ABSTRACT

LI, JINGJING. The Cytokine Balance During Canine Sepsis: Defining the Mechanisms of Amplification and Potential Targets for Intervention. (Under the direction of Drs. Shila Nordone, Adam Birkenheuer and Michael Levy).

Sepsis, the systemic inflammatory response to infection, is an aggressive multifactoral disease associated with high morbidity and mortality in the dog. Consensus opinion is that dysregulation of proinflammatory cytokines is responsible for the majority of pathology associated with septic shock and severe sepsis. Early diagnosis and intervention are critical for the survival of septic patients. Therefore, identification of potential biomarkers for diagnosis and therapeutics for the regulation of cytokine responses are essential to reduce sepsis-associated mortality.

We successfully characterized the cytokine responses in an induced endotoxemia model, identified a key regulator of cytokine amplification in the dog, Triggering Receptor Expressed on Myeloid Cells-1 (TREM-1), and identified a potential therapeutic point of intervention that broadly suppresses cytokine production. Collectively, our data represent the early stage of biomarkers for the diagnosis of canine sepsis and therapeutic discovery for the treatment of sepsis in the dog.

The Cytokine Balance During Canine Sepsis: Defining the Mechanisms of Amplification and Potential Targets for Intervention

by
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Literature Review

1. Introduction to sepsis

1.1 Sepsis definition

Sepsis, the systemic inflammatory response to infection, is an aggressive, multifactoral syndrome associated with high morbidity and mortality in patients. Sepsis is diagnosed when infection is suspected or proven, and at least two or more Systemic Inflammatory Response Syndrome (SIRS) criteria are present (1, 2). To help standardize clinical trials and better understand the pathophysiology of sepsis, several groups have proposed nomenclature to describe sepsis with different clinical syndromes: severe sepsis and septic shock. Severe sepsis is defined as sepsis with organ dysfunction. Septic shock is defined as severe sepsis with hypotension and unresponsiveness to fluid resuscitation (1, 2). It is consensus opinion that both clinicians and basic scientists should use the standardized nomenclature to help compare studies performed in different laboratories and clinics across the world.

1.2 Impact of sepsis

Sepsis has an important impact worldwide. Despite twenty-years of research, sepsis continues to cause high mortality and morbidity in humans and dogs and also has substantial financial impact on the healthcare system. The estimated annual incidence of human sepsis in the United States is around 715,000 cases and it costs the healthcare more than 17 billion dollars a year (3). Sepsis is often lethal. The mortality rate is less than 20% in patients with

sepsis, but increases dramatically to 25% - 50% in patients with severe sepsis. Patients with septic shock have the highest mortality rate of greater than 50% (3). This high mortality rate makes sepsis among the 10 leading causes of death in the United States (4-6). Similarly, the morbidity and mortality in canine sepsis are high. The morbidity is estimated to be 6% - 10% in critically ill patients and the mortality is estimated to be 50% -75% (7). The cost of canine sepsis has not been formally defined, but it may also be staggeringly high. The high mortality rate of sepsis in both human and canine patients is due to the unique pathogenesis of sepsis. Understanding the pathogenesis of sepsis is the key to improving survival in septic patients and to reduction of healthcare cost.

2. Sepsis pathogenesis

2.1 Pathogens that cause sepsis

Bacterial infection is the major cause of sepsis and accounts for more than 95% of sepsis cases. From 1979 to 1987, gram-negative bacteria were the predominant organisms causing sepsis. After 1987, gram-positive bacteria were reported as the predominant organisms. In 2000, gram-positive bacteria accounted for 52.1% of all sepsis cases and gram-negative bacteria accounted for 37.6% of sepsis cases (5). Several groups have proposed that the shift to gram-positive bacterial predominance in sepsis is due to nosocomial infection related to wide usage of intravascular catheter and the emergence of antibiotic resistant gram-positive bacteria (8, 9).

In addition to bacterial infection, fungi, parasites (e.g. malaria) and viruses can also cause sepsis. Among these organisms, fungal infection accounts for 4.6% of all sepsis cases and the incidence of sepsis caused by fungi is increasing by 10% per year. This trend is likely to continue due to the increasing number of immunocompromised patients, including human immunodeficiency virus (HIV)-infected patients, chemotherapy-treated cancer patients and immunosuppressive therapy-treated transplant recipients (10-12). In addition to its high incidence, fungal infection is also associated with higher mortality rates compared with typical bacterial infection (3, 5).

Clinicians and basic scientists need to be aware of the changes in the type of organisms causing sepsis so corresponding treatment and research can be directed towards the most likely pathogens.

2.2 Two stage theory of sepsis

Human sepsis is recognized to have two distinct phases: the hyper-inflammatory phase and the hypo-inflammatory phase (13, 14) (Figure. 1). In early phase of sepsis, patients have hyper-inflammatory response to infection. This hyper-inflammatory phase is characterized by excessive activation of immune cells and overproduction of proinflammatory cytokines (15). With standard treatment, some patients still die from organ dysfunction due to "cytokine storm." Approximately 50% of patients survive this phase, and a subset of this cohort enters into a protracted hypo-inflammatory phase (14). This late phase of sepsis is characterized by apoptosis of dendritic cells, B cells and $CD4^+$ T cells (16, 17). Due to the apoptotic depletion of cells from both innate and adaptive immune systems, patients are often unable to eradicate the primary infection or at the risk of developing secondary infection (13, 14). The majority of deaths occur in the hypo-inflammatory phase of sepsis (18). This hypo-inflammatory phase is not currently recognized in the dog. However, to our knowledge there are no studies investigating whether or not dogs have this late phase of sepsis.

2.3 Cells involved in sepsis

When the pathogens mentioned above enter a sterile site of the body, the resident macrophages and dendritic cells are the first cells to respond to the invaders and initiate the

innate immune system by phagocytosing bacteria and producing proinflammatory cytokines and chemokines. These chemokines attract neutrophils and monocytes from circulation to the site of infection. Neutrophil extravasation includes 1) initial rolling of neutrophils along the endothelium through interaction between sulfated sialyl-Lewis^x moieties on neutrophils and selectins expressed on endothelial cells (19-21), 2) subsequent tight adhesion of neutrophils to endothelial cells mediated largely by Mac-1 (CD11b/CD18) expressed on neutrophils and intercellular adhesion molecule 1 (ICAM-1) expressed on endothelial cells (22), and 3) final extravasation into the tissue. The process of neutrophil extravasation and migration requires actin polymerization and cytoskeleton rearrangement. Neutrophils are the first cells to extravasate from the circulation to the site of infection. Monocytes are also attracted to the site of infection by similar mechanisms and mature into macrophages to assist with the control of infection.

Once at the site of infection, neutrophils kill bacteria by phagocytosis and release of its arsenal of proteolytic enzymes, such as elastase and lysozyme, and reactive oxygen species. When infection is controlled by the innate immune response, inflammation resolves and tissues recover. However, when the infection is out of control and becomes systemic, it leads to sepsis. In sepsis, the innate immune response is dysregulated and characterized by massive activation of neutrophils and monocytes/macrophages. This results in excessive release of proinflammatory cytokines and neutrophil-mediated pathogenesis. These processes will be discussed later in more detail.

Innate immune cells, such as macrophages and dendritic cells, do not only act as phagocytic cells, they are also professional antigen presenting cells (APCs). They present microbial antigens on MHC II to T lymphocytes and bridge the innate and adaptive immune responses. Activation of the adaptive immune response is essential for the body to eradicate microbial invaders when the innate immune cells fail to contain the initial infection. However, septic patients often experience a significant decline of lymphocytes and dendritic cells by apoptosis (6, 23, 24) and impaired antigen presentation by monocytes (25, 26), which contribute to the immunosuppressive state observed in the later stage of sepsis.

To summarize, a broad range of immune cells, such as neutrophils, monocytes/macrophages, dendritic cells and lymphocytes, are involved in sepsis pathogenesis. Importantly, sepsis is characterized by dysregulation of innate immune response and deficiency in adaptive immune response.

2.4 Danger signal receptors

As mentioned above, tissue resident macrophages are the first cells to engage microbial invaders. How do macrophages recognize microbes? It is through the binding of pathogen recognition receptors (PRRs) expressed on innate immune cells to the highly conserved microbial products known as pathogen-associated molecular patterns (PAMPs). The best characterized examples of PRRs are Toll-like receptors (TLRs) (27).

TLRs are germline-encoded pattern-recognition receptors (PRRs) (27). They are widely expressed on innate immune cells including neutrophils and monocytes (28). To date, there are 11 human TLRs, 13 mouse TLRs and 9 canine TLRs identified (27, 29-32). They recognize various PAMPs derived from bacteria, fungi, viruses and protozoa. For example, TLR4, coupled to CD14 and MD2, recognizes lipopolysaccharide (LPS), which is mainly expressed on Gram-negative bacteria (33, 34); TLR2 forms a heterodimer with TLR1 or TLR6 and recognizes triacyl-lipopeptide or diacyl-lipopeptide expressed on Gram-positive bacteria; TLR5 recognizes flagellin found in some motile bacteria; TLR3, 7, 8 and 9 are located intracellularly and recognizes viral double-stranded RNA (TLR3), viral single-stranded RNA (TLR7 and TLR8) and microbial specific unmethylated CpG oligonucleotide sequences (TLR9), respectively (27, 28).

Activation of TLRs by their respective ligands expressed on microbes leads to activation of proinflammatory transcription factors, such as nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) (27, 28, 35), which ultimately result in release of proinflammatory cytokines to control the infection.

2.5 Cytokine response in sepsis

As mentioned above, recognition of PAMPs by TLRs transfers danger signals to the cell and triggers a cascade which leads to release of a broad range of proinflammatory cytokines. When the body's response to infection is balanced the amount of cytokines secreted matches the amount of cytokines needed to confine infection. Once the infection is resolved, the body

returns to a state of homeostasis. However, in sepsis, the body's response to infection is unbalanced with excessive release of proinflammatory cytokines, leading to tissue and vascular system damage and subsequent shock or multi-organ failure (36). Interestingly, human patients who survive the hyper-inflammatory phase of sepsis may enter an extended hypo-inflammatory phase that is characterized by lack of response to PAMPs, resulting in decreased cytokine production (37). In this section, we will address the featured cytokine response in different phases of sepsis and identify potential points of therapeutic intervention.

As stated above, the hyper-inflammation phase of sepsis is defined by excessive production and systemic release of proinflammatory cytokines. In this early stage of disease, the cytokine response is characterized by overproduction of a broad range of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, CXCL-1 and MCP-1. Two of the best characterized cytokines are TNF-α and IL-1β, which have been directly implicated in the pathogenesis of sepsis. Animal studies found neutralization of TNF-α improves survival in endotoxin and gram-negative bacteria-induced septic shock (36, 38, 39); however, these results did not translate to studies of humans with sepsis. Investigators using both anti-TNF antibodies and TNF-receptor fusion proteins to treat septic patients did not observe improved survival in clinical trials (40-44). Further research revealed that the release of TNF-α is short-term in septic patients. TNF-α is released within minutes after LPS administration, peaks at 90 min but becomes almost undetectable after 4-6 hr (45, 46), and can be only detected in patients with early sepsis. Since septic patients may be admitted to the hospital hours or days after the onset of sepsis, long after the release of TNF-

α, it is not surprising that these anti-TNF-α molecules failed to provide protection for septic patients in clinical trials. Similar results were obtained in clinical trials designed to neutralize IL-1β. These early therapeutic trials suggest: 1) When targeting specific cytokines for neutralization, timing of treatment may be critical, and 2) neutralization of a broad range of cytokines is more likely to significantly improve survival rates during the first phase of sepsis. It will be important to identify proinflammatory mediators that are expressed later and last longer during the hyper-inflammatory phase of sepsis as these will be potential targets for diagnosis and intervention.

One such target is high mobility group box 1 (HMGB-1). HMGB-1 was originally described as an intracellular transcription factor. Recently, HMGB-1 has been found to be released from necrotic cells and its soluble form functions as a proinflammatory stimulus by binding to TLRs. Tissue necrosis is common during sepsis. HMGB-1 is released 8 hr after endotoxin exposure in the mice and 18 hr after cecal ligation and puncture (CLP)-induced sepsis. And the increase of HMGB-1 could last several days after the onset of sepsis. In the CLP-induced sepsis model, administration of anti-HMGB-1 antibody as late as 24 hr after the induction of sepsis still enhances survival (47-49). Therefore, inhibition of the proinflammatory effect of HMGB-1 may be an excellent target for the treatment of sepsis.

As mentioned above, some of those patients who survive the hyper-inflammatory phase of sepsis may enter into a hypo-inflammatory state. In this phase, the patients have reduced ability to produce proinflammatory cytokines and are therefore at risk of secondary infection

(37, 50). The reduced capacity to produce cytokines in the late phase of sepsis is due to hypo-responsiveness of innate immune cells to PAMPs. The mechanism leading to the hypo-responsive state is not completely clear, but down-regulation of TLRs expression and impaired recycling of TLRs in monocytes and neutrophils are implicated in the hypo-responsive state (51, 52). In this phase, stimulation of the innate immune system may be beneficial to the patients. Indeed, stimulation of monocytes from septic patients with IFN-γ restores its TNF-α production after LPS stimulation and treatment of septic patients with IFN-γ improves survival (37).

In summary, cytokine response in sepsis is unbalanced with overproduction of cytokines in early phase and reduced ability to produce cytokines in late phase. Therefore, defining the cytokine response in different phases of naturally occurring sepsis is essential to guide the clinicians to use either anti-inflammatory strategies or immune stimulants to fine-tune the immune system and eventually promote survival in septic patients.

2.6 Neutrophil-mediated sepsis pathogenesis

When infection occurs, neutrophils are the first line of defense against bacteria that invade the body (53). In a typical response to infection, neutrophils migrate to the site to kill bacteria and cause local inflammation. However, in sepsis, due to extended stimulation with microbial products and proinflammatory cytokines, the ability of neutrophils to extravasate is impaired, resulting in systemic inflammation. In this section, we will discuss the mechanisms leading to neutrophil-mediated damage to septic patients.

In sepsis, adhesion molecules (such as CD11b) expressed on neutrophils and their ligands (such as ICAM-1) expressed on endothelial cells are up-regulated, preparing neutrophils for extravasation (54-58). However, expression of the high-affinity chemoattractant receptor CXCR2 on neutrophils is greatly decreased in septic patients due to extended stimulation by TLR agonists and proinflammatory cytokines, resulting in impaired migration of neutrophils to the site of infection (59-64). As a result, neutrophils adhere tightly to the endothelial cells, leading to neutrophil-mediated damage to the body.

Excessively activated neutrophils have two major detrimental effects in septic patients. First, the body's ability to control infection is substantially impaired because neutrophils have decreased ability to extravasate to the site of infection to control bacterial expansion. Secondly, these tightly adhered cells are devastating to the vascular system: 1) they occlude the capillary vessels, leading to tissue hypoperfusion and hypoxia (65, 66), and 2) damage the intercellular junctions by releasing their lytic factors, leading to increased vascular permeability (67).

Further worsening the neutrophil-mediated damage is the extremely extended lifespan of neutrophils through TLR agonists and proinflammatory cytokines stimulation (68-70). Indeed, it has been reported that 1) TLR agonists LPS and lipoteichoic acid (LTA) delay neutrophil apoptosis in a dose-dependent manner (71-73); 2) neutrophil lifespan is prolonged after exposure to IL-1β, TNF-α, GM-CSF, G-CSF or IFN-γ (72). As a result, neutrophil-

mediated tissue hypoxia, hypoperfusion and enhanced vascular permeability are prolonged, leading to septic shock and severe sepsis with multiple organ failure.

In summary, neutrophils are double-edged swords: on one hand, they are essential to eradicate pathogens by phagocytosing and lysing bacteria; on the other hand, over-activation of neutrophils by excessive stimulation with TLR agonists and proinflammatory cytokines leads to vascular and tissue damage, and subsequent septic shock or multi-organ failure. So understanding the role of neutrophils in sepsis will help us find a way to modulate neutrophil functions in septic patients: maximizing its killing ability to control infection and minimizing its harmful effects on the body.

3. The mechanism of cytokine amplification: triggering receptor expressed on myeloid cells-1 (TREM-1)

As discussed above, sepsis is characterized by an overwhelming production of proinflammatory cytokines, leading to tissue and vascular system damage and subsequent shock or multi-organ failure (36). In this section, we will address one of the mechanisms of cytokine amplification in sepsis – activation of triggering receptor expressed on myeloid cells-1 (TREM-1) (74).

3.1 Introduction to TREM-1

TREM-1 was first described in 2000 by Bouchon *et al*. as a cell surface transmembrane receptor exclusively expressed on myeloid cells, such as monocytes/macrophages and neutrophils (74). TREM-1 is up-regulated upon TLR agonist stimulation and functions as a cytokine amplifier. Over time, a soluble form of TREM-1 (sTREM-1) is released and serves as a negative feedback molecule to dampen the inflammatory response. sTREM-1 has good potential to be used as a biomarker for the diagnosis of sepsis. Lastly, recombinant sTREM-1 has been shown to improve survival in experimental sepsis models.

3.2 Regulation of TREM-1 expression

Previous research has shown that expression of TREM-1 is strongly associated with bacterial or fungal infection and appears to be downstream of TLR activation. However, how TREM-1 expression is regulated is still a mystery. In this section, we will discuss data from different laboratories regarding regulation of TREM-1 expression.

Numerous observations support that TREM-1 expression is highly associated with infection (75-77). For example, TREM-1 is highly expressed in inflammatory lesions caused by bacteria or fungi (such as folliculitis caused by *S. aureus* and granuloma caused by *Aspergillus fumigatus*) and is minimally expressed in non-microbial inflammations (such as psoriasis and vasculitis caused by immune complexes) (75). These observations suggest that TREM-1 is associated with pathogen infection rather than inflammation in general.

Subsequent experiments determined that TREM-1 expression is strongly associated with recognition of bacterial PAMPs by TLRs. In vitro, LPS, lipoteichoic acid (LTA), flagellin, heat-inactivated *Staphylococcus aureus* and heat-inactivated *Pseudomonas aeruginosa* increases TREM-1 expression on human monocytes and neutrophils (74, 75). However, non-bacterial ligands, such as poly(I:C) (TLR3) and imidazoquinoline S27609 (TLR7), as well as CpG oligodeoxynucleotide (TLR9), do not have major impact on TREM-1 expression (78). In vivo, TREM-1 is up-regulated on human monocytes in induced endotoxemia, and on mouse monocytes and neutrophils in caecal ligation and puncture (CLP)-induced sepsis (77, 78).

To further confirm if TLR agonist-mediated TREM-1 up-regulation is through the specific activation of TLRs, Zheng *et al*. used bone marrow-derived macrophages (BMM) from TLR4 and TLR2 knock-out mice. They found that LPS and lipoteichoic acid (LTA) failed to induce expression of TREM-1 message and protein from TLR4 KO and TLR2 KO BMM,

respectively. Their research indicates that up-regulation of TREM-1 by TLR agonists is through the specific activation of TLRs.

Although data clearly suggest activation of TLR induces up-regulation of TREM-1 expression, data are conflicting regarding the signaling pathways for TREM-1 up-regulation. Zanzinger *et al*. demonstrated that BMM from MyD88 (myeloid differentiation primary response gene 88)-knockout mice failed to up-regulate TREM-1 expression in response to LPS stimulation, suggesting that LPS-mediated TREM-1 up-regulation is dependent on the MyD88 pathway (79). However, data from Zheng *et al*. showed that expression of TREM-1 message and protein is similar between BMM derived from WT mice and BMM derived from MyD88-knockout mice after LPS treatment, suggesting that LPS-mediated TREM-1 up-regulation is independent of the MyD88 pathway (80). Moreover, they demonstrated that RAW cells with TRIF knock-down failed to up-regulate TREM-1 expression in response to LPS. Their data suggest that LPS-induced TREM-1 expression is independent of the MyD88 pathway and dependent on the TRIF pathway (80). The key difference between these two experiments is the purity of TLR agonists used. Zheng *et al*. used a highly purified LPS which allowed them to discriminate between TLR specific signaling pathways. In contrast, Zanzinger et al. used crudely purified LPS known to activate multiple TLRs (81). This discrepancy suggests that interpretation by Zheng *et al.* is accurate.

In addition to the conflicting data regarding signaling pathways involved in TREM-1 expression, data are also conflicting regarding whether TREM-1 expression is regulated

transcriptionally or post-transcriptionally. Zeng *et al*. found that the promoter region of TREM-1 gene contains NF-κB and PU.1 binding sites. Further, they demonstrated that PU.1 and NF-κB bind to their putative binding sequences in the promoter region of the TREM-1 gene using chromatin immunoprecipitation (ChIP) assay. This experiment was performed using RAW cells after LPS treatment. Finally they demonstrated that NF-κB is a positive regulator and PU.1 is a negative regulator of TREM-1 expression by doing the following experiments: 1) NF-κB inhibitor abolished LPS-induced TREM-1 expression, 2) RAW cells deficient in PU.1 had enhanced TREM-1 expression after LPS treatment, 3) Overexpression of PU.1 in RAW cells inhibited LPS-induced TREM-1 expression (82). However, the experimental results from Wong-Baeza *et al.* suggest that LPS-induced TREM-1 up-regulation is mediated by a post-transcriptional mechanism since TREM-1 mRNA level is not changed in monocytes after LPS treatment but surface TREM-1 expression is up-regulated (83). The results from these two laboratories sound controversial, but they may complement each other. In fact, our unpublished data indicate that TREM-1 expression is regulated both transcriptionally and post-transcriptionally: 1) TREM-1 mRNA is up-regulated after LPS treatment in canine neutrophils, and 2) TREM-1 protein is stored in neutrophil vesicles/granules and is expressed on the cell surface upon LPS treatment.

TREM-1 expression is not only regulated by TLR agonists, it is also regulated by prostaglandins. Murakami *et al.* reported that prostaglandin E_2 (PGE_2) up-regulated TREM-1 expression on human monocytes, suggesting that TREM-1 may also be associated with non-infectious inflammation. Moreover, they demonstrated that LPS-induced TREM-1 expression

is partly regulated by endogenous PGE_2 (84). However, other types of prostaglandins, such as prostaglandin D_2 (PGD_2), cyclopentenone prostaglandins PGJ_2 and 15-$dPGJ_2$, inhibited TREM-1 expression in response to LPS or *Pseudomonas aeruginosa* stimulation (85). These observations indicate that TREM-1 expression is not only regulated by microbial products but also regulated by endogenous inflammatory mediators.

In summary, TREM-1 expression is mainly regulated by TLR activation but can also be mediated by endogenous prostaglandins. More research needs to be done to fully define the signaling pathways regulating TREM-1 expression.

3.3. TREM-1 structure and function

TREM-1 was originally identified in a human cDNA database as a potential DAP12 associated receptor and its homologue was subsequently discovered in the mouse (74, 75). TREM-1 appears to be highly conserved as its mRNA sequence identity is high between the mouse, human, pig, cow and dog. (76, 86, 87). Structurally, TREM-1 has a single extracellular immunoglobulin (Ig) domain of the variable (V) type and forms "head-to-tail" homodimers when expressed on the cell surface (Figure. 2) (88). These extracellular homodimers are believed to be where the TREM-1 ligand binds. However, the TREM-1 ligand has yet to be identified. TREM-1 has a short cytoplasmic tail and is incapable of transducing intracellular signals on its own. Rather, TREM-1 binds to the adapter molecule-DNAX-activating protein 12 (DAP12) to initiate signal transduction events (74).

The function of TREM-1 was originally described by Bouchon *et al.* where they demonstrated that cross-linking of TREM-1 by an anti-TREM-1 antibody induced myeloperoxidase (MPO) secretion from human neutrophils and MCP-1 and TNF-α secretion from human monocytes (74). These effects were synergistically up-regulated with co-stimulation of TLR4 by LPS (74). Subsequent experiments from other laboratories showed that TREM-1 activation also synergizes with TLR2 and TLR3 stimulation to amplify the secretion of a broad range of proinflammatory cytokines, such as TNF-α, IL-1β, MCP-1 and GM-CSF (74, 75, 89). However, TREM-1 activation inhibited LPS-induced anti-inflammatory IL-10 secretion (89). These observations suggest that TREM-1 functions as an amplifier of the proinflammatory cytokine response to microbial products.

In addition to its role in cytokine amplification, TREM-1 regulates other functions of innate immune cells in response to infection. Radsak *et al*. demonstrated that stimulation of TREM-1 by the agonistic antibody triggers immediate neutrophil degranulation and enhances neutrophil phagocytosis and respiratory burst (90). TREM-1-induced degranulation and respiratory burst are potentiated by TLR agonists stimulation, such as LPS and Pam3Cys.

Furthermore, stimulation of TREM-1 up-regulates expression of cell surface molecules, such as adhesion molecules (CD11c and CD18 on human neutrophils and monocytes) and co-stimulatory molecules (CD40 and CD86 on human monocytes) (74). These results suggest that TREM-1 activation prepares innate immune cells to migrate to the site of infection to

enhance pathogen killing by degranulation and respiratory burst and prepares monocytes to present antigens to lymphocytes.

In summary, TREM-1 is a cell surface molecule with an immunoglobulin-V domain that is believed to form a "head-to-tail" homodimer to bind to its ligand. Unfortunately, its ligand has yet to be defined. Once bound to its ligand, it signals through DAP12. Functionally, TREM-1 activation synergizes with TLR stimulation to amplify proinflammatory cytokine secretion and enhance the functions of innate immune cells.

3.4 TREM-1 signaling pathways

Even though numerous experiments indicate that TREM-1 is an amplifier of the cytokine response to TLR stimulation, little is known about the signaling pathways downstream of TREM-1 activation.

TREM-1 has a short cytoplasmic tail and is associated with DAP12, an ITAM (immunoreceptor tyrosin-based activation motif)-containing adaptor protein, for the initiation of signaling cascade (74). Upon activation by anti-TREM-1 mAb, TREM-1 co-localizes with TLR4 in cell lipid raft (91), and induces recruitment and phosphorylation of phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ (PLC-γ), p38 mitogen-associated protein kinase (p38MAPK) and extracellular signal-related kinase 1 (ERK)1/2 (74, 92).

Activation of these signaling pathways leads to activation of transcription factors (such as NF-κB and AP-1) and ultimately leads to cytokine amplification (93, 94).

Recently, Ormsby *et al*. demonstrated that bruton tyrosine kinase (Btk) is a positive regulator of TREM-1 signaling since production of proinflammatory cytokines after TREM-1 stimulation is significantly reduced in bone marrow-derived dendritic cells (BMDCs) from Btk deficient mice compared to wild type mice. Moreover, they showed that cross-linking of TREM-1 induces significantly less TNF-α production in PBMCs from patients with X-linked agammaglobulinemia (a hereditary disease caused by *BTK* gene mutation). All of these evidence indicate that Btk is a positive regulator of TREM-1/DAP12 signaling pathway (95).

In summary, activation of TREM-1 leads to phosphorylation of a broad range of signal transduction proteins and ultimately leads to cytokine amplification. Among these transduction proteins, Btk acts as a positive regulator of TREM-1/DAP12 signaling pathways, suggesting that inhibition of Btk might be useful to reduce the TREM-1-mediated "cytokine storm" during sepsis. More research needs to be done to fully elucidate the signaling pathways downstream of TREM-1 activation.

