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

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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. Five healthy male beagle dogs were infected with *B. rossi*, three with a high infectious dose (HD group) and two with 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 when predetermined endpoints were reached. Parasitemia occurred on day 1 and 3 in the HD group and LD group 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 not only fails to curb the inflammatory response but may enhance it. Finally, we found that there is an imbalance in pro/anti-inflammatory cytokine concentrations during infection which may promote parasite replication.

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 (1-3). Babesiosis is a complex multi-systemic disease that can be classified as either uncomplicated or complicated (2, 4, 5). 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 (2). *B. rossi* infection, like *Plasmodium falciparum* malaria in humans, results in a protozoal sepsis with a severe systemic inflammatory response (6-8). The concept of a 'cytokine storm' is well established in human inflammatory and infectious conditions, such as malaria and sepsis (9). 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 (10). 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 (11, 12). 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) (13-15). Complications associated with this disease are likely the result of an unbalanced inflammatory cytokine response (13, 14, 16-18). 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 (2, 14, 19).

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, and found, that *B. rossi* infection initiates a pronounced, unbalanced inflammatory response and that infectious dose as well as treatment alter disease progression.

Materials and methods

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).

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 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 (19). 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.

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. The experiment lasted a total of 8 days from point of inoculation to termination, with treatment being required from day 4. 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.

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).

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.

Chemotherapeutic intervention

149 The infection was allowed to run its course until one of the following endpoints were identified:
150 hematocrit <15%, collapsed habitus, nervous signs (such as seizure activity), clinical evidence of lung
151 pathology with arterial blood gas evidence of acute respiratory distress syndrome (arterial partial
152 pressure of oxygen [PaO2] <60mmHg), serum creatinine > 200mmol/L (normal <140 mmol/L) and
153 hemoconcentration (PCV >55%). The infection ran its course for 4 days prior to intervention. The HD
154 group was treated on day 4 in the morning (at 96 hours). Due to the unexpected death of one dog in
155 the HD group, to avoid any further losses, the LD group was treated 12 hours later (at 108 hours) even
156 though they had not reached the same degree of disease severity as the HD group. All dogs were drug
157 cured with diminazene aceturate (3.5 mg/Kg subcutaneously) and provided with supportive treatment
158 as needed. The remaining 5 dogs (including the splenectomized dog) recovered completely and were
159 rehomed as pets.
160

161 # Statistical analysis

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

173

174

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.

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.

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 ($r = -0.674$, $p < 0.001$). Hematocrit (Hct) (Fig 2) declined significantly decline compared to baseline at 96 hours ($p < 0.001$) in the HD group and 120 hours ($p = 0.003$) in the LD group and both groups

198 demonstrated a progressive decrease in Hct after treatment. Hemoglobinemia was visibly present from
199 72 hours in the HD group.

200

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

203

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

205

206 For the HD group, increases in CRP concentrations (Fig 3) above baseline (14.33 mg/L, 10 – 21.5)
207 peaked at 72 hours (150 mg/L, 135 – 163, $p < 0.001$) and remained significantly increased at 96 hours
208 (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,
209 54 – 64, $p < 0.001$). The LD group showed a marked increase in CRP above baseline (25 mg/L, 10 –
210 40) at 108 hours (175 mg/L, 160 – 197, $p < 0.001$), declining thereafter but remaining significantly
211 increased for the remainder of the study. The CRP concentrations peaked 36-hours earlier in the HD
212 group. C-reactive protein was significantly correlated with temperature ($r = 0.722$, $p = 0.003$) and the
213 correlation between CRP and parasitemia approached significance ($r = 0.646$, $p = 0.056$). A
214 significant decrease in mature neutrophil (Fig 4) count was seen from 72 hours (1.79 X 10^9/L, 1.36 –
215 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, $p < 0.001$).
216 The neutrophil nadirs for the HD and LD groups were seen at 96 (1.57 X 10^9/L, 1.12 – 1.88, $p <$
217 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
218 marked increase in the mature neutrophil counts after treatment, exceeding laboratory reference
219 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
220 10^9/L, 23.75 – 30.94, $p < 0.001$). After treatment there was a gradual recovery of the mature
221 neutrophil count in the LD group, returning to within laboratory reference intervals at 192 hours. A
222 significant increase in band neutrophil counts (Fig 4) above baseline (0.14 X 10^9/L, 0.13 – 0.17) was
223 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,

224 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
225 exceeded the laboratory reference interval (0 – 0.5 x 10^9/L) consistently after treatment in the HD
226 group. At no point during the study did the band neutrophil count in the LD group increase
227 significantly above baseline values or exceed the laboratory reference interval. A significant reduction
228 in monocyte count compared to baseline (0.52 X 10^9/L, 0.46 – 0.65) was seen in the HD group at 24
229 (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
230 treatment, the monocyte counts were increased at 144 (1.86 X 10^9/L, 1.27 – 2.45, $p < 0.001$), 168
231 (3.13 X 10^9/L, 2.69 – 3.57, $p < 0.001$) and 192 hours (3.04 X 10^9/L, 1.8 – 4.28, $p < 0.001$) in the HD
232 group, exceeding the laboratory reference interval (0.15 – 1.35x 10^9/L) from 144 hours onwards.

233

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

235

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

237

238 Cytokine kinetics

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

240 a. Cytokines that increased during infection and decreased after treatment

241 The cytokines which fall into this category included IFNγ and KC-like (Table 1). The HD group had a
242 significant increase in IFNγ concentrations (Fig 5) above baseline ($p = 0.002$) and above the LD group
243 ($p < 0.001$) at 48 hours. The LD group had peak concentrations 48-hours later, at 96 hours ($p <$
244 0.001). There was a progressive and significant increase in KC-like concentrations (Fig 6) in the HD
245 group above baseline at 24 hours ($p < 0.001$), 48 hours ($p < 0.001$), 72 hours ($p < 0.001$) and 96 hours
246 ($p < 0.001$) declining significantly at 144 hours ($p = 0.004$) and 192 hours ($p < 0.001$). The LD group
247 only had significant increase in KC-like concentrations above baseline at 96 hours ($p < 0.001$). Strong

248 correlations were identified between KC-like and parasitaemia ($r = 0.888$, $p < 0.001$) as well as KC-
249 like and mature neutrophil count ($r = -0.817$, $p < 0.001$).
250
251 Fig 5. IFN γ concentrations during *B. rossi* infection and after treatment
252
253 Fig 6. KC-like concentrations during *B. rossi* infection and after treatment
254
255 **b. Cytokines that increased during infection and remained high after**
256 **treatment**
257 This category included the following cytokines MCP-1, IL-6, IL-8 and IL-10 (Table 1). The
258 chemokine MCP-1 concentrations (Fig 7) increased above baseline in the HD group from 24 hours
259 onwards reaching significance after treatment at 144 hours ($p < 0.001$) and 192 hours ($p = 0.001$). The
260 LD group did not have significant increases in MCP-1 concentrations throughout the study but one
261 dog in this group did demonstrate increased concentrations after treatment. Interleukin-6
262 concentrations (Fig 8) were moderately increased above baseline at 96 hours ($p = 0.049$) in the HD
263 group and progressively increased after treatment, at 144 hours ($p < 0.001$) and 192 hours ($p < 0.001$).
264 Like changes seen in MCP-1, one dog in the LD group had increased IL-6 at 192 hours but this did
265 not reach significance. Interleukin-6 and MCP-1 were strongly correlated ($r = 0.792$, $p < 0.001$). The
266 HD group had significantly reduced IL-8 concentrations (Fig 9) compared to baseline at 72 hours ($p =$
267 0.004), with a marked increase at 96 hours ($p < 0.001$). The LD group only had significantly increased
268 IL-8 concentrations at 192 hours ($p = 0.003$). Interleukin-10 concentrations (Fig 10) increased
269 significantly above baseline at 24 hours ($p = 0.019$), 72 hours ($p < 0.001$) and 96 hours ($p < 0.001$) in
270 the HD group. The LD group showed no significant increase in IL-10 but both dogs demonstrated a
271 progressive increase in concentrations from 72 hours after inoculation until treatment, remaining
272 increased in one dog after treatment. A strong positive correlation was identified between
273 parasitaemia and IL-10 ($r = 0.674$, $p = 0.009$) as well as between IL-10 and MCP-1 ($r = 0.828$, $p <$
274 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

c. Cytokines that increased after treatment

The next category of cytokines, GM-CSF (Fig 11), TNFα (Fig 12), IL-2 (Fig 13) and IL-7, 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

d. 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.

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

	Baseline	24 hours	48 hours	72 hours	96 hours	144 hours	192 hours
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*
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 **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 0.049*	0.226 1.000 <0.001*	1.000 1.000 <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 **LD vs Base** **HD vs Base**	1.000 NA NA	0.650 0.134 1.000	1.000 0.836 1.000	0.522 1.000 0.004*	<0.001* 1.000 <0.001*	1.000 0.897 1.000	1.000 0.003* 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 **LD vs Base** **HD vs Base**	1.000 NA NA	1.000 1.000 0.294	1.000 1.000 0.019*	1.000 1.000 <0.001*	1.000 0.074 <0.001*	1.000 1.000 0.207	1.000 1.000 1.000
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*

* 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) and TNFα (tumor necrosis factor alpha).

