteritis

Arianna Maiolini

Steroid-responsive Meningitis-Arteritis (SRMA) is a systemic inflammatory disease affecting mostly the cervical vessels and meninges. Clinical and laboratory findings include a marked neutrophilic pleocytosis reflecting the meningitis; fever, leukocytosis and increased production of acute-phase proteins characterize the systemic immune response.

The current study aims to increase the knowledge of aetiology and pathogenic mechanisms involved in this disease. A concurrent elevation of immunoglobulin A (IgA) concentrations in serum and cerebrospinal fluid (CSF) is one of the hallmarks of SRMA. However, it has been controversially discussed if such laboratory findings can be used for diagnostic purposes.

The first part of the study focused on the validation of previous results concerning IgA production and the examination of the diagnostic value of IgA determination in SRMA. A large population of dogs affected with SRMA in different stages was evaluated and compared to dogs suffering from various neurological diseases. A concurrent elevation of IgA in CSF and serum had a sensitivity of 91% with a specificity of 78%. In conclusion, the measurement of IgA in CSF and serum supports the diagnosis of SRMA, but cannot be used as a single diagnostic test.
concentrations in serum and in CSF were higher in SRMA than in any other disease confirming that IgA plays an important role in the pathogenesis of the disease.

The second part of the study consequently focused on possible mediators responsible for IgA production, but also for increased acute-phase protein induction, fever and systemic arteritis. Transforming growth factor beta-1 (TGF-β₁) and interleukin-6 (IL-6) are the most important cytokines controlling the antibody production. Our results showed that in SRMA IL-6 and TGF-β₁ are indeed increased intrathecally, while systemically IL-6 is increased and TGF-β₁ decreased. TGF-β₁ positively correlated with IgA concentration in CSF, IL-6 with the degree of pleocytosis. High concentrations of bioactive IL-6 in serum are very likely to be responsible for the production of acute-phase proteins and acting as endogenous pyrogens triggering fever in SRMA patients. We additionally propose that the synergistic effect associated with intrathecal presence of both cytokines leads to a Th17-skewed immune response in SRMA. Vascular Endothelial Growth Factor (VEGF) is a potent regulator of blood vessel permeability. We found increased concentrations of systemic and intrathecal VEGF during acute SRMA and relapses. We propose therefore that VEGF is responsible for the vascular changes observed in both, the acute and chronic phases of SRMA.

Various studies implemented the hypothesis that SRMA is caused by dysfunction of the adaptive immune system, but studies on the innate immune system are still missing. Toll-like receptors (TLRs) are pattern recognition receptors which play an important role in innate immunity, but can also influence the adaptive immune response. The activation profile of TLRs in SRMA was tested to support the hypothesis that these receptors are stimulated by infectious antigens or endogenous proteins (self-antigens). Indeed, our results showed a relatively high expression of
TLR4 and TLR9 on monocytes in acute SRMA, suggesting that these two receptors might be involved in the inflammatory process in SRMA, enhancing the autoimmune reaction.

In conclusion, IgA, IL-6, VEGF, TGF-β_1_ and TLRs are involved in different aspects of the pathogenesis of SRMA. All findings support the hypothesis, that an infectious agent can only trigger the disease. SRMA itself seems to be maintained by multiple alterations of the immune system resulting in an autoimmune disease.
Chapter 7: Zusammenfassung

IgA-Produktion, Signalproteine und Toll-like Rezeptoren in der Pathogenese der steril-eitrigen Meningitis-Arteriitis des Hundes