3.5 Soluble TREM-1

Over time soluble TREM-1 (sTREM-1) is released from neutrophils and monocytes, and it is found in culture supernatants after LPS treatment and in the serum of septic patients. Soluble TREM-1 is of particular interest because it has important biological, diagnostic and

therapeutic values. In this section, we will address the function of sTREM-1 and its potential as a biomarker for the diagnosis of sepsis. The therapeutic role of sTREM-1 will be addressed in the section named "Modulating TREM-1 signaling in sepsis models".

Soluble TREM-1 functions as a negative feedback by out-competing cell surface TREM-1 for engagement with its ligand, thereby inhibiting signaling through cell surface TREM-1 that amplifies cytokine production. sTREM-1 is up-regulated in the plasma of humans with induced endotoxemia (78), in the plasma and peritoneal fluid of mice with caecal ligation and puncture (CLP)-induced sepsis (77), and in the lung of mice with pneumonia caused by *Streptococcus pneumoniae* (96). These observations suggest that up-regulation of sTREM-1 is strongly associated with infection and may be used for the diagnosis of sepsis.

Indeed, Gibot *et al.* reported that plasma sTREM-1 levels have a sensitivity of 96% and a specificity of 89% for differentiating patients with SIRS from those with sepsis (97). They also reported that sTREM-1 levels in bronchoalveolar-lavage fluid have a sensitivity of 98% and a specificity of 90% to differentiate patients with bacterial or fungal pneumonia from those without pneumonia (98). These reports from Gibot *et al.* suggest that sTREM-1 is a reliable biomarker for the diagnosis of sepsis and pneumonia.

However, two observations from other laboratories showed that serum sTREM-1 levels have a sensitivity of 60%-70% and a specificity of 60% to differentiate patients with SIRS from patients with sepsis (99, 100). Additionally, Bopp *et al*. reported that plasma sTREM-1

concentrations were not different between healthy controls and patients with SIRS, severe sepsis or septic shock (101). Finally, Anand *et al.* reported that sTREM-1 in BAL yielded a poor sensitivity (42.1%) and specificity (75.6%) for the diagnosis of pneumonia (102). These observations suggest that sTREM-1 may not serve as a reliable biomarker for the diagnosis of sepsis or pneumonia.

The discrepancy in these studies may be due to 1) different methods used for the detection of sTREM-1, 2) different patient populations included and/or 3) different methods used for the collection of samples. Overall, the reliability of sTREM-1 as a biomarker for the diagnosis of sepsis needs further investigation.

3.6 Regulation of soluble TREM-1

There are two leading hypotheses about the regulation of soluble TREM-1 formation. The first hypothesis states that sTREM-1 is from a TREM-1 mRNA splice variant and the second hypothesis states that sTREM-1 is from the cleavage of cell surface TREM-1.

Research from Gingras *et al.* supports the first hypothesis. They detected an alternative mRNA TREM-1 splice variant (TREM-1sv) missing the transmembrane region in $CD14^+$ human monocytes (103). This TREM-1sv mRNA has the potential to be translated into sTREM-1. In contrast, results from Gomez-pina *et al.* support the second hypothesis. They found that metalloproteinase inhibitors reduced sTREM-1 release and increased the stability of surface TREM-1. Furthermore, they were unable to detect any splice variant of TREM-1

mRNA. So they concluded that sTREM-1 is exclusively generated from mature, membrane-bound TREM-1 by metalloproteinases-mediated cleavage (104).

Collectively, the origin of sTREM-1 is still a mystery based on these conflicting data. However, these data may not be exclusive and both hypotheses could be true, suggesting that sTREM-1 could originate from TREM-1 mRNA splice variants or cleavage of surface TREM-1.

3.7. TREM-1 ligand

To date, the ligand for TREM-1 remains undefined. By using different methods, investigators reported that the TREM-1 ligand is detected on neutrophils, monocytes and/or platelets.

Gibot *et al.* reported that TREM-1 ligand is detected on mouse peritoneal exudate neutrophils as early as 3 hr post-CLP and on peripheral neutrophils 15 hr post-CLP by using an mTREM-1/IgG1 tetramer (105). Similarly, Zanzinger *et al.* reported that TREM-1 ligand was detected on murine Gr-1^+ granulocytes and Gr-1^+ monocytes after LPS administration by using an mTREM/IgG1 fusion protein (79). Haselmayer *et al.* demonstrated that TREM-1 ligand is expressed on human platelets by using human TREM-1/IgG1 (106). Additionally, there may be a soluble form of the TREM-1 ligand. This is supported by an experiment showing that TREM-1/Fc fusion protein decreased the ability of sera from septic patients to activate monocytes (83).

Based on the data discussed above, the identity of TREM-1 ligand is still not defined. However, these data suggest that TREM-1 ligand is expressed on mouse neutrophils and monocytes after TLR ligand stimulation and on human platelets and also present in the sera of septic patients.

3.8 Modulating TREM-1 signaling in sepsis models

Given the role of TREM-1 activation in proinflammatory cytokine production, TREM-1 became an immediate target for treatment of sepsis that is characterized by overproduction of proinflammatory cytokines. In this section, we will address how modulating TREM-1 signaling affects disease pathogenesis and outcomes in sepsis.

3.8.1 Blocking TREM-1 in sepsis models

The effect of blocking TREM-1 in septic animals was first demonstrated by Bouchon *et al*. They showed that blocking TREM-1 by mTREM-1/IgG1 fusion protein improved survival in endotoxemic mice. Importantly, this fusion protein was still protective even when injected 4 hours after the induction of endotoxemia. The protective effect of the mTREM-1/IgG1 fusion protein was accompanied by decrease of TNF-α and IL-1β secretion in the plasma and decrease of neutrophils and monocytes infiltrating the peritoneum. Subsequent experiments showed that blocking TREM-1 by using this fusion protein protected mice against lethal *E. coli* peritonitis and caecal ligation and puncture (CLP)-induced septic shock. All the above results demonstrate that inhibition of TREM-1 function by mTREM-1/IgG1 decreases

proinflammatory cytokine secretion and thus protects mice from cytokine-mediated septic shock and death (75).

TREM-1 has also been blocked by a short synthetic peptide called LP17. This peptide mimics part of the extracellular domain of TREM-1. Pretreatment with LP17 protected mice from lethal LPS administration in a dose-dependent manner. This peptide also significantly protected endotoxemic mice from death even when injected 4 hours after LPS administration. Furthermore, LP17 improved survival in mice when administered 24 hours post completion of caecal ligation and puncture (CLP). Subsequent examination revealed that the protection provided by LP17 is associated with reduced secretion of TNF-α and IL-1β in the serum. (107).

Blocking TREM-1 has further been explored by using siRNA. Gibot *et al.* demonstrated that partial silencing of TREM-1 by siRNA provided significant protection in mice with peritonitis-induced sepsis. This protection was found to be associated with reduced proinflammatory cytokine secretion. However, more complete silencing of TREM-1 by a higher dose of siRNA increased mortality. Further examination revealed that the increased mortality was due to impaired bacterial clearance. Interestingly, both partial and more complete silencing of TREM-1 significantly reduced proinflammatory cytokine secretion and improved survival in endotoxemic mice (108). This study suggests that fine-tuning TREM-1 activation is the key to reducing mortality induced by excessive release of cytokines while maintaining an inflammatory response that is adequate to clear bacteria during infection.

3.8.2 Activation of TREM-1 in a mouse model of pneumococcal pneumonia

While blocking TREM-1 can reduce mortality in septic models, activation of TREM-1 can also be beneficial during infection. Lagler *et al*. demonstrated that activation of TREM-1 by an anti-TREM-1 mAb enhanced bacterial clearance and promoted survival in a murine model of pneumococcal pneumonia. Subsequent investigations revealed that this protective effect provided by TREM-1 activation is associated with enhanced proinflammatory cytokine secretion at early time points and accelerated resolution of inflammation at late time points (96).

In summary, the above experiments showed that fine-tuning of TREM-1 signaling by partial blocking, complete blocking or activation has the potential to be used as a therapeutic method to balance cytokine secretion in septic patients.

3.9 Disregulation of TREM-1 in chronic inflammatory disease

While TREM-1 is believed to function primarily as a regulator of innate immune responses to pathogens, aberrant expression of TREM-1 is known to contribute to chronic inflammatory bowel disease. In healthy individuals, greater than 80% of monocytes and macrophages in systemic secondary lymphoid tissue (lymph nodes and tonsils) express TREM-1, whereas less than 10% of resident intestinal macrophages express TREM-1. Importantly, intestinal macrophages are refractory to LPS-, TNF-α- and PMA-induced TREM-1 up-regulation. Further, cross-linking of TREM-1 on intestinal macrophages did not induce oxidative burst. Collectively, lack of TREM-1 expression on intestinal macrophages

and lack of responses to TREM-1 cross-linking is likely to prevent excessive inflammatory reaction in the intestine (109).

Interestingly, the percentage of TREM-1 positive intestinal macrophages is significantly increased in the lesions of inflammatory bowel diseases (IBD) patients and cross-linking of TREM-1 on these macrophages significantly up-regulated proinflammatory cytokine secretion (TNF-α, IL-1β, IL-6, IL-8 and MCP-1). These observations suggest that TREM-1-mediated cytokine amplification may contribute to IBD and blocking TREM-1 may alleviate IBD severity. Indeed, blocking TREM-1 by LP17 peptide significantly attenuated clinical course and histopathological alterations in mouse models of colitis. This study suggests that activation of TREM-1 contributes to the excessive inflammatory reactions in the patients with IBD and blocking TREM-1 may represent a target for the treatment of IBD patients (110).

In summary, TREM-1 expression is up-regulated upon TLR stimulation and it functions as an amplifier of cytokine production during infection. Diagnostically, soluble TREM-1 has good potential to be used as a biomarker for the diagnosis of sepsis. Therapeutically, fine-tuning of TREM-1 signaling may serve as a good target to help balance cytokine production in septic patients and IBD patients.

4. Role of MARCKS protein in inflammation

As described above, TREM-1 is a critical amplifier of cytokine production, and multiple therapeutics are under development to inhibit TREM-1 signaling. We have identified an alternate point of intervention that also inhibits cytokine production: MARCKS protein. MARCKS is an intracellular, membrane-associated protein that acts as both a PKC and calcium/calmodulin (Ca^{2+}/CaM) substrate, and thus plays a pivotal role in cell shape, motility, and exocytosis of vesicles and granules (111-114). It is now established that MARCKS function is critical for neutrophil migration, and inhibition of MARCKS significantly decreases neutrophil migration *in vitro* (115) and *in vivo* (116). Its function as a substrate for PKC places MARCKS protein in critical signal transduction pathways that perpetuate inflammation (Figure 3). Inflammation is initiated when highly conserved pathogen-associated molecular patterns (PAMPs), such as lipopolysaccaride (LPS), engage the Toll-like Receptors (TLR). TLR activation initiates a signal transduction cascade that culminates in NF-κB activation and initiation of cytokine production. TLR activation is the priming event for innate immune cells (Figure 3, panel A), while autocrine/paracrine activation of cytokine receptors (Figure 3 panel B) or activation of additional immunoreceptor tyrosine-based activation motifs (ITAM)-containing receptors (e.g. FcR and TREM-1) amplifies and perpetuates inflammation (Figure 3, panel C). **In the context of these established signaling events, we have identified a plausible role for MARCKS in the amplification and perpetuation of cytokine production.**

In resting cells, MARCKS is bound to the plasma membrane and locally sequesters phosphatidyl inositol 4,5,bisphosphate (PIP2) (111). PIP2 is the major substrate for receptor-stimulated phospholipaseCγ (PLC-γ) and forms inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) when cleaved by PLC-γ (Figure 1 Panel B). Once formed, DAG activates PKC-δ (117), which, in turn, phosphorylates MARCKS and induces its translocation into the cytosol where MARCKS interacts with actin (111). The removal of MARCKS from the plasma membrane frees more PIP2 to be cleaved by PLC-γ, thus perpetuating the formation of DAG and IP3. Downstream of DAG are two critical events that lead to amplification of cytokine production: 1) PKC-δ mediated phosphorylation of Stat1 that allows formation of the Stat1/Stat3 heterodimer, and 2) activation of ERK, JNK and p38MAPK downstream of TREM-1 cross-linking. Cumulatively, activation and translocation of these signal transduction proteins results in profound amplification of inflammatory cytokines (118, 119). Therefore, amplification of cytokines by activation of cytokine receptors or TREM-1 cross-linking depends largely on the release of PIP2 by MARCKS protein. **Thus, we hypothesize that MARCKS protein is a "gatekeeper" in cytokine amplification.**

4.1. MARCKS protein

Myristoylated alanine-rich C-kinase substrate (MARCKS) was first discovered in rat brain synaptosomes as a major, specific substrate of protein kinase C (PKC) in 1982 (120). MARCKS is a 32 kDa, rod-shaped, elongated, acidic protein (121, 122). Due to its non-globular, elongated form and the weak binding of SDS molecules to the acidic protein, it

migrates with a molecular weight of 80-87 kDa on SDS-PAGE (111, 121, 122). Phosphorylation of MARCKS is regulated by both PKC and calcium/calmodulin (Ca^{2+}/CaM) (120). MARCKS is found ubiquitously in eukaryotic cells. It is expressed in the organs of brain, spinal cord, spleen and lung (121), in the cell types of leukocytes (114, 123, 124), neurons (125), fibroblasts (126) and epithelial cells (127). It has been found in chicken (128), mouse (122), rat (129), cow (130) and human (131, 132). MARCKS protein has been implicated in neurosecretion, membrane trafficking, cell motility, phagocytosis, the regulation of cell cycle, transformation and is required for embryogenesis (111, 133, 134).

4.2. Structure and membrane trafficking regulation

MARCKS contains three evolutionarily conserved domains: the amino-terminal myristoylated domain, the multiple homology 2 domain (MH2 domain) and the phosphorylation site domain (PSD) or effector domain (ED). The amino-terminal myristoylated domain represents a consensus sequence for myristoylation, which is a co-translational lipid modification. In this modification, myristic acid is covalently attached to the amino group of the N-terminal glycine residue via an amide bond. The amino-terminal myristoylated domain is essential for the anchorage of MARCKS protein to the plasma membrane. The function of MH2 domain is unknown. The phosphorylation site domain (PSD) contains all three serine residues, which can be phosphorylated by PKC. The PSD is also highly basic since it contains a high percentage of basic amino acids, such as lysine and arginine residues, and no acidic residues (111, 135).

In resting cell, MARCKS protein is located in the plasma membrane. Both the amino-terminal myristoylated domain and the PSD are required for the protein to be anchored to the plasma membrane. The amino-terminal myristoylated domain is hydrophobic, which helps insert the protein into the hydrophobic phospholipid bilayer of the cell membrane. The PSD is basic and helps the protein attach to the acidic head-group of the membrane phospholipids by electrostatic attraction. Both mechanisms are required to anchor MARCKS on the cell membrane since neither myristoylation alone nor PSD mediated electrostatic interaction alone is sufficient for membrane binding (111, 135-138).

MARCKS cycles between the plasma membrane and cytoplasm in various cell types, such as neutrophils (124), macrophages (139, 140), neurons (125) and fibroblasts (126, 141). Phosphorylation of MARCKS protein by PKC adds negatively charged phosphate groups to the serine residues (Ser152, Ser156, Ser 163) within the PSD (142-144), which neutralizes the positively charged PSD and abolishes the contribution of PSD mediated electrostatic interaction between MARCKS and cell membranes. Since myristoylation alone is not sufficient for the membrane binding of MARCKS, MARCKS is detached from the cell membrane and enters the cytoplasm (136-139, 145, 146). Subsequent dephosphorylation of MARCKS by phosphatases, such as protein phosphatase 1, protein phosphatase 2A or calcineurin, allows MARCKS to return to the cell membrane (147-149).

In addition to PKC, calcium/calmodulin (Ca^{2+}/CaM) could also mediate MARCKS protein cycling between the plasma membrane and cytoplasm. In this case, activation of cells leads

to increased intracellular Ca^{2+} concentration, which activates calmodulin. Calcium-bound calmodulin is able to bind the PSD of MARCKS and dissociate the protein from the membrane. And this process is also reversible, as decrease of the Ca^{2+} concentration to normal level releases MARCKS from calmodulin and returns it to the cell membrane (150, 151).

However, activation of MARCKS by PKC or calcium/camodulin is mutually exclusive. One of the hypothesis suggested that there is a competition between PKC and camodulin for their interaction with MARCKS. If PKC is activated first, it phosphorylates MARCKS and dissociates it from the cell membrane, which decreases the binding affinity of subsequently activated camodulin to MARCKS. As a result, activated camodulin activates other substrates more strongly. In contrast, if camodulin is activated first, the binding of camodulin to the PSD of MARCKS sterically hinders the binding of PKC to MARCKS, thus preventing phosphorylation of MARCKS by PKC. As a result, activated PKC phosphorylates other substrates more strongly (134, 152). The competition of PKC and calmodulin for MARCKS mediates crosstalk between the PKC and calmodulin signaling pathways.

MARCKS protein is involved in the regulation of the actin cytoskeleton after being phosphorylated by PKC or binding to activated calmodulin. There are two hypothetical mechanisms explaining the regulation of actin cytoskeleton by MARCKS. The first mechanism proposes that membrane bound MARCKS cross-links actin to the plasma membrane and phosphorylation of MARCKS by PKC or binding by calmodulin releases both MARCKS and actin from the membrane, leading to increased plasticity of the local actin

cytoskeleton. The second mechanism proposes that membrane bound MARCKS sequesters phosphatidylinositol 4, 5-bisphosphate (PIP2) in the plasma membrane and release of MARCKS by PKC or calmodulin releases PIP2 from the membrane. Free PIP2 binds to actin-binding proteins and thus regulates cytoskeletal movement (113, 133, 153). The specific mechanisms of MARCKS in cytoskeletal movement is unknown but it is clear that MARCKS is critical in 1) migration of chalaniocarcinoma cells (154), 2) normal myoblast migration (155) and 3) platelet-derived growth factor-induced chemotaxis in activated human hepatic stellate cells (156).

Other than these two mechanisms of how MARCKS may be involved in the regulation of cytoskeletal movement, another hypothetical mechanism has been proposed to explain how MARCKS acts as a transportation protein by using actin to regulate exocytosis in goblet cells. In this mechanism, stimulation of cells activates PKC and subsequently leads to MARCKS phosphorylation. Phosphorylated MARCKS translocates to the cytoplasm and binds to the chaperone heat shock protein 70 (HSP70), which results in binding of MARCKS to the secretory granules through the affinity between MARCKS bound HSP70 and granule bound cysteine string protein (CSP). Dephosphorylation of MARCKS by protein phosphatase 1 or protein phosphatase 2A allows MARCKS to bind to the actin/myosin contractile system while still attached to the granule. Contraction of the actin/myosin system cycles MARCKS back to the cell membrane and also moves the granule to the membrane for exocytosis (135, 157). Indeed, it was found that phosphorylation of MARCKS is involved in 1) PMA- or neutrophil elastase-induced mucin secretion from both primary human bronchial

epithelial cells and the human bronchial epithelial 1 cell line (158), 2) arginine vasopressin-induced adrenocorticotropin secretion from ovine anterior pituitary (159), 3) PMA- and bombesin-mediated neurotensin secretion from the BON human endocrine cell line (160), 4) exocytosis of oxytocin from bovine large luteal cells (161), 5) bovine chromaffin cell secretion (162), 6)glucose-stimulated insulin secretion from rat pancreatic islets (163) and 7) PMA or thrombin-induced serotonin release from platelets (164, 165), All of these experiments described above demonstrated that MARCKS plays a role in cell adhesion/migration and degranulation and suggest the inhibition of MARCKS function could inhibit these inflammatory events .

Prior to 2001, investigators have used a synthetic peptide identical to the PSD sequence of MARCKS and siRNA to inhibit MARCKS function. In 2001, a myristoylated N-terminal sequence peptide (MANS peptide), whose sequence is identical to the first 24 amino acids of human MARCKS, was designed to inhibit MARCKS function. The Adler laboratory observed that pre-incubation with this peptide for 15 min significantly decreased UTP or PMA and 8-Br-cGMP (a nonhydrolyzable cGMP analogue) induced mucin secretion from cultured normal human bronchial epithelial (NHBE) cells. However, the scrambled control peptide (RNS peptide), which has the same amino acids composition as MANS peptide but arranged in random order, does not have such an effect (127).

In summary, MARCKS is a ubiquitously expressed protein and plays an important role in cytoskeletal movement. Numerous observations demonstrated that MARCKS is involved in

cell adhesion/migration and degranulation. Since these functions are important in inflammatory cells, inhibition of MARCKS may be a target to reduce inflammation.

4.3. MARCKS and inflammation

During infection, innate immune cells, such as neutrophils and monocytes, migrate to the site of infection to kill bacteria by phagocytosis and release of its arsenal. Excessive infiltration and degranulation of these innate immune cells can do more harm than benefit to the body, as seen in sepsis. As discussed above, inhibition of MARCKS may be a target to reduce inflammation by reducing excessive cell adhesion/migration and degranulation. Indeed, numerous experiments have shown that inhibition of MARCKS by MANS peptide greatly reduces degranulation in a broad range of cell types in vitro and reduces cell migration both in vivo and in vitro.

Beyond inhibition of mucin secretion, disruption of MARCKS protein function with MANS peptide greatly reduced leukocyte degranulation, which shares a similar mechanism with mucin exocytosis. PMA-induced myeloperoxidase secretion from freshly isolated human neutrophils was decreased by MANS peptide treatment in a dose dependent manner. In an eosinophil-like cell line HL-60 clone 15, MANS peptide significantly inhibited eosinophil peroxidase release. Similarly, in a human monocytic cell line U937 and the lymphocyte natural killer cell line NK-92, MANS peptide significantly reduced lysozyme and granzyme secretion from these two cell lines respectively (114).

Disruption of MARCKS function by MANS peptide also inhibited leukocyte migration both in vitro and in vivo. Eckert *et al*. demonstrated that MANS peptide inhibited N-formyl-methionyl-Leucyl-phenylalanine (fMLP), PMA and immune complexes (IC)-induced human neutrophil adhesion and migration towards the chemoattractants fMLP, leukotriene B4 (LTB4) and IL-8 in vitro (124). In a neutrophil elastase-induced bronchitis mouse model, MANS peptide decreased infiltration of neutrophils, eosinophils and lymphocytes in the bronchoalveolar lavage (BAL) fluid. Interestingly, pre-treatment with MANS peptide decreased BAL levels of CXCL-1, IL-1β, IL-6, MCP-1 and TNF-α by 50-60% (166). Similarly, neutrophil influx and BAL levels of CXCL-1, IL-6 and TNF-α were significantly decreased by MANS peptide in a mouse model of ozone-induced inflammation (167). Although both in vivo experiments showed that MANS peptide reduced proinflammatory cytokines in BAL fluid, it is unclear that the reduced cytokine level is due to reduced leukocyte infiltration or reduced ability of leukocytes to produce/secrete cytokines or both.

Thus, we sought to define whether inhibition of MARCKS protein could alter the ability of activated cells to produce cytokines, and firmly place MARCKS in the inflammation machinery and represent a putative target for anti-inflammatory therapeutics.

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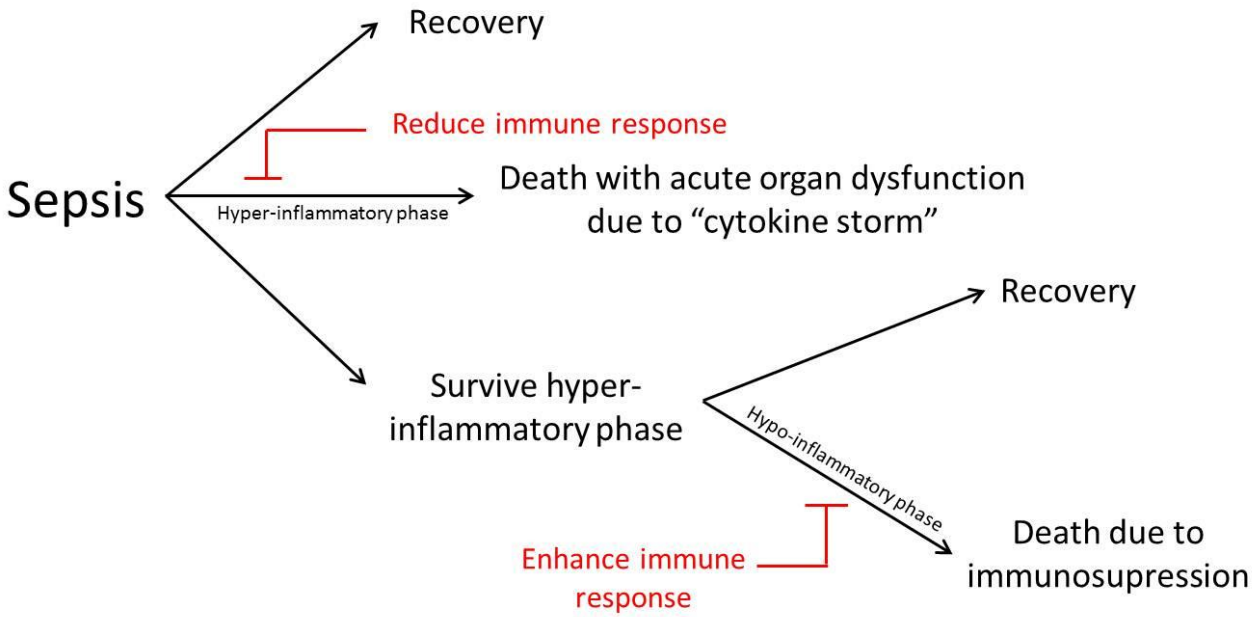

Figure 1. Two stage theory of sepsis

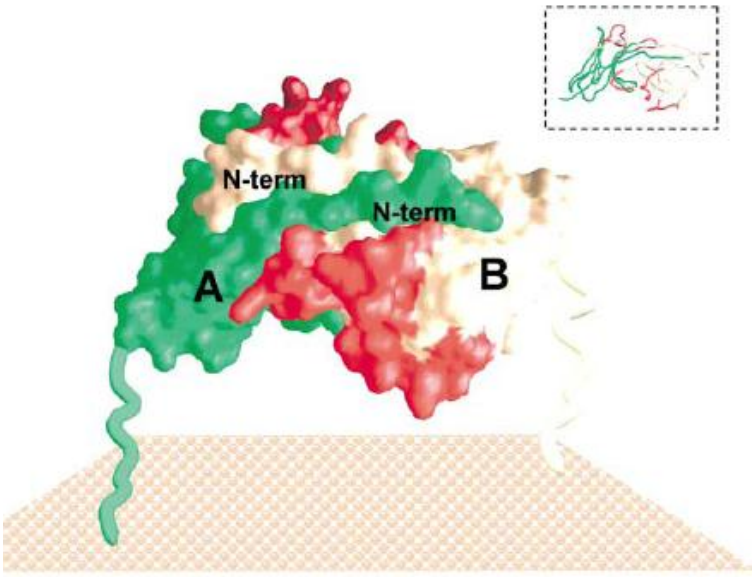

Figure 2. Cartoon representing TREM-1 “head-to-tail” homodimer with the neck regions bound to the cell membrane (88).

