Kinetics of the inflammatory response during experimental *Babesia rossi* infection of beagle dogs

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Highlights

- Experimental *Babesia rossi* infection using a cryopreserved wild-type strain.
- Markers of inflammation including cytokines, C-reactive protein, and leukocytes.
- Progression of inflammation during infection and after treatment.
- Influence of infectious dose on progression of clinical disease and inflammation.

Abstract

Babesia rossi causes severe morbidity and mortality in dogs in sub-Saharan Africa, and the complications associated with this disease are likely caused by an unfocused, excessive inflammatory response. During this experimental *B. rossi* study we investigated inflammatory marker and cytokine kinetics during infection and after treatment. We aimed to determine whether infectious dose and treatment would influence the progression of the inflammatory response and clinical disease. Six healthy male beagle dogs formed the study population, one was used to raise the infectious inoculum, three were administered a high *B. rossi* infectious

dose (HD group) and two a low infectious dose (LD group). Clinical examination, complete blood count (CBC) and C-reactive protein (CRP) were determined daily. Cytokines were quantified on stored plasma collected during the study, using a canine specific cytokine magnetic bead panel (Milliplex©). The experiment was terminated and treatment administered when predetermined experimental or humane endpoints were reached. Parasitemia occurred on day 1 and 3 in the HD and LD groups respectively. The rate of increase in parasitemia in the HD group was significantly faster than that seen in the LD group. Significant differences were found in heart rate, blood pressure, interferon gamma (INFγ), keratinocyte chemoattractant (KC), INFγ-induced protein 10 (IP10), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP1), tumor necrosis factor alpha (TNFα), interleukin 2 (IL-2), IL-6, IL-7, IL-8, IL-10 IL-15, IL-18, CRP, neutrophils and monocytes between groups at multiple time points during the course of the infection. Our findings suggest that the initiation of inflammation occurs before the onset of clinical disease in *B. rossi* infection and infectious dose influences the onset of the inflammatory response. Treatment enhances the inflammatory response in the immediate post-treatment period which may contribute to disease associated complications. Finally, we found that there is an imbalance in pro/anti-inflammatory cytokine concentrations during infection which may promote parasite replication.

Keywords: Babesia rossi; Markers of inflammation; Cytokines; Influence of treatment; Inoculum dose

1. Introduction

Babesia rossi, a virulent *Babesia* species, causes a severe form of babesiosis in the domestic dog associated with a high rate of morbidity and mortality (Leisewitz et al., 2019b; Penzhorn, 2011; Schoeman, 2009). Babesiosis is a complex multi-systemic disease that can be classified as either uncomplicated or complicated (Jacobson, 2006; Jacobson and Clark, 1994; Leisewitz et al., 2019b). Complicated babesiosis occurs when the pathology noted cannot be attributed purely to the anemia or when the anemia becomes severe enough to perpetuate organ dysfunction (Leisewitz et al., 2019b). *B. rossi* infection, like *Plasmodium falciparum* malaria in humans, results in a protozoal sepsis with a severe systemic inflammatory response (Bone et al., 1992; Clark et al., 2006; Jacobson et al., 2002). The concept of a 'cytokine storm' is well established in human inflammatory and infectious conditions, such as malaria and sepsis (Clark, 2007). This theory proposes that systemic illness and the course of disease is not solely caused by the microbes themselves but is also the result an unbalanced cytokine response to microbe antigens (Clark et al., 1997). The disease course seen in *B. rossi* infections bears a striking resemblance to that seen in falciparum malaria, leading one to hypothesize that a similar 'cytokine storm' may be an essential mechanism in the pathogenesis of this disease (Clark and Jacobson, 1998; Reyers et al., 1998). It is clear that *B. rossi* initiates a marked inflammatory response characterized by increased circulating markers such as C- reactive protein (CRP) and cytokines including monocyte-chemotactic protein-1 (MCP-1), interleukin (IL)-2, IL-6, IL-10, IL-18 and tumor necrosis factor alpha (TNFα) (Goddard et al., 2016; Koster et al., 2009; Leisewitz et al., 2019a). Complications associated with this disease are likely the result of an unbalanced inflammatory cytokine response (Brown et al., 2015; Galán et al., 2018; Goddard et al., 2016; Leisewitz et al., 2019a; Zygner et al., 2014). Additional clinical and

systemic indicators of inflammation, used to monitor affected dogs, which are associated with poor outcome in *B. rossi* infections include increased band neutrophil count, clinical collapse, presence of cerebral neurological signs and high parasitaemia (Bohm et al., 2006; Leisewitz et al., 2019a; Leisewitz et al., 2019b).

Although we have some understanding of the inflammatory response triggered by *B. rossi*, all the existing research was performed in natural infections in dogs of various breeds, presented at variable disease stages and severity. In this prospective longitudinal experimental study, we aimed to investigate changes in markers of inflammation (cytokine concentrations, neutrophil count, monocyte count and CRP) and indicators of disease severity (including habitus, appetite, vital parameters, parasitaemia and hematocrit) over time in an experimental *B. rossi* infection of beagle dogs. We also aimed to investigate the influence infectious dose and treatment would have on disease progression. Finally, we wanted to identify if any significant correlations existed among the markers of inflammation and indicators of disease severity. We hypothesized that *B. rossi* infection would initiate a pronounced, unbalanced inflammatory response and that infectious dose as well as treatment would influence disease progression.

2. Materials and methods

2.1. Animals

This prospective longitudinal experimental study included six 6-month-old purpose bred sterilized male beagle dogs. The dogs were obtained from a commercial breeder (StudVet Beagles, RSA) and microchipped to allow for accurate identification. Vaccination and

deworming programs were current. All dogs were clinically healthy and free of regional tick-borne diseases at the start of the experimental study, confirmed by hematology, serum biochemistry and polymerase chain reaction and reverse line blotting (PCR-RLB) for *Babesia, Ehrlichia, Theileria* and *Anaplasma*. One dog was splenectomized and used to raise a viable parasite inoculum from cryopreserved wild type *B. rossi*. The remaining five dogs were randomly assigned to one of two groups, namely the 3 dogs in the high dose (HD) and 2 dogs in the low dose (LD) groups, and experimentally infected with the corresponding *B. rossi* parasite inoculum dose. The mean of samples collected at two separate time points, 4 and 2 weeks prior to inoculation, from each of the 5 remaining experimental dogs formed the baseline data against which changes were compared overtime. Samples from the dog selected for the splenectomy were not included in the baseline data set. This experimental study was approved by the Animal Ethics Committee of the Faculty of Veterinary Science at the University of Pretoria (REC048-19).

2.2. Study design

A dose of the stored wild-type inoculum was thawed and administered to the splenectomised dog with a second dose repeated 24 hours later. On the third day after the initial inoculation, viable *B. rossi* parasites were noted within the central venous blood smear of the splenectomised dog. Four days after the initial inoculum was administered, there were sufficient parasites in the central venous blood for collection and creation of the experimental infectious doses. The experimental infection was continued from Day 0 (day of infection), for 96 hours in the HD group and 108 hours in the LD, to Day 4 in both groups. The dogs were followed for a further 4 days after receiving treatment, to Day 8 (192 hours).

2.3. Preparation of the *Babesia rossi* inoculum and initiation of the infection

One randomly selected dog was splenectomized by a specialist surgeon and allowed 4 weeks recovery time after the surgery. The cryopreservate was created using blood from a dog naturally infected with *B. rossi* which was tested, and found to be negative for other blood-borne parasites using PCR-RLB and stored at -80°C. The cryopreserved *B. rossi* did not undergo any passages and was the original parasite collected from the naturally infected host, which was labelled for identification with the details of the host animal and date, tested for regional blood borne parasites and then stored until use for the experimental study. The splenectomized dog was then injected with 2 mL of thawed *B. rossi* cryopreservate intravenously, followed by a further 2 mL 24 hours later. Parasitemia was determined 12-hourly using a previously described technique, starting one day post-inoculation (Bohm et al., 2006). Parasitemias were all determined on central venous blood. Once a parasitemia was detected and quantified, citrated whole blood was collected from the splenectomized dog. Using culture media (Culture Media RPMI 1640, Hepes, filtered water, sodium bicarbonate, sodium pyruvate and gentamycin), the blood sample was serially diluted to obtain the two inoculum doses, 10^8 and 10^4 parasitized red blood cells for the high and low doses respectively. The dogs in the HD and LD groups were then inoculated intravenously with the respective doses.

2.4. Chemotherapeutic intervention and blood transfusions

The infection was allowed to run its course until one of the following endpoints were identified: hematocrit <15%, collapsed habitus, nervous signs (such as seizure activity), clinical evidence of lung pathology with arterial blood gas evidence of acute respiratory distress

syndrome (arterial partial pressure of oxygen [PaO2] <60mmHg), serum creatinine > 200mmol/L (normal <140 mmol/L) and hemoconcentration (PCV >55%). The infection ran its course for 4 days prior to intervention. The HD group was treated on day 4 in the morning (at 96 hours). Due to the unexpected death of one dog in the HD group, to avoid any further losses, the LD group was treated 12 hours later (at 108 hours) even though they had not reached the same degree of disease severity as the HD group. All dogs were drug cured with diminazene aceturate (3.5 mg/Kg subcutaneously). All three dogs in the HD group received a blood transfusion. The dog that died received blood on Day 4 immediately after sample collection (96 hours) and chemotherapy. One dog received a blood transfusion on Day 5 and the other on Day 6, after sample collection at 120 and 144 hours respectively. Additional supportive treatment was provided as needed. The remaining 5 dogs (including the splenectomized dog) recovered completely and were rehomed as pets.

2.5. Daily monitoring

All dogs were examined by a veterinarian once daily from the day of inoculation until the onset of clinical signs and thereafter as frequently as was deemed necessary to ensure adequate care until recovery. Habitus, appetite, temperature, heart rate, respiratory rate, mucous membrane color and blood pressure (using a non-invasive oscillometric technique – Vet-HDO® Monitor) were determined daily, at the same time each morning. Blood pressure was measured on the tail of each dog, whilst lying in lateral recumbency. All dogs were thoroughly acclimated to this process prior to initiation of the experimental study to reduce stress associated increases in blood pressure during handling and sample collection.