Discussion

Our study was the first to evaluate kinetics of markers of inflammation 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 and has also demonstrated that a pronounced inflammatory response continues and may even worsen despite clearance of the parasitemia. 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. Although not statistically significant, there was a tendency for rectal temperature to increase with increasing parasitemia. 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 findings were seen in the experimental infection of dogs with *B. canis* (20). One dog in the HD group demonstrated a marked reduction in rectal temperature of 2.8°C at 96 hours, followed shortly thereafter by collapse and death. In a recent study of dogs naturally infected with *B. rossi*, rectal temperatures were significantly lower in dogs that presented collapsed with both collapse and hypothermia being positively associated with an increased risk of death (2). Rectal temperature may act as a proxy for the onset and progression of inflammation as well as the onset of hypoperfusion and shock. The blood pressure changes seen in our study differed from those seen during an experimental infection of dogs with *B. canis*, where the mean arterial blood pressure, measured using a non-invasive oscillometric blood pressure meter, declined progressively after inoculation (20). Mild hypotension was only identified in the one dog that died at 96 hours from the HD group. In previous

347 studies hypotension worsened with disease severity (20, 21). The absence of hypotension in the
348 remaining dogs in our study may be due to the short duration of the experiment and the timing of
349 treatment. If the infection had been allowed to progress beyond our endpoints, we may have identified
350 hypotension in more dogs.

351 High parasitemia is positively associated with increased risk of complications and death in *B. rossi*
352 infections (14, 19). Venous parasitemia's up to 30% have been recorded in natural *B. rossi* infections
353 and this may be a contributor to the virulence of this *Babesia* species (19, 22). As with previous
354 studies on *B. rossi* infection, we demonstrated a progressive parasitemia which required
355 chemotherapeutic intervention. The infectious dose had a prominent impact on the progression of
356 parasitemia with levels increasing at a significant rate and to very high levels in the HD group, up to
357 59%, within 4 days of inoculation. The LD group demonstrated a more gradual rise in parasitemia,
358 more closely mirroring natural infection. The immune system may be overwhelmed and unable to
359 mount an effective and timeous response to the *B. rossi* parasites when faced with high infectious
360 doses or it is possible that these parasites actively suppress effective immune responses which may be
361 more effectively suppressed at a higher parasitemia. The concept of an ineffective immune response
362 may be supported by the positive correlation identified between parasitemia and IL-10, a prominent
363 anti-inflammatory cytokine. Immune evasion by protozoa is a well-known phenomenon and has been
364 demonstrated in *Plasmodium, Trypanosoma* and *Leishamania* among others (23). In *Leishamania*
365 infections, the parasites promote an immunosuppressive cytokine profile with high levels of IL-10,
366 allowing them unrestricted replication (23) and a similar interaction may take place in *B. rossi*
367 infections. Immune dysregulation with concurrent hyperinflammation and immunosuppression is
368 ubiquitous in human patients that are critically ill (24-26). It is also possible that, as in sepsis in
369 humans, there is a state of hyperinflammation that is ineffective at clearing the infection but
370 nevertheless does damage the host. The negative correlation between parasitemia and mature
371 neutrophil count may also point to a deficient innate immune response to the *B. rossi* infection in
372 these dogs. In addition to low neutrophil numbers a recent study on neutrophil function in *B. rossi*
373 infections also identified an association between higher concentrations of neutrophil myeloperoxidase

374 concentrations and poor prognosis suggesting possible diminished neutrophil burst function in the
375 remaining neutrophils (27). It has also been shown that *B. rossi* results in significant lymphopenia as a
376 result of a loss of $CD3^+$, $CD4^+$, $CD8^+$ and $CD21^+$ phenotypes (28). The degree of lymphocyte loss was
377 also correlated to disease severity, and this may be responsible for a state of immune dysfunction
378 despite hyperinflammation (28). Timing of treatment relative to parasitemia concentrations may have
379 a marked influence on the degree of cytokine response after treatment, with cytokines such as TNFα,
380 GM-CSF, IL-2 and IL-7 demonstrating marked increases after the parasites were damaged/killed by
381 the treatment in the HD group with mild to moderate increases in the LD group. Higher parasitemia at
382 the time of treatment may result in a more severe, unregulated pro-inflammatory response after
383 treatment. Damage to the parasites releases soluble parasite antigens into circulation which could be
384 efficient in stimulating this profound immune response. Although this response may increase the rate
385 of *B. rossi* parasite clearance, it could be redundant and lead to unnecessary widespread 'innocent
386 bystander' injury to host tissues.

387 Anemia is common in dogs infected with *B. rossi* and in a recent study up to 84% of dogs had
388 hematocrits below the laboratory reference interval at presentation (2). Although anemia is not a
389 reliable predictor of death, severe anemia does require treatment to avoid the systemic complications
390 of hypoxia and even death (2). The HD group demonstrated a significant anemia at 96 hours after
391 inoculation which worsened after treatment requiring multiple blood transfusions. The severity of the
392 anemia after treatment in this group was likely underestimated as a result of this intervention.
393 Although the decline in Hct was significant in the LD group after treatment, it only resulted in mild
394 anemia in these dogs and blood transfusions were not necessary. The absence of anemia during active
395 infection in the LD group was probably the result of insufficient time permitted for disease
396 progression to reach the same severity as that seen in the HD group. A marked decline in hematocrit
397 occurred after treatment in both groups similar to previous studies (29).

398 Mature neutropenia was identified as early as 24 hours post-inoculation in the HD group, but only
399 reached statistical significance from 72 hours. The mature neutropenia persisted until treatment,
400 thereafter, counts increased significantly above baseline and laboratory references intervals. A mature

401 neutropenia was seen in the LD group, 36-hours after the HD group, but neutrophilia did not develop
402 after treatment. Previous studies evaluating hematological changes in natural *B. rossi* infections found
403 that a large percentage of dogs present with a neutropenia (27, 30). A progressive band neutrophilia
404 was seen in the HD group after treatment and band neutrophilia in *B. rossi* infections has been
405 associated with lower hematocrits and blood transfusions, consistent with findings in our study (30).
406 A band neutrophil count of > 0.5 x 10^9/L at presentation carries an odds ratio for death of 5.9 (2).
407 Interestingly the only dog with a band neutrophil count above this level prior to treatment in our study
408 was the dog that died. In one study there was a higher proportion of dogs with a neutrophilia in the
409 group that received blood transfusions (30). The blood transfusions received by the HD group may
410 have contributed to the left shift neutrophilia but hemolysis and subsequent increases in cytokine
411 release and systemic inflammation are likely the main role players. There was a strong negative
412 correlation between mature neutrophil count and KC-like, a cytokine with a major role in neutrophil
413 migration and activation (31). Interleukin-8, another important cytokine in the migration and
414 activation of neutrophils, had a peak concentration at 96 hours in the HD group, coinciding with the
415 mature neutrophil nadir (32). The migration of neutrophils, under the influence of cytokine cues, out
416 of circulation to various sites of inflammation may contribute to the circulating neutropenia seen in *B.*
417 *rossi* infections. The cytokine, GM-CSF, stimulates the initiation of granulopoiesis in the bone
418 marrow, and this cytokine increased after treatment, particularly in the HD group, coinciding with
419 increases in neutrophil and monocyte counts (33). The HD group developed a mild monocytosis after
420 treatment, similar to natural *B. rossi* infections (30). It should be noted that *B. rossi* infected dogs
421 demonstrate monocyte/macrophage accumulation in the pulmonary interstitium and spleen (34, 35). A
422 large increase in MCP-1 after treatment in the HD group indicates increased demand for
423 monocyte/macrophage activity during this period and GM-CSF may have provided the bone marrow
424 stimulation to increase production of monocytes after treatment (33, 36).

425 Acute phase proteins are regularly used in the detection and monitoring of systemic inflammation. C-
426 reactive protein is an acute phase protein that is consistently elevated in canine babesiosis despite
427 levels not correlating with outcome (15, 20, 37). In one study of CRP in natural *B. canis* infection,

CRP had its peak concentration at presentation and declined progressively following treatment (37). In a *B. canis* experimental infection, CRP increased before the presence of a detectable parasitemia and the onset of the increase was inoculum dose dependent with the highest dose resulting in increased concentrations first (20). 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 experimental *B. canis* infection (20). This may however not have been the case had lower infectious doses been used. Treatment resulted in a progressive decline in CRP concentrations in both groups. Although CRP increased with parasitemia the correlation wasn't statistically significant in this small cohort, but rectal temperature and CRP were positively correlated. As seen in the *B. canis* experimental study, CRP concentrations in our study reached a ceiling and remained relatively stable despite progressive parasitemia (20). C-reactive protein levels remained high even 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 (38).

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, but these have only been evaluated in dogs at presentation, providing a single snap shot in time of a complicated and dynamic disease (13, 14). 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 (13, 14). Only IL-6 and IL-10 concentrations were significantly higher in dogs that died compared to survivors (13). Decreased concentrations of IL-8 were consistently identified in natural *B. rossi* infections, in contrast to *B. canis* infections (13, 14, 16).

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 (39). In malaria, IFNγ is considered an important mediator in the protective innate immune response during the blood stage and initial parasite replication (40). It

455 is possible that IFNγ is important in the initial immune response to *B. rossi* infection, suppressing early
456 replication of the parasite as this cytokine increased early in the course of the experimental infection,
457 coinciding with the initial increase in parasitemia in both groups. The concentrations, however, declined
458 acutely once the parasitemia exceeded 5% in HD group. The sudden decline in IFNγ concentrations in
459 the HD group coincided with a marked increase in parasitemia. The high levels of parasitemia seen in
460 the HD group may have induced a state of immune exhaustion (41). High concentrations of IL-10 may
461 also have contributed to the sudden decline in IFNγ, thereby suppressing its secretion and contributing
462 to the resultant unregulated parasite replication (42-44). Suppression of IFNγ secretion may be one
463 mechanism employed by the parasite allowing unrestrained increase in parasite biomass and it is
464 possible that a similar decline may have been seen in the LD group if infection had been allowed to
465 evolve. Keratinocyte chemoattractant-like increases in *B. gibsoni* and *B. canis* infections, and high
466 concentrations were able to differentiate complicated from uncomplicated *B. canis* cases (16). In our
467 study KC-like increased progressively during infection and declined following treatment, correlating
468 strongly to parasitemia. In addition to promoting neutrophil migration, KC-like may contribute to
469 increased risk of complications because of enhanced neutrophil release of reactive oxygen species and
470 neutrophil extracellular traps, important mechanisms by which host tissue may be damaged (45).