Arianna Maiolini

Die steril-eitrige Meningitis-Arteriitis (SRMA) ist eine systemische Erkrankung mit vornehmlicher Manifestation der Entzündung im Bereich der zervikalen Gefäße und Meningen. Die Befunde der Klinik und des Labors beinhalten eine deutliche Pleozytose, welche die Meningitis widerspiegelt; Fieber, Leukozytose und eine erhöhte Produktion der Akute-Phase Proteine charakterisieren die systemische Immunantwort. Die gegenwärtige Studie soll Erkenntnisse über Ätiologie und Pathomechanismen dieser Erkrankung ergänzen. Eine simultane Erhöhung der Immunoglobulin A (IgA) Konzentration in Serum und Liquor cerebrospinalis (CSF) ist charakteristisch für SRMA. Dennoch wurde der diagnostische Nutzen dieser Laborparameter bisher kontrovers diskutiert. Der erste Teil der Studie konzentrierte sich daher auf die Bestätigung vorheriger Resultate bezüglich der IgA Produktion und die Auswertung des diagnostischen Nutzens der IgA Bestimmung in der SRMA-Diagnostik. Eine große Anzahl an Hunden, die an SRMA unterschiedlicher Stadien erkrankt waren, wurde evaluiert und verglichen mit Hunden, die an anderen neurologischen Erkrankungen litten. Eine gleichzeitige IgA Erhöhung in CSF und Serum ergab für die Diagnostik eine Sensitivität von 91% mit einer Spezifität von 78%. Zusammenfassend unterstützt die IgA Messung in CSF und Serum die Diagnose SRMA, kann aber nicht als einziger diagnostischer Parameter genutzt werden. Die IgA Konzentrationen waren in Serum und CSF bei an SRMA erkrankten...
Hunden höher als bei allen anderen Erkrankungen, was die wichtige Rolle von IgA in der Pathogenese der SRMA bestärkt. Daher lag der Focus der zweiten Studie auf der Untersuchung möglicher Mediatoren, die verantwortlich für die Produktion von IgA und von Akute-Phase Proteinen sind bzw. die Befunde Fieber und systemische Arteritis erklären lassen. Transforming growth factor beta-1 (TGF-β₁) und Interleukin-6 (IL-6) sind die wichtigsten Zytokine für die Steuerung der Antikörper Produktion. Unsere Ergebnisse zeigen, dass sich bei SRMA IL-6 und TGF-β₁ intrathekal erhöhen, während IL-6 systemisch ansteigt und TGF-β₁ abfällt. TGF-β₁ korreliert positiv mit der IgA Konzentration im CSF und IL-6 mit dem Grad der Pleozytose. Hohe Werte von bioaktivem IL-6 im Serum sind sehr wahrscheinlich für die Produktion der Akute-Phase Proteine verantwortlich und können als endogenes Pyrogen angesehen werden, das Fieber in SRMA-Patienten auslöst. Wir vermuten, dass der synergistische Effekt assoziiert mit intrathekaler Anwesenheit beider Zytokine zu einer Th17-dominierten Immunantwort bei SRMA führt. Der Vascular Endothelial Growth Factor (VEGF) ist ein potenter Regulator der Gefäßpermeabilität. Eine erhöhte Konzentration des systemischen und intrathekalen VEGF in der akuten Form von SRMA und bei Rezidiven kann für die histopathologischen Blutgefäßveränderungen bei SRMA verantwortlich sein. Diverse Studien implementieren die Hypothese, dass SRMA durch eine Dysfunktion des adaptiven Immunsystems entsteht, Studien über das angeborene Immunsystem fehlen jedoch. Toll-like Rezeptoren (TLRs) sind Strukturerkennungsrezeptoren, die eine wichtige Rolle im angeborenen Immunsystem spielen, aber auch das adaptive Immunsystem beeinflussen. Das Aktivierungsprofil der TLRs in SRMA wurde getestet, um die Hypothese zu unterstützen, dass diese Rezeptoren durch infektiöse Antigene oder endogene Proteine (Selbstantigene) beeinflusst werden. Eine relativ
hohe Expression von TLR4 und TLR9 auf Monozyten in der akuten Phase von SRMA lässt vermuten, dass diese zwei Rezeptoren im Entzündungsprozess der SRMA eine Autoimmunreaktion fördern. Das TLR-Profil bei SRMA ähnelte nicht dem von bakteriellen Infektionen, daher kann eine aktive bakterielle Infektion vermutlich ausgeschlossen werden.

Zusammenfassend kann gesagt werden, dass IgA, IL-6, VEGF, TGF-β₁ und TLRs in verschiedenen Stadien der Pathogenese von SRMA beteiligt sind. Alle Ergebnisse unterstützen die Hypothese, dass ein infektiöses Agens diese Erkrankung höchstens initiieren kann; SRMA selbst scheint jedoch durch mehrere Veränderungen des Immunsystems unterhalten zu werden, die eine Autoimmunerkrankung auslösen.
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Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled “IgA production, Signalling proteins and Toll-like receptors involved in the pathogenesis of canine Steroid-responsive Meningitis-Arteritis”.

No third party assistance has been used.
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 institution:
Department of Small Animal Medicine and Surgery, 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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Arianna Maiolini