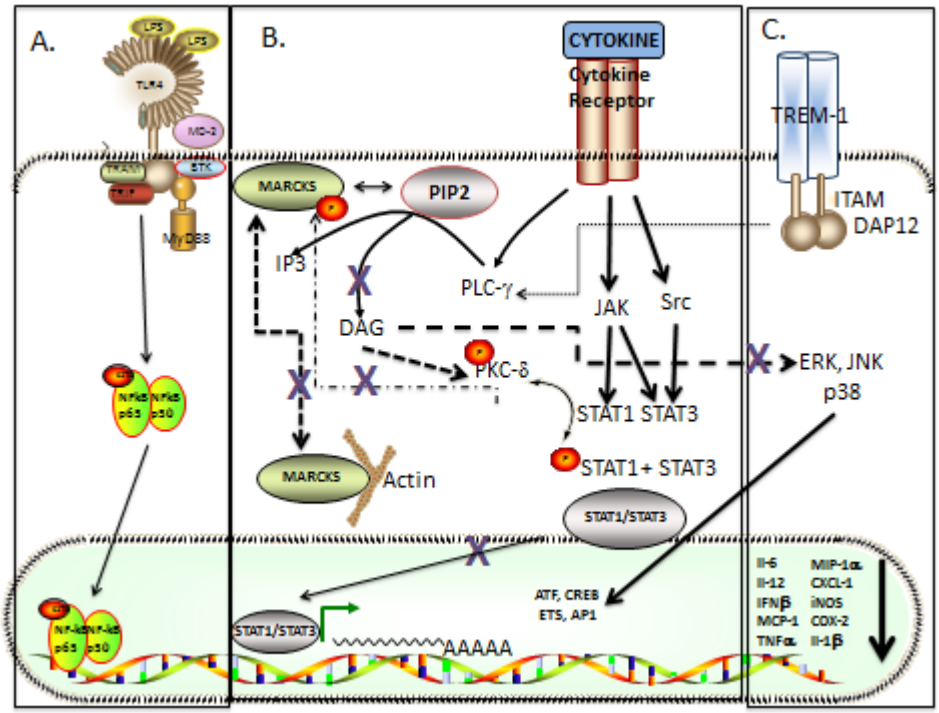

Figure 3. Putative role of MARCKS protein in inflammation

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Cytokine Responses in Dogs with Induced Endotoxemia and Naturally Occurring Sepsis

Abstract

Sepsis is characterized by dysregulation of cytokine production. Cytokine profiles in canine sepsis have not been described. Therefore, we measured cytokine concentrations in healthy dogs as well as dogs with induced endotoxemia, naturally occurring sepsis, recent surgery (sterile inflammation), or systemic inflammatory response syndrome (SIRS) with no evidence of infection. We found that IL-8, CXCL1 orthologue and MCP-1 were significantly increased in dogs with naturally occurring sepsis compared to healthy dogs, but were not significantly different compared to dogs with SIRS or dogs with recent surgery. However, since a small number of animals were included in our experiment, significant differences between dogs with sepsis and dogs with non-infectious inflammatory conditions (SIRS or recent surgery) may have been obscured. In the future, larger studies should be performed to evaluate the potential of MCP-1 as biomarkers of canine sepsis.

1. Introduction

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) with suspected or proven infection (1, 2). Despite advances in diagnosis and treatment of sepsis, it still causes a high mortality rate in dogs. The prevalence is estimated to be 6% - 10% in critically ill patients and the mortality rate is estimated to be 50% -75% (3).

Rapid diagnosis and intervention is critical for the survival of septic patients. Recent research using human subjects suggests that novel biomarkers may be used to help diagnose sepsis (4-6). In dogs, procalcitonin whole blood mRNA expression, serum/plasma concentrations of protein C, antithrombin, IL-6 and MCP-1 have failed to act as sensitive and specific diagnostic biomarkers of canine sepsis (7-11). Other cytokines have not been evaluated for this use in dogs. Thus, we sought to describe the kinetics of a panel of 14 cytokines during experimentally induced endotoxemia and evaluate if any of these cytokines appear to have potential as diagnostic markers for naturally occurring canine sepsis.

2. Materials and Methods

2.1 Animals

Endotoxemia was induced in six healthy mixed breed male dogs aged 6 to 12 months old (mean = 8.5 months) and weighing 18.7 to 22.4 kg (mean = 20.3 kg). All dogs were housed in animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and all care was provided in accordance with the principles outlined by the National Institutes of Health. All experimental procedures were reviewed and approved by the Animal Care and Use Committee at North Carolina State University.

Samples from healthy dogs as well as dogs with naturally occurring sepsis, SIRS and recent surgery were all obtained from the teaching hospital at college of veterinary medicine in North Carolina State University. The dogs with sepsis and dogs with SIRS met at least two of the SIRS criteria (Table 1). Dogs in the sepsis category had SIRS and cytological, histopathological or microbiological evidence of infection. Dogs in the SIRS category had SIRS but had no cytological, histopathological or microbiological evidence of infection. Dogs in the recent surgery category did not have SIRS.

2.2 Study design

Samples were collected from dogs with induced endotoxemia as described (12). Briefly, six healthy experimental dogs were injected with 1 ml 0.9% NaCl intravenously as control and after 5 days washout they were injected with 2 μg LPS O127:B8 per kilogram body weight to

induce endotoxemia. Blood samples were collected at 0 hr, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12 hr, 24 hr, 48 hr and 72 hr to measure the cytokine concentrations in the sera of the dogs with endotoxemia by Milliplex Map canine cytokine/chemokine kit (Milllipore, Billerica, MA) per manufacturer's instructions. The vital signs (rectal temperature, heart rate, respiratory rate and blood pressure) were monitored at 0 hr time point and every 30 minutes thereafter until 6 hr, and then at 8 hr, 10 hr, 12 hr and 24 hr. If the dogs became hemodynamically unstable (systolic arterial pressure < 90 mmHg, mean arterial pressure < 70 mmHg or heart rate > 180 beats per minuets) after LPS administration, they were given 10 ml isotonic crystalloid per kilogram body weight intravenously to maintain hemodynamic stability and the isotonic crystalloid was given as many times as necessary.

Blood from healthy dogs as well as dogs with sepsis, SIRS or recent surgery was collected at one time point when the animals were admitted to the teaching hospital at college of veterinary medicine in North Carolina State University. Serum was harvested and frozen at -80C until analysis. Cytokine concentrations in the sera were measured by Milliplex Map canine cytokine/chemokine kit (Milllipore, Billerica, MA) per manufacturer's instructions. Analytes evaluated included IL-8, CXCL1 orthologue, TNF-α, MCP-1, CXCL10, IL-6, IL-10, GM-CSF, IL-2, IFN-γ, IL-7, IL-15, IL-18 and IL-4. Based on affirmative data in preliminary studies, the multiplex analysis was reduced to only include IL-8, CXCL1 orthologue, TNF-α, MCP-1 and GM-CSF.

2.3 Statistics

Data were analyzed by using paired t-test to compare body temperature and mean blood pressure in the dogs after LPS injection to the parameters in the dogs after saline injection at the corresponding time points. Unpaired t-test was used to compare the cytokine concentrations in healthy dogs, dogs with sepsis, dogs with SIRS and dogs with recent surgery. In cases where cytokine concentrations were below the assay Lower Limit of Detection (LLOD), values were assigned that were half of the LLOD for the purpose of statistical analysis (13-18). The data were analysed by using SAS software (SAS, Cary, NC) or prism 5 (GraphPad Software, San Diego, CA). The level of significance used was $p \leq 0.05$. Error bars represent the standard error of the mean (SEM).

3. Results

3.1 Endotoxemia model

The body temperature was significantly increased in dogs within 2 hr post LPS administration compared to the saline control (Figure 1A). The mean blood pressure was decreased within 1 hr after LPS administration (Figure 1B). These effects were similar to those observed in other canine endotoxemia studies (19, 20).

3.2 Kinetics of cytokine concentrations in dogs with endotoxemia

The kinetics of cytokine concentrations were determined in the serum of dogs post LPS administration. Concentrations of IL-8, CXCL1 orthologue, MCP-1, CXCL10, IL-6, IL-10, TNF- α and GM-CSF were consistently increased post LPS administration (Figure 2A-H), whereas detection of IL-4, IL-2, IFN-γ, IL-7, IL-15 and IL-18 was highly variable (Table 2-7).

3.3 Cytokine responses in naturally occurring canine sepsis

As concentrations of IL-8, CXCL1 orthologue, MCP-1, CXCL10, IL-6, IL-10, TNF-α and GM-CSF were consistently increased in dogs with induced endotoxemia, we sought to determine whether these cytokines were similarly elevated in dogs with naturally occurring sepsis and evaluate if these cytokines could be used as biomarkers for the diagnosis of sepsis.

Cytokine concentrations in the serum from six healthy dogs, five dogs with sepsis and four dogs with SIRS were measured in a fourteen-cytokine panel. We found that CXCL10, IL-6,

IL-10, IL-15, IL-2, IFN-γ, IL-18, IL-4, IL-7 and GM-CSF were variably detected in the serum from these dogs. Based on our results from the fourteen-cytokine panel, we measured IL-8, CXCL1 orthologue, TNF-α, MCP-1 and GM-CSF in the serum from the remaining dogs. Concentrations of GM-CSF were variably detected in the serum of all the clinical dogs. TNF-α was above the limit of detection (3.2 pg/ml) in one dog with sepsis (13.42 pg/ml) and two dogs with SIRS (17.84 pg/ml and 18.29 pg/ml). Concentrations of IL-8, CXCL1 orthologue and MCP-1 were significantly increased in dogs with sepsis compared to healthy dogs, but were not significantly different compared to dogs with SIRS or recent surgery (Figure 3A-C).

4. Discussion

The kinetics of TNF-α and IL-6 in dogs with induced endotoxemia have been described previously. The peak times and kinetics of IL-6 and TNF-α we observed were similar to those described by other investigators (Figure 2E and G) (19-21). Minor differences may be due to different types of LPS (O127:B8 or O111:B4), different dosages of LPS and/or different assays used for the measurement of TNF-α and IL-6.

Concentrations of IL-8, MCP-1 and CXCL-1 orthologue were significantly increased in dogs with sepsis compared to healthy dogs, but were not significantly increased compared to dogs with SIRS or dogs with recent surgery (Figure 3A-C). One limitation of our study is that only a small number of animals were included, therefore we may not be able to detect small differences between dogs with sepsis and dogs with non-infectious inflammatory conditions (SIRS or recent surgery). Interestingly, concentrations of MCP-1 in two dogs with sepsis were higher than those in any dogs with SIRS or dogs with recent surgery, and were close to the peak concentrations of MCP-1 in dogs with induced endotoxemia. This suggests that MCP-1 may have utility as a component of a biomarker panel for the diagnosis of sepsis. Larger studies to evaluate MCP-1 as a biomarker for the diagnosis of canine sepsis are indicated.

In dogs with induced endotoxemia, concentrations of eight cytokines were initially increased after a bolus injection of LPS, and were rapidly decreased presumably due to clearance of LPS from circulation. It seems unlikely that the cytokine kinetics in dogs with this type of

induced endotoxemia fully represent cytokine kinetics in dogs with naturally occurring sepsis. However, results from dogs with induced endotoxemia appear to be useful for the identification of potential biomarkers of canine sepsis. To fully understand cytokine kinetics in dogs with naturally occurring sepsis and evaluate if certain cytokines or a panel of cytokines could be used for the diagnosis of canine sepsis, serial measurement of cytokine concentrations in the dogs with naturally occurring sepsis should be determined.

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Table 1. SIRS criteria in both humans and dogs

Systemic Inflammatory Response Syndrome Criterion (SIRS)		
Criteria	**Definition in humans**	**Definition in dogs**
Temperature	**> 38 °C or < 36°C**	**> 39.4 °C or < 37.2°C**
White blood cell count	**< 4,000/µl or > 12,000/µl**	**< 5,000/µl or > 19,000/µl**
Heart Rate (beats/minute)	**> 90**	**> 140**
Respiratory rate (breaths/minute)	**> 20**	**> 40**

Table 2. IL-4 concentrations in dogs with induced endotoxemia (pg/ml)

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
0h	180.25					
2h				426.85		
4h				4009.75		
6h				2336.02		
8h	88.89			135.6		
10h				1854.34	88.54	290.97
12h				2176.75	946.34	
24h				801.13		
48h				88.89		
72h				1492.06		

Table 3. IL-2 concentrations in dogs with induced endotoxemia (pg/ml)

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
0h	70.66	11.75	4.26		3.92	
2h	66.66	0.65	0.65			
4h	62.66	6.11	4.26	23.26	15.59	
6h	31.03	15.56	19.39	13.65	1.42	2.66
8h	38.87	19.39	11.75			5.19
10h	31.03	19.39		4.26	0.22	0.22
12h	38.87	38.87	4.26	11.75	3.29	
24h	42.81	31.03		27.14	2.66	1.42
48h	31.03	7.97	9.86	48.74		1.42
72h	50.72	31.03	19.39	62.66	7.11	

Table 4. IFN-γ concentrations in dogs with induced endotoxemia (pg/ml)

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
0h	223.33		25.66	181.41		
2h	223.33			659.97		
4h	181.41		25.66	1727.86		
6h	87.08		25.66	1951.35		
8h				263.26		
10h	25.66			579.46		
12h	87.08			546.65	8.55	8.55
24h	223.33		25.66	445.52		
48h	87.08			546.65		
72h	87.08			738.67		

Table 5. IL-7 concentrations in dogs with induced endotoxemia (pg/ml)

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
0h	66.65	3.19				
2h	71.8					
4h	51.13			107.66	5.64	
6h	14.23	6.01		3.19		
8h	3.19	24.93	8.78			17.04
10h	19.6	51.13				
12h	24.93	61.49				
24h	27.58	66.65				
48h	16.92			3.19		10.07
72h	30.22	27.58		35.48		

Table 6. IL-15 concentrations in dogs with induced endotoxemia (pg/ml)

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
0h	177.06	104.24				
2h	177.06	60.43		120.17	95.38	10.49
4h	156.61	114.89		256.85		
6h	71.61	146.29	37.41	372.38		
8h	109.58	166.87	49.05	37.41		6.4
10h	66.04	217.31		49.05		
12h	71.61	329.52	12.78	135.9		16.35
24h	93.49	305.49		197.28	8.46	16.35
48h	82.62	125.44		237.16		
72h	114.89	237.16		217.31	16.35	

Table 7. IL-18 concentrations in dogs with induced endotoxemia (pg/ml)

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
0h	77.54	32.18				8.79
2h	99.75		2.14	8.45		
4h	77.54	43.69				6.83
6h	32.18	43.69	20.48			
8h	32.18	14.52	20.48			22.11
10h	26.36	55.06				18.35
12h	32.18	83.11	8.45			33.25
24h	43.69	110.77		20.48		36.92
48h	49.39	43.69		49.39		22.11
72h	55.06	55.06	2.14		49.69	

Figure 1. Kinetics of body temperature (A) and mean blood pressure (B) in dogs after LPS administration to induce endotoxemia (red line) or after saline administration as control (black line). Data are shown as mean ± SEM. n = 6 dogs.

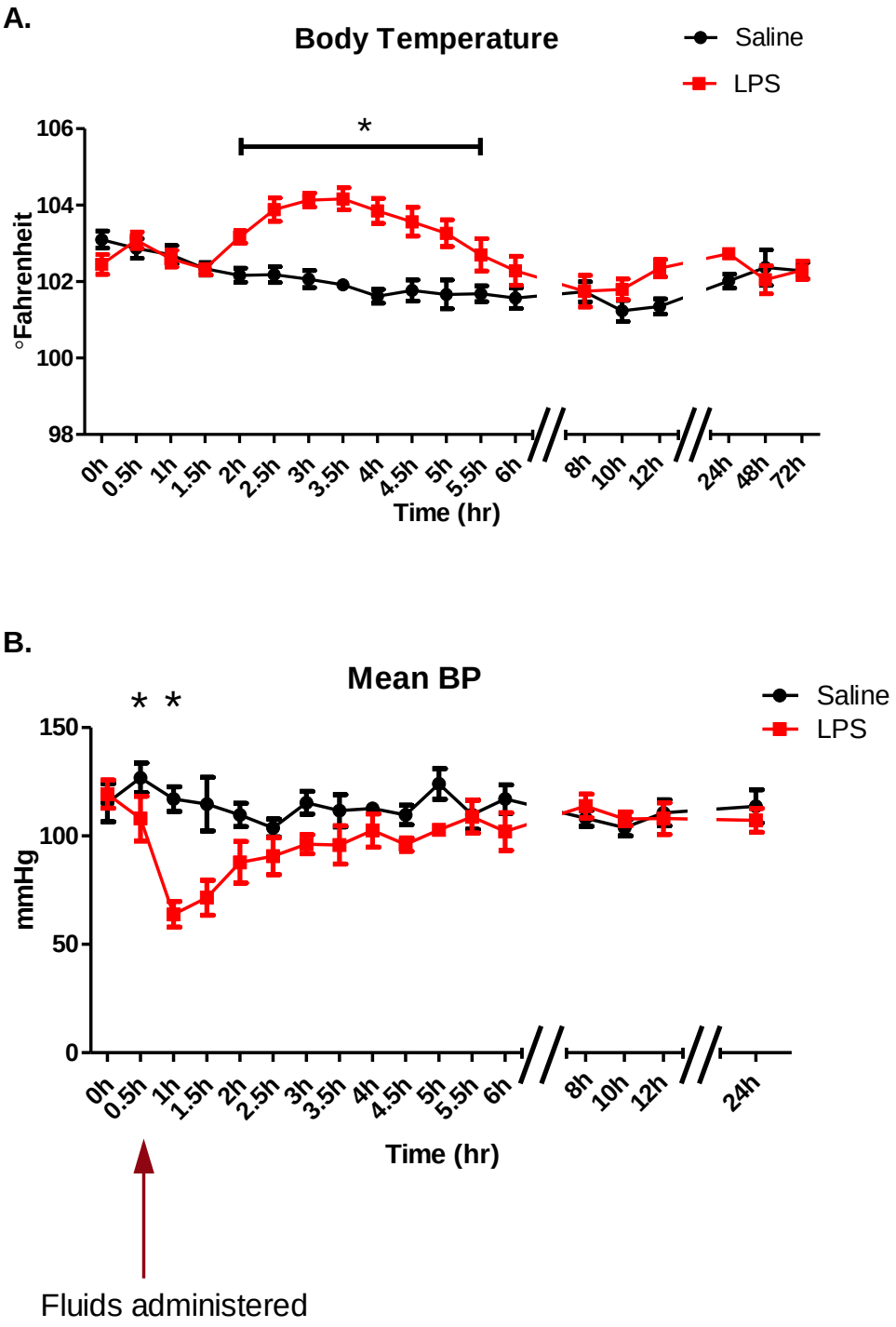
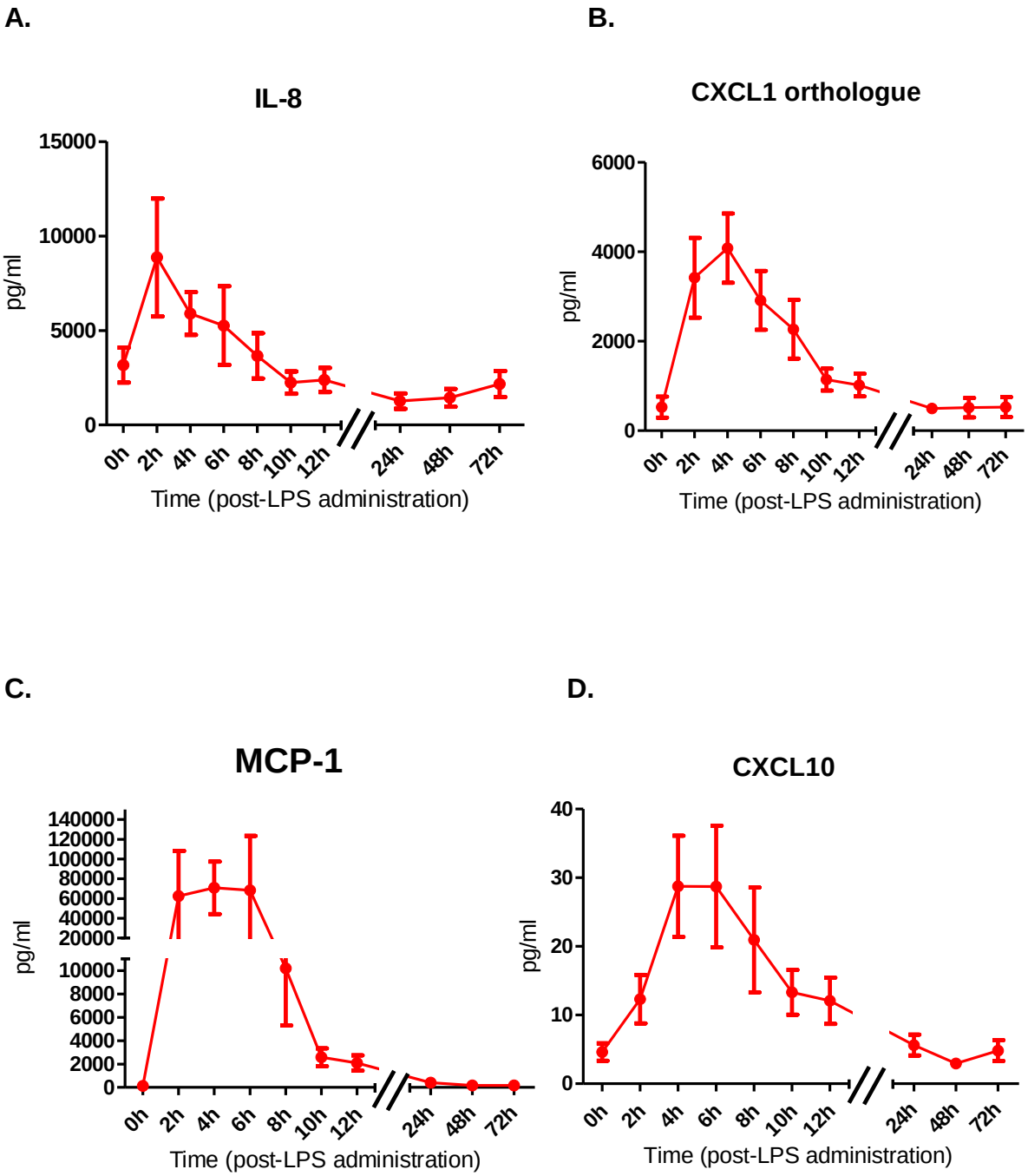
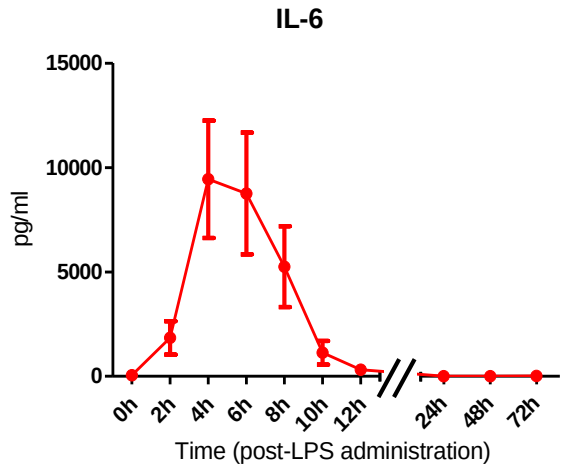

A.
Body Temperature
Saline
LPS
*
106
104
102
100
98
°Fahrenheit
0h
0.5h
1h
1.5h
2h
2.5h
3h
3.5h
4h
4.5h
5h
5.5h
6h
8h
10h
12h
24h
48h
72h
Time (hr)
B.
Mean BP
* *
Saline
LPS
150
100
50
0
mmHg
0h
0.5h
1h
1.5h
2h
2.5h
3h
3.5h
4h
4.5h
5h
5.5h
6h
8h
10h
12h
24h
Time (hr)
Fluids administered

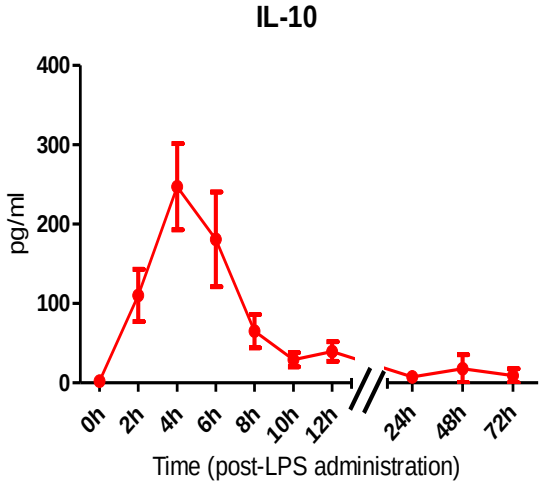
Figure 2. Cytokine kinetics in the serum of dogs with induced endotoxemia. The eight cytokines which are consistently detected in dogs with induced endotoxemia include (A) IL-8, (B) CXCL1 orthologue, (C) MCP-1, (D) CXCL10, (E) IL-6, (F) IL-10, (G) TNF-α and (H)GM-CSF. Data are shown as mean ± SEM. n = 6 dogs


A.
IL-8
15000
10000
5000
0
pg/ml
0h
2h
4h
6h
8h
10h
12h
24h
48h
72h
Time (post-LPS administration)
B.
CXCL1 orthologue
6000
4000
2000
0
pg/ml
Time (post-LPS administration)
C.
MCP-1
140000
120000
100000
80000
60000
40000
20000
10000
8000
6000
4000
2000
0
pg/ml
Time (post-LPS administration)
D.
CXCL10
40
30
20
10
0
pg/ml
Time (post-LPS administration)

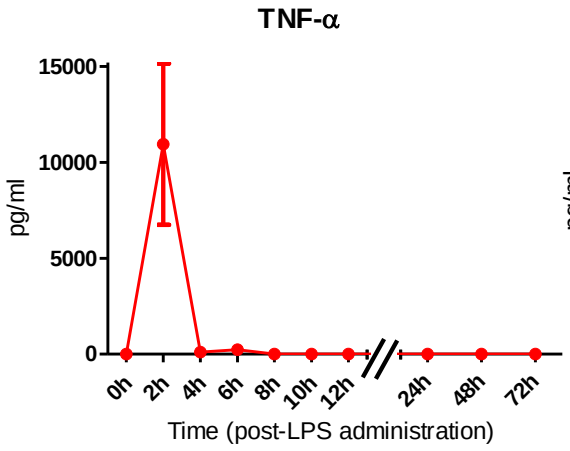
E.


IL-6
15000
10000
5000
0
pg/ml
0h
2h
4h
6h
8h
10h
12h
24h
48h
72h
Time (post-LPS administration)

F.


IL-10
400
300
200
100
0
pg/ml
0h
2h
4h
6h
8h
10h
12h
24h
48h
72h
Time (post-LPS administration)

G.


TNF-α
15000
10000
5000
0
pg/ml
0h
2h
4h
6h
8h
10h
12h
24h
48h
72h
Time (post-LPS administration)

H.