471 Cytokine increases after treatment could reflect a role in the 'run-away' inflammation that persists even
472 after the initial trigger has been removed. Two pro-inflammatory cytokines, MCP-1 and IL-6 had a
473 similar pattern of change and showed a strong positive correlation with one another. Both cytokines
474 displayed progressive increases from the point of inoculation increasing markedly after treatment in the
475 HD group. No significant increases were noted in the LD group throughout the study period, but
476 concentrations of MCP-1 were trending upwards 24 hours prior to treatment. In previous studies on
477 natural *B. rossi* infections MCP-1 and IL-6 were increased in infected dogs at presentation and higher
478 concentrations were associated with increased risk of mortality (13, 14). The concentrations of MCP-1
479 and IL-6 in the dog that died were considerably higher than any other dog in our study just prior to
480 treatment indicative of a negative prognosis. Monocyte chemoattractant protein-1 recruits and activates
481 monocytes/macrophages and would be a vital host mechanism in the immune response to babesia

parasites by amplifying inflammatory signals and enhancing phagocytosis of parasites and damaged erythrocytes (36). Persistently high levels however could contribute to an unregulated inflammatory response and increased risk of complications such as acute lung injury seen in some dogs that die as a result of *B. rossi* infection (34). A potent stimulator of IL-6 production is TNFα, and a strong positive correlation was detected between IL-6 and TNFα in this study. The role of IL-6 in septic conditions is poorly understood, although it does play a role in many pro-inflammatory activities such as stimulating the production of acute phase proteins like CRP from hepatocytes (well known to be raised in *B. rossi* infection), activation of lymphocytes and acting as a pyrogen (15, 46). Interleukin-6 is also thought to link inflammation with thrombosis in sepsis (46, 47). 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, contributing to increased risk of complications such as cerebral babesiosis, myocardial dysfunction and death (48-50). 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 (51-53).

In previous studies on the cytokine changes in *B. rossi* infections, IL-8 was decreased at presentation when compared to healthy control dogs (13, 14). 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 (16). 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. Interleukin 8 concentrations remained high after treatment in this group. 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. Once treated, IL-8 concentrations increased progressively until 192 hours in this group. 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 (32). 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 (32, 54). The final cytokine in this group, IL-10, is a prominent anti-inflammatory cytokine. Concentrations

509 of IL-10 progressively increased over the course of the experimental infection and decreased gradually
510 after treatment. High IL-10 concentrations have been noted in natural *B. rossi* infections (13, 14).
511 Interleukin-10 is essential in the modulation of the inflammatory response and plays a key role in
512 preventing excessive inflammation as well as promoting the resolution of inflammation once the
513 inciting pathogen has been eliminated (54). Although the anti-inflammatory effects of IL-10 are critical,
514 excessive or inappropriately timed production of IL-10 may prevent an effective immune response to
515 an organism, allowing persistence or even unregulated replication in the host (54). This has been seen
516 in *Leishmania spp.* and *Plasmodium spp.* infections in which high IL-10 concentrations can lead to
517 fulminant fatal infections or chronic persistent infections (54). Human septic patients with continuous
518 over production of IL-10 and high IL-10:TNFα ratio develop marked immunosuppression and have a
519 higher risk of mortality (55). A strong positive correlation was seen between IL-10 and parasitemia
520 supporting the notion that IL-10 may have a permissive effect on the replication of *B. rossi* parasites.

521 The cytokines GM-CSF, TNFα, IL-2 and IL-7 only increased significantly after treatment. Increased
522 levels of GM-CSF have been identified in *B. rossi* infections, particularly in dogs presented earlier in
523 the course of disease (13). The rise in GM-CSF concentrations after treatment noted in our study may
524 act as a double-edged sword, on the one side replenishment of neutrophil and monocyte counts is
525 essential but on the other, excess production, adhesion and activation of granulocytes and macrophages
526 after the parasites are eliminated may contribute to widespread tissue damage (33). The initiator pro-
527 inflammatory cytokine TNFα is one of the most studied cytokines in human medicine and is an
528 important mediator in the protection against microbial infections (56). It can however lead to pathology
529 in cases where there is disproportionate and dysregulated immune response to an infection by the host
530 (56). It is also a potent stimulator of the production of other pro-inflammatory cytokines such as IL-1β,
531 IL-6 and IL-8, serving as co-ordinator in the inflammatory response (46). Increased concentrations
532 were found in natural *B. rossi* infections and higher levels were associated with increased risk of
533 complicated disease and death (14). Concentrations of TNFα were only increased after treatment in this
534 study and reached very high levels in one dog in the HD group. There was also a strong positive
535 correlation with IL-6, GM-CSF, IL-2 and IL-7. The excessive production of TNFα following treatment

536 may be indicative of a dysregulated immune response. Both IL-2 and IL-7 act on lymphocytes and were
537 positively correlated with each other in this study (57, 58). Interleukin-2 did not increase when *B. rossi*
538 infected dogs were compared to healthy control dogs in one study but higher concentrations were noted
539 in infected dogs presented within 48 hours of clinical illness (13, 14). Increases in IL-7 has not been
540 identified in previous studies of *B. rossi* infections (14). Both cytokines only displayed significant
541 increases after treatment in the current study. Previously, significant reduction in T-helper lymphocytes
542 and cytotoxic T-lymphocytes were identified in complicated *B. rossi* infections and decreased
543 concentrations of cytotoxic T-lymphocytes was also noted in uncomplicated cases at presentation (28).
544 In that study it was hypothesised that a state of functional immune suppression may be present, and this
545 is supported by the apparent deficiency of cytokines involved in lymphocyte proliferation and activation
546 identified in our study prior to treatment (28). Treatment and subsequent lysis of the parasites may have
547 interrupted the immunosuppressive state and the release of soluble antigens was able to stimulate the
548 adaptive immune response triggering production of these cytokines.

549

550 Three cytokines showed no distinct pattern of change or only demonstrated changes in one dog. A
551 strong positive correlation was identified between IL-15 and IL-18 in this study. Both these cytokines
552 only increased significantly in one dog in the LD group after treatment. A trend in the increase of IL-
553 15 concentrations early on in disease course of *B. rossi* infection was identified in one study (13). In
554 the current study IP-10 concentrations were mildly increased in both groups.

555

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

563

564 Our study has found that infectious dose influenced the onset and dynamics of the inflammatory
565 response. Most variables shared similar kinetic patterns between groups, differing with respect to the
566 timing of the onset of disease only. If the infection in the LD group had been allowed to progress, it is
567 likely these variables would have reached similar degrees of change to those seen HD group. There
568 were however exceptions, where kinetic patterns differed during infection between the two groups
569 such as those seen in IL-8. Concentrations of CRP, KC-like, IL-15, IL-18 and IP-10 in the LD group
570 exceeded those of the HD group after treatment. These findings suggest that not only will infectious
571 dose influence the onset of inflammation, but it may influence the kinetics and nature of the
572 inflammatory response to *B. rossi* infection. Moreover, the level of parasitemia may be a contributor
573 to the development of complications after treatment. We also highlighted the influence
574 chemotherapeutic damage to the parasites had on the progression of the inflammatory response. We
575 found that not only was treatment unsuccessful in curbing the inflammatory response, but it may
576 augment it by triggering the production of several pro-inflammatory cytokines and proliferation of
577 inflammatory cells. Progression of the inflammatory response after treatment would be redundant and
578 may even lead to unnecessary host tissue damage. It is clear from the findings of our study that *B.*
579 *rossi* infection and treatment triggers a classical 'cytokine storm' in which the host's response is
580 characterized by severe inflammation and tissue damage beyond that induced by the parasite itself (9,
581 59).