2.6. Hematology and biochemistry

Blood was collected atraumatically from the jugular vein into EDTA Vacutainer Brand Tubes (Beckton Dickinson Vacutainer Systems, UK) for a daily CBC (ADVIA 2120i, Siemens, Germany) and the EDTA plasma was then stored at -80°C. Blood samples were collected into serum Vacutainer brand tubes (Beckton Dickinson Vacutainer Systems, UK) every second day for CRP measurements. The CRP was analyzed using canine specific immunoturbidimetric CRP method[h] (Gentian, Norway) run on the Cobas Integra 400 plus (Roche, Switzerland).

2.7. Cytokine analysis

Once the experiment was concluded, the stored batched EDTA plasma samples were thawed at room temperature and used to determine granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (INFγ), IL-2, IL-6, IL-7, IL-8, IL-15, IL-10, IL-18, TNF-a, INFγ-induced protein 10 (IP-10), keratinocyte chemoattractant (KC-like) and MCP-1. Concentrations were determined in duplicate by fluorescent-coded magnetic beads (MagPlex-C; MILLIPLEX. MAP Kit, Canine Cytokine Magnetic Bead Panel, 96-Well Plate Assay, CCYTO-90K, Millipore, Billerica, MA), based on the Luminex xMAP technology (Luminex 200, Luminex Corporation, Austin, TX). Two quality controls were included in the plate as internal quality controls. The assay was performed according to the manufacturer's instructions. Cytokine concentrations were determined by comparing the optical density of the samples to the standard curves, produced from standards run on the same plate. The minimum detectable concentrations of the cytokines provided by the manufacturer were regarded as the detection limits in this study. Measurable values below the detection limit were assigned a value equal to the minimum detectable concentration for the respective cytokine and those

with no measurable values were set as zero.

2.8. Statistical analysis

For the statistical analysis, variables that were shown to be non-normally distributed, were log-transformed; these were parasitemia, the leukocyte counts, CRP, GM-CSF, IFNγ, KC-like, all the interleukins, MCP-1 and TNFα. The other variables were not transformed. The means of the variables were then compared between the HD group and the LD group at each time point as well as between each time point and the mean baseline value within each group using linear mixed models, with animal identity as a random effect and the Bonferroni adjustment for multiple comparisons was applied. Pairwise correlations between variables were assessed using Spearman's rank correlation. Significance was assessed at $P<0.05$. Statistical analysis was done using Stata 15 (StataCorp, College Station, TX, U.S.A.). Significant values in the text will be presented as the mean followed by the range and P value. Graphical presentation of some variables are included with error bars representing the standard deviation.

3. Results

The demographic characteristics of the experimental group of dogs were as follows: All dogs were 6-month-old sterilized male beagle dogs. All 6 dogs tested negative for *Babesia, Ehrlichia, Anaplasma* and *Theileria* based on PCR-RLB done prior to the initiation of the experimental study. No significant difference was noted between the LD group and HD group for baseline data for any variable.

3.1. Clinical variables

The HD group demonstrated changes in the clinical variables including habitus, appetite, temperature, heart rate and respiratory rate between 36 to 48 hours earlier than the LD group, indicating a more rapid onset of clinical disease in this group. Increases in diastolic (76 mmHg, range 74 – 77 vs baseline: 65 mmHg, range 63 – 67; $p = 0.013$), systolic (156 mmHg, range 145 – 161 vs baseline: 124 mmHg, range 122 – 125; $p < 0.001$) and mean arterial pressures (105 mmHg, range 101 – 110 vs baseline: 86 mmHg, range 74 – 87; $p < 0.001$) above baseline were noted in the HD group at 72 hours.

3.2. Clinicopathological variables

The HD group had a detectable parasitemia 48 hours earlier than the LD group (Fig 1). There was a rapid increase in parasitemia thereafter, peaking at 46.76% (range 34.95 – 59.8) at 96 hours in the HD group and 5.76% (range 4.71 – 6.81) at 108 hours in the LD group. Parasitemia was strongly correlated to KC-like ($r = 0.888$, $p < 0.001$), IL-10 ($r = 0.676$, $p = 0.009$) and mature neutrophil count ($r = -0.674$, $p < 0.001$). Hemoglobinemia was visibly present from 72 hours in the HD group. Hematocrit (Hct) (Fig 2) declined significantly compared to baseline at 96 hours ($p < 0.001$) in the HD group and 120 hours ($p = 0.003$) in the LD group. Both groups demonstrated a progressive decrease in Hct after treatment.

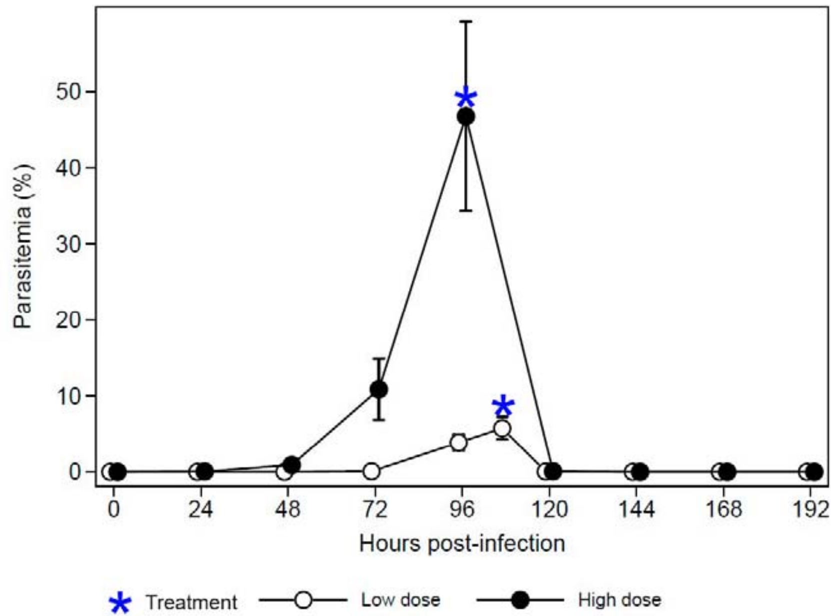

Fig 1. Parasitemia from inoculation of *B. rossi* until 4 days after treatment (Error bars represent there SD)

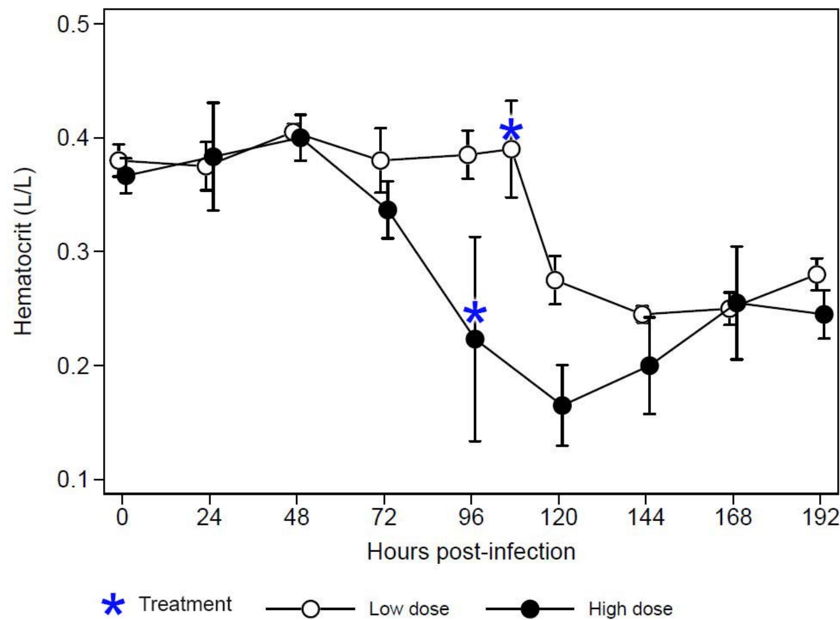

Fig 2. Hematocrit during *B. rossi* infection and after treatment (Error bars represent there SD)

For the HD group, increases in CRP concentrations (Fig 3) above baseline (14.33 mg/L, 10 – 21.5) peaked at 72 hours (150 mg/L, 135 – 163, $p < 0.001$) and remained significantly

increased at 96 hours (125 mg/L, 92 – 160, $p < 0.001$), 120 hours (81.5 mg/L, 81 – 82, $p < 0.001$) and 144 hours (59 mg/L, 54 – 64, $p < 0.001$). The LD group showed a marked increase in CRP above baseline (25 mg/L, 10 – 40) at 108 hours (175 mg/L, 160 – 197, $p < 0.001$), declining thereafter but remaining significantly increased for the remainder of the study. The CRP concentrations peaked 36-hours earlier in the HD group. C-reactive protein was significantly correlated with temperature ($r = 0.722$, $p = 0.003$) and the correlation between CRP and parasitemia approached significance ($r = 0.646$, $p = 0.056$). A significant decrease in mature neutrophil (Fig 4) count was seen from 72 hours (1.79 X 10^9/L, 1.36 – 2.44, $p < 0.001$) in HD group and 108 hours in the LD group (1.49 X 10^9/L, 1.17 – 1.81, $p < 0.001$). The neutrophil nadirs for the HD and LD groups were seen at 96 (1.57 X 10^9/L, 1.12 – 1.88, $p < 0.001$) and 108 hours (1.49 X 10^9/L, 1.17 – 1.81, $p < 0.001$) respectively. In the HD group there was a marked increase in the mature neutrophil counts after treatment, exceeding laboratory reference intervals (3 – 11.5 X 10^9/L) at 168 (17.64 X 10^9/L, 12.21 – 23.07, $p < 0.001$) and 192 hours (27.35 X 10^9/L, 23.75 – 30.94, $p < 0.001$). After treatment there was a gradual recovery of the mature neutrophil count in the LD group, returning to within laboratory reference intervals at 192 hours. A significant increase in band neutrophil counts (Fig 4) above baseline (0.14 X 10^9/L, 0.13 – 0.17) was seen in the HD group at 120 hours (1.81 X 10^9/L, 0.88 – 2.74, $p < 0.001$), 168 hours (2.77 X 10^9/L, 2.28 – 3.25, $p < 0.001$) and 192 hours (6.45 X 10^9/L, 4.32 – 8.57, $p < 0.001$). Band neutrophils counts exceeded the laboratory reference interval (0 – 0.5 x 10^9/L) consistently after treatment in the HD group. At no point during the study did the band neutrophil count in the LD group increase significantly above baseline values or exceed the laboratory reference interval. A significant reduction in monocyte count compared to baseline (0.52 X 10^9/L, 0.46 – 0.65) was seen in the HD group at 24 (0.28 X 10^9/L, 0.2 – 0.32, $p = 0.044$) and 48 hours (0.27 X 10^9/L, 0.2 – 0.34, $p = 0.024$). Following treatment, the monocyte

counts were increased at 144 (1.86×10^9/L, 1.27 – 2.45, $p < 0.001$), 168 (3.13×10^9/L, 2.69 – 3.57, $p < 0.001$) and 192 hours (3.04×10^9/L, 1.8 – 4.28, $p < 0.001$) in the HD group, exceeding the laboratory reference interval ($0.15 – 1.35 \times 10^9$/L) from 144 hours onwards.