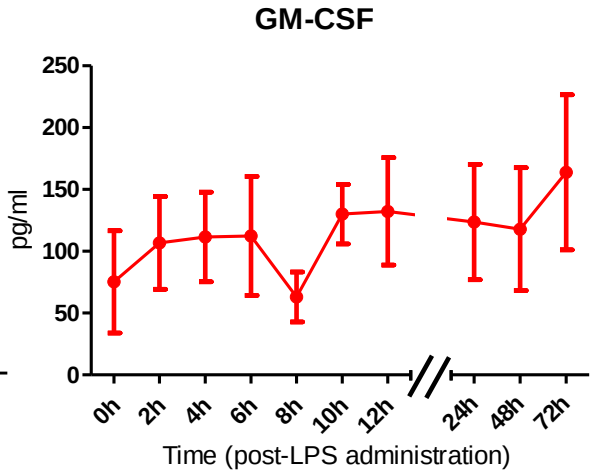
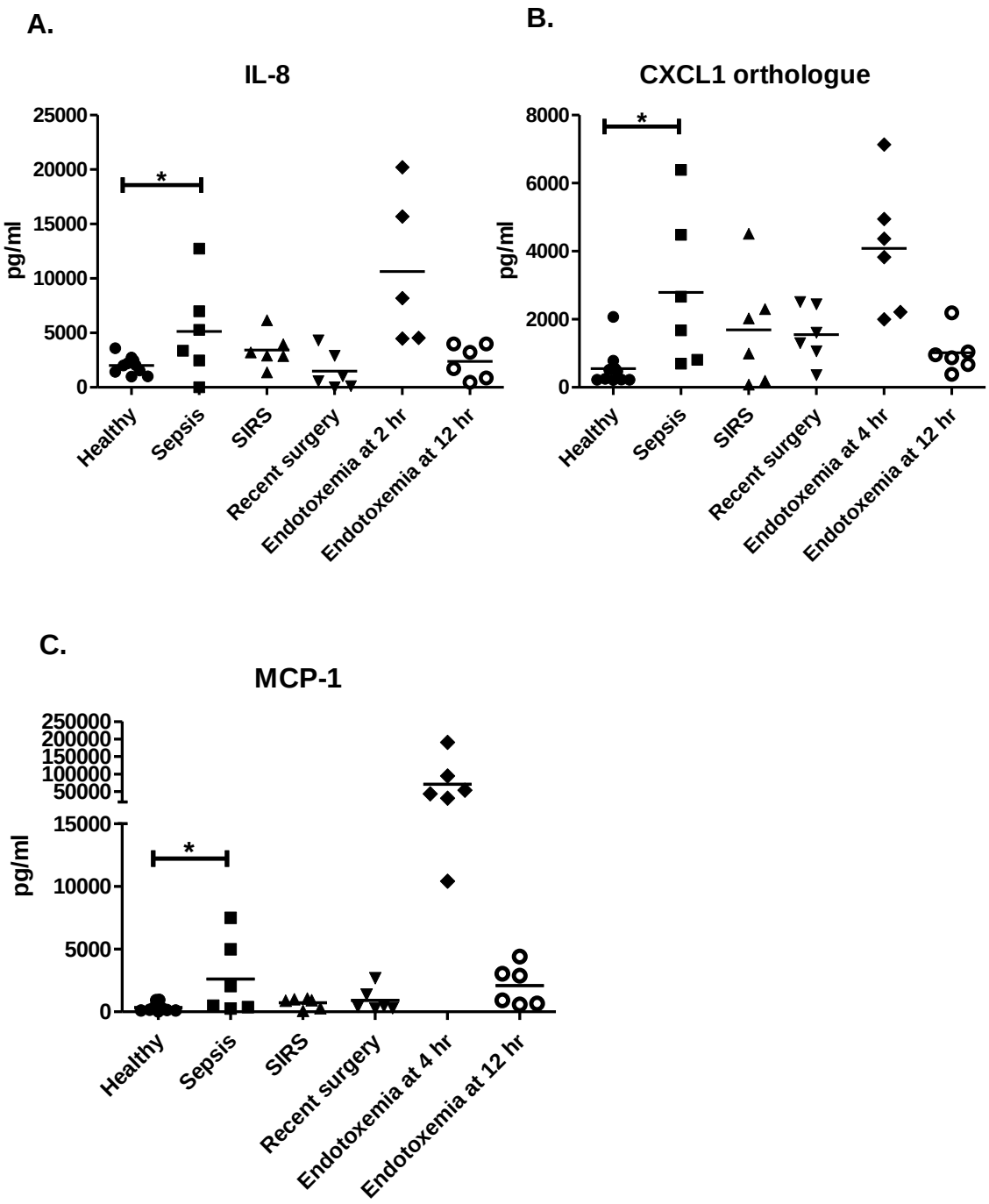

GM-CSF
250
200
150
100
50
0
pg/ml
0h
2h
4h
6h
8h
10h
12h
24h
48h
72h
Time (post-LPS administration)

Figure 3. Cytokine concentrations in the serum of healthy dogs and dogs with sepsis, SIRS or recent surgery. The three cytokines which are consistently detected include (A) IL-8, (B) CXCL1 orthologue and (C) MCP-1. Each datum point represents the cytokine concentration for an individual dog, and horizontal bar through the points indicates the mean of the values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$


A.
IL-8
pg/ml
25000
20000
15000
10000
5000
0
*
Healthy
Sepsis
SIRS
Recent surgery
Endotoxemia at 2 hr
Endotoxemia at 12 hr
B.
CXCL1 orthologue
pg/ml
8000
6000
4000
2000
0
*
Healthy
Sepsis
SIRS
Recent surgery
Endotoxemia at 4 hr
Endotoxemia at 12 hr
C.
MCP-1
pg/ml
250000
200000
150000
100000
50000
15000
10000
5000
0
*
Healthy
Sepsis
SIRS
Recent surgery
Endotoxemia at 4 hr
Endotoxemia at 12 hr

Expression and Function of Triggering Receptor Expressed on Myeloid Cells -1 (TREM-1) on Canine Neutrophils

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Abstract

The dog is both a valued veterinary species and a widely used translational model for sepsis research. However, relatively little work has been performed evaluating potential biomarkers present during canine infection. Triggering receptor expressed on myeloid cells-1 (TREM-1) has shown promise as a biomarker for infection and pneumonia in humans. Here we describe, for the first time, the expression and function of the canine orthologue of TREM-1. Expression of TREM-1 on canine neutrophils is significantly up-regulated by stimulation with microbial agonists of TLR2/6, TLR1/2, and TLR4/MD2. Kinetics of TREM-1 protein up-regulation are rapid, with significant increases observed within 2 hr of neutrophil activation. Functionally, canine TREM-1 synergistically enhances LPS-induced production of IL-8, TNF-α and a canine orthologue of CXCL1. Collectively, these data suggest that TREM-1 expression in dogs, as it is in humans, is an amplifier of proinflammatory responses to microbial products. These results have direct application to veterinary diagnostics as well as the potential to enhance the utility of canine disease models in the assessment of potential therapeutics in the treatment of human sepsis.

Key words: TREM-1, TLR, neutrophils, sepsis, canine

1. Introduction

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a cell surface transmembrane receptor that has been characterized in humans and mice and is expressed on neutrophils, monocytes and macrophages (1). TREM-1 encodes a single extracellular immunoglobulin (Ig) domain of the variable (V) type and possesses a positively charged residue within its transmembrane domain permitting the partnering with and signaling through the adaptor protein DAP12 (2). TREM-1 is a molecule that is strongly associated with inflammatory lesions induced by bacterial or fungal organisms, but minimally associated with non-infectious inflammatory lesions, such as psoriasis, ulcerative colitis and vasculitis (3). Expression of TREM-1 increases after activation of toll-like receptors (TLRs), suggesting it is an innate immune system marker that is downstream of early pathogen recognition. Functionally, activation of TREM-1 leads to the amplification of proinflammatory cytokine production (1, 3).

Cytokine amplification is a key characteristic of sepsis, and a role for TREM-1 in the instigation of the "cytokine storm" during septicemia was considered after elucidation of its function. Early experiments found that blockade of TREM-1 signaling significantly reduced cytokine production and enhanced survival of mice in both lipopolysaccharide (LPS) and bacterial models of sepsis (3, 4). Importantly, these studies clarified that the beneficial effect of TREM-1 blockade was not limited to pre-treatment before onset of sepsis, but rather, was effective up to 4 hours after mice became symptomatic.

Two mechanisms have been postulated for the regulation of TREM-1 signaling *in vivo*: secretion of soluble TREM-1 via splice variant production at the mRNA level or proteolytic cleavage of existing protein from the cell surface (5). Mechanistically, this soluble TREM-1 is thought to dampen cytokine production by out-competing cell surface TREM-1 for engagement by an as of yet uncharacterized ligand, thereby inhibiting signaling through cell surface TREM-1 that amplifies cytokine production. This soluble TREM-1 is detected in the biological fluids of septic patients, suggesting it may have utility as an early biomarker of sepsis in clinically ill patients (6-9).

We report, for the first time, the identification of TREM-1 in the dog. We have identified and cloned a canine TREM-1 cDNA, produced recombinant canine TREM-1 protein, and demonstrated the expression of native TREM-1 protein on the surface of canine neutrophils. We show that TREM-1 expression is up-regulated on neutrophils after exposure to bacterial products known to activate TLRs and functions as an amplifier of cytokine production. From these observations, we hypothesize that TREM-1 may serve as a mediator of cytokine-based inflammation during canine sepsis, and thus serve as a specific biomarker of infection and inflammation in the dog.

2. Materials and Methods

2.1 Bioinformatics and phylogenetics

Sequence alignments were generated with ClustalW (10). The chromosomal locations of human, mouse and canine genes were defined with Entrez Gene (http://www.ncbi.nlm.nih.gov/gene). For phylogenetic analyses, variable (V) domains from members of the TREM and TREM-like families were aligned by ClustalW and neighbor-joining trees constructed from pairwise Poisson correction distances with 2000 bootstrap replications by MEGA4 software (11). TREM-1 sequences include: human (NP_061113), chimpanzee (XP_001174149), orangutan (Q5RDA5), macaque (XP_001082517), horse (XP_001501031), pig (NP_998921), rat (XP_217336), cow (NP_996853) and mouse (NP_067381). Other TREM family members include: human TREM-2 (NP_061838), mouse TREM-2 (NP_112544), mouse TREM-3 (NP_067382), human TREM-4 (NP_660316) and mouse TREM-4 (NP_848911). TREM-like family members include: human CMRF35L7/TREM-5 (NP_777552), mouse Cmrf35L2/TREM-5 (NP_742047), human TREML1 (NP_835468), mouse TREML1 (NP_082039), human TREML2 (NP_079083), mouse TREML2 (NP_001028577), human TREML4 (NP_937796), mouse TREML4 (NP_001157267), and human NCR2/NKp44 (NP_001186439).

2.2 Cloning canine TREM-1

RNA was isolated from canine whole blood using ZR Whole-Blood Total RNA Kit (Zymo Research, Irvine, CA). cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystem, Branchburg, NJ) according to the manufacturer's

recommendations. Primers for amplification of TREM-1 (5'-caccACAAGAAGGATGAGGAAG-3' and 5'-CAATTGCCAGAGGGATGTAG-3') were designed based on the putative canine sequence. The forward primer includes the predicted start codon (underlined), four nucleotides at the 5′ end (lower case) that are required for directional cloning into pET100/D-TOPO® vector (Invitrogen, Carlsbad, CA) and a mutation of T to A at position 8 to alter an endogenous stop codon upstream of the start codon (double underlined). The reverse primer was designed downstream of the predicted stop codon. pET100/D-TOPO® introduces an amino-terminal polyhistidine (6xHis) tag. The reaction conditions were: 1× PCR reaction buffer, 1.5 mM $MgSO_4$, 200 μM each dNTP, 0.5 μM of each primer and 25 U/ml of SuperTaq™ Plus Polymerase (Applied Biosystem, Carlsbad, CA). Thermal cycling conditions consisted of 40 cycles of: 95 ℃ for 20 sec, 57 ℃ for 20 sec, and 68 ℃ for 60 sec, with a final extension at 68 ℃ for 10 min. The PCR product was ligated into pET100/D-TOPO®, generating the pHis-cfTREM-1 plasmid, and sequenced.

2.3 Expression and detection of His-cfTREM-1

The pHis-cfTREM1 plasmid was transformed into BL21 Star™ cells (Invitrogen, Carlsbad, CA) and expression of recombinant His-cfTREM-1 induced with 1 mM IPTG. Cells were lysed in 5× reducing sample buffer (Pierce, Rockford, IL). After centrifugation, the cell lysate was subjected to SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel; Invitrogen, Carlsbad, CA) and transferred to PVDF membranes (Millipore, Billerica, MA). After blocking (Superblock blocking buffer, Pierce), membranes were incubated with an anti-His antibody as described by the vendor (Roche, Basel, Switzerland) or with various commercially

available anti-TREM-1 primary antibodies diluted 1:200 in 3% BSA-PBST for 1 hr at room temperature. After five consecutive five minute washes, the membrane was incubated in HRP-labeled secondary antibodies against the species used in primary antibodies for 1 hr. Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) was used for signal detection. Recombinant human TREM-1-GST (Abnova, Walnut, CA) was used as a positive control for Western analyses of anti-TREM-1 antibodies.

2.4 Expression and detection of FLAG-cfTREM-1

A eukaryotic expression vector encoding FLAG-tagged canine TREM-1 was generated. The coding sequence of canine TREM-1 (excluding the peptide leader sequence) was amplified from pHis-k9TREM-1 using a blunt forward primer (5′-GAACCAGATGAAATAAAGTATGTCTTAGCAGAG-3′), a reverse primer that introduces a 3′ *Not*I restriction site (5′- GCATGCGGCCGCTCATGGCTTCACCAGCCTAGG-3′) and Pfu DNA polymerase (Stratagene, La Jolla, CA). This amplicon was digested with *Not*I and ligated into the *Eco*RV/*Not*I sites of the plasmid pLF, that introduces an amino terminal peptide leader sequence and FLAG tag (12). The leader-FLAG-k9TREM-1 sequence expression cassette was then excised with *Eco*RI/*Xho*I and shuttled into the *Eco*RI/*Sal*I restriction sites of pIRES2-EGFP (Clontech, Mountain View, CA). The pFLAG-k9TREM-1-IRES2-EGFP plasmid was transfected into HEK 293T cells for dual expression of the FLAG-tagged TREM-1 and EGFP. pIRES2-EGFP expressing an irrelevant protein (zebrafish immune-related lectin-like receptor 3, illr3) was employed as a negative control. Transfected cells were harvested, incubated with allophycocyanin labeled TREM-26 (anti-TREM-1) mAb

and analyzed on a LSR II flow cytometer (BD Biosciences, Mountain View, CA) using FACSDiva software (BD Biosciences, Mountain View, CA).

2.5 Blood samples

All experiments involving dogs were performed in accordance with relevant institutional and national guidelines and regulations, and were approved by the North Carolina State University Institutional Animal Care and Use Committee. Peripheral blood samples were collected from healthy dogs via jugular venipuncture into vacutainer™ tubes containing ACD (Becton-Dickinson, Franklin Lakes, NJ). De-identified human blood units were acquired from the American Red Cross (Durham, NC) in accordance with the NCSU Institutional Review Board (IRB) standards.

2.6 Neutrophil preparation, processing and flow cytometry

Canine peripheral blood samples were collected as described above. Whole blood was centrifuged over Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) at 400 ×g for 25 min and neutrophils were harvested from the bottom of histopaque gradient tubes. Erythrocytes were lysed with ammonium chloride lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM Na_2EDTA) and cells were washed twice in endotoxin-free PBS (Gibco, Carlsbad, CA). The purity of neutrophils was determined by microscopic examination of stained cytospin preparations and flow cytometry using a monoclonal antibody specifically against canine neutrophils (VMRD, Inc., catalog number CADO48A, Pullman, WA). Human neutrophils were isolated in a similar manner with two modifications; diluted blood was underlayed with

endotoxin-tested Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) and red blood cells were lysed using water as the hypotonic solution. Purity of isolated neutrophils is described in Supplememtal Fig. S1.

Freshly isolated canine neutrophils were seeded at a density of 1×10^6 cells/well in 12 well plates and cultured in RPMI 1640 supplemented with endotoxin-tested 10% fetal bovine serum (Gibco, Carlsbad, CA), 1 mM sodium pyruvate, 15 mM HEPES, 55 μM 2-mercaptoethanol, 10 IU/ml penicillin and 10 μg/ml streptomycin for 1 hr. After an initial 1 hr rest period, cells from each dog were treated with five different TLR agonists or medium alone in the presence of 30 pg/ml recombinant canine granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) to enhance neutrophil survival. TLR agonists were optimized for use (data not shown) at the following concentrations: 50 ng/ml Pam_2CSK_4 (TLR2/6) (InvivoGen, catalog number: tlrl-pam2, Carlsbad, CA), 300 ng/ml Pam_3CSK_4 (TLR1/2) (InvivoGen, catalog number: tlrl-pms, Carlsbad, CA), 30 ng/ml crudely purified LPS from *E.Coli* O111:B4 (contains agonists for TLR2,TLR4,TLR9, as described in (13) (Sigma-Aldrich, catalog number: L3024, St. Louis, MO) , 30 ng/ml ultra-pure LPS from *E.Coli* O111:B4 (TLR4) (InvivoGen, catalog number: tlrl-pelps, Carlsbad, CA), and 30ng/ml flagellin from *Salmonella typhimurium* (TLR5) (InvivoGen, catalog number: tlrl-pstfla, Carlsbad, CA). Cells were harvested at 1, 2, 4 and 6 hr post-exposure to TLR agonists.

Surface expression of TREM-1 on freshly isolated human and canine neutrophils was determined by flow cytometric analysis. Cells were incubated with biotinylated TREM-26 (anti-TREM-1) monoclonal antibody in 1× PBS containing 10% FBS on ice for 30 min. After incubation, cells were washed with PBS and incubated with Strepavidin-PerCP (Biolegend, San Diego, CA) for 15 min on ice, washed twice, and fixed with 4% paraformaldehyde. TREM-1 expression was analyzed on a LSR II flow cytometer (BD Biosciences, Mountain View, CA) using FACSDiva software (BD Biosciences, Mountain View, CA).

2.7 Cross-linking of canine TREM-1 and measurement of cytokines and chemokines

96-well round-bottom plates were coated with goat-anti-mouse IgG-F(ab')$_2$ antibody in PBS (5 μg/ml, Novus Biologicals, Littleton, CO) overnight, followed by LEAF^{TM} TREM-26 (anti-TREM-1 monoclonal antibody, 5 μg/ml, Biolegend) or anti-human-CD25 monoclonal antibody BC96 (5 μg/ml, Biolegend) as an isotype control. Freshly purified canine neutrophils were rested for 1 hr and plated at a concentration of 2×10^5 cells/well. Ultra pure LPS from *E.Coli* O111:B4 (InvivoGen, catalog number: tlrl-pelps, Carlsbad, CA) was added to wells at a concentration of 1 ng/ml, 10 ng/ml or 100 ng/ml when cells were plated. After 18 hr, cell culture supernatants were harvested and evaluated for the production of IL-8, monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), Tumor Necrosis Factor-α (TNF-α) and a canine orthologue of Chemokine (C-X-C motif) ligand 1 (CXCL1) by Milliplex Map canine cytokine/chemokine kit (Millipore, Billerica, MA) per manufacturer's instructions. The dynamic range (upper and

lower limits of detection in pg/ml) for cytokines were: IL-8 (52282.85, 77.551), CXCL1 orthologue (2000.968, 3.126), TNF-α (7099.934, 3.220), GM-CSF (10,351.528, 3.093) and MCP-1 (2012.860, 14.215) respectively.

2.8 Statistics

Data were analyzed by two-way ANOVA followed by Duncan's post test using the General Linear Models Procedure of SAS (SAS, Cary, NC). In cases where cytokine concentrations were below the assay Lower Limit of Detection (LLOD), values were assigned that were one half of the LLOD (defined above) for the purpose of statistical analysis (14-19). The level of significance used was $p \leq 0.05$. Error bars represent the SEM.

3. Results and Discussion

3.1 Canine TREM-1

Electronic searches (tBLASTn) of the dog genome with human TREM-1 (Genbank NM_018643) as a query identified a V domain on chromosome 12 as a candidate TREM-1 sequence that corresponded to the predicted canine TREM-1 gene (LOC608994). A comparison of the sequence encoded by LOC608994 to human and mouse TREM-1 sequences indicated that the peptide leader sequence encoded by LOC608994 was not well conserved with other mammalian TREM-1 sequences. Examination of the genomic sequence upstream of the V domain of the putative canine TREM-1 revealed an alternative peptide leader sequence within LOC608994, but out of frame with its sequence. Further examination of this genomic region indicated that an intron (between the peptide leader sequence and V domain) was inadvertently included in LOC608994. Using primers that spanned the alternative start codon and the stop codon of canine TREM-1, the complete coding sequence was amplified from peripheral blood cDNA and cloned. Sequence analyses of this cDNA revealed that LOC608994 does include an aberrant intron. The presence of an in-frame stop codon upstream of the start codon of the canine TREM-1 transcript suggests that this is indeed the translational start site. On the amino acid level, canine TREM-1 is 56% identical and 68% similar to human TREM-1. As observed in other mammals, canine TREM-1 encodes a single extracellular V domain and includes a positively charged residue (lysine) within the transmembrane domain (Figure 1). In other species, this charged lysine permits the association of TREM-1 with the signaling adaptor protein DAP12 (20), and it is predicted that canine TREM-1 also signals through this adaptor. In human and mouse, TREM-1 is one

of multiple V domain receptors encoded by TREM and TREM-like multi-gene families on human chromosome 6p21 and mouse chromosome 17. Canine TREM-1 is also encoded within a similar gene cluster on chromosome 12 and in all three species, the TREM/TREM-like gene clusters are flanked by the *FOXP4* and *NFYA* genes suggesting conserved synteny. In order to confirm that canine TREM-1 is indeed orthologous to human and mouse TREM-1, a phylogenetic comparison of the V domain from canine TREM-1 was compared to V domains from human and mouse TREM and TREM-like proteins. As seen in Figure 2 the sequence identified here belongs to the TREM, and not to the TREM-like, family and shares the most similarity to TREM-1. These observations support the classification of this sequence as canine TREM-1.

3.2 Validation of a cross-reactive anti-TREM-1 monoclonal antibody

Canine TREM-1 was expressed in *Escherichia coli* as a His-fusion protein. Western-blot analysis with anti-His antibody revealed that recombinant canine TREM-1 had an approximate MW of 29 kDa, close to the predicted MW of 31.8 kDa (Figure 3A). In order to identify an antibody that recognizes canine TREM-1, eight anti-TREM-1 monoclonal antibodies (Supplementary data, Table S1) were screened for cross-reactivity with recombinant canine TREM-1 by Western blot. One monoclonal, clone TREM-26, recognized both recombinant human TREM-1 and recombinant canine TREM-1 (Figure 3B). When analyzed by flow cytometry, monoclonal antibody TREM-26 successfully recognized recombinant canine TREM-1 on transfected HEK 293T cells (Figure 3C), validating the use of this monoclonal antibody for analysis of cell surface canine TREM-1.

3.3 TREM-1 is up-regulated on canine neutrophils after stimulation with TLR agonists

TREM-1 is constitutively expressed on human monocytes/macrophages and neutrophils and is up-regulated by various stimuli such as the TLR ligands LPS and lipoteichoic acid (LTA) (1, 3). Using monoclonal antibody TREM-26, we compared expression of TREM-1 on freshly isolated canine and human neutrophils. Approximately 90% of human and canine neutrophils constitutively express TREM-1, suggesting a similar expression and membrane localization in both species (Figure 4).

In order to evaluate TLR agonist induced up-regulation of canine TREM-1, we first had to determine the lifespan of neutrophils in vitro. In vivo, neutrophils have a relatively short lifespan, circulating for 12-14 hours before undergoing apoptosis (21). Therefore, we examined the in vitro lifespan of canine neutrophils using Annexin V staining as an indicator of apoptosis, and determined whether the addition of recombinant canine GM-CSF enhanced their viability. After six hr of culture, approximately 35 % of neutrophils were Annexin V^+, and thus entering early apoptosis (Supplementary data, Figure S2). In contrast, the addition of recombinant canine GM-CSF reduced the number of early apoptotic cells to approximately 6%. GM-CSF was shown not to alter TREM-1 expression (data not shown). Therefore, all subsequent cultures were performed with the addition of GM-CSF to maintain neutrophil viability.

As canine neutrophils express TLR2, TLR4 and TLR5 (22), we hypothesized that agonists for these TLRs would modulate TREM-1 expression on canine neutrophils. TREM-1

expression on canine neutrophils was evaluated in the presence or absence of diacylated lipopeptide (Pam_2CSK_4, a TLR2/6 agonist), triacylated lipopeptide (Pam_3CSK_4, a TLR1/2 agonist), ultra pure LPS (a TLR4/MD2 agonist), crudely purified LPS (containing TLR2, TLR4 and TLR9 agonists) (13) and flagellin (a TLR5 agonist) (23) over a 6 hr period. Treatment with the lipopeptides and LPS resulted in a slight, albeit insignificant, increase in the percentage of cells expressing TREM-1 (Figure 5A & B). However, the relative density of TREM-1 on the surface of neutrophils, as indicated by mean fluorescence intensity (MFI), increased significantly within two hr of exposure to Pam_2CSK_4 and Pam_3CSK_4 (Figure 5C) and within 4 hr of exposure to both ultra-pure and crudely purified LPS (Figure 5D) ($p<0.05$). TREM-1 surface expression was not observed to change after exposure to flagellin (Supplementary data, Figure S3).

The observed LPS- and lipopeptide-induced up-regulation of TREM-1 protein on canine neutrophils suggests that, in the dog, TREM-1 expression is coupled to anti-bacterial responses as it is in humans. Importantly, the kinetics of TREM-1 up-regulation on canine neutrophils is rapid, occurring within two to four hr of exposure to TLR agonists, suggesting that TREM-1 is linked to early anti-bacterial responses.

Expression of TLR5 has been reported on both human and canine neutrophils (22, 24). Administration of flagellin to mice leads to an increase in serum levels of soluble TREM-1, suggesting TLR5 activation *in vivo* is capable of altering TREM-1 expression (25). *In vitro*, treatment of human neutrophils with flagellin enhances cell survival and prevents

spontaneous apoptosis (26). In contrast, in our *in vitro* system canine neutrophils treated with flagellin had the same rate of spontaneous apoptosis as untreated cells (data not shown). Our results may therefore 1) be an artifact of *in vitro* activation, 2) suggest activation of TLR5 on canine neutrophils is multi-factorial, requiring additional factors such as binding proteins or additional forms of activation, or 3) may result in soluble TREM-1 which is not measurable by flow cytometry. Although we confirmed that the flagellin used in these studies is biologically active and can activate NF-κB in human HEK293-TLR5^{+} cells (Supplemental data, Figure S4), we cannot rule out that the lack of flagellin-induced TREM-1 expression may be a reagent-specific effect. Indeed, flagellin from some microbial sources, such as *Campylobacter jejuni*, *Helicobacter pylori*, and *Bartonella bacilliformis*, is not recognized by human TLR5 (27). Likewise, it is possible that flagellin may vary in its interaction with canine TLR5 based on the microbial source.