582

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588

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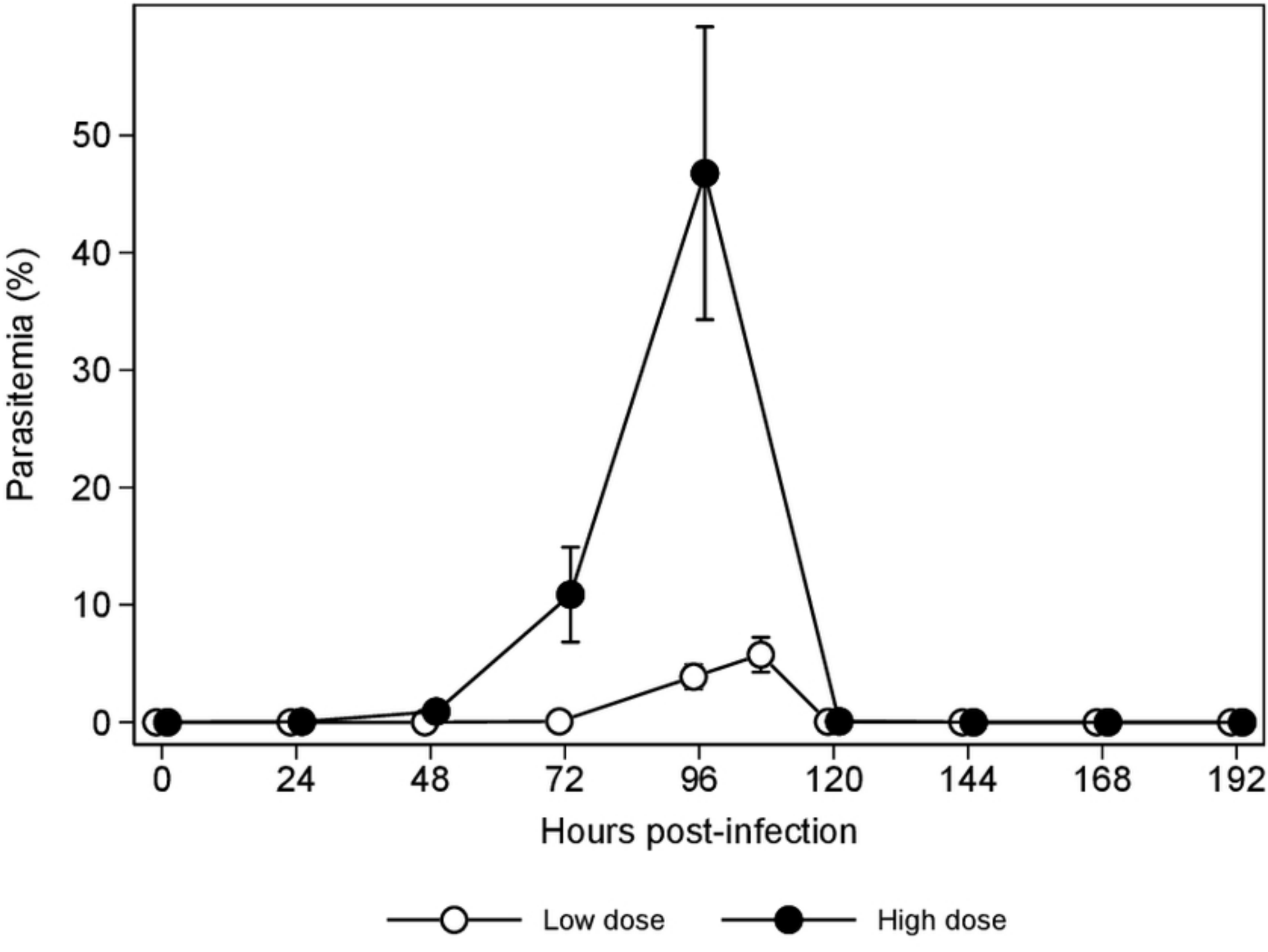
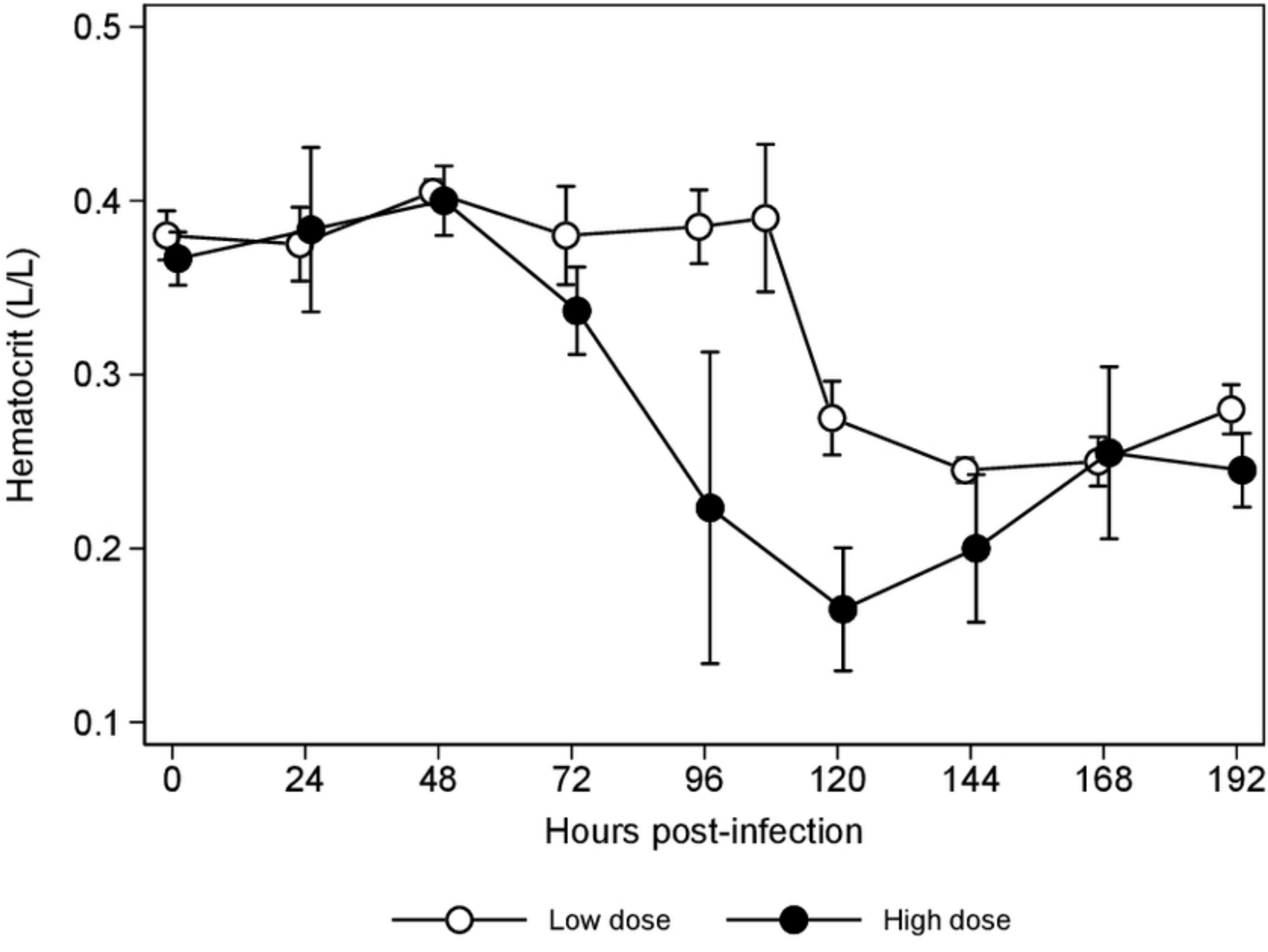
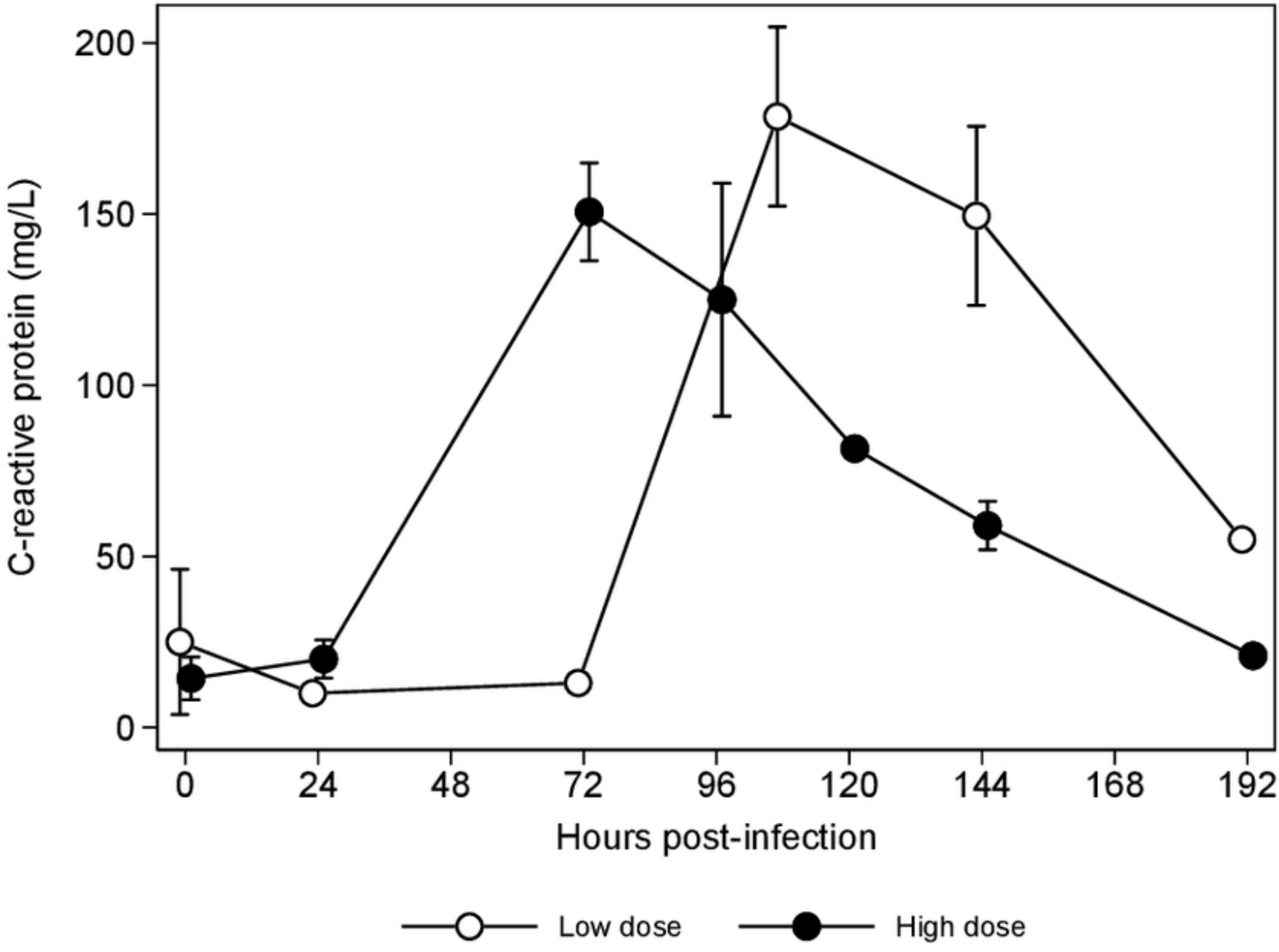
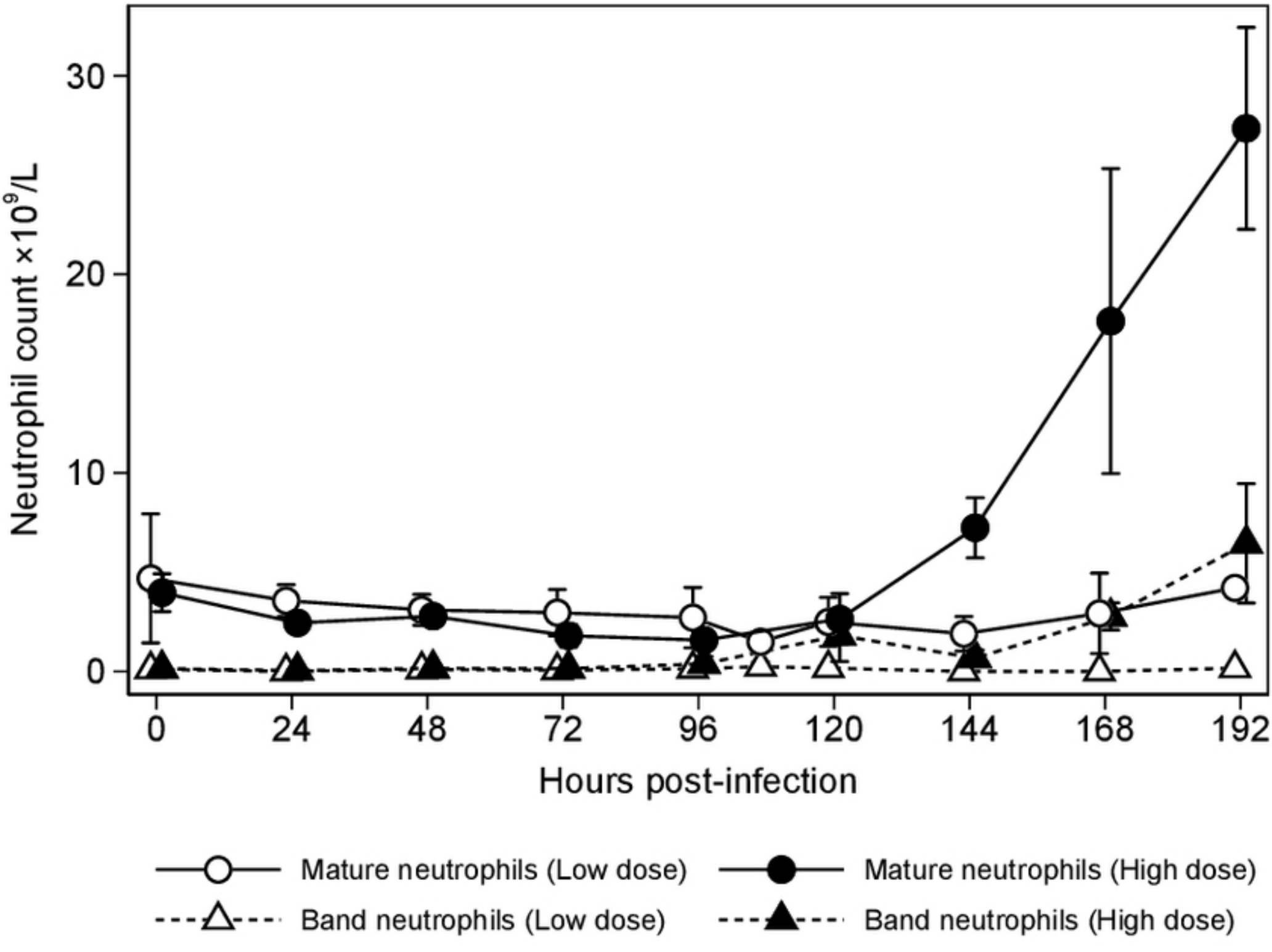
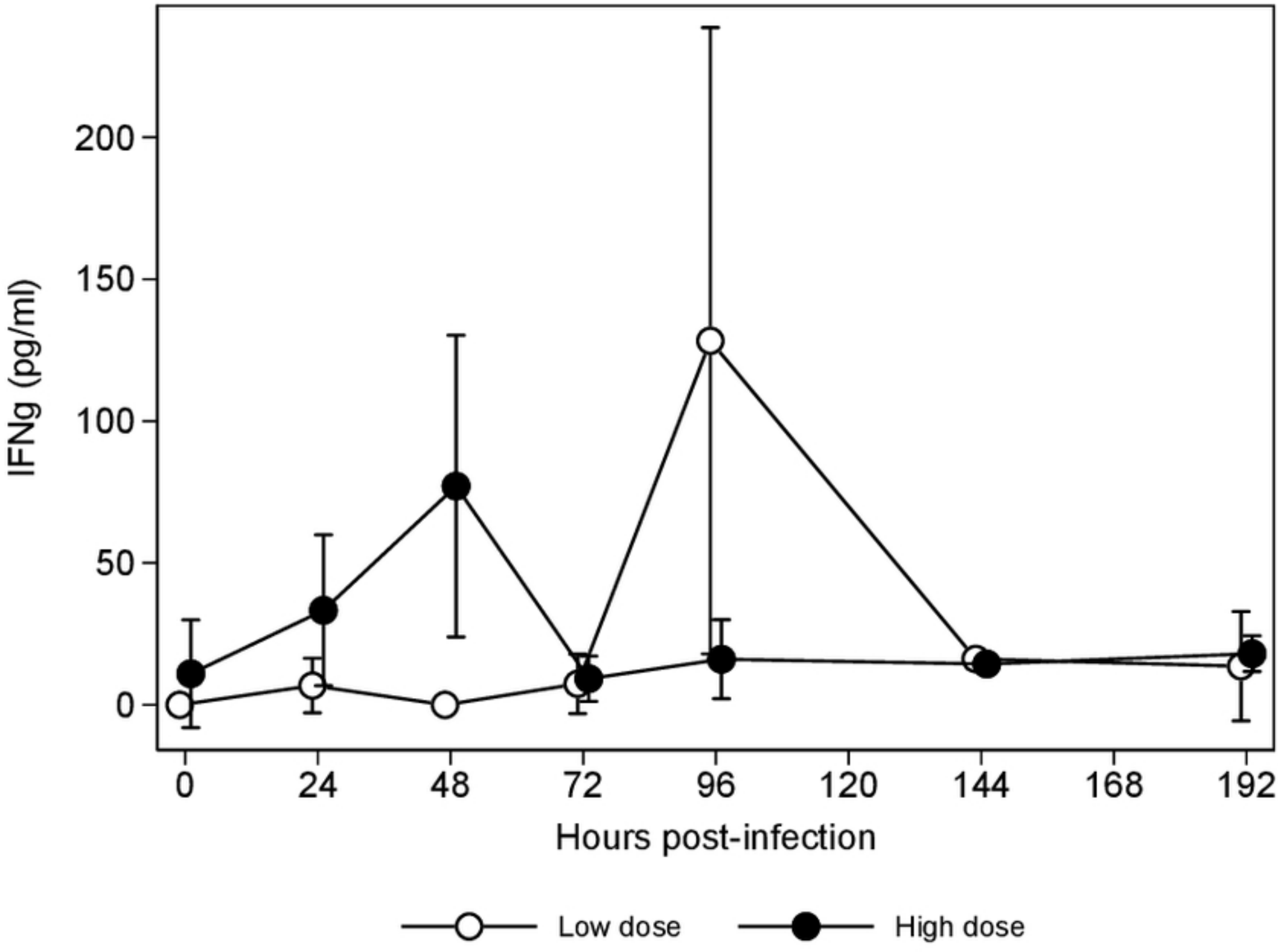
724