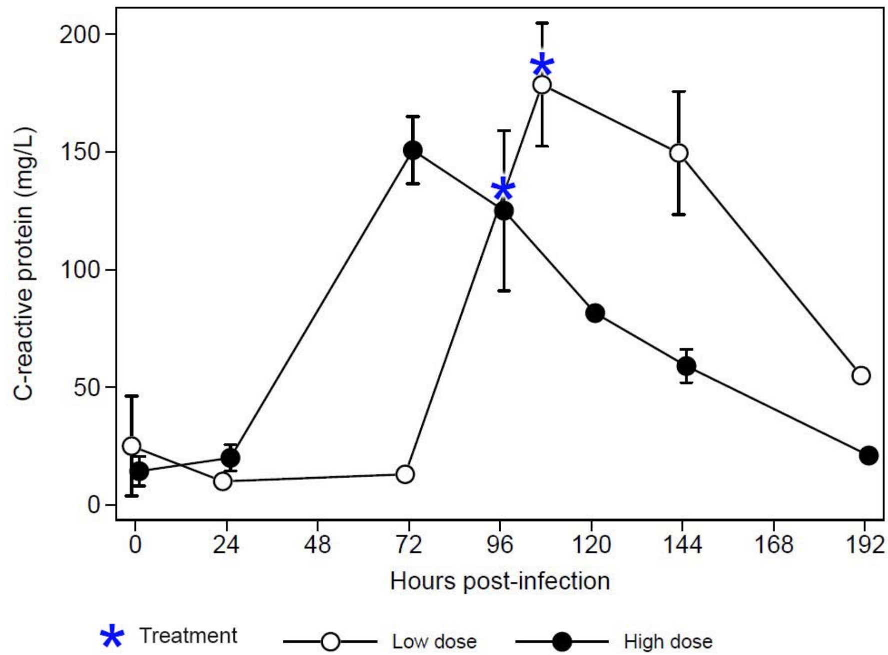

Fig 3. C-reactive protein concentrations during *B. rossi* infection and after treatment

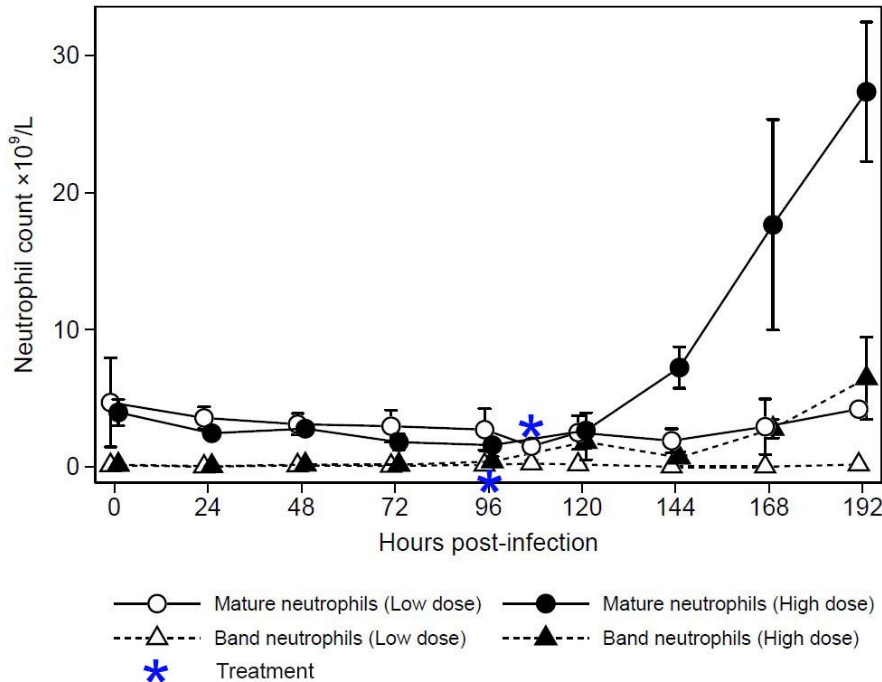

Fig 4. Mature and band neutrophil counts during *B. rossi* infection and after treatment

3.3. Cytokine kinetics

Thirteen cytokines were evaluated, and the results were divided into 4 groups by pattern of change.

3.3.1. Cytokines that increased during infection and decreased after treatment

The cytokines which fall into this category included IFNγ and KC-like (Table 1). The HD group had a significant increase in IFNγ concentrations (Fig 5) above baseline ($p = 0.002$) and above the LD group ($p < 0.001$) at 48 hours. The LD group had peak concentrations 48-hours later, at 96 hours ($p < 0.001$). There was a progressive and significant increase in KC-like concentrations (Fig 6) in the HD group above baseline at 24 hours ($p < 0.001$), 48 hours ($p < 0.001$), 72 hours ($p < 0.001$) and 96 hours ($p < 0.001$) declining significantly at 144 hours ($p =$

0.004) and 192 hours (p < 0.001). The LD group only had significant increase in KC-like concentrations above baseline at 96 hours (p < 0.001). Strong correlations were identified between KC-like and parasitaemia (r = 0.888, p < 0.001) as well as KC-like and mature neutrophil count (r = -0.817, p < 0.001).

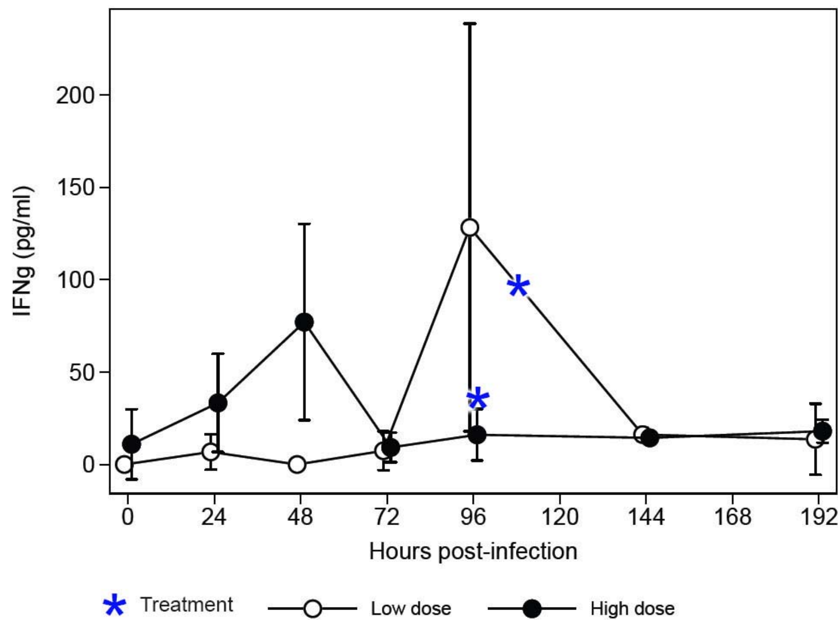

Fig 5. IFN γ concentrations during *B. rossi* infection and after treatment

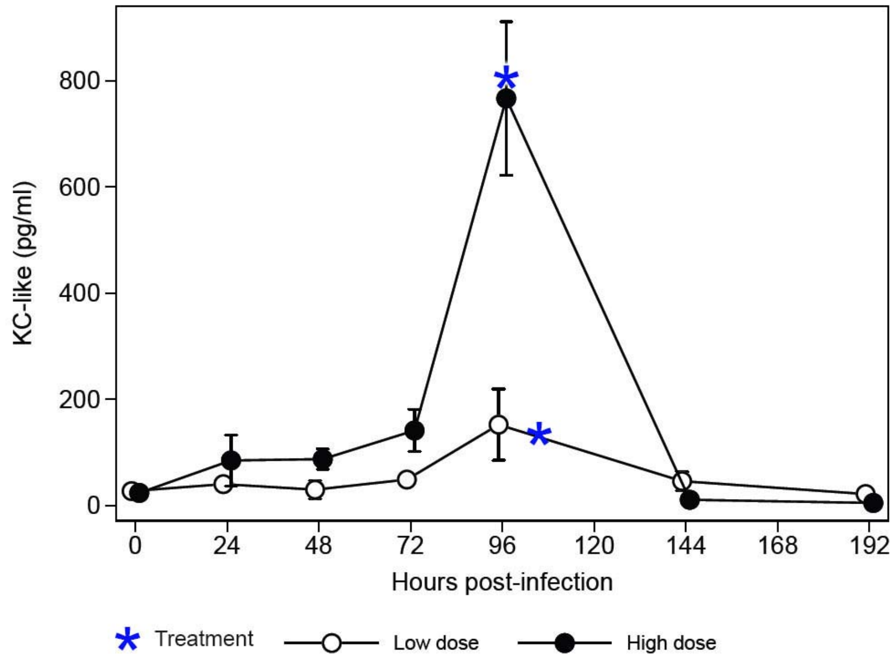

Fig 6. KC-like concentrations during *B. rossi* infection and after treatment

3.3.2. Cytokines that increased during infection and remained high after treatment

This category included the following cytokines MCP-1, IL-6, IL-8 and IL-10 (Table 1). The chemokine MCP-1 concentrations (Fig 7) increased above baseline in the HD group from 24 hours onwards reaching significance after treatment at 144 hours ($p < 0.001$) and 192 hours ($p = 0.001$). The LD group did not have significant increases in MCP-1 concentrations throughout the study but one dog in this group did demonstrate increased concentrations after treatment. Interleukin-6 concentrations (Fig 8) were moderately increased above baseline at 96 hours ($p = 0.049$) in the HD group and progressively increased after treatment, at 144 hours ($p < 0.001$) and 192 hours ($p < 0.001$). Like changes seen in MCP-1, one dog in the LD group had increased IL-6 at 192 hours but this did not reach significance. Interleukin-6 and MCP-1 were strongly correlated ($r = 0.792$, $p < 0.001$). The HD group had significantly reduced IL-8 concentrations (Fig 9) compared to baseline at 72 hours ($p = 0.004$), with a