3.4 Cross-linking of canine TREM-1 up-regulates proinflammatory cytokines and chemokines

Cross-linking of TREM-1 on human neutrophils synergistically enhances LPS-triggered IL-8 secretion and myeloperoxidase release, suggesting that TREM-1 signaling amplifies proinflammatory responses to microbial products (1). Thus, we next sought to determine whether canine TREM-1 was functionally equivalent to its human and murine orthologues. To this end, TREM-1 on canine neutrophils was cross-linked by plate-bound antibody in the presence or absence of crudely purified LPS, and proinflammatory cytokine secretion was evaluated. Cross-linking TREM-1 in the absence of LPS resulted in modest secretion of IL-8

and a canine orthologue of CXCL1 (Figure 6A and 6B). Marked synergistic up-regulation of IL-8, TNF-α and the CXCL1 orthologue was observed with concentrations of LPS that are suboptimal (1 & 10 ng/ml) and optimal (100 ng) for induction of cytokine secretion (Figure 6A-C). In contrast, culturing cells in medium alone or in the presence of a plate-bound isotype control had minimal or no effect on cytokine/chemokine secretion. The observed synergistic enhancement of proinflammatory cytokine production suggests that canine TREM-1 functions as an amplifier of inflammatory cytokines during infection in a manner analogous to that observed in humans and mice (1). Interestingly, we did not detect GM-CSF secretion after TREM-1 cross-linking and/or LPS treatment (data not shown). Our observation is supported by Smedman et al., who recently reported that highly purified preparations of human neutrophils fail to produce and secrete GM-CSF (28). Their data and ours suggest that previous reports of neutrophil-derived GM-CSF (29) may be the result of monocyte contamination.

MCP-1 secretion was observed to be TREM-1-independent (Figure 6D). MCP-1 is an early and potent regulator of monocyte migration and a potent proinflammatory mediator. MCP-1 production is regulated cooperatively by the nuclear transcription factors NF-κB and AP-1 in human endothelial cells (30). Although the transcription factors involved in MCP-1 production in canine neutrophils have yet to be described, it appears that LPS alone, even at suboptimal concentrations, is able to stimulate canine neutrophils to secrete MCP-1.
The functional role of TREM-1 as an amplifier of cytokine production makes this molecule a potential key regulator of the ‘cytokine storm’ associated with sepsis. Clinically, the

specificity of TREM-1 up-regulation as a single diagnostic indicator of bacterial infection is questionable, in that several reports in humans suggest it can be found in its soluble form in serum of patients with conditions unrelated to bacterial infection (31, 32). In veterinary species, tissue expression of TREM-1 must be established in order to define the role of TREM-1 in the pathology of disease. It is highly likely that similar to humans, TREM-1 may have utility when used as part of a panel of multiple diagnostic biomarkers for bacterial sepsis. Towards this end, research is ongoing in our laboratory to develop an ELISA to measure soluble canine TREM-1 in the serum of systemic inflammatory response syndrome (SIRS) and sepsis patients.

3.5 Summary

The data presented herein are the first to characterize the expression of canine TREM-1 expression and function. These results show promise for the utility of TREM-1 as a biomarker and potential therapeutic target in canine patients suffering from uncontrolled inflammation. As the clinical manifestations and treatments for naturally occurring canine sepsis mirror those in human patients, dogs with naturally occurring sepsis represent an opportunity for diagnostic and therapeutic comparative and translational research. Future studies evaluating the *in vivo* expression and function of TREM-1 in dogs are clearly indicated.

Conflict of interest

The authors declare that they have no conflicts of interest

Acknowledgement

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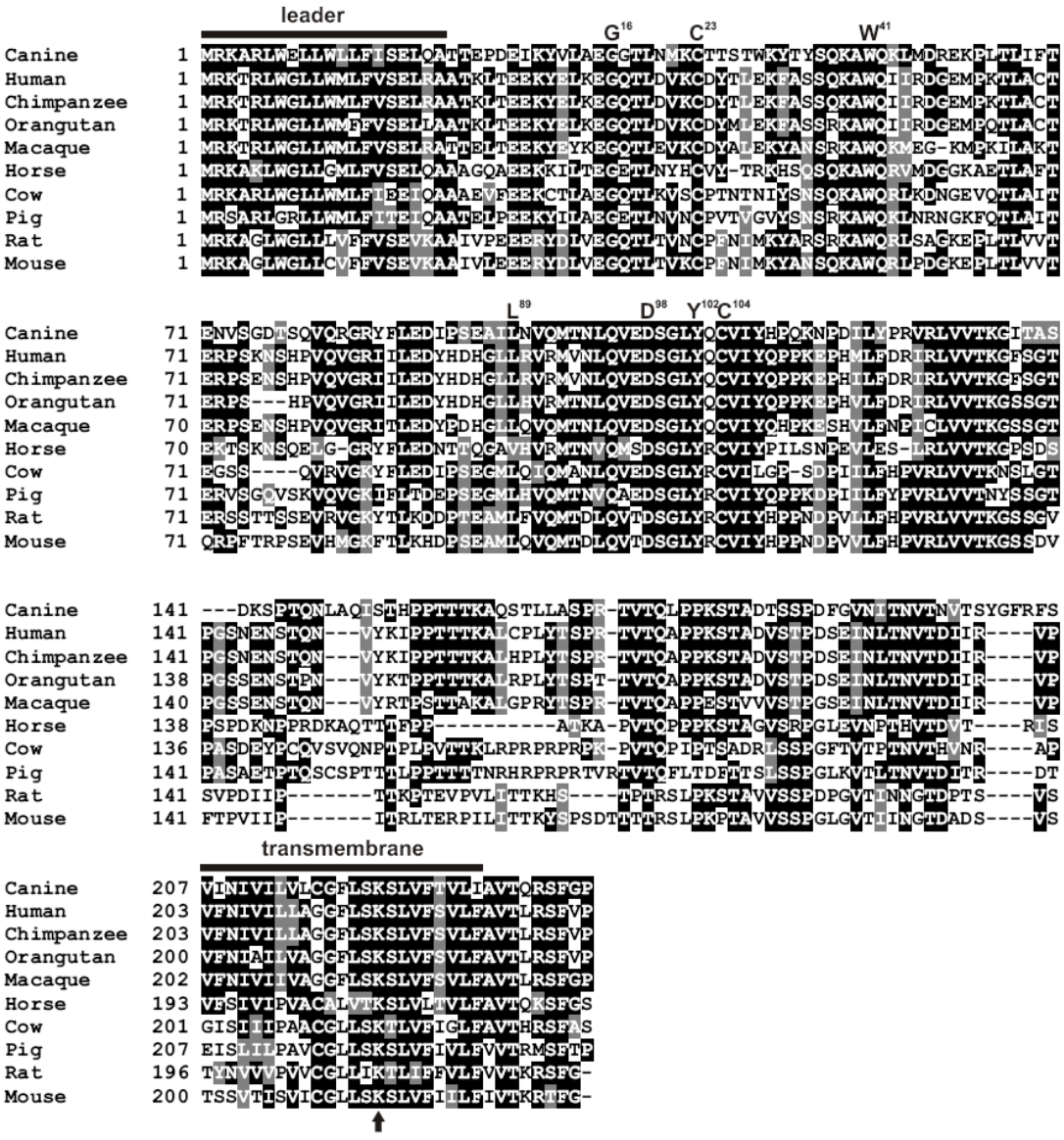
Figure 1. Protein alignment of canine TREM-1 with other mammalian TREM-1 amino acid sequences in Genbank. Identities (exact match) are shaded black, and similarities are shaded grey. The location of the peptide leader sequence and transmembrane domains are indicated. The conserved lysine required for association with DAP12 is indicated with an arrow. Conserved residues indicative of a V domain are shown above the alignment (IMGT numbering) (33).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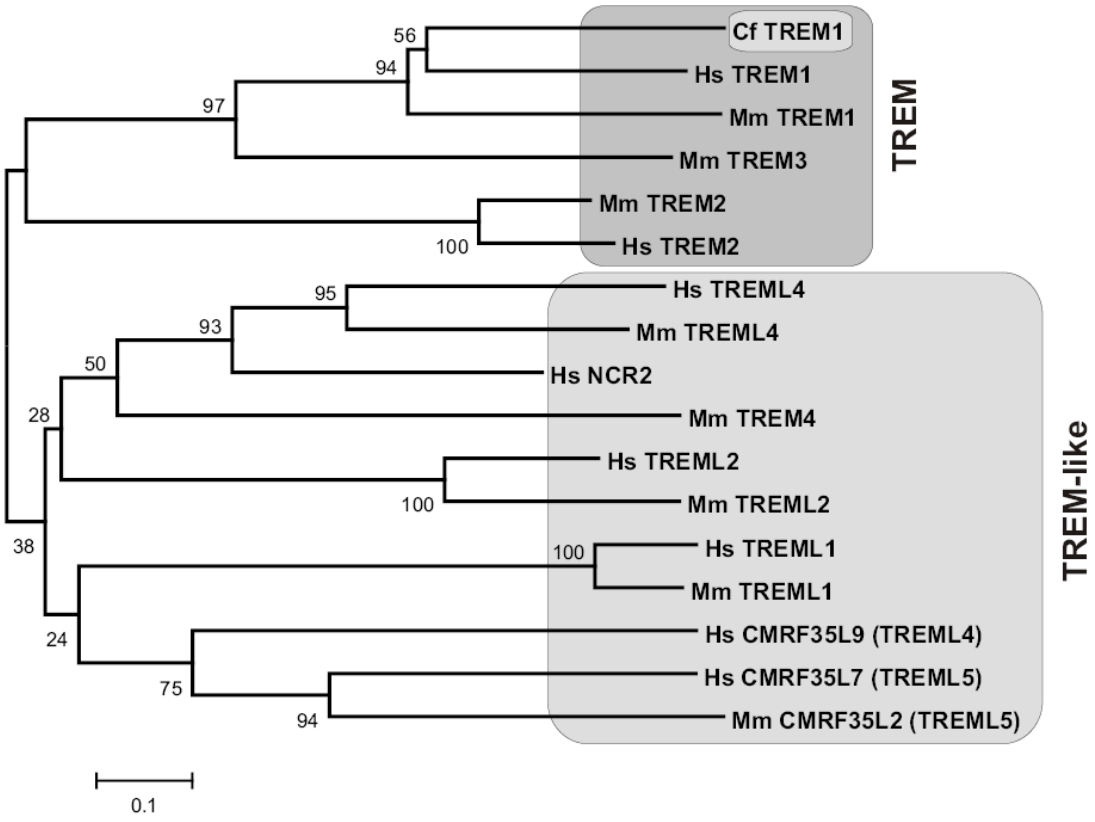
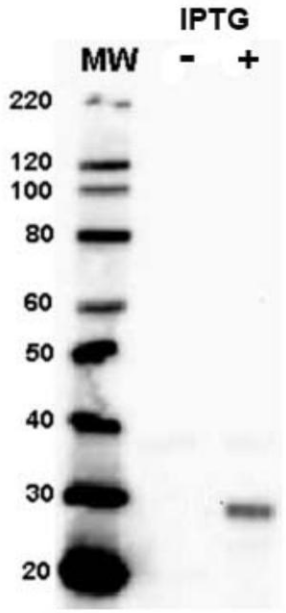


Figure 2. Phylogenetic comparison of the predicted canine TREM-1 protein to members of the TREM and TREM-like families. Neighbor-joining trees were constructed with 2000 bootstrap replication using MEGA4 software (11). Branch lengths are measured in terms of amino acid substitutions, with the scale indicated below the tree.

Figure 3. The anti-human TREM-1 monoclonal antibody TREM-26 binds canine TREM-1 in Western and flow cytometric analyses. (A) His-cfTREM-1 expression was induced in transformed bacteria (IPTG +) and detected by Western analysis with an anti-His antibody. A His-fusion protein of 29 kD, close to the approximated MW of 31 kD, was identified. Lysates from uninduced bacteria were included as a negative control (IPTG -). (B) Recombinant human TREM-1-GST and bacterial lysates expressing canine TREM-1-His were subjected to Western analyses with detection by monoclonal clone TREM-26. The expected MW of recombinant human TREM-1-GST is 50 kD. (C) Recombinant canine TREM-1 was expressed on HEK 293T cells and detected by flow cytometry. The plasmid pFLAG-k9TREM-1-IRES2-EGFP was transfected into HEK 293T cells for dual expression of k9TREM-1 and EGFP (EGFP+ cell were expected to be TREM-1+). Cells transfected with pIRES2-EGFP encoding an irrelevant surface protein were not bound by mAb TREM-26 (left). Cells transfected with pFLAG-k9TREM-1-IRES2-EGFP were dually positive for EGFP and TREM-1 surface expression (right).

A.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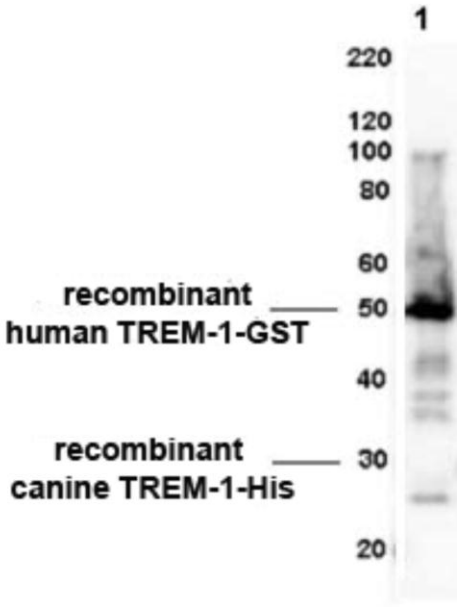
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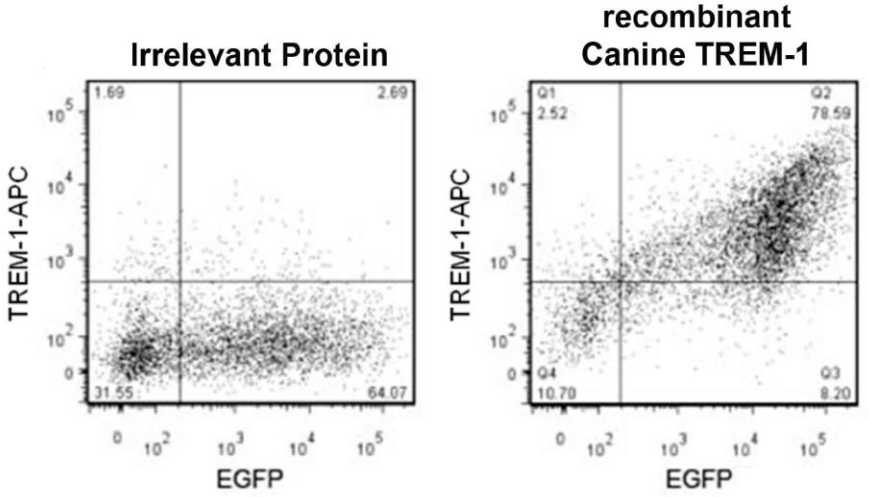
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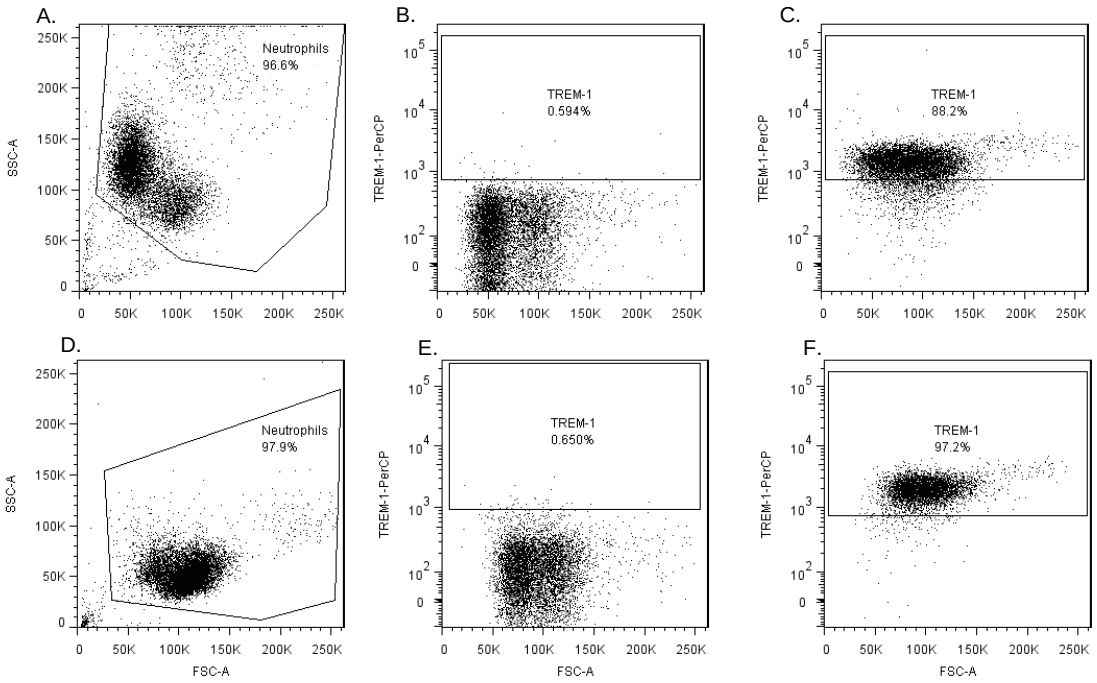


Figure 4. TREM-1 is constitutively expressed on the surface of human and canine neutrophils. Representative flow cytometric analysis of TREM-1 expression on canine (A-C) and human (D-F) neutrophils. Forward and side scatter characteristics of purified canine (A) and human (D) neutrophils. Neutrophils were labeled with isotype control antibody (B, E) or TREM-26 (C, F).

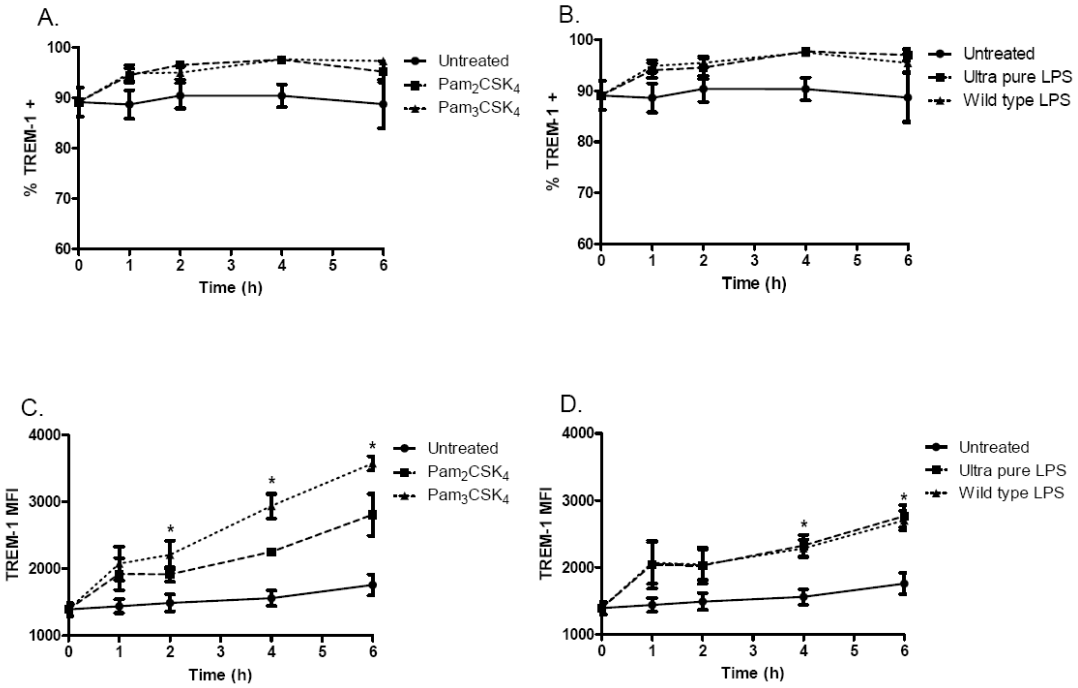
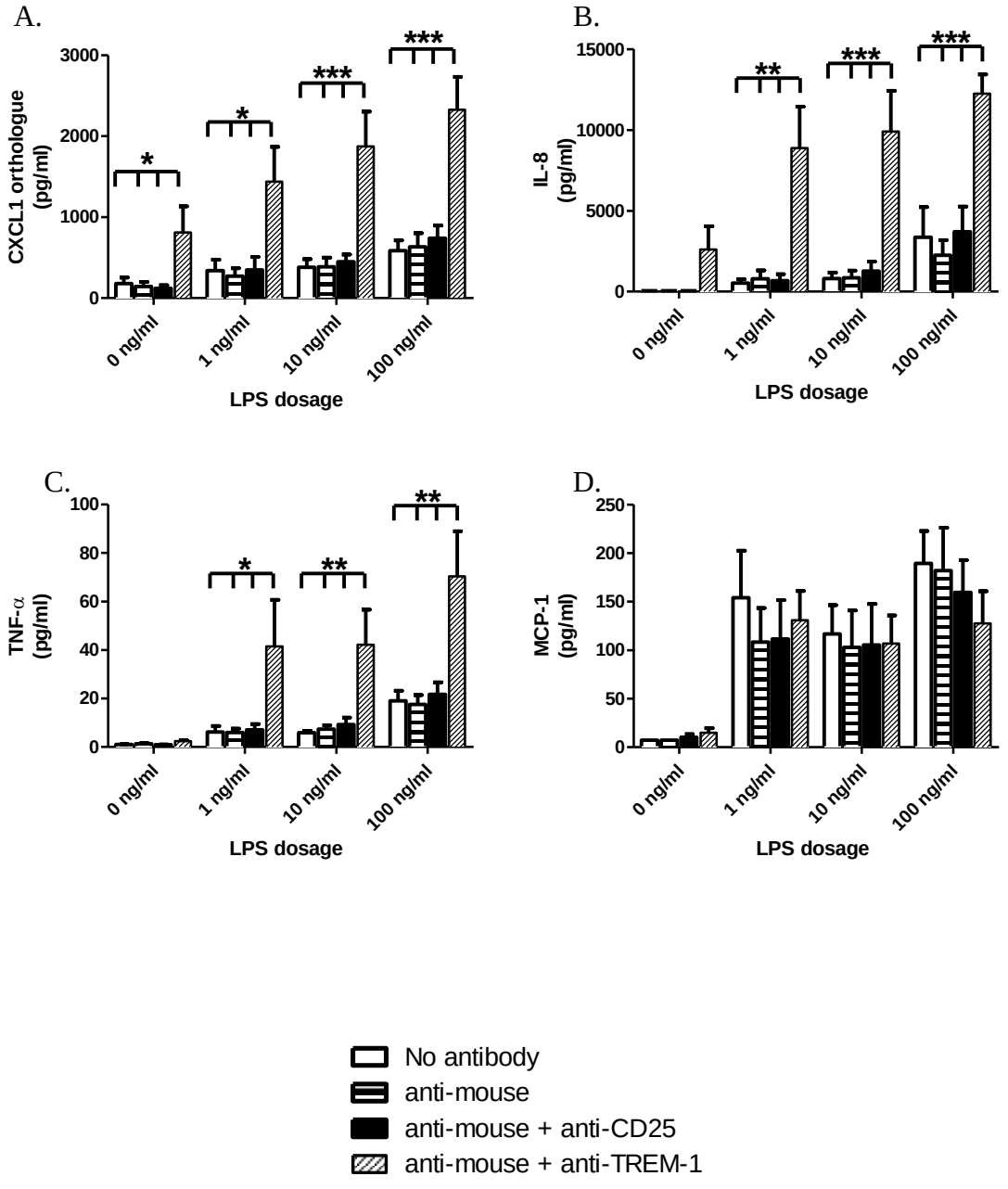


Figure 5. TREM-1 expression is up-regulated after stimulation with Pam2CSK4, Pam3CSK4, ultra pure LPS or crudely purified LPS. The percentage of TREM-1 positive neutrophils was slightly increased after (A) Pam2CSK4, Pam3CSK4, (B) ultra pure LPS or crudely purified LPS stimulation. The relative density of TREM-1 expressed on the surface of neutrophils (MFI) was significantly up-regulated within 2 hr after (C) Pam2CSK4 or Pam3CSK4 stimulation and (D) within 4 hr after ultra pure LPS or crudely purified LPS stimulation. *, $p<0.05$. n = 5 dogs.

Figure 6. Canine TREM-1 amplifies cytokine/chemokine secretion. Cross-linking of TREM-1 synergistically enhances LPS-induced production of (A) a canine CXCL1 orthologue, (B) IL-8 and (C) TNF-α. TREM-1 ligation did not have significant effect on (D) MCP-1 secretion. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. n = 5 dogs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LPS dosage
C.
TNF-α (pg/ml)
100
80
60
40
20
0
*
**
**
0 ng/ml
1 ng/ml
10 ng/ml
100 ng/ml
LPS dosage
D.
MCP-1 (pg/ml)
250
200
150
100
50
0
0 ng/ml
1 ng/ml
10 ng/ml
100 ng/ml
LPS dosage
No antibody
anti-mouse
anti-mouse + anti-CD25
anti-mouse + anti-TREM-1

Supplemental Information

Expression and function of triggering receptor expressed on myeloid cells - 1 (TREM-1) on canine neutrophils

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Table S1. Cross-reactivity[1] of commercially available anti-human TREM-1 antibodies with recombinant canine TREM-1.

Company	Location	Clone	Reactivity with human TREM-1	Reactivity with canine TREM-1
Abcam	Cambridge, MA	44CT	Yes	No
Biolegend	San Diego, CA	TREM-37	Yes	No
Biolegend	San Diego, CA	TREM-26	**Yes**	**Yes**
Gene Tex	Irvine, CA92606	2E2	No	No
Gene Tex	Irvine, CA92606	3F5	Yes	No
Novus	Littleton, CO	3F5	Yes	No
R&D Systems	Minneapolis, MN	Unreported	No	No
Santa Crus Biotechnology	Santa Crus, CA	Unreported	No	No

[1] Reactivity defined by Western analyses

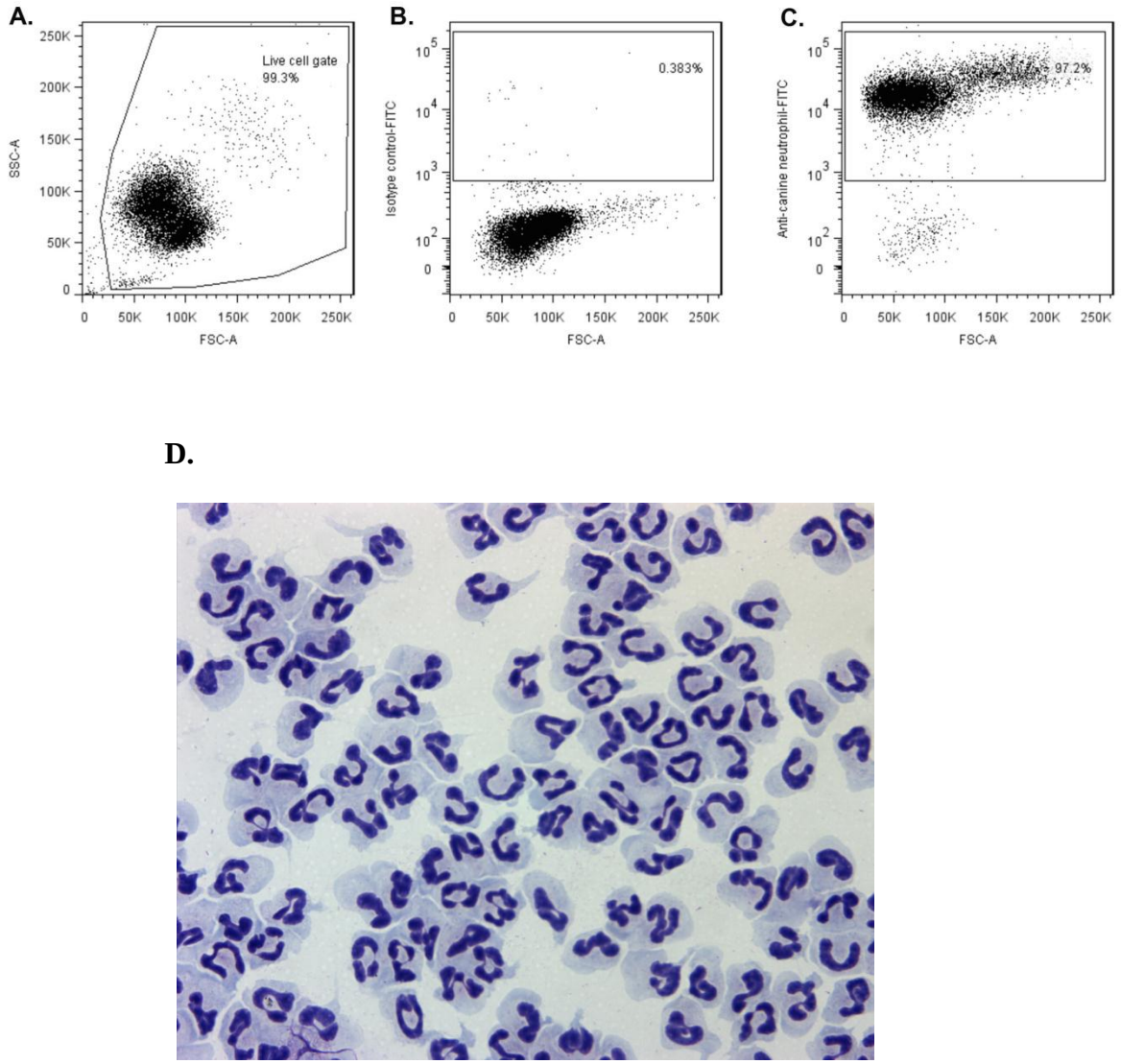

Figure S1: Purity of isolated canine neutrophils. Neutrophils were isolated as described in the materials and methods. (A) Post-isolation neutrophils Forward and Side Scatter. Neutrophils were stained with (B) Isotype control or (C) Anti-canine neutrophil antibody to determine purity. (D) Diff-Quick staining of isolated neutrophils, magnification 63×.

