Original Article

Lipopolysaccharide and toll-like receptor 4 in dogs with congenital portosystemic shunts

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Abstract

Surgical attenuation of a congenital portosystemic shunts (CPSS) results in increased portal vein perfusion, liver growth and clinical improvement. Portal lipopolysaccharide (LPS) is implicated in liver regeneration via toll-like receptor (TLR) 4 mediated cytokine activation. The aim of this study was to investigate factors associated with LPS in dogs with CPSS.

Plasma LPS concentrations were measured in the peripheral and portal blood using a limulus amoebocyte lysate (LAL) assay. LPS concentration was significantly greater in the portal blood compared to peripheral blood in dogs with CPSS (P = 0.046) and control dogs (P = 0.002). LPS concentrations in the peripheral (P = 0.012) and portal (P = 0.005) blood of dogs with CPSS were significantly greater than those for control dogs. The relative mRNA expression of cytokines and TLRs was measured in liver biopsies from dogs with CPSS using quantitative PCR. TLR4 expression significantly increased following partial CPSS attenuation (P = 0.020). TLR4 expression was significantly greater in dogs that tolerated complete CPSS attenuation (P = 0.011) and those with good portal blood flow on pre-attenuation (P = 0.004) and post-attenuation (P = 0.015) portovenography. Serum IL-6 concentration was measured using a canine specific ELISA and significantly increased 24 h following CPSS attenuation (P < 0.001).

Portal LPS was increased in dogs with CPSS, consistent with decreased hepatic clearance. TLR4 mRNA expression was significantly associated with portal blood flow and increased following surgery. These findings support the concept that portal LPS delivery is important in the hepatic response to surgical attenuation. Serum IL-6 significantly increases following surgery, consistent with LPS stimulation via TLR4, although this increase might be non-specific.

Keywords: Lipopolysaccharide; Toll like receptor 4; IL-6; Liver; Dog; congenital portosystemic shunts

Introduction

A congenital portosystemic shunt (CPSS) is an abnormal vessel connecting the portal venous system to the systemic venous system (Payne et al., 1990). A CPSS allows blood from the splanchnic viscera to bypass the liver, resulting in portal vein hypoperfusion and hence liver hypoplasia and hepatic insufficiency. Surgical CPSS attenuation is recommended to restore normal portal blood flow. Successful CPSS attenuation results in resolution of clinical signs, improvements in hepatic function and portal perfusion and increased liver volume (Hunt and Hughes, 1999; Kummeling et al., 2010; Lee et al., 2006; Stieger et al., 2007). These findings suggest that the return of normal hepatic size and function is achieved by liver regeneration. We have previously shown that markers of hepatocyte replication are associated with the degree of liver development and the response to surgery in dogs with CPSS, supporting a role for hepatic regeneration (Tivers et al., 2014a).

Hepatic portal blood flow contributes 80% of the afferent liver blood flow and is vital for normal liver regeneration in people, rodents and dogs (Mathie et al., 1979; Michalopoulos, 2007). In experimental partial hepatectomy (PH) in pigs and rats, removal of two thirds of the liver mass caused an effective increase in hepatic portal blood flow (Kahn et al., 1984; Rice et al., 1977). It is unclear whether liver regeneration is stimulated by the increase in blood flow or by increased delivery of hepatotrophic substances in the portal blood (Mortensen and Revhaug, 2011). This increase in portal blood flow relative to liver mass is similar to that observed following CPSS attenuation. Intuitively, the response to CPSS attenuation is likely to be governed by similar factors.

Lipopolysaccharide (LPS) or endotoxin is a component of the cell wall of Gram-negative bacteria and is released following bacterial death. Gram-negative bacteria are present in the small intestine and therefore LPS is absorbed from the gut and into the portal vein (Peterson et al., 1991; Howe et al., 1997). LPS has been shown to play a positive role in liver regeneration in rodent models (Cornell, 1985a, b, 1990; Gao et al., 1999). Kupffer cells are specialised hepatic macrophages that bind LPS entering the liver via the portal vein (Freudenberg et al., 1982). LPS acts on Kupffer cells by binding to toll-like receptor (TLR) 4 (Fenton and Golenbock, 1998). Kupffer cells produce IL-6 and tumour necrosis factor (TNF) α after stimulation with LPS in rodents and these cytokines are implicated in the early stages of liver regeneration (Carswell et al., 1975; Shirahama et al., 1988; Decker et al., 1989; Hori et al., 1989; Busam et al., 1990). Activation of the cytokine network via LPS stimulation of Kupffer cells has been suggested as the stimulus for liver regeneration (Fausto, 2006a). Therefore, it is possible that LPS contributes to triggering the hepatic response to CPSS attenuation.