marked increase at 96 hours ($p < 0.001$). The LD group only had significantly increased IL-8 concentrations at 192 hours ($p = 0.003$). Interleukin-10 concentrations (Fig 10) increased significantly above baseline at 24 hours ($p = 0.019$), 72 hours ($p < 0.001$) and 96 hours ($p < 0.001$) in the HD group. The LD group showed no significant increase in IL-10 but both dogs demonstrated a progressive increase in concentrations from 72 hours after inoculation until treatment, remaining increased in one dog after treatment. A strong positive correlation was identified between parasitaemia and IL-10 ($r = 0.674$, $p = 0.009$) as well as between IL-10 and MCP-1 ($r = 0.828$, $p < 0.001$). Similar kinetic profiles were seen between the HD and LD groups for MCP-1 and IL-10, varying in onset but not necessarily severity. IL-8 and IL-6 concentrations however appeared to follow different kinetic pathways between the HD and LD groups with the LD group demonstrating higher concentrations for the first 72 hours after inoculation. Interestingly the dog that died in the HD group had considerably higher concentrations of MCP-1 and IL-6 than any other dog at 96 hours.

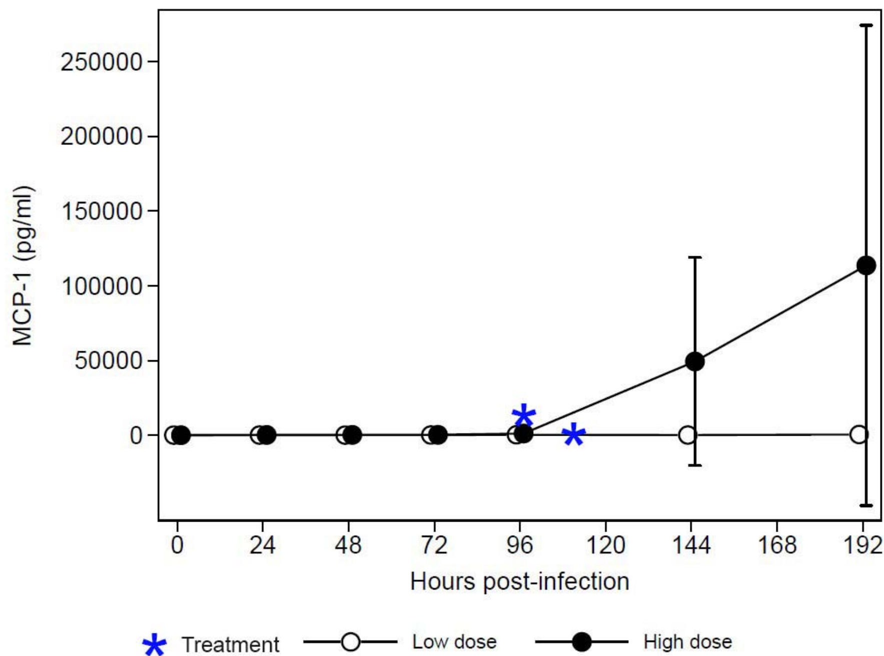

Fig 7. MCP-1 concentrations during *B. rossi* infection and after treatment

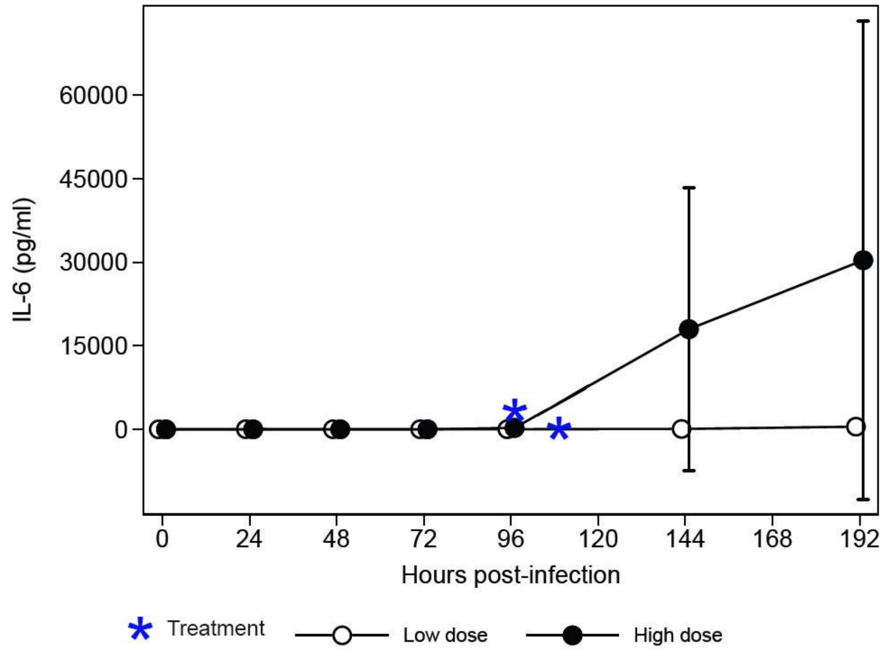

Fig 8. IL-6 concentrations during *B. rossi* infection and after treatment

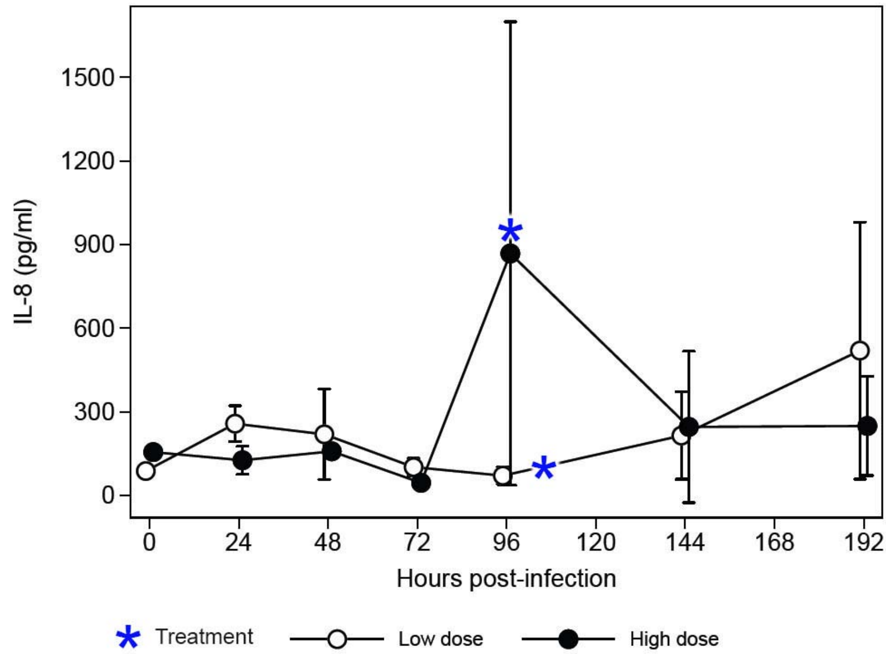

Fig 9. IL-8 concentrations during *B. rossi* infection and after treatment

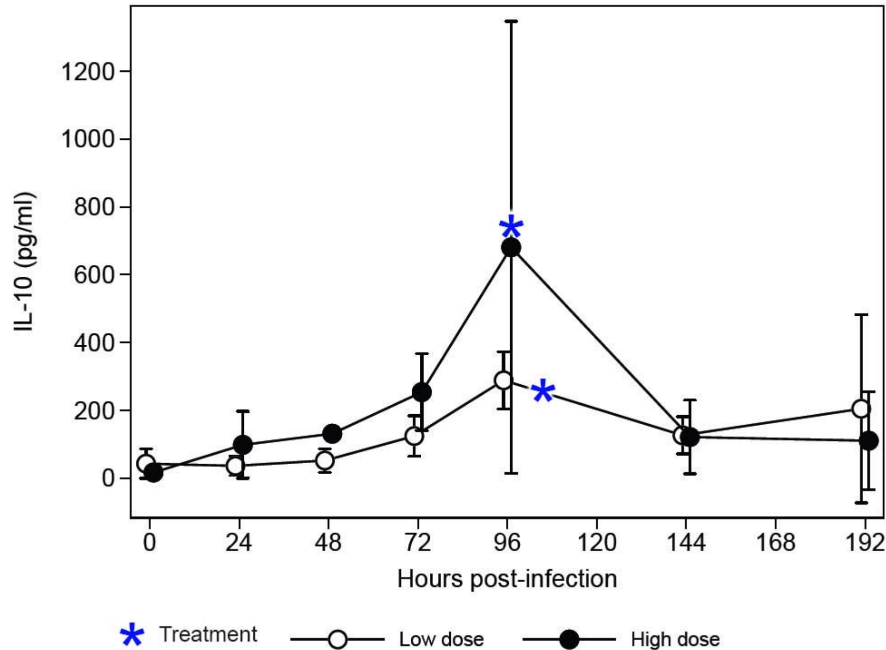

Fig 10. IL-10 concentrations during *B. rossi* infection and after treatment

3.3.3. Cytokines that increased after treatment

The next category of cytokines, GM-CSF (Fig 11), TNFα (Fig 12), IL-2 (Fig 13) and IL-7 (Fig 14), had very similar patterns of change and were all markedly increased after treatment in the HD group, particularly in one dog (Table 1). Significant increases in these cytokines were seen after treatment, at 144 (GM-CSF $p < 0.001$; TNFα $p < 0.001$; IL-2 $p < 0.001$ and IL-7 $p = 0.002$) and 192 hours (GM-CSF $p < 0.001$; TNFα $p < 0.001$; IL-2 $p < 0.001$ and IL-7 $p = 0.004$). Although not statistically significant, one dog from the LD group showed a similar profile after treatment, with marked increases in all 4 cytokines although not to the same degree as seen in the HD group. The dogs with the highest parasitemia in each group demonstrated the greatest increases in cytokine concentrations after treatment. Tumour necrosis factor alpha demonstrated strong correlations with IL-6 ($r = 0.925$, $p < 0.001$), GM-CSF ($r = 0.811$, $p <$

0.001), IL-2 ($r = 0.810$, $p < 0.001$) as well as IL-7 ($r = 0.810$, $p < 0.001$). Interleukin 2 and IL-7 were also strong correlated ($r = 0.872$, $p < 0.001$).