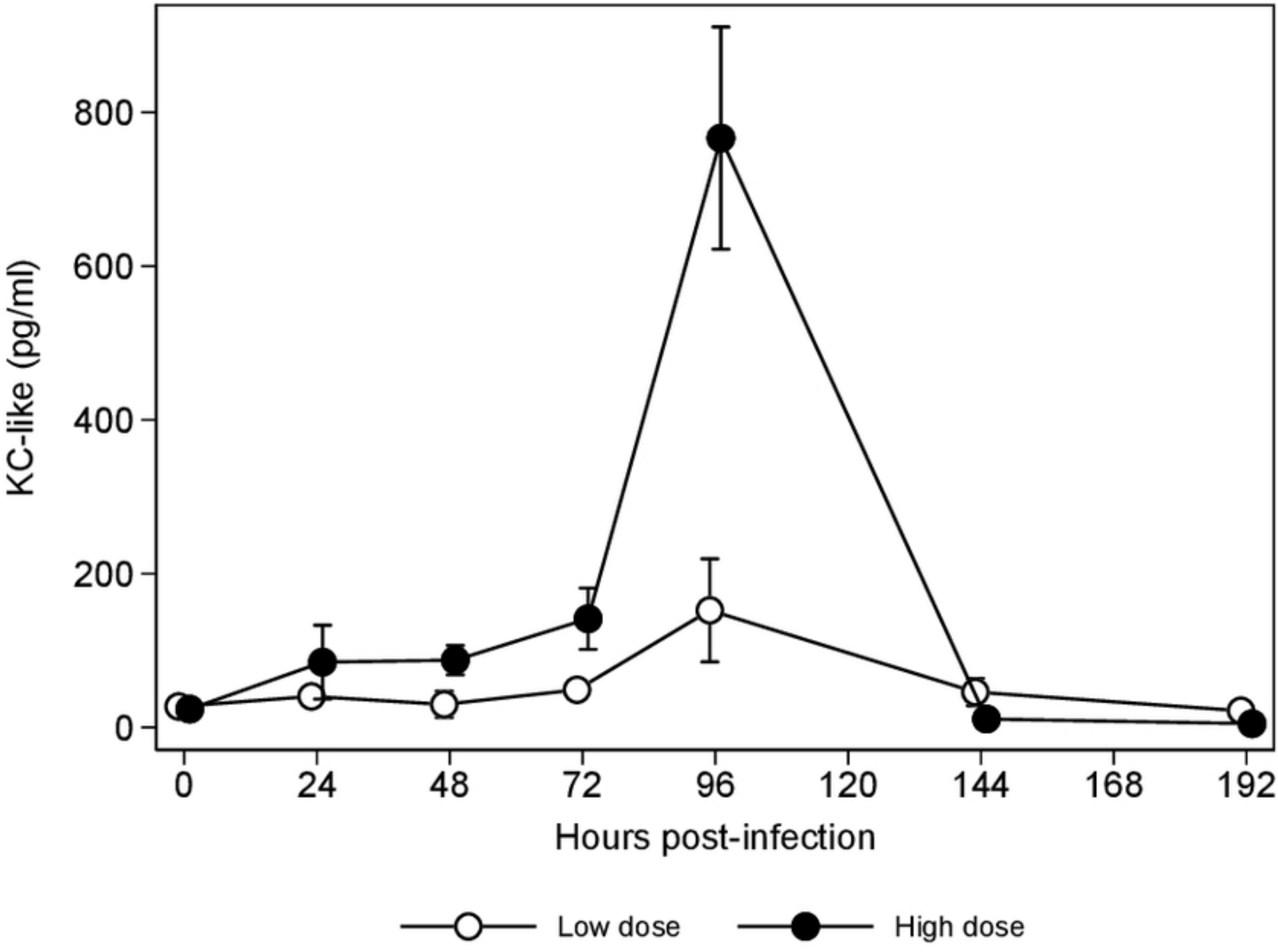

Parasitemia (%)
0
10
20
30
40
50
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose

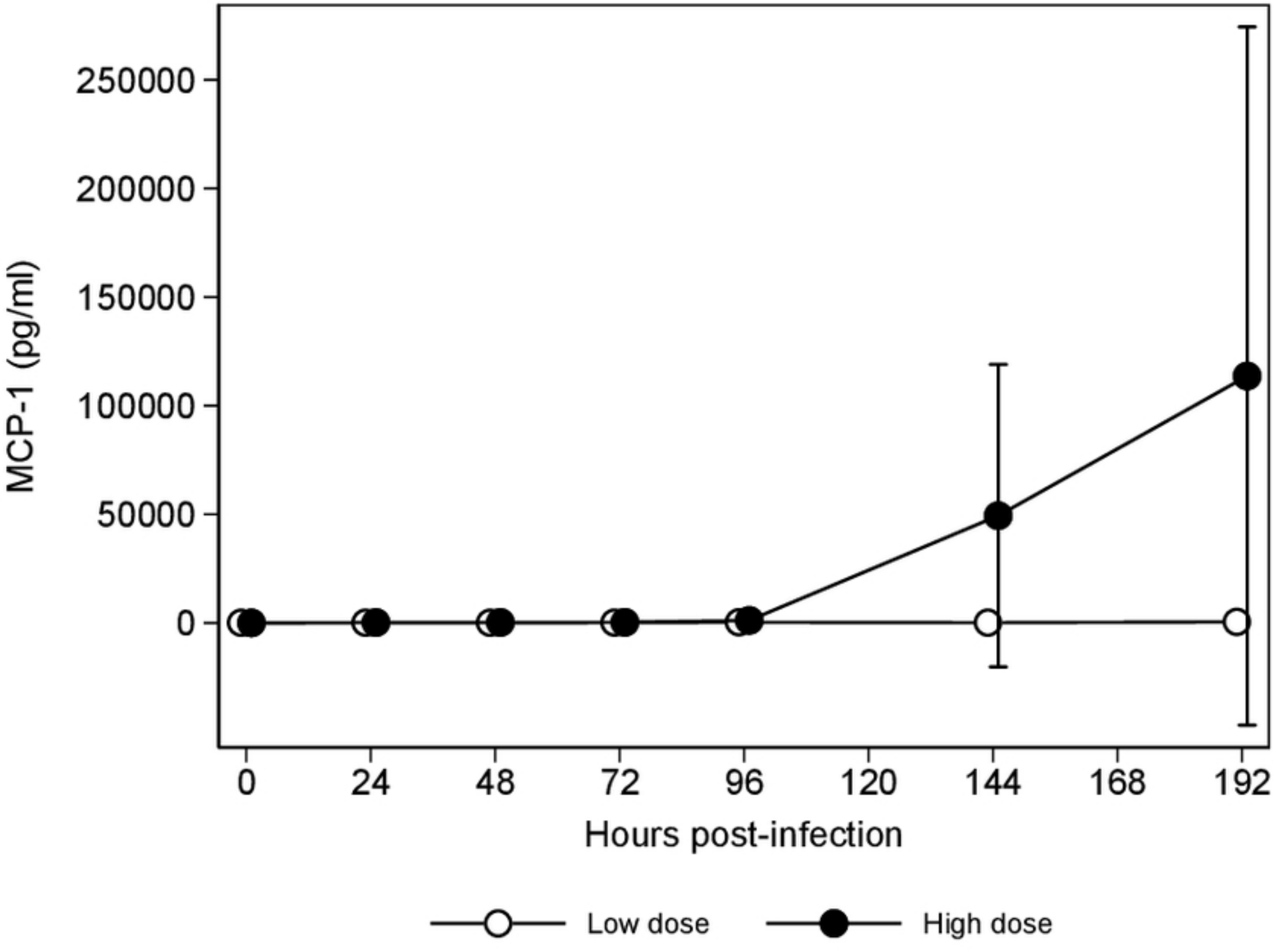

0.5
0.4
0.3
0.2
0.1
Hematocrit (L/L)
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose

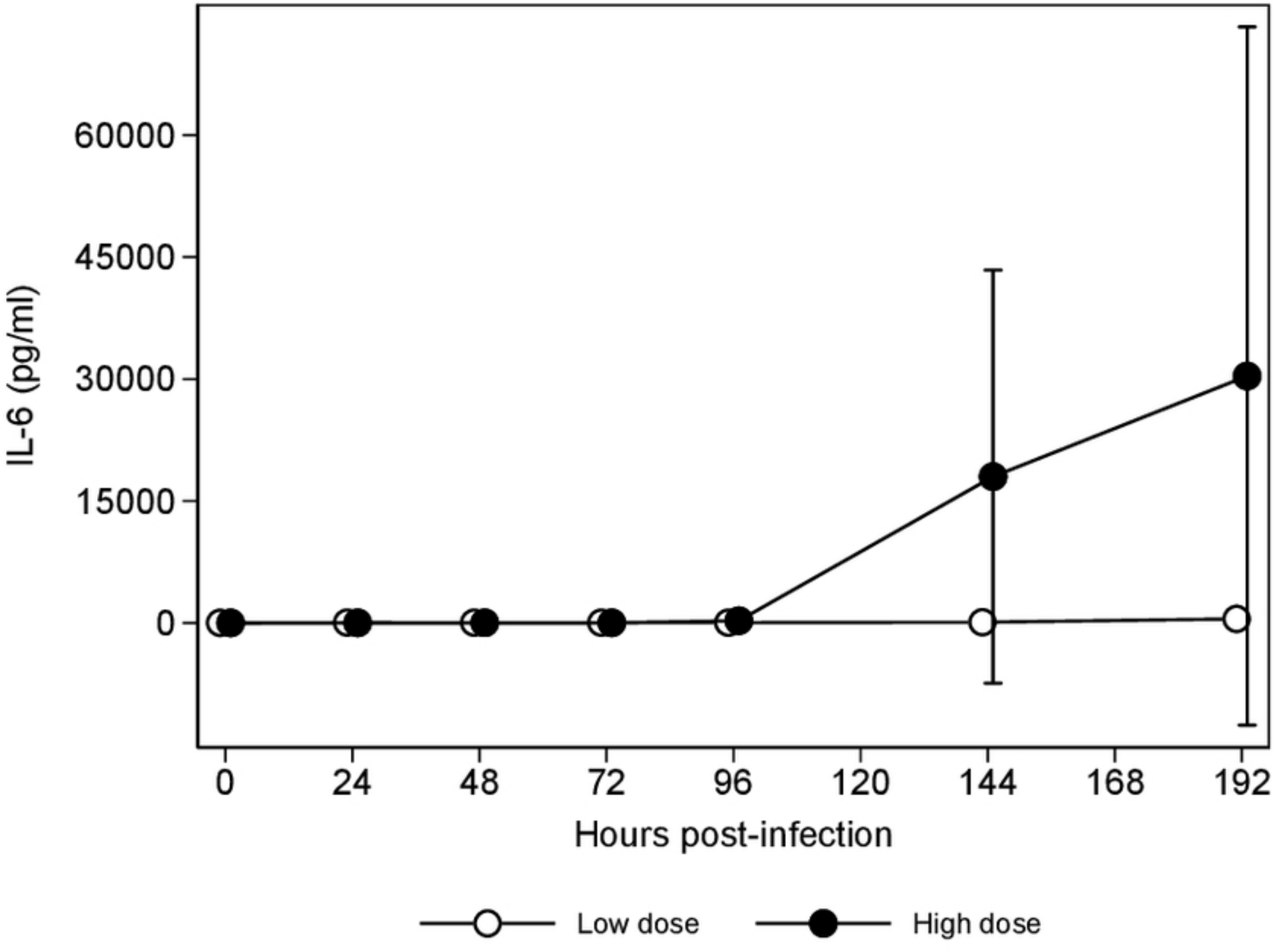

C-reactive protein (mg/L)
200
150
100
50
0
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose