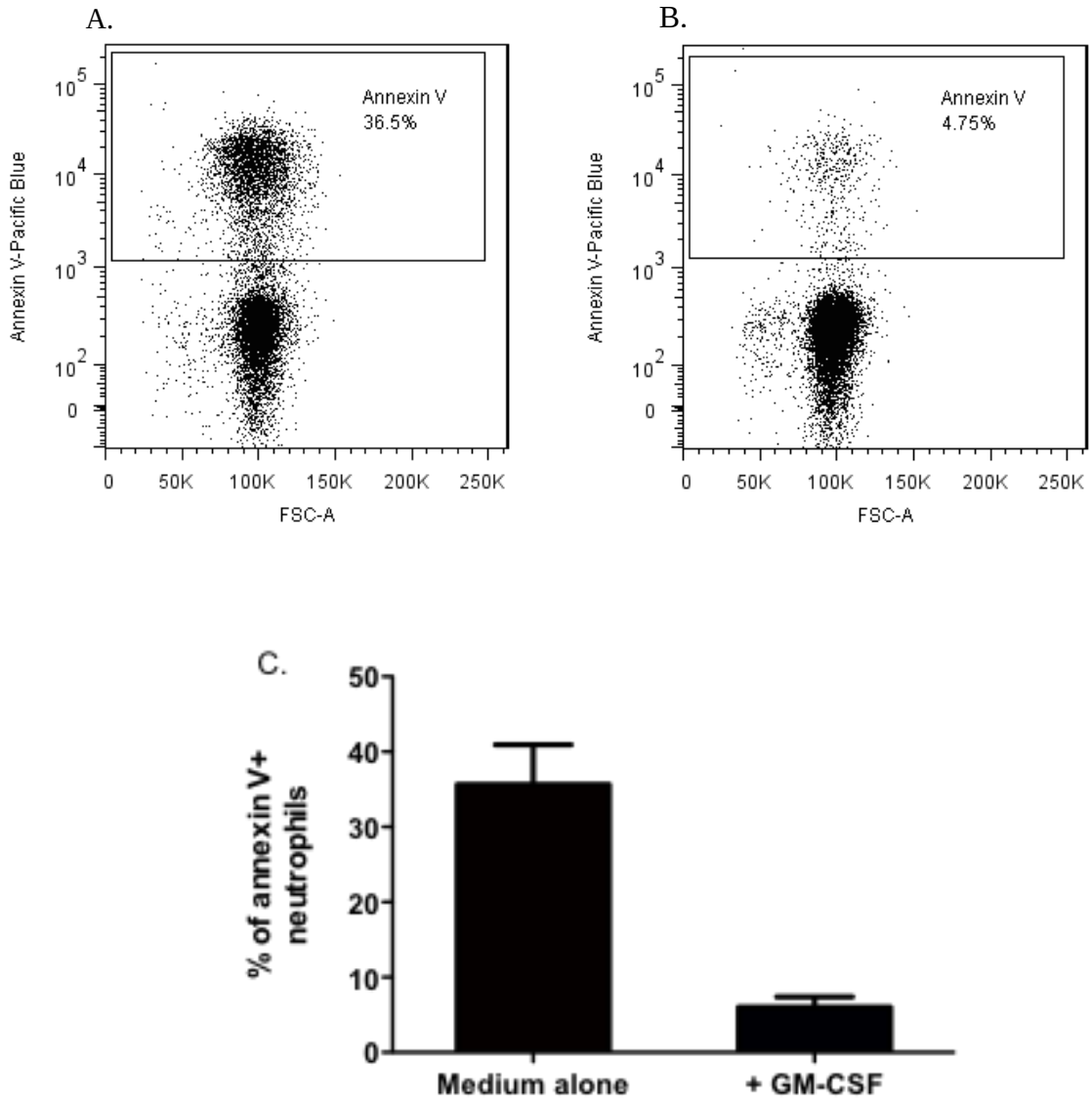

Figure. S2. GM-CSF enhances canine neutrophil viability *in vitro*. Canine neutrophils were cultured (A) without GM-CSF or (B) with 30 pg/ml GM-CSF for 6 hr. Cells were stained with Annexin V-Pacific Blue and analyzed by flow cytometry. (C) Culturing canine neutrophils with recombinant canine GM-CSF reduced the percentage of Annexin V positive cells from 35.6 ± 11.9% to 6.0 ± 3.1%, thus enhancing neutrophil viability. n= 5 dogs.

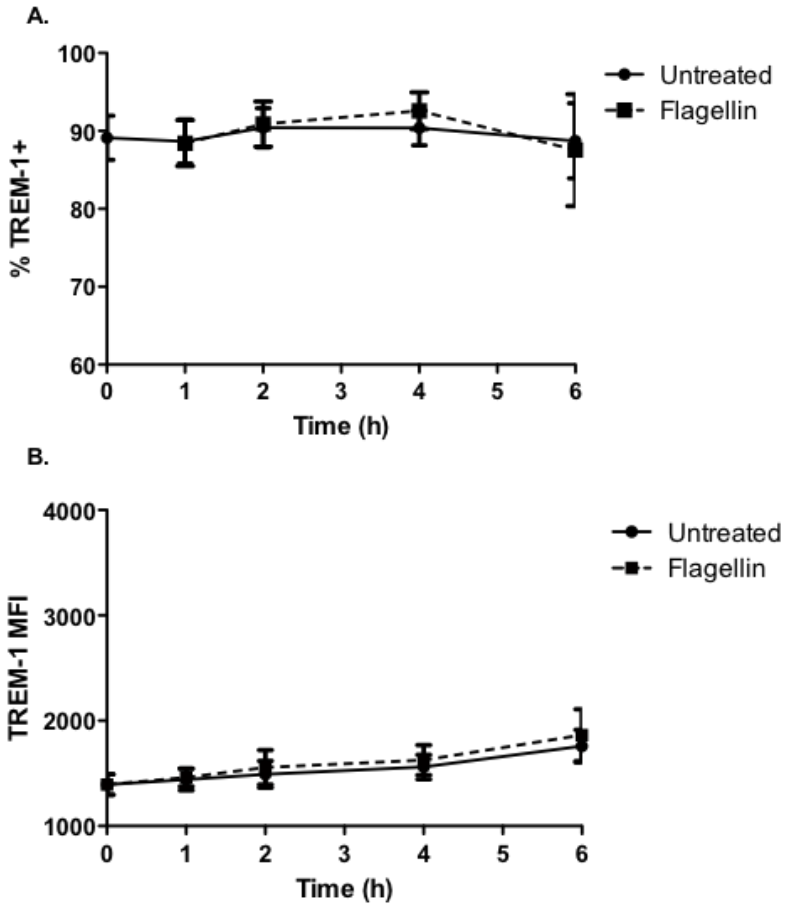

Figure. S3. Exposure of canine neutrophils to flagellin does not influence surface TREM-1 expression. Canine neutrophils were either (A) exposed to 30 ng/ml flagellin or (B) untreated for 6 hr. TREM-1 surface expression was assessed by flow cytometry with the anti-TREM-1 mAb, TREM-26. Flagellin exposure did not significantly up-regulate the percentage of TREM-1 positive cells or TREM-1 MFI. n= 5 dogs.

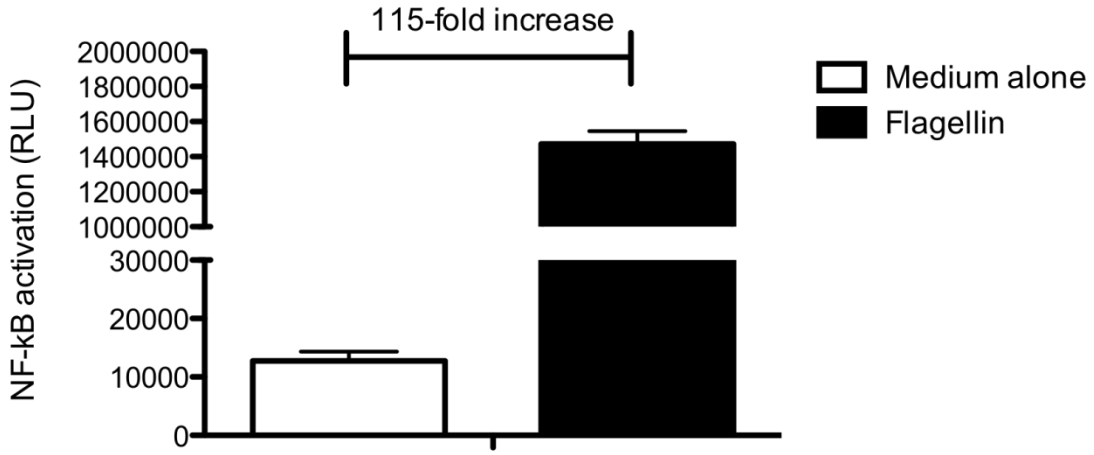

Figure S4: Ultrapure flagellin is biologically active. HEK293 cells over-expressing TLR5 were transfected with an NF-κB reporter and exposed to 30 ng/ml ultrapure flagellin from *Salmonella typhimurium.* Supernatant was harvested 24h after exposure to flagellin and NF-κB driven SEAP-reporter gene expression, as measured by relative light units (RLU), was determined to be 115±46-fold greater in flagellin-exposed HEK293 TLR5 cells in comparison to cell exposed to medium alone. n=4 separate experiments.

Supplemental Methods.

Validation of neutrophil purity

Neutrophils were stained with anti-human-CD25 monoclonal antibody BC96 as isotype control (Biolegend, San Diego, CA) or anti-canine neutrophil antibody (VMRD, catalog # CADO48A) as described in materials and methods. FITC-conjugated Goat anti-Mouse IgG1 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as secondary antibody.

GM-CSF treatment of neutrophils.

Freshly isolated canine neutrophils were seeded at a density of 1x 10^6 cells/well in 12 well plates and cultured in RPMI 1640 supplemented with endotoxin-tested 10% fetal bovine serum (Gibco, Carlsbad, CA), 1mM sodium pyruvate, 15mM HEPES, 55μM 2-mercaptoethanol, 10 IU/ml penicillin and 10μg/ml streptomycin for one hr. Cells were then cultured in the presence or absence of 30 pg/ml recombinant canine granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) for six hr. Cells were then incubated with AnnexinV-Pacific Blue per manufacturer's instructions and TREM-1 expression was analyzed on a LSR II flow cytometer (BD Biosciences, Mountain View, CA) using FACSDiva software (BD Biosciences, Mountain View, CA). GM-CSF was shown not to alter TREM-1 expression (data not shown).

Neutrophil exposure to flagellin.

Freshly isolated canine neutrophils were seeded at a density of 1x 10^6 cells/well in 12 well plates and cultured as described above. After the initial one hr rest period, cells were incubated in medium supplemented with 30 pg/ml GM-CSF in the presence or absence of ultrapure flagellin (TLR5 agonist) from *Salmonella typhimurium* (Invivogen, catalog number tlrl-pstfla, San Diego, CA). Cells were harvested at 1, 2, 4 and 6 h post-exposure to TLR agonists. TREM-1 surface expression determined by flow cytometry as described in the manuscript.

Validation of Flagellin bioactivity

HEK 293 cells over-expressing TLR5 were transfected with pNF-kB-NIFTY-SEAP (Invivogen) using Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN). Cells were exposed to 30 ng/ml ultrapure flagellin from *Salmonella typhimurium* (Invivogen, catalog number: tlrl-pstfla, Carlsbad, CA). Supernatant was harvested 24h after exposure to flagellin and NF-κB driven SEAP-reporter gene expression was assayed according to manufacturer's instructions (Chemiluminescent SEAP Reporter Gene Assay, Roche Applied Science, Indianapolis, IN). All data were normalized to an external GFP control.

MARCKS protein plays a critical role in proinflammatory cytokine production

Jingjing Li, Melissa A. D'Annibale, Adam J. Birkenheuer, Michael G. Levy, Jeffrey A. Yoder, Sam L. Jones, Kenneth B. Adler and Shila K. Nordone

Abstract

Myristoylated alanine-rich C kinase substrate (MARCKS) is a ubiquitously expressed protein kinase C substrate that has emerged as a potential therapeutic target for amelioration of mucin secretion during chronic obstructive pulmonary disease. MARCKS also plays a key role in regulation of neutrophil adhesion, migration and degranulation. Given its biological role in immune cells, we hypothesized that MARCKS may play an integral role in cytokine secretion. MARCKS protein is highly conserved between the dog and human, and importantly, the N-terminal 24 amino acids are virtually identical between species, allowing us to use the well-characterized myristoylated N-terminal sequence peptide (MANS peptide) to inhibit canine MARCKS function through the N-terminus. Using isolated neutrophils, we evaluated the effect of MARCKS inhibition on LPS-induced cytokine production. We found that pretreatment of canine neutrophils with MANS peptide significantly reduces the secretion of a broad range of LPS-induced cytokines, including IL-8, a CXCL1 orthologue and TNF-α, in comparison to untreated cells or those treated with a scrambled control peptide. This reduction in cytokine secretion is maintained when MANS is administered 2 hours after LPS-treatment of cells. Reduction in cytokine secretion is found not to be due to protein retention or cell death, but rather, to cytokine production. The reduction in cytokine production is found to be associated with reduced cytokine mRNA transcript synthesis. Our observations identify MARCKS protein as a promising therapeutic target in treatment of inflammatory disease, particularly those syndromes attributed to neutrophil influx and inflammatory cytokine production.

1. Introduction

During infection, neutrophils are the first cells to migrate to the site of infection to kill bacteria by phagocytosis and release of their cytotoxic arsenal. At the site of infection, neutrophils secrete various proinflammatory cytokines upon stimulation of their toll-like receptors (TLRs) by pathogen-associated molecular patterns (PAMPs). Excessive neutrophil infiltration, degranulation and secretion of proinflammatory cytokines can do more harm than benefit to the body, as seen in septic shock and severe sepsis. Therefore, inhibition of neutrophil-mediated excessive inflammation is likely to be critical to improving survival during sepsis.

Recent studies have identified myristoylated alanine-rich C-kinase substrate (MARCKS) as a potential target to reduce neutrophil-associated pathology in induced bronchitis and chronic obstructive pulmonary disease (COPD) (1, 2). MARCKS is an intracellular, membrane-associated protein that acts as both a PKC and calcium/calmodulin (Ca^{2+}/CaM) substrate (3). It is ubiquitously expressed in eukaryotic cells including human neutrophils (4) and plays a pivotal role in cell shape, motility, transmembrane transport, and vesicles and granule exocytosis (3, 5-7). Disruption of MARCKS function by a myristoylated N-terminal sequence peptide (MANS peptide), whose sequence is identical to the first 24 amino acids of human MARCKS, inhibited neutrophil degranulation (5) and adhesion/migration both in vitro (4) and in vivo (1, 2). Disruption of MARCKS by MANS peptide also has been shown to decrease leukocyte infiltration and bronchoalveolar lavage (BAL) levels of various proinflammatory cytokines in a neutrophil elastase-induced bronchitis mouse model and a

mouse model of ozone-induced inflammation (1, 2). Although both in vivo experiments showed that MANS peptide reduced proinflammatory cytokines in BAL fluid, it was unclear whether the reduced cytokine level was due to reduced leukocyte infiltration or reduced ability of leukocytes to produce/secrete cytokines or both.

In the current study we sought to define whether disruption of MARCKS function directly altered the ability of activated neutrophils to produce cytokines. Using isolated canine neutrophils, we demonstrate, for the first time, that inhibition of MARCKS by MANS peptide pretreatment reduces secretion of a broad range of LPS-induced proinflammatory cytokines, including IL-8, a CXCL1 orthologue and TNF-α. Importantly, this reduction is maintained even when MANS peptide is administered 2 hours after LPS-induced inflammation is initiated. Furthermore, we demonstrate that inhibition of cytokine secretion is not due to cytokine retention or cell death, but is the result of reduced cytokine protein production. This MANS-induced reduction in cytokine protein production is associated with reduced cytokine mRNA transcript synthesis.

2. Materials and Methods

2.1 Bioinformatics and phylogenetics

Sequence alignments were generated with ClustalW (8). The chromosomal location of human, canine and mouse genes were defined with Entrez Gene (http://www.ncbi.nlm.nih.gov.prox.lib.ncsu.edu/gene). For phylogenetic analyses, members of the MARCKS and MARCKS-related protein families were aligned by ClustalW and neighbor-joining trees constructed from pairwise Poisson correction distances with 2000 bootstrap replications by MEGA4 software (9). MARCKS sequences include: human (NP_002347), dog (XP_855257), mouse (NP_032564), xenopus (NP_001080075) and chicken (NP_990811). MARCKS-related protein family members include: human (NP_075385), dog (XP_854637), mouse (NP_034937) and chicken (NP_001074187).

2.2 Preparation of canine neutrophils

All experiments involving dogs were performed in accordance with relevant institutional and national guidelines and regulations, and were approved by the North Carolina State University Institutional Animal Care and Use Committee. Peripheral blood samples were collected from healthy dogs via jugular venipuncture into vacutainer™ tubes containing ACD (Becton-Dickinson, Franklin Lakes, NJ). Canine neutrophils were prepared as described previously (10). In brief, whole blood was centrifuged over endotoxin-tested Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) at 400 ×g for 25 min and neutrophils were harvested from the bottom of histopaque gradient tubes. Erythrocytes were lysed with ammonium chloride lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA)

and cells were washed twice in endotoxin-free PBS (Gibco, Carlsbad, CA). The purity of neutrophils was determined by microscopic examination of stained cytospin preparations and flow cytometry using a monoclonal antibody specifically against canine neutrophils (VMRD, Inc., catalog number CADO48A, Pullman, WA). The purity of canine neutrophils is greater than 98%.

2.3 Western blot

Canine neutrophils and peripheral blood mononuclear cells (PBMCs) were lysed in RIPA lysis buffer (Pierce, Rockford, IL) containing a cocktail of protease inhibitors (1 mM iodoacetamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml pepstatin A, 1 mM Phenylmethanesulfonyl fluoride and 5 mM Diisopropylfluorophosphate, Sigma-Aldrich, St. Louis, MO) on ice for 30 min. After lysis, cells were centrifuged at 12000 × g for 10 min at 4 ℃. Supernatant was recovered and treated with 5× reducing sample buffer (Pierce, Rockford, IL) at 95 ℃ for 5 min. The lysate was then subjected to SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel; Invitrogen, Carlsbad, CA) and transferred to PVDF membranes (Millipore, Billerica, MA). After blocking with 5% milk-TBST, membranes were incubated with goat anti-human-MARCKS antibody N-19 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-human-phospho-MARCKS (Ser152/156) antibody (Cell Signaling Technology, Danvers, MA) at 4 ℃ overnight. After five consecutive five minute washes, the membrane was incubated in HRP-labeled secondary antibodies against the species used in primary antibodies for 1 hr. Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) was used for signal detection.

2.4 Peptides

Cell-permeant MARCKS myristoylated aminoterminus peptide (MANS peptide) and the scrambled missense control peptide (RNS peptide) were synthesized by Genemed Synthesis, Inc. (San Francisco, CA). The sequence of the MANS peptide is identical to the first 24 amino acids of human MARCKS: MA-GAQFSKTAAKGEAAAERPGEAAVA (MA = N-terminal myristate chain). And the RNS peptide has the same amino acid composition as MANS peptide but arranged in a random order: MA-GTAPAAEGAGAEVKRASAEAKQAF.

2.5 Treatment of canine neutrophils with MARCKS inhibitor

Freshly isolated canine neutrophils were seeded at a density of 2×10^5 cells/well in 96-well plates and cultured in RPMI 1640 supplemented with endotoxin-tested 10% fetal bovine serum (Gibco, Carlsbad, CA), 1 mM sodium pyruvate, 15 mM HEPES, 55 μM 2-mercaptoethanol, 10 IU/ml penicillin and 10 μg/ml streptomycin. To enhance cell viability, 30 pg/ml recombinant canine granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) was added to the culture medium as previously described (10).

To determine whether MANS peptide pretreatment could inhibit cytokine secretion, canine neutrophils were rested in culture medium described above for 30 min and then were incubated with indicated concentrations of MANS/RNS peptide for 30min. After the initial 30 min rest and 30 min peptide pretreatment, cells were treated with indicated concentrations

of crudely purified LPS from *Escherichia coli* O111:B4 (contains agonists for TLR2, TLR4, TLR9) (Sigma–Aldrich, catalog number: L3024, St. Louis, MO) (11). After 18 hr incubation with LPS, cell culture supernatant were harvested and evaluated for the production of IL-8, tumor necrosis factor-α (TNF-α), a canine orthologue of Chemokine (C-X-C motif) ligand 1 (CXCL1) and monocyte chemoattractant protein-1 (MCP-1) by Milliplex Map canine cytokine/chemokine kit (Millipore, Billerica, MA) per manufacturer's instructions (Figure 1A).

To determine whether MANS peptide could inhibit cytokine secretion from cells already activated by LPS, canine neutrophils were rested in the culture medium described above for 1 hr and treated with LPS as described above. After 2 hr pretreatment by LPS, indicated concentrations of MANS/RNS peptide were added to the cell culture medium. After 18 hr incubation with LPS (16h incubation with MANS/RNS peptide), cell culture supernatant were harvested and cytokine levels were measured as described above (Figure 1B).

To determine whether the MANS-induced decrease of IL-8 secretion was due to inhibition of cytokine production, canine neutrophils were prepared as described above with the exception that culture was maintained for 16h rather than 18h, and intracellular IL-8 production was evaluated by flow cytometry. Brefeldin A was added to cultures 4 hr post LPS stimulation to facilitate intracellular protein retention (Figure 1C and 1D).

2.6 Flow cytometry

Cytokine production in canine neutrophils was determined by flow cytometry. Cells were permeabilized by using BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution (BD Biosciences, Mountain View, CA) according to the manufacturer's instructions. After permeabilization, cells were incubated with biotinylated anti-canine IL-8 monoclonal antibody (clone number: 258911, R&D Systems, Minneapolis, MN) in 1 × Perm/Wash buffer on ice for 30 min. Cells were then washed with 1 × Perm/Wash buffer and incubated with Strepavidin-PerCP (Biolegend, San Diego, CA) for 15 min on ice. After washed once with 1 × Perm/Wash buffer and once with PBS, cells were analyzed on a LSR II flow cytometer using FACSDiva software (BD Biosciences, Mountain View, CA). Anti-human-CD25 monoclonal antibody BC96 (Biolegend, San Diego, CA) was used as an isotype control in this experiment.

To determine if MANS/RNS peptide and/or brefeldin A enhanced cell death, canine neutrophils from the above experiments were also stained by pacific-blue labeled annexin V (Biolegend, San Diego, CA) according to the manufacturer's instructions and analysed on a LSR II flow cytometer using FACSDiva software (BD Biosciences, Mountain View, CA).

2.7 RNA isolation and Real-time RT-PCR

Canine neutrophils were rested in medium for 1 hr and pre-treated with 100 μM MANS peptide or RNS peptide for 30 min. After the initial rest and MANS/RNS pretreatment, crudely purified LPS from *Escherichia coli* O111:B4 was added at a concentration of 100

ng/ml. After incubation with LPS for 4 hr, cells were harvested, RNA from these cells was extracted by using ZR whole-blood RNA miniprep (Zymo research, Irvine, CA) and cDNA was synthesized by using RT2 first strand kit (SABiosciences, Frederick, MD). To quantify mRNA expression, primers for amplification of canine IL-8 (5'-ACTTCCAAGCTGGCTGTTGC-3' and 5'-GGCCACTGTCAATCACTCTC-3'), TNF-α (5'-CCAAGTGACAAGCCAGTAGC-3' and 5'-TCTTGATGGCAGAGAGTAGG-3') and primers for amplification of canine beta-actin (5'-GAC CCT GAA GTA CCC CAT TGA G -3' and 5'-TTG TAG AAG GTG TGG TGC CAG AT -3') were used in real-time PCR with SYBR Green qPCR master mix (SABiosciences, Frederick, MD). PCR amplification was performed at 95 ℃ for 10 sec, 63 ℃ for 30 sec with a total of 50 cytcles. IL-8 and TNF-α mRNA levels were normalized to beta-actin and fold change in expression levels were determined using delta delta Ct methods (12).

2.8 Statistics

Data were analysed by paired t-test using prism 5 (GraphPad Software, San Diego, CA). In cases where cytokine concentrations were below the assay Lower Limit of Detection (LLOD), values were assigned that were one half of the LLOD (defined above) for the purpose of statistical analysis (13-18). The level of significance used was $p \leq 0.05$. Error bars represent the SEM.

3. Results

3.1 Canine MARCKS

Comparison of MARCKS protein sequence across dog, human and mouse revealed three evolutionarily conserved domains: 1) the amino-terminal myristoylated domain, 2) the multiple homology 2 domain (MH2 domain) and 3) the phosphorylation site domain (PSD domain) (Figure 2A) (3). Importantly, the first 24 amino acids of MARCKS representing the amino-terminal myristoylated domain were identical with the exception of one amino acid. This N-terminal domain is essential for the anchorage of MARCKS protein to the plasma membrane (3) and contains the sequence of the MANS peptide. We detected canine MARCKS from neutrophils using an antibody against the amino terminus of human MARCKS on western blot, suggesting that the amino terminus of canine and human MARCKS are indeed highly homologous at the protein level (Figure 3A). The high level of homology between species at the N-terminus justified our use of the well-characterized MARCKS inhibitory peptide MANS (5, 19-21) in canine cells.