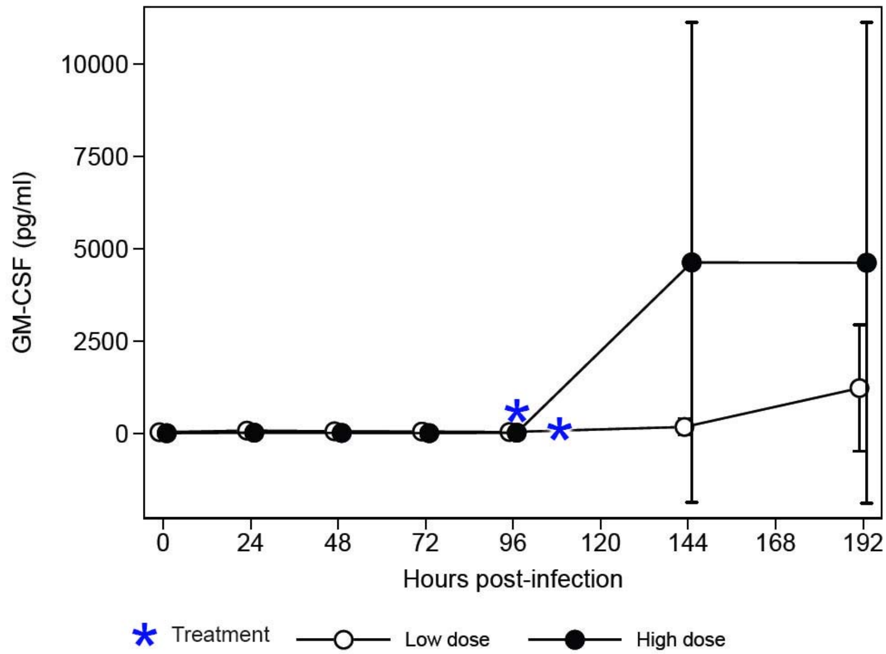

Fig 11. GM-CSF concentrations during *B. rossi* infection and after treatment

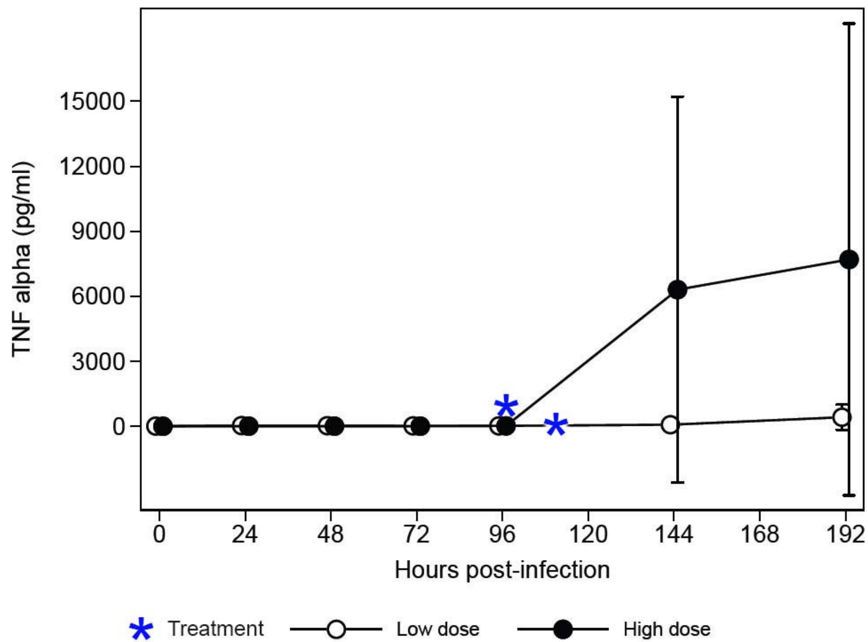

Fig 12. TNFα concentrations during *B. rossi* infection and after treatment

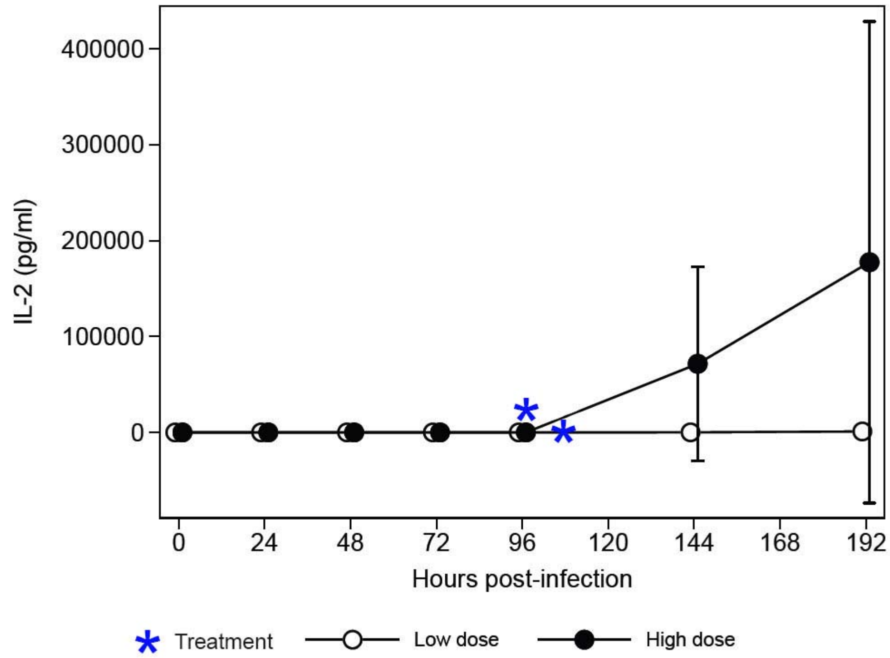

Fig 13. IL-2 concentrations during *B. rossi* infection and after treatment

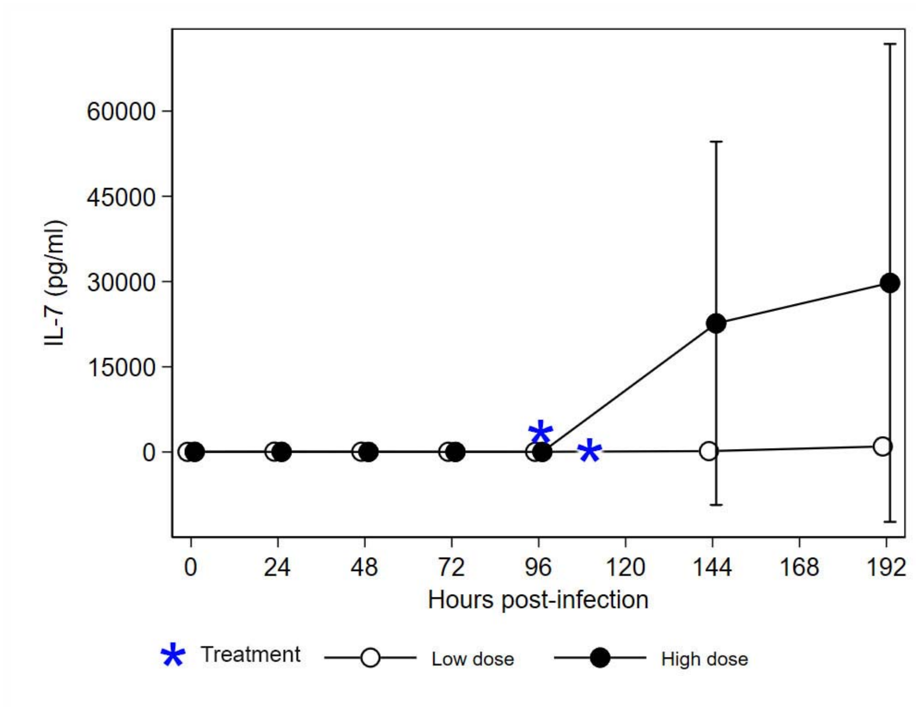

Fig 14. IL-7 concentrations during B. rossi infection and after treatment

3.3.4. Cytokines that showed no distinct pattern of change

The last three cytokines, IL-15, IL-18 and IP-10 showed minor changes in their concentrations during the course of the experiment. IL-15 (LD: 3043.38 pg/mL, 64 – 6022.76 vs HD: 23.36 pg/mL, 0 – 46.76, $p < 0.001$) and IL-18 (LD: 792.75 pg/mL, 18.86 – 1566.64 vs HD: 7.59 pg/mL, 0 – 15.17, $p = 0.016$) concentrations were significantly increased at 192 hours in the LD group. Interleukin 15 and IL-18 were strongly correlated ($r = 0.981$, $p < 0.001$). Finally, IP-10 showed mild increases in both groups during the study period.