The aim of this study was to investigate whether factors involved in LPS metabolism are increased after surgical attenuation of canine CPSSs. The first aim was to measure the concentration of LPS in portal blood compared with peripheral blood in dogs with CPSS at the time of surgery and control dogs. The second aim was to measure the hepatic mRNA expression of inflammatory cytokines and TLRs in dogs with CPSS, before and after partial attenuation. The third aim was to measure the serum IL-6 concentration before and immediately after CPSS attenuation. The hypotheses tested were that plasma LPS concentration would be significantly greater in the portal blood compared with the peripheral blood and that gene expression and

IL-6 concentration would significantly change in response to surgical CPSS attenuation.

Materials and methods

Clinical management

Dogs with CPSS were prospectively recruited between August 2007 and October 2011. The Ethics Committee of the Royal Veterinary College granted ethical approval (original approval on 4th June 2004 and updated 22nd October 2010, URN 2010 1058) and owners gave full, informed consent. Dogs were treated surgically via suture attenuation of their CPSS as previously described (Lee et al., 2006). Dogs that did not tolerate complete attenuation, due to intra-operative portal hypertension, had partial suture attenuation. Dogs treated with partial attenuation had repeat surgery approximately 3 months post-operatively.

Portovenography was performed before and after temporary complete CPSS attenuation at each surgery to assess the development of the intrahepatic portal vasculature as previously described (Lee et al., 2006). Grade was determined according to the number of generations of intrahepatic portal vessels that were visible on a scale of 1 - 4 (Lee et al., 2006). Portovenogram grades of 1 and 2 represented poor portal blood flow and portovenogram grades of 3 and 4 represented good portal blood flow (Tivers et al., 2014b).

Healthy experimental Beagle dogs that had been humanely destroyed for reasons unrelated to hepatic disease were used as controls for all parts of the study.

Dogs undergoing exploratory laparotomy for reasons unrelated to CPSS were also included as controls for the measurement of serum IL-6 only.

Plasma LPS concentration

Paired residual blood samples were taken peri-operatively from the jugular vein and mesenteric vein of dogs with CPSS. Residual samples were available as a consequence of placing a jugular central venous catheter pre-operatively and a mesenteric catheter intra-operatively for the measurement of portal pressures and for mesenteric portovenography. Blood samples were taken from Beagle control dogs from the jugular vein immediately before and from the portal vein immediately following euthanasia. Samples were collected into heparinised, glass, pyrogen free tubes (Associates of Cape Cod) and the plasma was separated and stored at -80 °C.

A limulus amebocyte lysate (LAL) assay using pyrochrome chromogenic reagent, reconstituted with glucashield beta glucan inhibiting buffer (Associates of Cape Cod) was used to measure the plasma LPS concentration. Samples were heated at 75 °C for 10 min and diluted 1:200 with LAL reagent water (Associates of Cape Cod). Samples were analysed in triplicate using an ELx808 absorbance microplate reader (BioTek). Sample concentration was calculated from the standard curve using Gen5 V1.07.5 software (BioTek).

qPCR Gene Expression

For dogs with CPSS, at each surgery a liver biopsy was taken for routine diagnostic purposes and a portion was placed in RNAlater (Sigma-Aldrich) and stored according to manufacturer's instructions. Liver tissue was taken from Beagle control

158 dogs immediately following euthanasia and stored in the same way. Routine
159 histopathology was performed on sections stained with haematoxylin and eosin.
160
161 RNA was extracted from approximately 20-30 mg of each hepatic sample
162 using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). The tissue
163 was homogenised in 500 µL Lysis Solution using a Mixer Mill MM 300 (Retsch). An
164 in-solution DNase digestion was performed using the Ambion TURBO DNA-free Kit
165 (Life Technologies) to remove any contaminating DNA. RNA quality and quantity
166 was assessed by microfluidic capillary electrophoresis using the Agilent 2100
167 Bioanalyser (Agilent Technologies). The median RNA integrity number was 8.3
168 (range, 7.1-9.2). No samples had genomic DNA contamination. Two separate cDNA
169 were synthesised from each RNA sample using a mixture of random hexamer and
170 oligo $(dT)_{15}$ primers (Promega) and IMProm-II reverse transcriptase enzyme
171 (Promega). Where possible, the amount of RNA template for cDNA synthesis was
172 standardised at 1 µg. The cDNA was diluted to a final volume of 100 µL with
173 nuclease-free water and stored at -20 °C before further use.
174
175 Relative hepatic expression of five genes related to hepatic LPS signalling (IL-
176 1β, IL-6, TNFα, TLR2 and TLR4) was measured using quantitative polymerase chain
177 reaction (qPCR). Published canine specific primers for the genes of interest (Wang et
178 al., 2007; House et al., 2008) and four liver specific reference genes, hydroxymethyl-
179 bilane synthase, ribosomal protein L13a, ribosomal protein L32 and ribosomal protein
180 S18, were used (Peters et al., 2007; Table 1).
181