Further, the amino acid sequence of the PSD domain is identical between human MARCKS and canine MARCKS (Figure 2A), suggesting that they have similar function as a substrate for PKC or a binding site for calcium/calmodulin (3). Using an antibody specific for human phospho-MARCKS (Ser152/156), we detected canine phospho-MARCKS in both neutrophils and PBMCs treated with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) (Figure 3B), thereby verifying high homology at the PSD domain.

In order to confirm the predicted canine MARCKS is indeed orthologous to human MARCKS, a phylogenetic comparison of canine MARCKS was compared to MARCKS protein and MARCKS-related protein from human and other species. As seen in Figure 2B the predicted canine MARCKS sequence belongs to the MARCKS, and not to the MARCKS-related protein family. These observations support the classification of this sequence as canine MARCKS.

3.2 Pretreatment of neutrophils with MARCKS inhibitor significantly reduced secretion of a broad range of LPS-induced cytokines

In order to elucidate whether MARCKS is directly implicated in cytokine secretion, highly purified neutrophils were pretreated with a titrating dose of the MANS peptide or a scrambled control RNS peptide prior to activation with LPS. As mentioned above, the MANS peptide is derived from the highly conserved amino terminus of MARCKS and has previously been shown to inhibit MARCKS-associated neutrophil degranulation (5) and methacholine-induced mucin secretion (20, 21). Canine neutrophils secreted minimal amount of cytokines in the absence of stimulation, but once activated with LPS, secreted high levels of multiple proinflammatory cytokines including CXCL1 orthologue, IL-8, TNF-α and MCP-1 (Figure 4A-D). Pretreatment of neutrophils with 100 μM MANS peptide prior to the addition of LPS significantly reduced secretion of CXCL1 orthologue, IL-8 and TNF-α in the supernatant compared to supernatant from cells treated with the same dose of RNS control peptide (Figure 4A-C). While pretreatment of neutrophils with MANS peptide

trended towards lowering MCP-1 levels, differences were not significant between treatment groups.

3.3 MANS peptide maintains its ability to diminish cytokine secretion when administered 2 hr after LPS activation

In order to determine whether MARCKS inhibition remains effective in reducing cytokine secretion in the face of ongoing inflammation, titrating doses of MANS or RNS were added to neutrophil cultures 2 hr after LPS addition. Secretion of CXCL1 orthologue, IL-8 and TNF-α in cellular supernatant of LPS pretreated neutrophils remained significantly lower in cells treated with 100 μM of MANS when compared to supernatant of cells treated with RNS (Figure 5A-C). Similar to observations made with pretreatment of neutrophils with peptides, the addition of MANS peptide to LPS pretreated neutrophils did not significantly diminish MCP-1 secretion (Figure 5D).

3.4 MARCKS inhibition reduces the production of cytokines by neutrophils

As previously mentioned, MARCKS is known to play an integral role in cell motility and migration (19). Mechanistically, the effect of MARCKS on cellular migration is likely exerted at the level of cytoskeletal rearrangement as MARCKS has been shown to associate with actin as it recycles on and off the plasma membrane (3). Cytoskeletal rearrangement is critical for expulsion of cytokines from neutrophils, therefore, we hypothesized that disruption of MARCKS by MANS peptide was likely contributing to retention of cytokines within neutrophils rather than directly affecting production of protein. In order to evaluate

cytokine retention, LPS-induced IL-8 was measured by intracellular flow cytometry in the presence or absence of MANS or RNS peptide. Surprisingly, intracellular IL-8 levels were significantly lower in cells pretreated with MANS peptide in comparison to those pretreated with the RNS control peptide (Figure 6B and D). MANS peptide was also able to significantly reduce IL-8 production after 2 hr of LPS pretreatment (Figure 6C and E). Annexin V staining was used to confirm that reduced production of IL-8 was not due to induction of cell death. Indeed, treatment of neutrophils with MANS peptide slightly reduced the percentage of Annexin V^+ neutrophils, thereby ruling out induction of cell death as an explanation for reduced cytokine production (Figure 7A and B). Collectively these data suggest that inhibition of cytokine secretion from MANS-treated canine neutrophils can be attributed to reduced cytokine production.

3.5 Disruption of MARCKS by MANS peptide reduces LPS-induced cytokine mRNA synthesis in neutrophils

To determine if MANS peptide-induced reduction in cytokine production was associated with changes in mRNA transcript levels, we measured IL-8 and TNF-α mRNA levels in MANS/RNS pretreated canine neutrophils. Our result showed that MANS peptide pretreatment at a concentration of 100 μM significantly reduced LPS-induced IL-8 (Figure 8A) and TNF-α (Figure 8B) mRNA synthesis compared to RNS peptide control or no peptide treatment control.

4. Discussion

The purpose of this study was to determine whether inhibition of MARCKS function has a direct effect on cytokine secretion and production after LPS-induced inflammation. We observed that inhibition of MARCKS protein with MANS peptide resulted in a significant reduction of a broad range of LPS-induced proinflammatory cytokine secretion from canine neutrophils. Importantly, MANS-induced reduction of cytokine secretion is achievable 2 hr after the onset of LPS-induced inflammation. Reduction of cytokine secretion was not due to cytokine retention or cell death, but was the result of reduced cytokine protein production that was associated with reduced cytokine mRNA transcript synthesis.

Using cytokine multiplex techniques, we found pretreatment of neutrophils with MANS peptide reduced LPS-induced secretion of IL-8, CXCL1 orthologue and TNF-α. While MCP-1 concentrations tended to be decreased in the presence of MANS peptide, cytokine concentrations were not significantly reduced compared to the no peptide or RNS peptide control. Previous studies in our laboratory suggested that canine MCP-1 production is regulated differently from IL-8, CXCL1 orthologue and TNF-α production (10). As IL-8 and CXCL1 orthologue are potent neutrophil chemoattractants and activators, and are produced and used by neutrophils in an autocrine or paracrine manner (22-24), their production and secretion may be tightly regulated by multiple signaling pathways (including TREM-1-mediated pathway and MARCKS-mediated pathway) in neutrophils. In contrast, as MCP-1 is chemoattractant for monocytes and T cells (25, 26), its production and expulsion by neutrophils may be primarily regulated by early TLR signaling that acts as a priming event

for cellular activation. Collectively, these data suggest alternative pathways of cytokine regulation in canine neutrophils.

MARCKS protein itself constitutes nearly 90% of newly synthesized protein in neutrophils post-LPS stimulation (27). This substantial increase in MARCKS synthesis suggests that MARCKS may have an important role in neutrophil function during infection with Gram-negative pathogens. Further, recent data suggest MARCKS may directly associate with LPS once it is internalized by TLR4, thus playing a role in signal transduction and/or regulation of LPS-induced responses (28). Disruption of MARCKS function by MANS peptide inhibits multiple aspects of neutrophil function, including degranulation (5) and adhesion/migration in vitro (4) and in vivo (1, 2). Our data extend the role of MARCKS protein into cytokine production and the regulation of mRNA transcript synthesis.

The mechanism of the action of the MANS peptide used in this study to inhibit MARCKS protein function is not fully defined. Two theories exist to explain the mechanism of action of MANS peptide. Eckert *et al*. propose that MANS peptide may function as a dominant 'negative peptide', occupying specific MARCKS binding sites on the plasma membrane and thereby preventing MARCKS from reassociating with the membrane after phosphorylation (4). Indeed, this group has shown that treatment of resting human neutrophils with MANS peptide resulted in translocation of MARCKS from the membrane to the cytosol (4). In contrast, Singer *et al*. and Li *et al*. propose that inhibition of MARCKS by MANS peptide is mediated at the level of cytoskeleton (21, 29). As we observed a marked effect on cytokine

protein production and specific mRNA transcript synthesis, our data suggest that the MANS peptide is interfering with MARCKS function at level of signaling transduction rather than simply through interfering with expulsion of proteins. Cytoskeletal proteins regulate lipid raft formation and facilitate the assembly of signaling complexes (30). It is possible that the effect of MANS peptide on cytokine mRNA transcript synthesis may be through its effect on actin-mediated assembly of signaling complexes. Further research is necessary to unravel the role of MARCKS protein in signal transduction events, and more specifically, the mechanism of action of MANS peptide in negation of cytokine production.

Given the newly identified role of MARCKS protein in neutrophil cytokine production, its use as a therapeutic target in neutrophil-mediated inflammatory disease warrants substantial consideration. Excellent examples of this are acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). In ALI and ARDS the pathological changes associated with irreversible lung damage are primarily mediated by neutrophil influx and proinflammatory cytokine secretion (31-34). Neutrophil concentration in ARDS patient bronchial lavage fluid strongly correlates with disease severity and prognosis (35, 36) and disease severity is reduced upon neutrophil depletion in mice (37). Further, reduction of IL-8 protects animals from severe lung damage (32). Therefore, we suggest that managing neutrophil influx and the proinflammatory cytokine ‘storm’ via inhibition of MARCKS protein is a potential strategy to reduce ALI/ARDS morbidity and mortality.

In summary, we are the first to show that disruption of MARCKS function by MANS peptide inhibits cytokine production in inflamed canine neutrophils, and that this reduction in the cytokine ‘storm’ remains possible hours after inflammation is initiated. Mechanistically, the decrease in cytokine production is associated with a decrease in cytokine mRNA synthesis. Further research is necessary to clarify the mechanism of MARCKS protein in interfering with cytokine signaling pathways.

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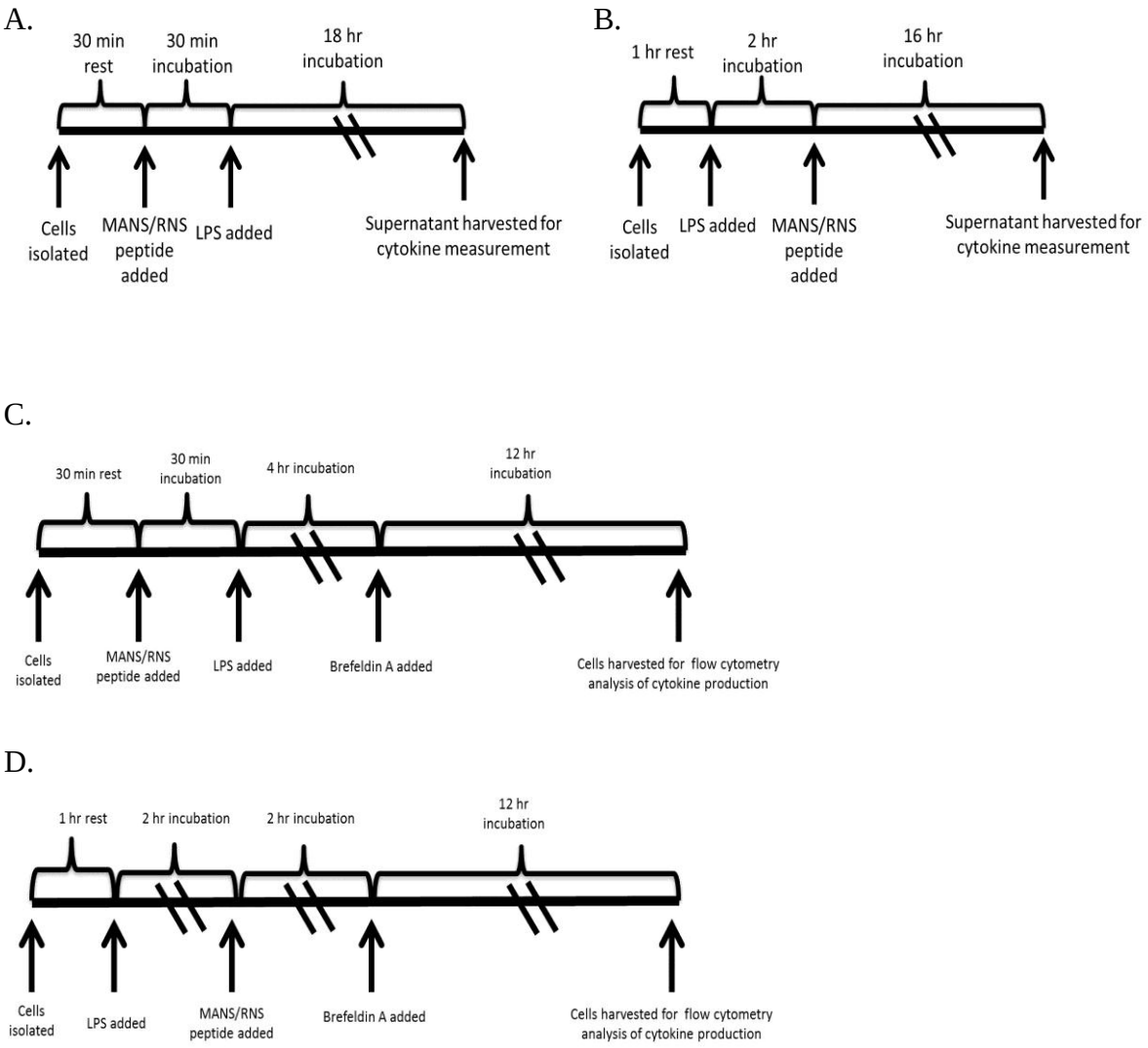
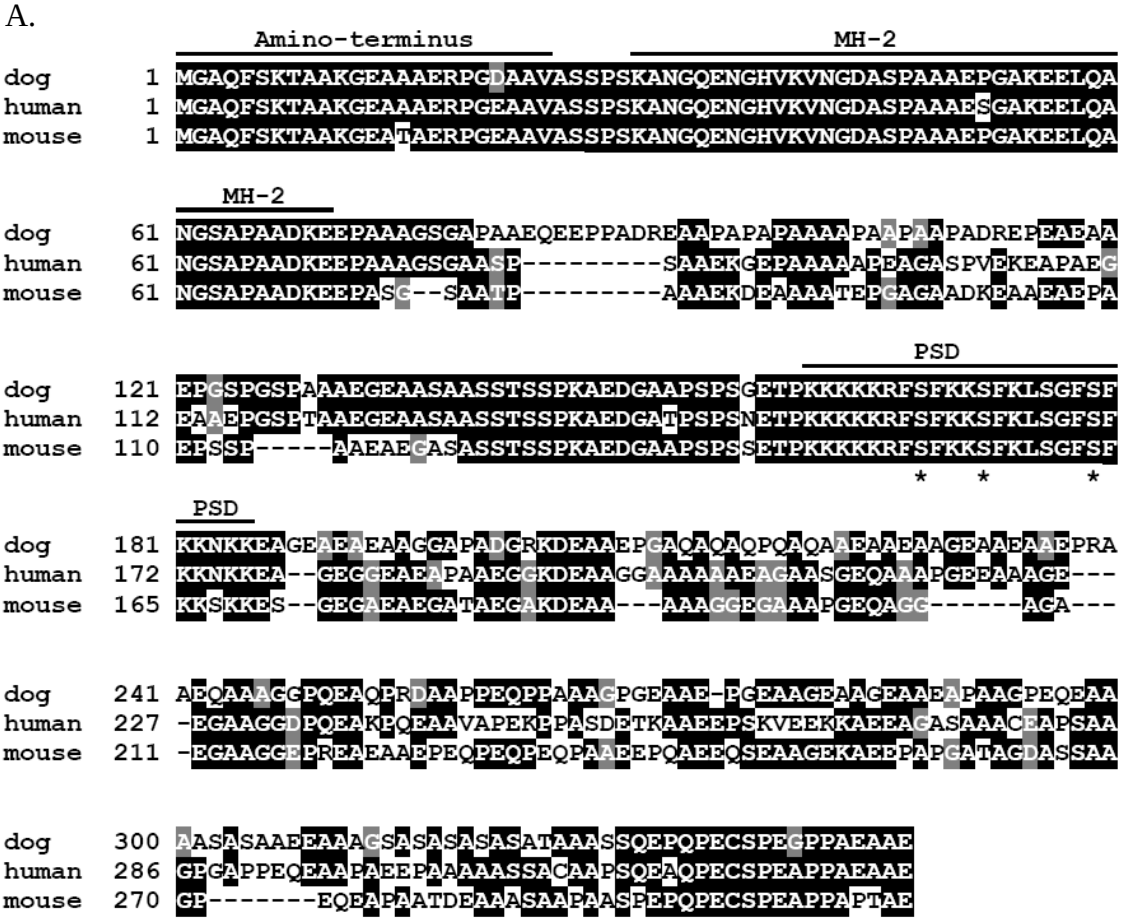


Figure 1. Timeline of MANS peptide and LPS treatment on canine neutrophils.

Figure 2. Phylogenetic comparison of the predicted canine MARCKS protein to members of the MARCKS and MARCKS-related protein families. Neighbor-joining trees were constructed with 2000 bootstrap replication using MEGA4 software (9). Branch lengths are measured in terms of amino acid substitutions, with the scale indicated below the tree.

A.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human 1 MGAQFSKTAAKGEAAAERPGEAAVASSPSKANGQENGHVKVNGDASPAAAESGAKEELQA
mouse 1 MGAQFSKTAAKGEATAERPGEAAVASSPSKANGQENGHVKVNGDASPAAAEPGAKEELQA
MH-2
dog 61 NGSAPAADKEEPAAAGSGAPAAEQEEPPADREAAPAPAPAAAAPAAPAAPADREPEAEAA
human 61 NGSAPAADKEEPAAAGSGAASP---------SAAEKGEPAAAAAPEAGASPVEKEAPAEG
mouse 61 NGSAPAADKEEPASG--SAATP---------AAAEKDEAAAATEPGAGAADKEAAEAEPA
PSD
dog 121 EPGSPGSPAAAEGEAASAASSTSSPKAEDGAAPSPSGETPKKKKKRFSFKKSFKLSGFSF
human 112 EAAEPGSPTAAEGEAASAASSTSSPKAEDGATPSPSNETPKKKKKRFSFKKSFKLSGFSF
mouse 110 EPSSE-----AAEAEGASASSTSSPKAEDGAAPSPSSETPKKKKKRFSFKKSFKLSGFSF
* * *
PSD
dog 181 KKNKKEAGEAEAEAAGGAPADGRKDEAAEPGAQAQAQPQAQAAEAAEAAGEAAEAAEPRA
human 172 KKNKKEA--GEGGEAEAPAAEGGKDEAAGGAAAAAAEAGAASGEQAAAPGEEAAAGE---
mouse 165 KKSKKES--GEGAEAEGATAEGAKDEAA---AAAGGEGAAAPGEQAGG------AGA---
dog 241 AEQAAAGGPQEAQERDAAPPEQPPAAAGPGEAAE-PGEAAGEAAGEAAEAPAAGPEQEAA
human 227 -EGAAGGDPQEAKEQEAAVAPEKPPASDETKAAEEPSKVEEKKAEEAGASAAACEAPSAA
mouse 211 -EGAAGGEPREAEAAEPEQPEQPEQPAAEEPQAEEQSEAAGEKAEEPAPGATAGDASSAA
dog 300 AASASAAEEAAAGSASASASASASATAAASSQEPQPECSPEGPPAEAAE
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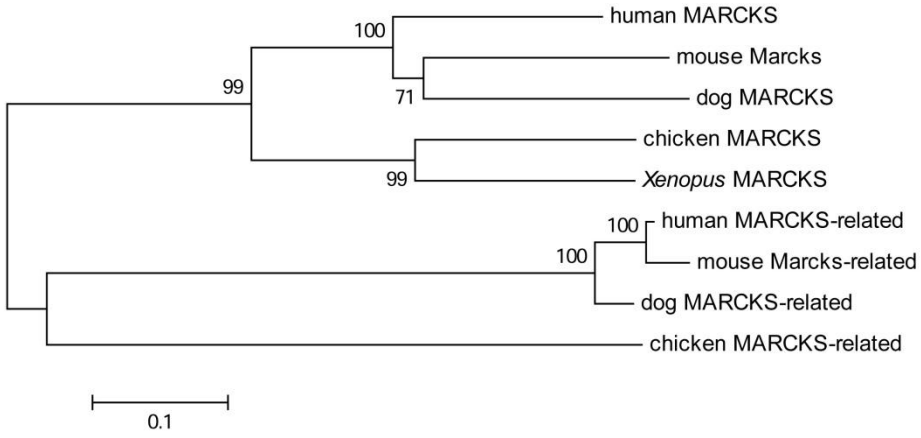
mouse 270 GP-------EQEAPAATDEAAASAAPAASPEPQPECSPEAPPAPTAE

B.


human MARCKS
mouse Marcks
dog MARCKS
chicken MARCKS
Xenopus MARCKS
human MARCKS-related
mouse Marcks-related
dog MARCKS-related
chicken MARCKS-related
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99
71
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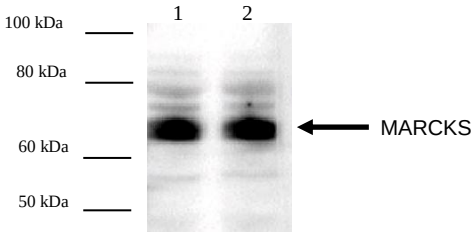
100
100
0.1

A.



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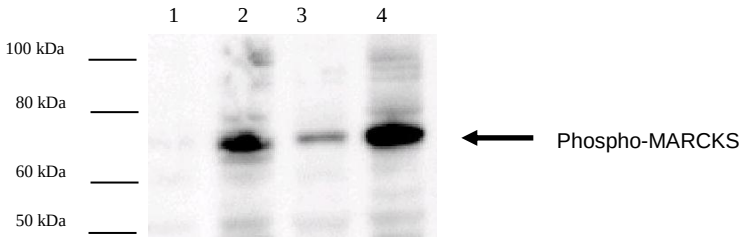


Figure 3. Canine MARCKS and canine phospho-MARCKS were detected on western blot. (A) Canine MARCKS from canine neutrophils was detected on western blot by using an antibody against the N-terminus of human MARCKS. Lane 1 and Lane 2 show canine MARCKS. (B) Canine phospho-MARCKS was detected on western blot by using an antibody against human phospho-MARCKS (Ser152/156). Lane 1: canine neutrophils were left untreated; Lane 2: canine neutrophils were treated with PMA for 20 min; Lane 3: canine PBMCs were left untreated; Lane 4: canine PBMCs were treated with PMA for 20 min.

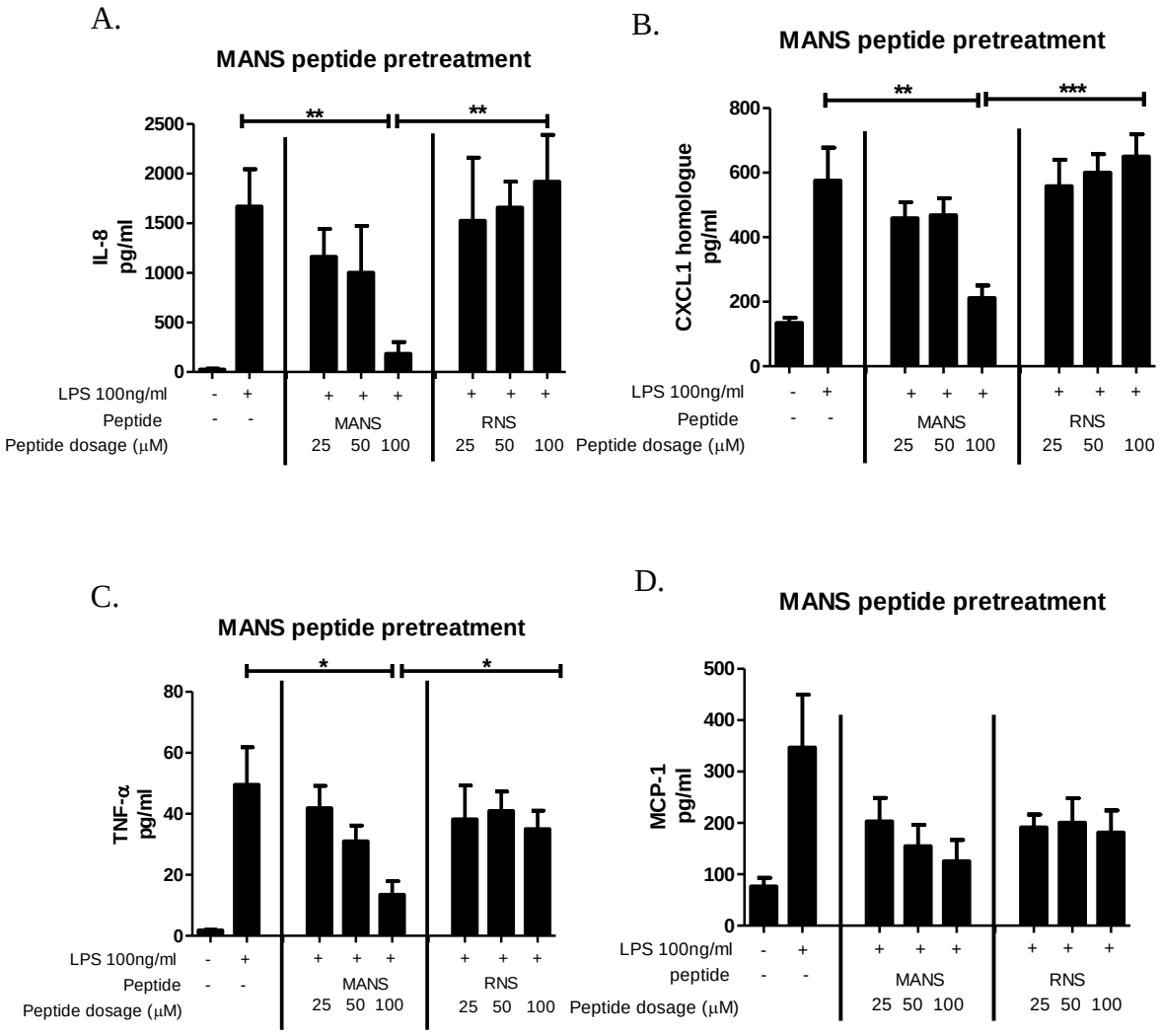


Figure 4. MANS peptide pretreatment reduces LPS-induced cytokine secretion from canine neutrophils. MANS peptide at 100 μM significantly reduced LPS-induced secretion of CXCL1 orthologue (A), IL-8 (B) and TNF-α (C) from canine neutrophils compared to the RNS peptide control. MANS peptide did not have significant effect on MCP-1 secretion (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n = 6 dogs.