Table 1: Cytokine concentrations during *B. rossi* infection and after treatment

	Baseline	24 hours	48 hours	72 hours	96 hours Treatment: HD 96 hours LD 108 hours	144 hours	192 hours
Cytokines that increased during infection and decreased after treatment							
IFNγ:	**Units pg/mL**						
LD mean (Range)	0	6.8 (0–13.6)	0	7.42 (0–14.83)	128.3 (50.28–206.32)	16.2 (13.6–18.79)	13.62 (0–27.24)
HD mean (Range)	10.96 (0–32.885)	33.32 (13.6–63.54)	77.08 (19.48–124.28)	9.22 (0–14.07)	16.07 (0–24.11)	14.4 (13.6–15.2)	18.05 (13.6–22.49)
P value: LD vs HD	1.000	0.271	<0.001*	1.000	0.071	1.000	1.000
LD vs Base	NA	1.000	1.000	1.000	0.000*	0.040*	1.000
HD vs Base	NA	0.073	0.002	1.000	1.000	0.815	0.503
KC-like:	**Units pg/mL**						
LD mean (Range)	27.36 (19.58–35.14)	40.45 (36.18–44.71)	30.07 (17.88–42.25)	49.05 (44.6–53.49)	152.37 (104.96–199.77)	46.09 (33.52–58.66)	21.83 (16.14–27.52)
HD mean (Range)	23.87 (18.19–31.38)	84.97 (52–140.06)	87.58 (67.16–104.96)	141.4 (103.74–183.18)	766.48 (625.08–913.78)	11.01 (8.13–13.89)	5.02 (4.2–5.84)
P value: LD vs HD	1.000	0.050*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
LD vs Base	NA	0.763	1.000	0.084	<0.001*	0.273	1.000
HD vs Base	NA	<0.001*	<0.001*	<0.001*	<0.001*	0.004*	<0.001*
Cytokines that increased during infection and remained high after treatment							
MCP-1:	**Units pg/mL**						
LD mean (Range)	156.19 (123.49–188.9)	174.05 (103.86–244.23)	144.31 (56.05–232.56)	212.77 (196.48–229.06)	360.59 (253.52–467.65)	195.97 (98.85–293.09)	541.64 (70.61–1012.66)
HD mean (Range)	67.1 (64.67–68.86)	244.06 (225.92–264.24)	282.53 (241.14–321.89)	336.13 (258.86–404.03)	1217.07 (523.22–2271.2)	49402.62 (201.93–98603.3)	113660 (74.19–113660)
P value: LD vs HD	0.335	1.000	1.000	1.000	1.000	0.078	0.232
LD vs Base	NA	1.000	1.000	1.000	1.000	1.000	1.000

HD vs Base	NA	1.000	1.000	1.000	0.050	<0.001*	0.001*
IL-6:	**Units pg/mL**						
LD mean (Range)	20.42 (18.49–22.36)	51.21 (17.32–85.09)	43.89 (7.14–80.63)	39.69 (14.27–65.1)	39.16 (15.81–62.51)	93.75 (16.32–171.18)	500.38 (13.06–987.69)
HD mean (Range)	8.95 (4.09–15.29)	18.88 (8.5–29.58)	24.33 (14.96–34.98)	22.49 (20.46–25.9)	280.67 (40.22–644.35)	17999.95 (39.47– 5960.43)	30369.21 (10.56–60727.85)
P value: LD vs HD	1.000	1.000	1.000	1.000	1.000	0.226	1.000
LD vs Base	NA	1.000	1.000	1.000	1.000	1.000	1.000
HD vs Base	NA	1.000	1.000	1.000	0.049*	<0.001*	<0.001*
IL-8:	**Units pg/mL**						
LD mean (Range)	87.02 (71.8–102.25)	256.96 (211.61–302.31)	218.71 (103.39–334.03)	101.11 (78.62–123.59)	70.27 (48.04–92.5)	214.17 (103.39–324.95)	519.26 (193.33 – 845.19)
HD mean (Range)	154.91 (139.24–165.98)	125.58 (76.02–176)	157.68 (144.41–164.83)	44.78 (37.03–55.44)	867.64 (271.26–1817.67)	245.34 (53.25–437.43)	248.71 (123.03 – 374.39)
P value: LD vs HD	1.000	0.650	1.000	0.522	<0.001*	1.000	1.000
LD vs Base	NA	0.134	0.836	1.000	1.000	0.897	0.003*
HD vs Base	NA	1.000	1.000	0.004*	<0.001*	1.000	1.000
IL-10:	**Units pg/mL**						
LD mean (Range)	42.65 (12.1–73.19)	36.2 (16.1–56.29)	51.85 (27.29– 6.41)	124.03 (81.89–166.16)	288.42 (229.09–347.74)	126.24 (87.43–165.05)	204.61 (8.5–400.72)
HD mean (Range)	16.57 (16.05–23.34)	98.13 (36.57–211.81)	131.01 (109.22–147.73)	253.56 (182.15–384.13)	680.69 (150.44– 1429.07)	121.15 (44.27–198.02)	110.26 (8.5–212.01)
P value: LD vs HD	1.000	1.000	1.000	1.000	1.000	1.000	1.000
LD vs Base	NA	1.000	1.000	1.000	0.074	1.000	1.000
HD vs Base	NA	0.294	0.019*	<0.001*	<0.001*	0.207	1.000
Cytokines that increased after treatment							
GM-CSF:	**Units pg/mL**						
LD mean (Range)	35.07 (34.06–36.09)	78.89 (33.23–124.54)	60.68 (14.13–107.23)	53.87 (24.78–82.95)	39.78 (11.25–68.31)	177.71 (22.96–332.46)	1228.75 (18.95–2438.55)
HD mean (Range)	9.2 (9.2–9.2)	22.53 (0–58.38)	15.64 (0–37.71)	9.1 (0–27.3)	22.36 (11.25–32.07)	4629.59 (38.37–9220.8)	4620.24 (19.67–9220.8)

P value: LD vs HD **LD vs Base** **HD vs Base**	1.000 NA NA	0.847 1.000 1.000	1.000 1.000 1.000	0.337 1.000 1.000	1.000 1.000 1.000	1.000 1.000 <0.001*	1.000 0.788 <0.001*
TNFα:	**Units pg/mL**						
LD mean **(Range)**	12.6 (12.28–12.93)	36.38 (8.74–64.02)	32.15 (6.1–58.2)	24.58 (6.97–42.18)	23.34 (6.1–40.63)	76.88 (7.96–145.8)	422.07 (6.77–837.37)
HD mean **(Range)**	6.1 (6.1–6.1)	9.55 (6.1–14.19)	9.46 (6.1–12.61)	10.62 (6.1–13.72)	22.95 (13.08–37.97)	6306.60 (13.77–12599.51)	7699.37 (6.1–15392.63)
P value: LD vs HD **LD vs Base** **HD vs Base**	1.000 NA NA	1.000 1.000 1.000	1.000 1.000 1.000	1.000 1.000 1.000	1.000 1.000 0.637	0.652 1.000 <0.001*	1.000 1.000 <0.001*
IL-2:	**Units pg/mL**						
LD mean **(Range)**	21.24 (18.67–23.82)	65.94 (12.19–119.68)	53.62 (0–107.23)	41.11 (7.13–75.08)	31.68 (0–63.35)	135.89 (7.65 264.13)	1052.81 (3.5–2102.12)
HD mean **(Range)**	7.64 (0–19.42)	20.34 (0–37.66)	14.43 (3.5–29.09)	5.44 (0–12.81)	5.48 (3.5–9.44)	71563.81 (29.33–143098.3)	177399 (7.91–354790.1)
P value: LD vs HD **LD vs Base** **HD vs Base**	1.000 NA NA	1.000 1.000 1.000	1.000 1.000 1.000	1.000 1.000 1.000	1.000 1.000 1.000	0.329 1.000 <0.001*	0.801 1.000 <0.001*
IL-7:	**Units pg/mL**						
LD mean **(Range)**	20.23 (15.64–24.82)	55.79 (18.26–93.31)	45.83 (7.5–84.15)	38.08 (12.89–63.26)	29.12 (7.5–50.74)	148.96 (16.14–281.77)	967.68 (22.91–1912.44)
HD mean **(Range)**	20.92 (7.5–47.77)	20.03 (7.5–32.12)	12.4 (7.5–17.84)	7.84 (3.39–13.57)	15.28 (7.83–23.08)	22636.76 (23.42–45250.1)	29754.71 (11.77–59497.64)
P value: LD vs HD **LD vs Base** **HD vs Base**	1.000 NA NA	1.000 1.000 1.000	1.000 1.000 1.000	1.000 1.000 1.000	1.000 1.000 1.000	0.522 1.000 0.002*	1.000 0.769 0.004*
Cytokines that showed no distinct pattern of change							
IL-15:	**Units pg/mL**						
LD mean **(Range)**	63.73 (49.71–77.75)	221.32 (68.19–374.45)	187.07 (21.42–352.71)	142.83 (45.11–240.55)	120.62 (23.29–217.95)	529.53 (63.16–995.9)	3043.38 (64–6022.76)

HD mean (Range)	22.61 (13.86–28.73)	51.02 (10.32–116.44)	37.7 (24.79–69.46)	20.94 (9–43.5)	31.26 (15.23 – 49.96)	64.28 (0–128.56)	23.36 (0–46.72)
P value: LD vs HD LD vs Base HD vs Base	1.000 NA NA	1.000 1.000 1.000	1.000 1.000 1.000	0.699 1.000 1.000	1.000 1.000 1.000	0.048* 1.000 1.000	<0.001* 0.272 1.000
IL-18:	**Units pg/mL**						
LD mean (Range)	20.18 (19.99–20.38)	54.65 (21.99–87.3)	42.66 (9.9–75.41)	31.22 (16.56–51.88)	28.95 (10.86–47.03)	140.81 (20.47–261.14)	792.75 (18.86–1566.64)
HD mean (Range)	9.42 (8.33–10.33)	20.6 (7.61–43.92)	14.6 (7.42–26.28)	9.34 (5.56–16.36)	11.43 (7.23–16.96)	87.87 (0–175.73)	7.59 (0–15.17)
P value: LD vs HD LD vs Base HD vs Base	1.000 NA NA	1.000 1.000 1.000	1.000 1.000 1.000	1.000 1.000 1.000	1.000 1.000 1.000	0.582 1.000 1.000	0.002* 0.268 1.000
IP-10:	**Units pg/mL**						
LD mean (Range)	1.84 (0–3.67)	1.94 (0–3.88)	2.34 (0–4.67)	5.16 (3.35–6.97)	16.78 (14.33–19.22)	11.63 (6.11–17.14)	14.59 (6.11–17.14)
HD mean (Range)	4.26 (0–12.77)	14.25 (10.68–17.51)	14.91 (13.66–15.81)	11.23 (10.96–11.52)	10.7 (8.65–13.07)	0	0
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	0.001* 1.000 <0.001*	0.001* 1.000 <0.001*	1.000 0.838 0.004*	1.000 0.002* 0.006*	<0.001* 0.032* 0.966	<0.001* 0.017* 0.966

* Significant difference when applying linear mixed models, $P<0.05$. IFNγ (interferon gamma), KC-like (keratinocyte chemoattractant), MCP-1 (Monocyte chemoattractant protein 1), IL (Interleukin), GM-CSF (granulocyte-macrophage colony-stimulating factor), TNFα (tumor necrosis factor alpha) and IP (interferon gamma-induced protein).