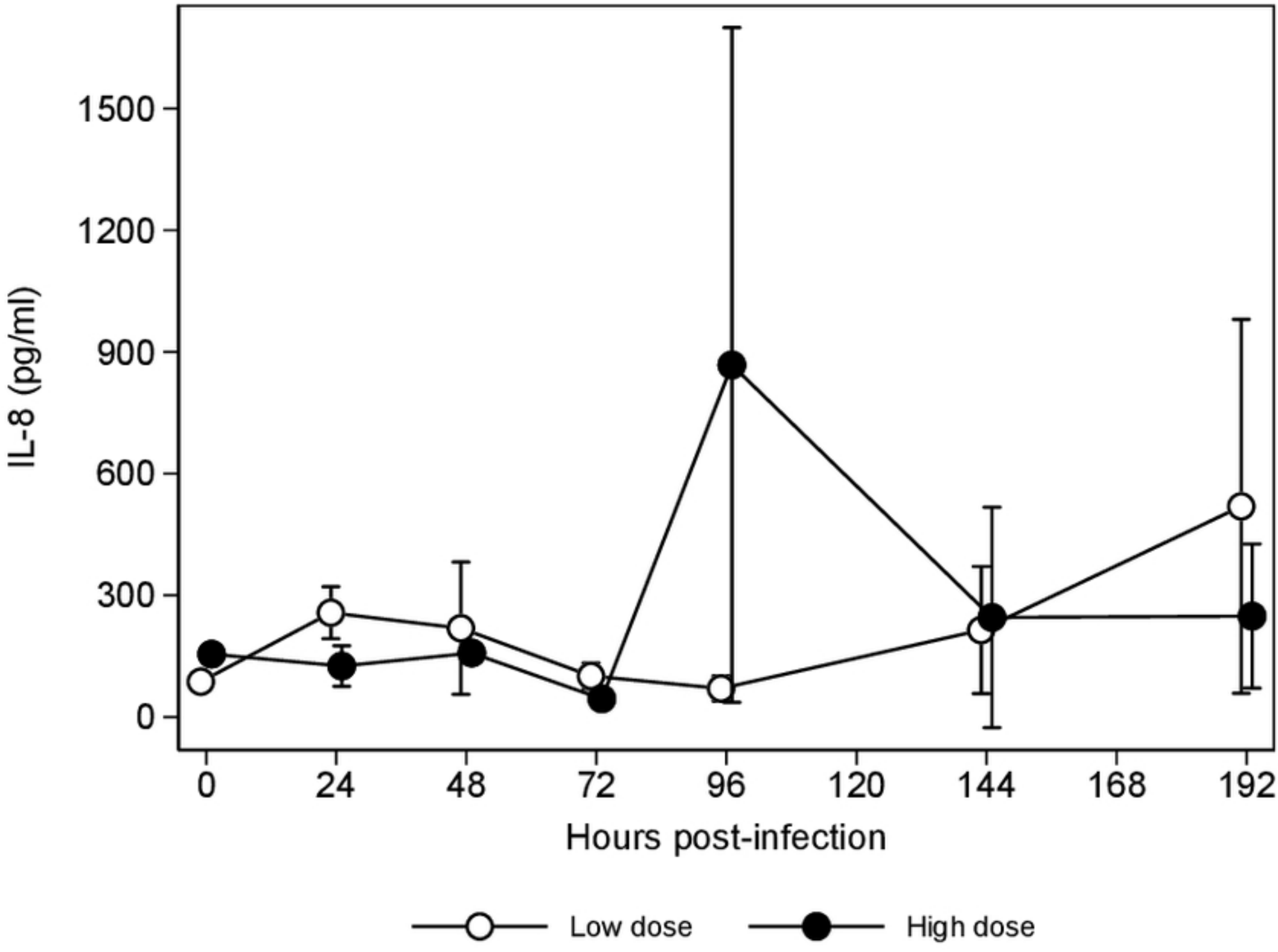

Neutrophil count ×10^9/L
30
20
10
0
0
24
48
72
96
120
144
168
192
Hours post-infection
Mature neutrophils (Low dose)
Mature neutrophils (High dose)
Band neutrophils (Low dose)
Band neutrophils (High dose)

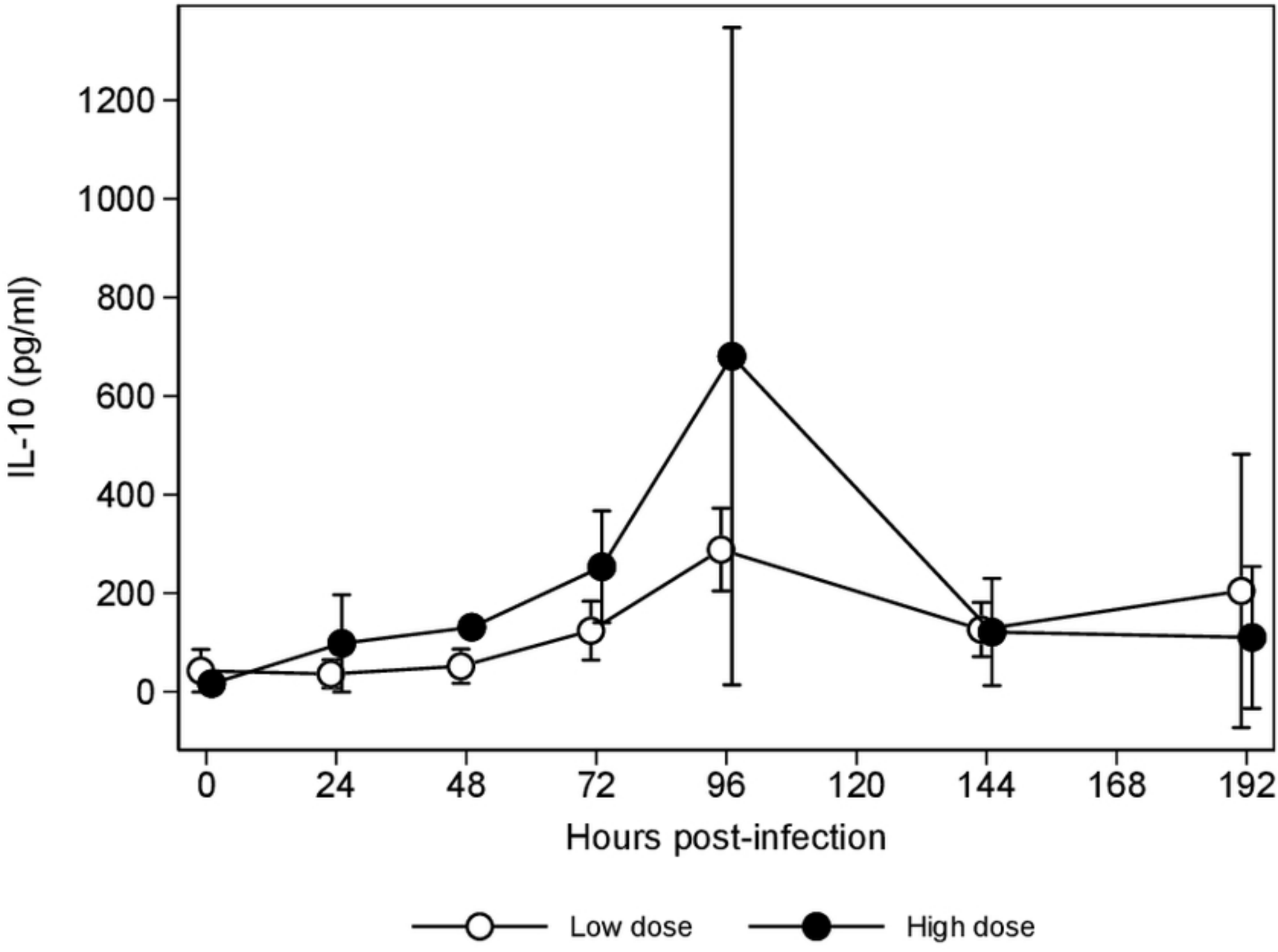

IFNg (pg/ml)
200
150
100
50
0
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose

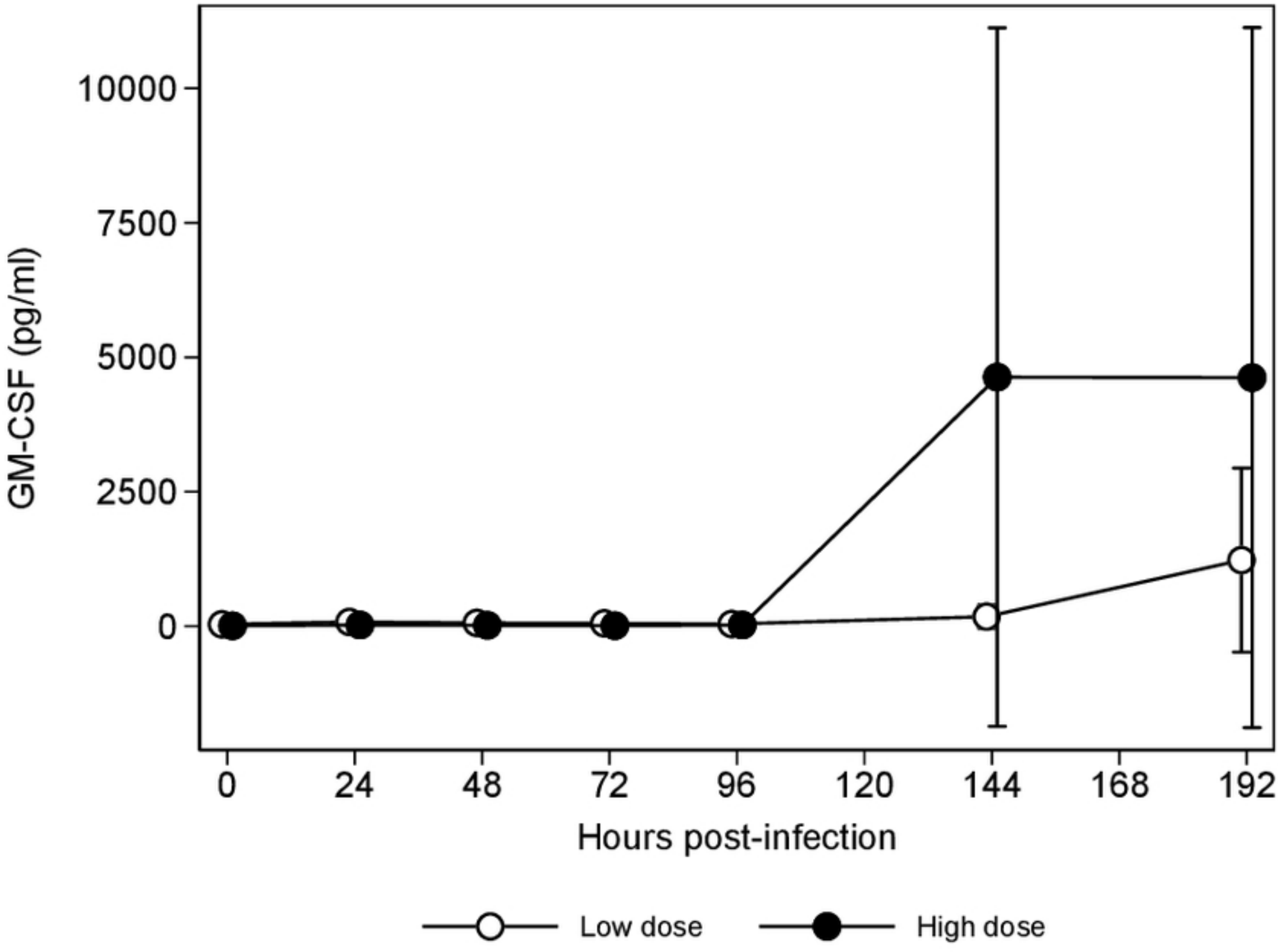

KC-like (pg/ml)
800
600
400
200
0
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose

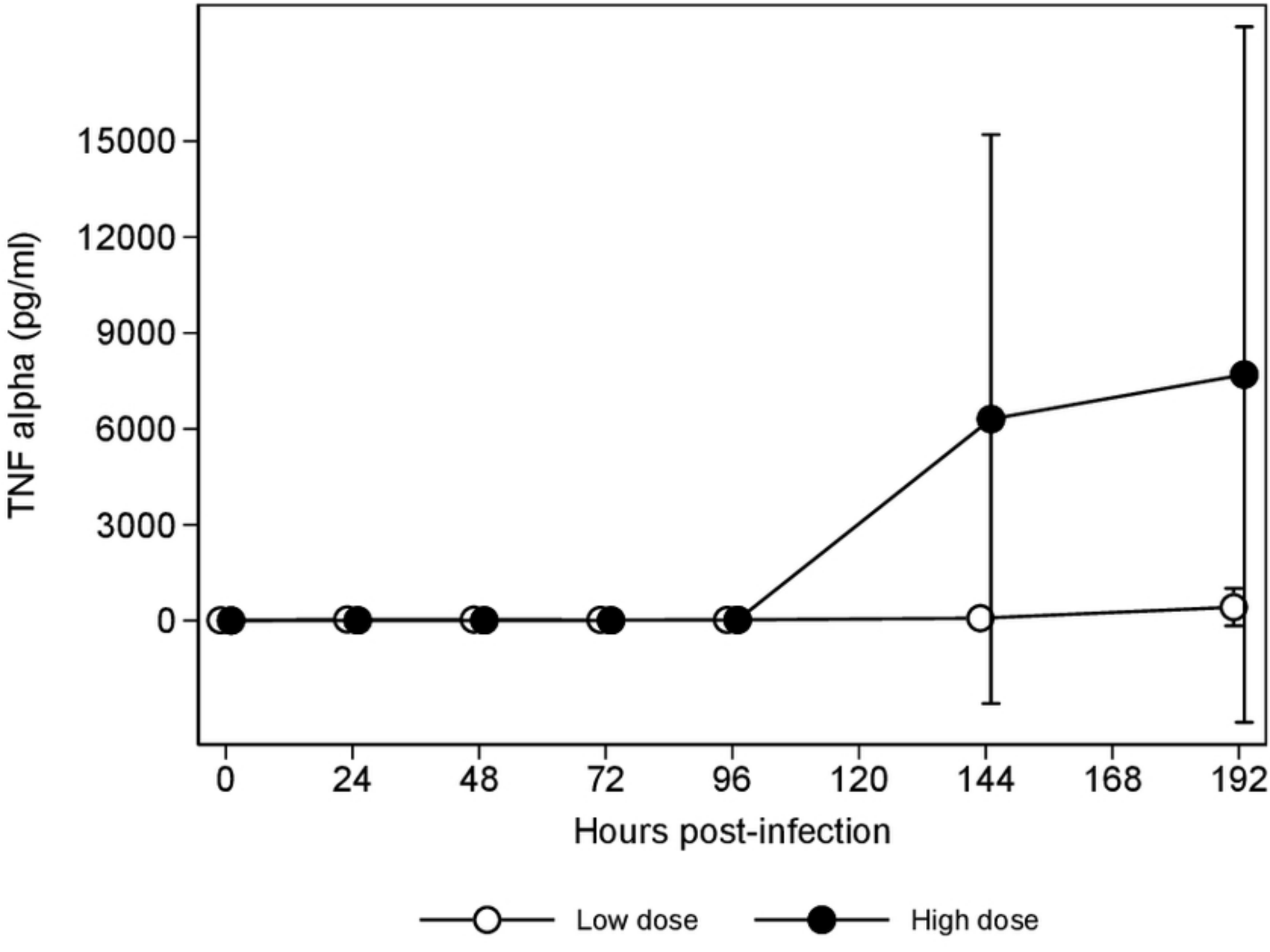

250000
200000
150000
100000
50000
0
MCP-1 (pg/ml)
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose

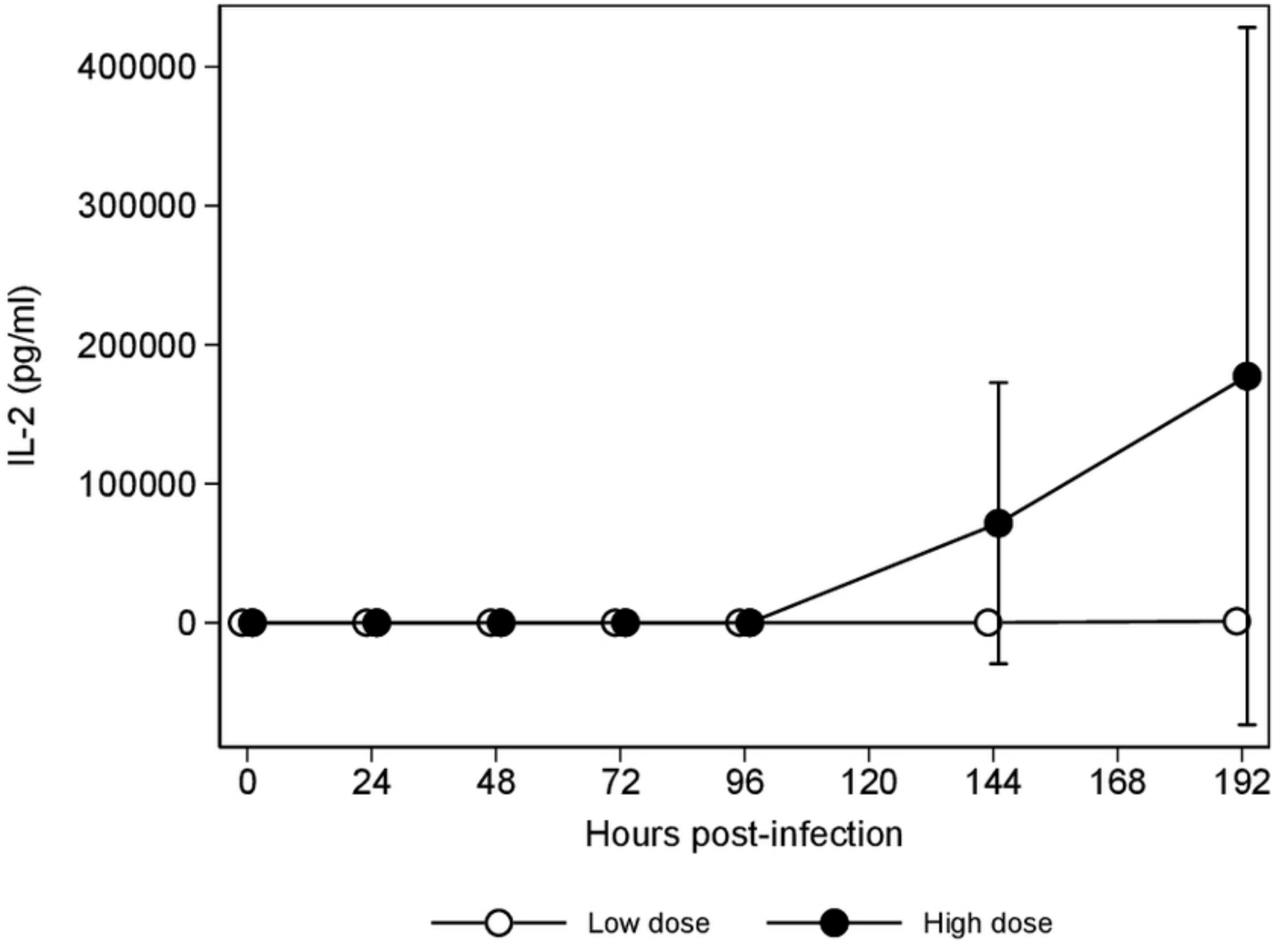

60000
45000
30000
15000
0
IL-6 (pg/ml)
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose


1500
1200
900
600
300
0
IL-8 (pg/ml)
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose


IL-10 (pg/ml)
1200
1000
800
600
400
200
0
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose


10000
7500
5000
2500
0
GM-CSF (pg/ml)
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose


15000
12000
9000
6000
3000
0
TNF alpha (pg/ml)
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose


400000
300000
200000
100000
0
IL-2 (pg/ml)
0
24
48
72
96
120
144
168
192
Hours post-infection
Low dose
High dose