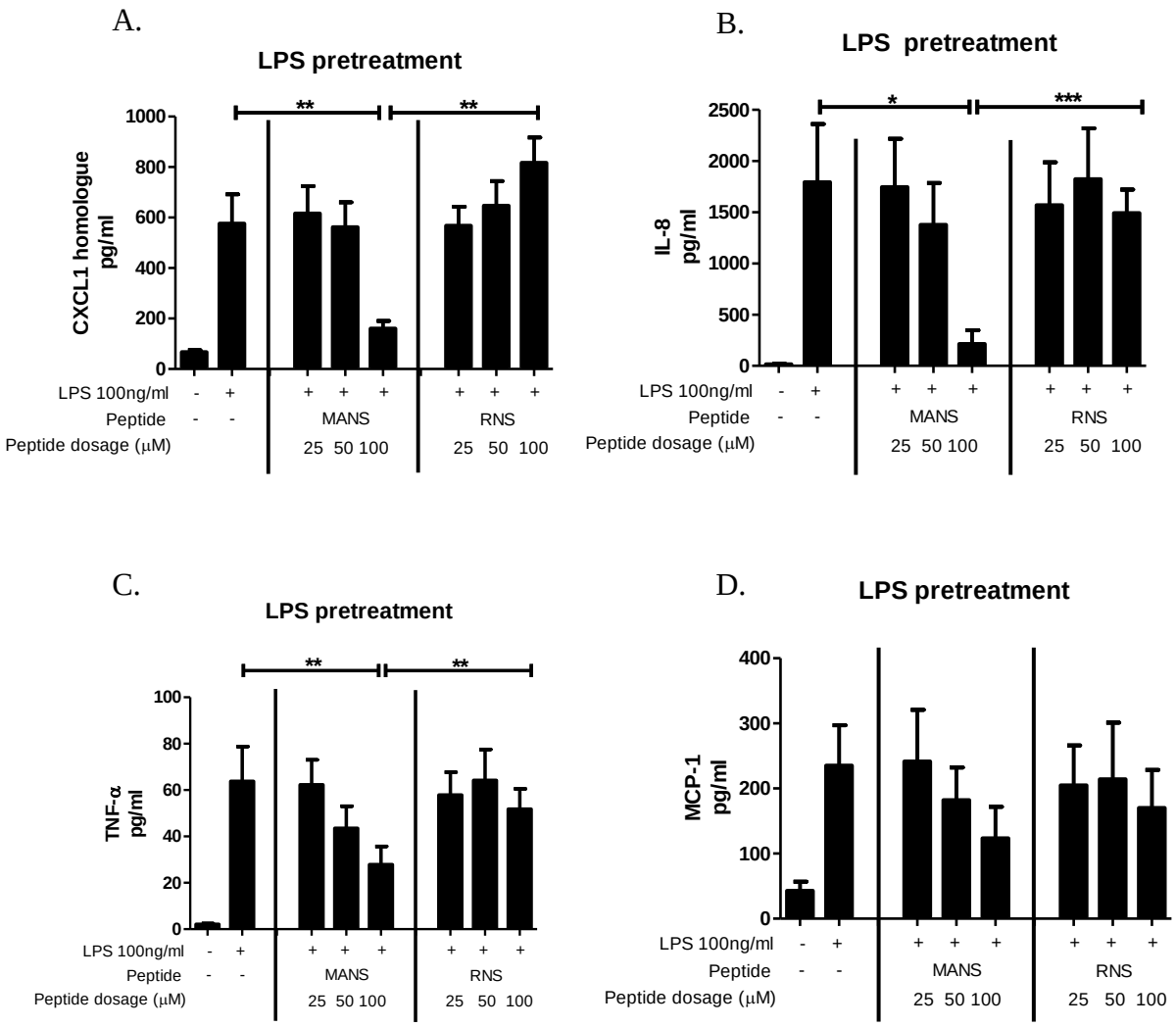
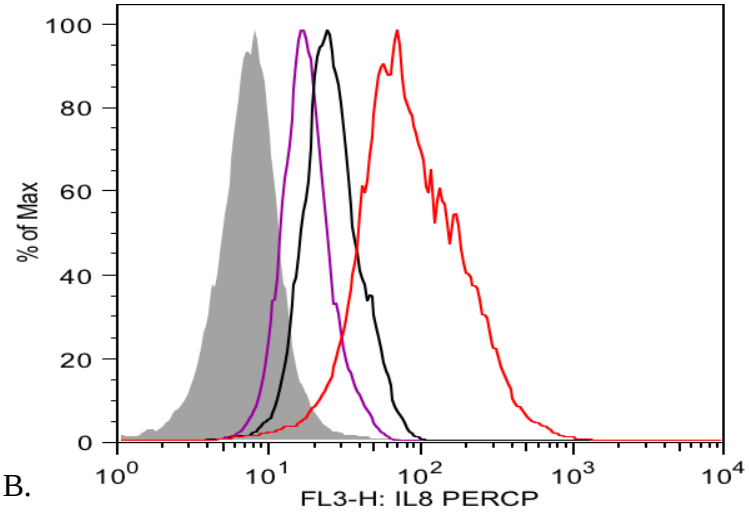


Figure 5. MANS peptide reduces cytokine secretion from canine neutrophils pretreated with LPS for 2 hr. MANS peptide at 100 μM significantly reduced CXCL1 orthologue (A), IL-8 (B) and TNF-α (C) secretion from canine neutrophils pretreated with LPS for 2 hr compared to the RNS peptide control. MANS peptide did not have significant effect on MCP-1 secretion (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n = 6 dogs.

Figure 6. MANS peptide reduces canine IL-8 production. (A) LPS induces the up-regulation of intracellular IL-8 in canine neutrophils. Untreated canine neutrophils were left unstained (grey shaded line), stained with isotype control antibody (purple line) or anti-canine IL-8 antibody (black line); LPS treated cells stained with anti-canine IL-8 antibody (red line). Intracellular IL-8 levels were measured by flow cytometry. (B, D) MANS peptide pretreatment reduces LPS-induced IL-8 production in canine neutrophils. Canine neutrophils were left untreated (black line); treated with LPS (red line); pretreated with MANS peptide 30 minutes prior to addition of LPS (blue line) or pretreated with RNS peptide 30 minutes prior to addition of LPS (green line). Intracellular IL-8 levels were measured by flow cytometry. (C, E) MANS peptide reduces IL-8 production in canine neutrophils pretreated with LPS. Canine neutrophils were left untreated (black line); treated with LPS (red line); pretreated with LPS 2 hr prior to addition of MANS peptide (blue line) or RNS peptide (green line). Intracellular IL-8 levels were measured by flow cytometry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n = 5 dogs.

A.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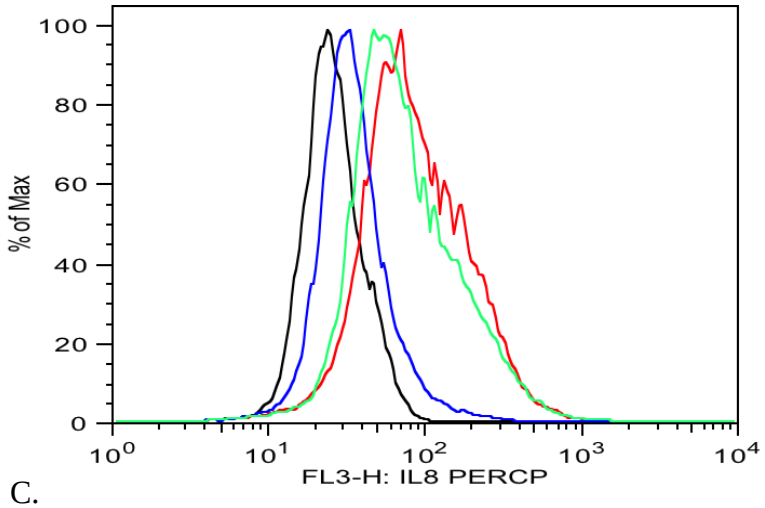
FL3-H: IL8 PERCP

B.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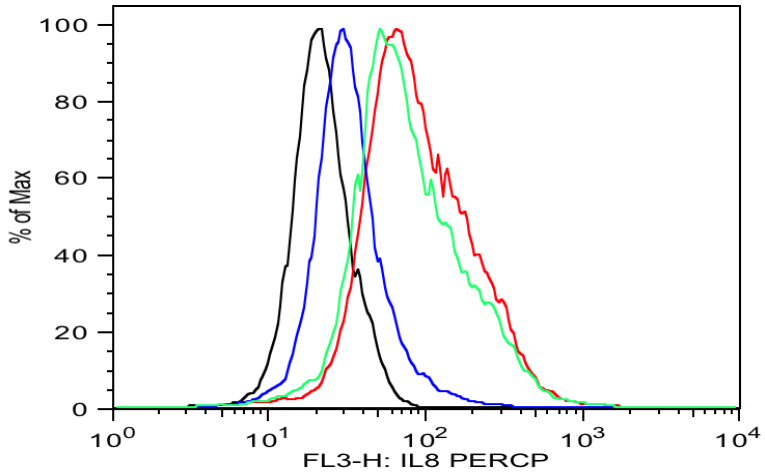
C.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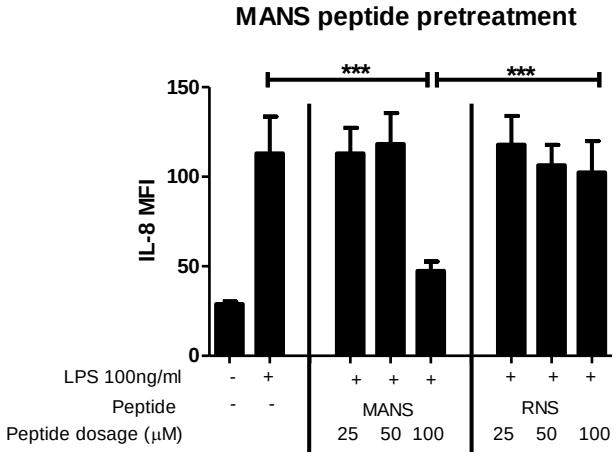
D.


MANS peptide pretreatment
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***
150
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50
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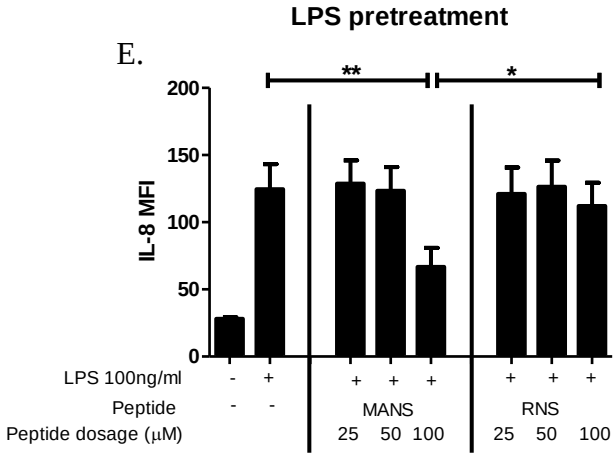
IL-8 MFI
LPS 100ng/ml - + + + + + + +
Peptide - - MANS RNS
Peptide dosage (μM) 25 50 100 25 50 100

E.


LPS pretreatment
**
*
200
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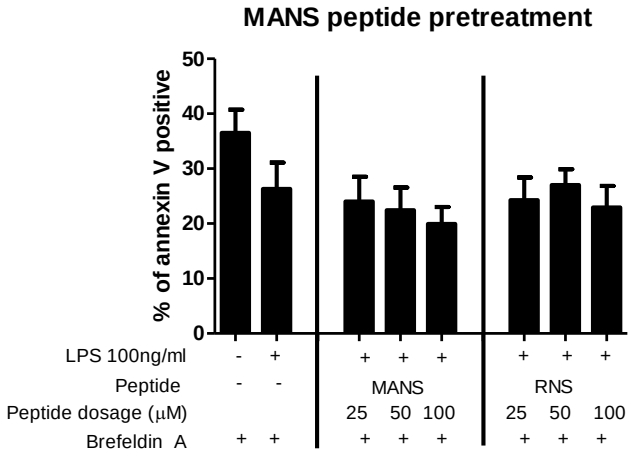
IL-8 MFI
LPS 100ng/ml - + + + + + + +
Peptide - - MANS RNS
Peptide dosage (μM) 25 50 100 25 50 100

A.



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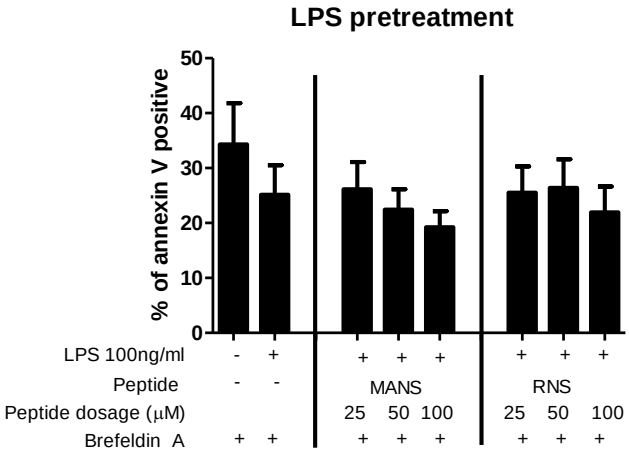

Figure 7. MANS peptide together with brefeldin A slightly reduces the percentage of apoptotic canine neutrophils in MANS peptide pretreatment experiment (A) and LPS pretreatment experiment (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n = 5 dogs.

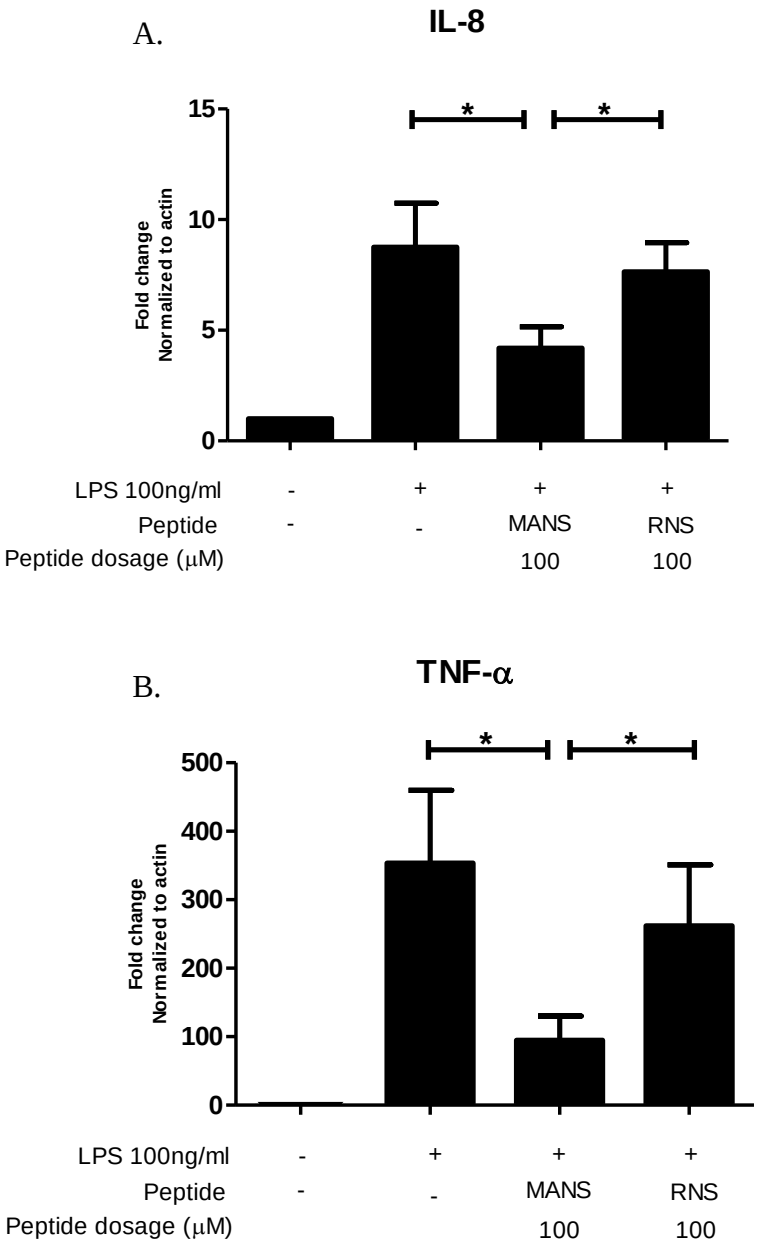

Figure 8. MANS peptide reduces specific cytokine mRNA synthesis. Pretreatment of canine neutrophils with MANS peptide reduced (A) IL-8 and (B) TNF-α mRNA synthesis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n = 8 dogs.

Dissertation Summary

The work in this study was performed under the direction of Drs. Shila Nordone, Adam Birkenheuer and Michael Levy. Chapter I reviewed cytokine- and neutrophil-mediated sepsis pathogenesis, and the role of TREM-1 and MARCKS protein play in infection-associated inflammation. Chapter II described cytokine concentrations in healthy dogs as well as dogs with induced endotoxemia, naturally occurring sepsis, recent surgery (sterile inflammation), or systemic inflammatory response syndrome (SIRS) with no evidence of infection. In this study, we found that IL-8, CXCL1 orthologue and MCP-1 were significantly increased in dogs with naturally occurring sepsis compared to healthy dogs, but were not significantly different compared to dogs with SIRS or dogs with recent surgery. Since concentrations of MCP-1 in two dogs with sepsis were higher than those in any dogs with SIRS or dogs with recent surgery, and were close to the peak concentrations of MCP-1 in dogs with induced endotoxemia, larger studies should be performed to evaluate MCP-1 as a biomarker for the diagnosis of canine sepsis. Furthermore, to fully understand cytokine kinetics in dogs with naturally occurring sepsis and evaluate if certain cytokines or a panel of cytokines could be used for the diagnosis of canine sepsis, serial measurement of cytokine concentrations in the dogs with naturally occurring sepsis should be determined.

In Chapter III we determined one mechanism of cytokine amplification – activation of the cell surface receptor TREM-1. TREM-1 expression was originally identified on neutrophils and monocytes in the human and mouse (1, 2). We are the first to show the expression of

TREM-1 in the dog. We determined the kinetics of TREM-1 expression on canine neutrophils upon TLR agonist stimulation both in vitro and in vivo. In vitro, TREM-1 is up-regulated within 2 hr after stimulation with various microbial TLR agonists; in vivo, TREM-1 is initially down-regulated and subsequently up-regulated post LPS administration (appendix I). Furthermore, we showed that cross-linking of TREM-1 on canine neutrophils in the presence of LPS synergistically amplifies cytokine secretion, suggesting that mechanistically canine TREM-1 functions as an amplifier of cytokine production, which is identical to what has been documented in the human (1). However, there are still two missing puzzles about canine TREM-1: 1) the expression of soluble TREM-1 (sTREM-1) in the dog has not been characterized due to the lack of antibodies; 2) the identity of TREM-1 ligand is still a mystery even though there are several papers showing the expression of TREM-1 ligand on different cell types (3-5). To understand the whole picture of canine TREM-1 in sepsis, these two missing puzzles need to be solved in the future.

Chapter IV identifies a novel therapeutic to inhibit cytokine production in inflamed canine neutrophils. We showed in this chapter that disruption of MARCKS protein function by using a cell-permeant peptide (MANS peptide) reduces cytokine secretion from LPS-activated canine neutrophils. Pretreatment of canine neutrophils with MANS peptide significantly reduced secretion of a broad range of LPS-induced cytokines, including IL-8, CXCL1 orthologue and TNF-α, in comparison to control peptide RNS. Importantly, the reduction in cytokine secretion was maintained even when MANS peptide was administered 2 hours after LPS-induced inflammation was initiated. Interestingly, reduction in cytokine

secretion was found not to be due to protein retention or cell death, but rather, to cytokine production. The reduction in cytokine production was found to be associated with reduced cytokine mRNA transcript synthesis. In the future, the mechanism of action of MANS peptide on canine neutrophils should be determined.

In summary, we defined cytokine responses in induced and naturally occurring canine sepsis and determined TREM-1 cross-linking as one mechanism for cytokine amplification in the dog. In terms of therapeutics, we identified MARCKS as a potential target to inhibit cytokine production. Our studies contribute to a better understanding of canine sepsis and set the stage for further research that will lead to better diagnosis and treatment of sepsis in both human and veterinary patients.

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APPENDIX

Appendix A

Kinetics of TREM-1 expression in dogs with induced endotoxemia

Materials and Methods

Induction of endotoxemia in the dog was described as in chapter II. Whole blood was harvested in tubes containing EDTA (BD Biosciences, Mountain View, CA) at 0 hr, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12 hr, 24 hr, 48 hr and 72 hr post LPS or saline administration. Two hundred microliter whole blood was stained using a Pacific Orange-labeled monoclonal antibody specific for canine neutrophils (VMRD, Inc., catalog number CADO48A, Pullman, WA) and biotinylated TREM-26 (anti-TREM-1) monoclonal antibody (Biolegend, San Diego, CA) on ice for 30 min. After incubation, erythrocytes were lysed with ammonium chloride lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM Na_2EDTA) and cells were washed twice in endotoxin-free PBS (Gibco, Carlsbad, CA). After wash, cells were incubated with Strepavidin-PerCP (Biolegend, San Diego, CA) for 15 min on ice, washed twice, and fixed with 4% paraformaldehyde. Cells were then analyzed on an LSR II flow cytometer (BD Biosciences, Mountain View, CA) using FACSDiva software (BD Biosciences, Mountain View, CA). TREM-1 expression was determined on canine neutrophils identified by the canine neutrophil specific antibody.

Results and discussion

The percentage of neutrophils in peripheral blood decreased within 2hr post LPS administration but rebounded by 4hr post-treatment and became significantly elevated in

comparison to saline controls from 8 hr to 48 hr (Figure 1). The percentage of TREM-1 positive neutrophils decreased within 2h post LPS administration and returned to the baseline at 72 hr (Figure 2). The relative density of TREM-1 on the surface of neutrophils, as indicated by mean fluorescence intensity (MFI), was significantly decreased from 6 hr to 12 hr, returned to baseline at 24 hr then significantly increased through 72 hr (Figure 3).

In contrast, in vitro exposure of canine neutrophils to LPS results in an increase in TREM-1 expression within 2 hr after LPS stimulation (Chapter III, Figure 5).The discrepancy between our in vitro and in vivo data regarding TREM-1 expression could be due to the following reasons: 1) Fast egress of immature neutrophils from the bone marrow after LPS administration might be responsible for the significantly lower TREM-1 MFI and lower percentage of TREM-1 positive cells at early time points; 2) In vivo, interaction of neutrophils with other cells (endothelial cells, monocytes etc.) might signal cleavage of TREM-1 or signal production of TREM-1splice variant; 3) Effect of cytokines might be responsible for the initial decrease and subsequent up-regulation of TREM-1 MFI over time.

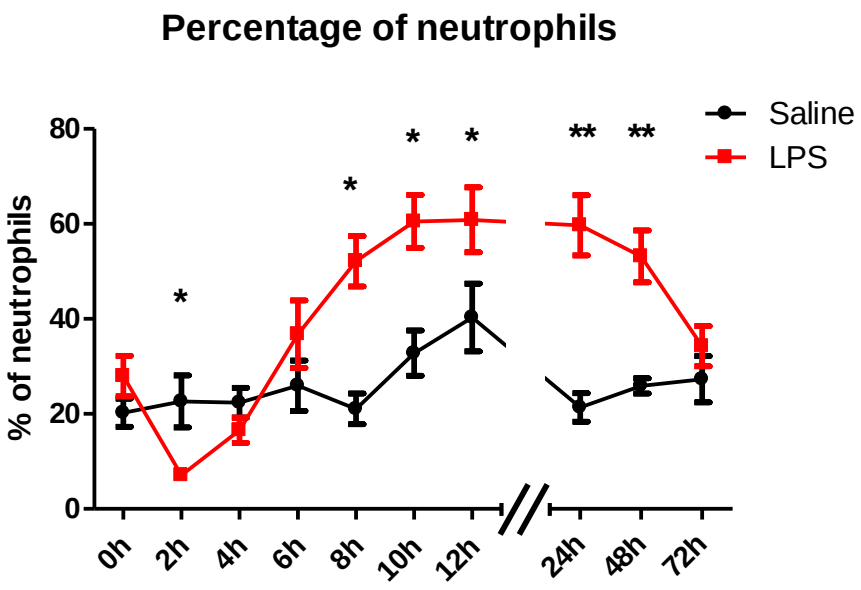

Figure 1. Neutrophil percentage over time in peripheral blood of dogs with induced endotoxemia. Red line: dogs treated with LPS to induce endotoxemia; black line: dogs treated with saline as control.

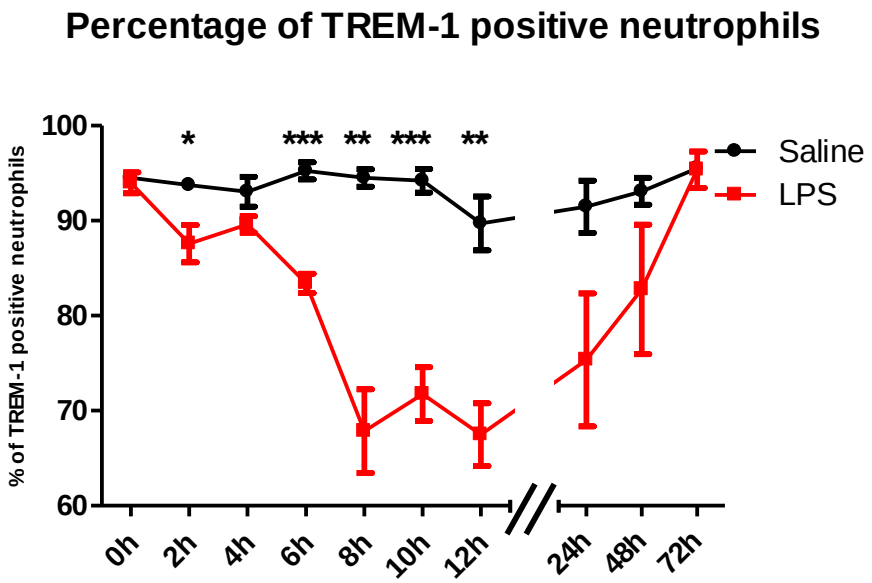

Figure 2. Percentage of TREM-1 positive neutrophils over time in dogs with induced endotoxemia. Red line: dogs treated with LPS to induce endotoxemia; black line: dogs treated with saline as control.

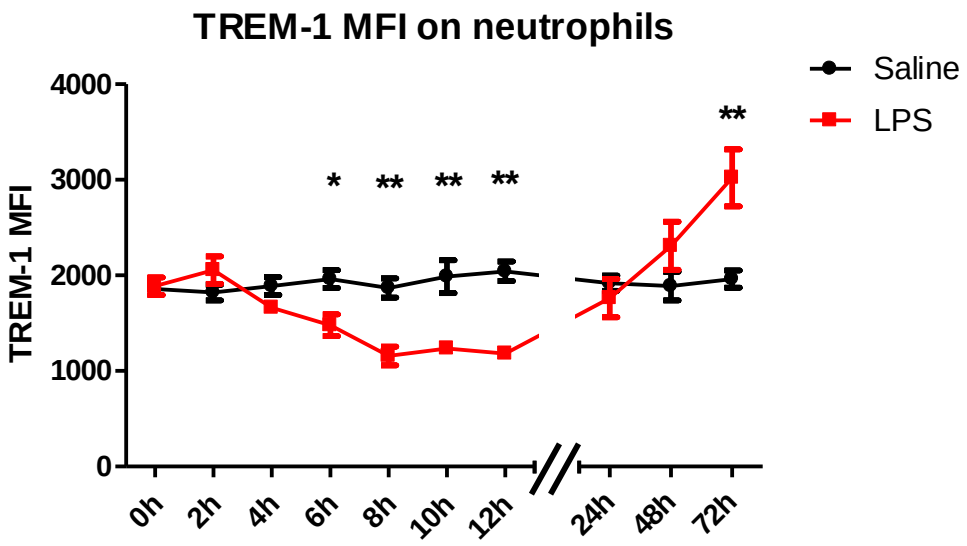

Figure 3. Relative density of TREM-1 expression on neutrophils over time in dogs with induced endotoxemia. Red line: dogs treated with LPS to induce endotoxemia; black line: dogs treated with saline as control.