4. Discussion

Our study was the first to evaluate the kinetics of inflammatory markers over the course of infection and recovery in a canine model experimentally infected with *Babesia rossi*. The results of this study illustrate the key role cytokines play in initiating and perpetuating inflammation in this disease. Also demonstrating continuation of the inflammatory response despite treatment. In addition to these findings the influence of inoculum dose was demonstrated, with a high infectious dose leading to an earlier onset of disease development and resulted in a more fulminant form of the disease. All the findings agreed with our original hypotheses, providing some insights into the pathogenesis of this hemolytic disease and even shedding additional light on the influence of treatment on the progression of the inflammatory response.

A progressive decline in both habitus and appetite was associated with higher infectious dose, rising parasitemia and worsening disease, improving with resolution of inflammation. As such, these clinical parameters can act as good indicators of disease onset and resolution. There was a tendency for rectal temperature to increase with increasing parasitemia, but it did not reach significance which may have been a consequence the small sample size. The inoculum dose influenced the onset of changes in temperature, heart rate and respiratory rate in this study, with the HD group demonstrating increases 24 to 36 hours earlier than the LD group, similar to the findings of an experimental *B.canis* infection (Schetters et al., 2009a). In a recent study of dogs naturally infected with *B. rossi*, collapse and hypothermia were positively associated with an increased risk of death, and both were identified in the dog that died in our study (Leisewitz et al., 2019b). The blood pressure changes seen in our study differed from those seen during the *B canis* experimental infection, where the mean arterial blood

pressure declined progressively after inoculation (Schetters et al., 2009a). Mild hypotension was only identified in the one dog that died at 96 hours from the HD group. In previous studies hypotension worsened with disease severity (Jacobson et al., 2000; Schetters et al., 2009a) and the absence of hypotension in this study may be due to the short duration of the experimental infection before intervention.

Anemia is common in dogs infected with *B. rossi* and in a recent study up to 84% of dogs had hematocrits below the laboratory reference interval at presentation (Leisewitz et al., 2019b). Although anemia is not a reliable predictor of death, severe anemia does require treatment to avoid the systemic complications of hypoxia and even death (Leisewitz et al., 2019b). The HD group demonstrated a significant anemia at 96 hours after inoculation which worsened after treatment, requiring blood transfusions. The severity of the anemia after treatment in this group was likely underestimated as a result of this intervention. Although the decline in Hct was significant in the LD group after treatment, it only resulted in mild anemia in these dogs. The absence of anemia during active infection in the LD group was probably the result of insufficient time permitted for disease progression to reach the same disease severity as that seen in the HD group.

High parasitemia is positively associated with increased risk of complications and death in *B. rossi* infections (Bohm et al., 2006; Leisewitz et al., 2019a). As with previous studies on *B. rossi* infection, we demonstrated a progressive parasitemia which required chemotherapeutic intervention (Bohm et al., 2006; Schetters et al., 2009b). The infectious dose had a prominent impact on the progression of parasitemia with levels increasing at a significant rate in the HD group, up to 59%, within 4 days of inoculation. The LD group demonstrated a gradual rise in parasitemia, more closely mirroring natural infection. At these high parasitaemias the

immune system may be overwhelmed or even actively suppressed by the parasites. The concept of an ineffective immune response may be supported by the positive correlation identified between parasitemia and IL-10, a prominent anti-inflammatory cytokine. In *Leishamania* infections, parasites promote an immunosuppressive cytokine profile with high levels of IL-10, allowing for unrestricted replication (Morrot, 2020) and a similar interaction may take place in *B. rossi* infections. Immune dysregulation with concurrent hyperinflammation and immunosuppression is ubiquitous in human patients that are critically ill and its possible that a similar process may be present in *B. rossi* infections (Matthay et al., 2019; Mira et al., 2017; van der Poll et al., 2017). The negative correlation between parasitemia and mature neutrophil count may also point to an inefficient innate immune response to the *B. rossi* infection. In addition to low neutrophil numbers, a recent study on neutrophil function in *B. rossi* infections also identified an association between higher concentrations of neutrophil myeloperoxidase concentrations and poor prognosis suggesting possible diminished neutrophil burst function in the remaining neutrophils (Celliers et al., 2020). It has also been shown that *B. rossi* results in significant lymphopenia as a result of a loss of $CD3^+$, $CD4^+$, $CD8^+$ and $CD21^+$ phenotypes (Rautenbach et al., 2017). The degree of lymphocyte loss was also correlated to disease severity, and this may contribute to the state of immune dysfunction despite hyperinflammation (Rautenbach et al., 2017).

A mature neutropenia persisted until treatment in both groups, with counts increasing significantly thereafter in the HD group. Previous studies evaluating hematological changes in natural *B. rossi* infections found that a large percentage of dogs present with a neutropenia (Celliers et al., 2020; Scheepers, 2013). A band neutrophil count of > 0.5 x 10^9/L at presentation carries an odds ratio for death of 5.9 (Leisewitz et al., 2019b). Interestingly the only dog with a band neutrophil count above this level prior to treatment in our study was

the dog that died. A strong negative correlation between mature neutrophil count and KC-like, a cytokine with a major role in neutrophil migration and activation, was noted (Ritzman et al., 2010). Interleukin-8, another important cytokine in the migration and activation of neutrophils, had a peak concentration at 96 hours in the HD group, coinciding with the mature neutrophil nadir (Harada et al., 1994). The migration of neutrophils, under the influence of cytokine cues, out of circulation to various sites of inflammation may contribute to the circulating neutropenia seen in *B. rossi* infections.

The acute phase protein CRP is consistently elevated in canine babesiosis despite levels not correlating with outcome (Koster et al., 2009; Matijatko et al., 2007; Schetters et al., 2009a). The inoculum dose in our study influenced the onset of increases in CRP concentration with the HD group showing a significant increases 36 hours earlier than the LD group. Low levels of parasitemia were detectable prior to significant increases in CRP concentrations in both groups, unlike findings in an experimental *B. canis* infection (Schetters et al., 2009a). Although CRP increased with parasitemia the correlation did not reach statistical significance but this may have been a consequence of the inadequate sample size and type II error. Rectal temperature and CRP were positively correlated. As seen in the *B. canis* experimental study, CRP concentrations in our study reached a ceiling, remaining stable despite progressive parasitemia (Schetters et al., 2009a). C-reactive protein concentrations remained high after parasitemia was undetectable and this delay was most likely due to the half-life (which is approximately 161 hours in dogs, with significant inter-individual variation) rather than continued production (Kuribayashi et al., 2015).

Cytokines are a group of proteins secreted by cells of the immune system which act as key signalling molecules in any inflammatory response. A number of cytokine changes have been

identified in *B. rossi* infections at presentation, providing a single snap shot in time of a complicated and dynamic disease (Goddard et al., 2016; Leisewitz et al., 2019a). Cytokines shown to increase during *B. rossi* infections include IL-6, IL-10, MCP-1 and TNFα, and their concentrations tended to be higher in dogs with complicated disease (Goddard et al., 2016; Leisewitz et al., 2019a). Only IL-6 and IL-10 concentrations were associated with increased risk of death (Goddard et al., 2016). Reduced IL-8 concentrations were consistently identified in natural *B. rossi* infections, in contrast to *B. canis* infections (Galán et al., 2018; Goddard et al., 2016; Leisewitz et al., 2019a).

Interferon gamma and KC-like increased with the start of infection and declined after treatment. These cytokines seem to be released by the host in an attempt to control the parasite biomass similar to the IFNγ response seen in falciparum malaria (King and Lamb, 2015). It is possible that IFNγ is important in the initial immune response to *B. rossi* infection, suppressing early replication of the parasite by increasing early in the course of the experimental infection. The concentrations, however, declined acutely with a marked increase in parasitemia. The high levels of parasitemia seen in the HD group may have induced a state of immune exhaustion (Yadav and Cartin-Ceba, 2016). High IL-10 concentrations may also have contributed to the sudden decline in IFNγ, suppressing its secretion and contributing to the unregulated parasite replication (D'Andrea et al., 1993; Fiorentino et al., 1991; Toliver-Kinsky et al., 2002). Keratinocyte chemoattractant-like increases in *B. gibsoni* and *B. canis* infections, and high concentrations were able to differentiate complicated from uncomplicated *B. canis* cases (Galán et al., 2018). In our study KC-like increased progressively during infection and declined following treatment, correlating strongly to parasitemia. In addition to promoting neutrophil migration, KC-like may contribute to increased risk of

complications because of enhanced release of reactive oxygen species and extracellular traps by neutrophils, mechanisms by which host tissue may be damaged (Jin et al., 2014).

Two pro-inflammatory cytokines, MCP-1 and IL-6 had a similar pattern of change and showed a strong positive correlation with one another. Both cytokines displayed progressive increases from the point of inoculation. In previous studies of natural *B. rossi* infections MCP-1 and IL-6 were increased in infected dogs at presentation and higher concentrations were associated with increased risk of mortality, and concentrations of both were higher in the dog that died than any other dog in this study just prior to treatment (Goddard et al., 2016; Leisewitz et al., 2019a). Monocyte chemoattractant protein-1 recruits and activates monocytes/macrophages which would be vital in the immune response to babesia parasites by amplifying inflammatory signals but may increase the risk of complications such as acute lung injury seen in some dogs that die as a result of *B. rossi* infection (Deshmane et al., 2009; Martin, 2020). A potent stimulator of IL-6 production is TNFα, and a strong positive correlation was detected between IL-6 and TNFα in this study. Interleukin 6 plays a role in many pro-inflammatory activities such as stimulating the production of acute phase proteins like CRP from hepatocytes, activation of lymphocytes and acting as a pyrogen (Blackwell and Christman, 1996; Koster et al., 2009). Interleukin-6 is also thought to link inflammation with thrombosis in sepsis (Blackwell and Christman, 1996; Song and Kellum, 2005). Widespread formation of microthrombi is a well-defined pathology in canine babesiosis, particularly in *B. rossi* infections, and increases in IL-6 may be an important trigger for this (Dvir et al., 2004; Goddard et al., 2013; Pardini, 2010). Interleukin-6 is also shown to play an important role in the acute endocrine response to infection which is well described and so typical of this disease (Karga et al., 2000; Schoeman et al., 2007a; Schoeman et al., 2007b).

In previous studies on the cytokine changes in *B. rossi* infections, IL-8 was decreased at presentation when compared to healthy control dogs (Goddard et al., 2016; Leisewitz et al., 2019a). In contrast to these findings, IL-8 increases in *B. canis* infections and even showed a progressive rise for at least 7 days after treatment (Galán et al., 2018). Our study demonstrated decreased IL-8 concentrations during the early stages of infection in the HD group followed by a considerable increase shortly before treatment, when parasitemia was very high. A mild transient increase in IL-8 concentrations was noted in the LD group 24-hours after infection, followed by a progressive decline in concentrations until treatment. The decline in IL-8 production in *B. rossi* infections is poorly understood but because this cytokine is not constitutively produced and requires inflammatory stimulus, it is possible that in the early stages of infection, before parasitemia and hemolysis are severe, there is insufficient stimulus (Harada et al., 1994). Suppression of IL-8 and the cytokines that stimulate its production (TNFα and IL-1) during the initial phase of infection may be, in part, due to high concentrations of IL-10 (Couper et al., 2008; Harada et al., 1994). The final cytokine in this group, IL-10, is a prominent anti-inflammatory cytokine. Concentrations of IL-10 progressively increased over the course of the experimental infection and decreased gradually after treatment. High IL-10 concentrations have been noted in natural *B. rossi* infections (Goddard et al., 2016; Leisewitz et al., 2019a). Interleukin-10 is essential in the modulation of the inflammatory response and plays a key role in preventing excessive inflammation as well as promoting the resolution of inflammation once the inciting pathogen has been eliminated (Couper et al., 2008). Although the anti-inflammatory effects of IL-10 are critical, excessive or inappropriately timed production of IL-10 may prevent an effective immune response to an organism, allowing persistence or even unregulated replication in the host (Couper et al., 2008). This has been seen in *Leishmania spp.* and *Plasmodium spp.* infections in which high

IL-10 concentrations can lead to fulminant fatal infections or chronic persistent infections (Couper et al., 2008). A strong positive correlation was seen between IL-10 and parasitemia supporting the notion that IL-10 may have a permissive effect on the replication of *B. rossi* parasites.

During the study we found that chemotherapeutic intervention and possibly blood transfusions impact the progression of the inflammatory response in these dogs. A marked decline in hematocrit occurred after chemotherapeutic treatment in both groups similar to previous studies (Jacobson et al., 1996). The HD group received blood transfusions which would have led to an underestimation of the anaemia in this group after 120 hours. Higher parasitemia at the time of treatment may result in a more severe, unregulated pro-inflammatory response after treatment. Cytokines such as TNFα, GM-CSF, IL-2 and IL-7 demonstrated marked increases after treatment in the HD group with lesser increases in the LD group. Damaged parasites release soluble antigens into circulation which likely stimulate a profound immune response increasing *B. rossi* parasite clearance, but may be excessive leading to unnecessary widespread 'innocent bystander' injury to host tissues. A progressive band neutrophilia was seen in the HD group after treatment. Band neutrophilia in *B. rossi* infections has been associated with lower hematocrits and blood transfusions, consistent with findings in our study (Scheepers, 2013). The blood transfusions received by the HD group may have contributed to the left shift neutrophilia but hemolysis and subsequent increases in cytokine release and systemic inflammation are likely the main role players. The cytokine, GM-CSF, stimulates the initiation of granulopoiesis in the bone marrow, and this cytokine increased after treatment, particularly in the HD group, coinciding with increases in neutrophil and monocyte counts (Hamilton, 2002). A large increase in MCP-1 after treatment in the HD group indicates increased demand for monocyte/macrophage activity during this

period and GM-CSF would have stimulated bone marrow production (Deshmane et al., 2009; Hamilton, 2002). Treatment resulted in a progressive decline in CRP concentrations in both groups. Cytokine increases after treatment could reflect a 'run-away' inflammation that persists even after the initial trigger has been removed. Both MCP-1 and IL-6 increased markedly after treatment in the HD group. Interleukin 8 concentrations were high in the HD group and progressively increased in the LD group after treatment.

The cytokines GM-CSF, TNFα, IL-2 and IL-7 only increased significantly after treatment. It must be said that the failure to identify significant increases in these cytokines prior to treatment may be the result of the small sample size decreasing the power of this study to detected more subtle changes. Increased levels of GM-CSF have been identified in *B. rossi* infections, particularly in dogs presented earlier in the course of disease (Goddard et al., 2016). The cytokine TNFα is one of the most studied cytokines in human medicine and is an important mediator in the protection against microbial infections, it can however lead to pathology in cases where there is dysregulated response to infection by the host (Idriss and Naismith, 2000). Increased concentrations were found in natural *B. rossi* infections and higher levels were associated with increased risk of complicated disease and death (Leisewitz et al., 2019a). The excessive production of TNFα following treatment in our study may be indicative of a dysregulated immune response. Tumor necrosis factor α is a potent stimulator of the production of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8, serving as co-ordinator in the inflammatory response (Blackwell and Christman, 1996). There was a strong positive correlation between TNFα and IL-6, GM-CSF, IL-2 and IL-7, respectively. Both IL-2 and IL-7 act on lymphocytes and were positively correlated with each other in this study (Gaffen and Liu, 2004; Hofmeister et al., 1999). Interleukin-2 did not increase when *B. rossi* infected dogs were compared to healthy control dogs but higher concentrations were noted in dogs presented

within 48 hours of clinical illness (Goddard et al., 2016; Leisewitz et al., 2019a). Increased IL-7 concentrations have not been identified in natural *B. rossi* infections (Leisewitz et al., 2019a). Both cytokines only displayed significant increases after treatment in the current study. A significant reduction in T-helper lymphocytes and cytotoxic T-lymphocytes were identified in natural *B. rossi* infections (Rautenbach et al., 2017). In that study it was hypothesised that a state of functional immune suppression may be present, and this is supported by the apparent deficiency of cytokines involved in lymphocyte proliferation and activation identified in our study prior to treatment (Rautenbach et al., 2017). Treatment and subsequent lysis of the parasites may have interrupted the immunosuppressive state and the release of soluble antigens was able to stimulate the adaptive immune response triggering production of these cytokines.

. A strong positive correlation was identified between IL-15 and IL-18 in this study. Both these cytokines only increased significantly in one dog in the LD group after treatment. A trend in the increase of IL-15 concentrations early on in disease course of *B. rossi* infection was identified in one study (Goddard et al., 2016). In the current study IP-10 concentrations were mildly increased in both groups.

The main limitation of this study was the small sample size. Six dogs we were used in the study, with only 5 dogs being inoculated. Every attempt was made to exclude any confounding or influencing factors. All dogs were the same age, sex and breed with identical vaccination and deworming protocols. They were housed in the same isolation housing and outdoor facilities. Diet, training, experimental procedures, sample collection and human interaction was consistent between all dogs. Due to the small sample size, it is possible that significant differences between the groups may have been missed appearing to confirm the null hypothesis when in fact the alternative may be true (type 2 error). The infection was

terminated in the LD group before reaching similar disease severity seen in the HD group, preventing direct comparisons of the timelines between high and low infectious doses. Despite the fact that all remaining dogs were monitored clinically and recovered after Day 8, we do not know the details of the resolution of the systemic inflammatory response beyond 192 hours, which is another limitation of this study. Finally, as only dogs in the HD group received blood transfusions, comparison of many variables between the two groups after this point may have created a bias. The blood transfusions would not only have altered the red blood cell parameters but may have had an impact in the inflammatory markers and cytokine profiles as well (Dani et al., 2017; Scheepers, 2013).

Our study has found that infectious dose influenced the onset and dynamics of the inflammatory response in *B. rossi* infections. Moreover, the level of parasitemia may be a contributor to the development of complications after treatment. Alternations in the progression of the inflammatory response as a result of chemotherapeutic damage to the parasites was also highlighted. We found that treatment may augment inflammation by triggering the production of several pro-inflammatory cytokines and proliferation of inflammatory cells. Progression of the inflammatory response after treatment would be redundant and may even lead to unnecessary host tissue damage. It is clear from the findings of our study that *B. rossi* infection and subsequent treatment trigger a classical 'cytokine storm' in which the host's response is characterized by severe inflammation and tissue damage beyond that induced by the parasite itself (Clark, 2007; Clark et al., 2008).

Acknowledgements

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Contributions: Assistance in the running of the cytokine analysis.

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