For quantification, each liver sample had two cDNA samples analysed in duplicate. Reactions were carried out in 25 µL volumes using a Bio-Rad CFX96 Real-Time PCR Detection System thermocycler (Bio-Rad Laboratories). Each reaction consisted of 1 µL cDNA as the template with Immobuffer (1 × concentration), Hi-Spec Additive (1 × concentration), dNTP (final concentration 1 mM), magnesium chloride (final concentration 2.5mM for genes of interest, 4.5mM for reference genes), 1 unit Immolase DNA polymerase (Bioline) and EvaGreen dye (Biotium; 0.06 × diluted 1:4 with nuclease-free water). Samples were incubated at 95 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 10 s. An appropriate primer-dimer melting temperature for 1 s was programmed before fluorescence readings were taken at the end of each cycle. A melting curve analysis from 65 ^{o}C to 95 ^{o}C with a plate read every 0.5 ^{o}C was performed at the end of 40 cycles. Bio-Rad CFX Manager Software (Bio-Rad) was used for initial qPCR analysis.

Analysis of raw real-time data was performed using GenEx professional version 4.4.2 software (Multid Analyses). Relative gene expression was quantified as previously described (Vandesompele et al., 2002). Quantification cycle (Cq) values were corrected using the calculated efficiencies for each primer set. Normalisation of each sample Cq for the genes of interest was performed relative to the geometric normalisation of the four reference genes. The relative expression of the mRNA of each gene of interest in each cDNA sample was then calculated using the normalised Cq of each sample relative to the average Cq of all of the samples. For each gene the following comparisons were made: CPSS compared to control; partial attenuation

compared to complete attenuation; before and after partial attenuation (paired samples).

Serum IL-6 concentration

Blood samples were taken from both dogs with CPSS and control dogs that underwent exploratory laparotomy pre-operatively for diagnostic purposes and after surgery in dogs with CPSS for post-operative monitoring. Where available, residual blood was used for the study. Residual blood samples were also taken immediately before euthanasia in Beagle control dogs. The serum was separated and stored at -80 °C.

A Quantikine Canine ELISA Kit (R and D Systems) was used to measure the serum concentration of IL-6 (Song et al., 2012). Samples were analysed in duplicate using an ELx808 absorbance microplate reader (BioTek). Sample concentration was calculated from the standard curve using Gen5 V1.07.5 software (BioTek).

Statistical analysis

Analysis was performed using PASW Statistics 18.0.0 statistical software package (Education SPSS, IBM). Continuous data were visually assessed for normality. Median and range were reported for skewed data, which was compared with the Mann Whitney U test or the Wilcoxon signed-ranks test as appropriate. Repeated measures were compared with the Friedman's two-way analysis of variance by ranks with pair-wise comparison. The LPS concentration and qPCR data was transformed to normal distribution (square root or logarithm). The data was then

compared with an independent *t* test or paired sample *t* test. Significance was set at the 5% level ($P \leq 0.05$).

Results

Plasma LPS concentration

Paired peripheral and portal plasma samples from 13 dogs with CPSS were included. The following breeds were included: Bichon Frise (*n*=2), Labrador (*n*=2), Border terrier (*n*=1), Cavalier King Charles spaniel (*n*=1), Crossbreed (*n*=1), Dogue de Bordeaux (*n*=1), German shepherd dog (*n*=1), Miniature Schnauzer (*n*=1), Springer spaniel (*n*=1), West Highland white terrier (*n*=1), Yorkshire terrier (*n*=1). The median age was 295 days (range, 125-1835 days). Nine dogs (69.2%) had an extrahepatic CPSS and four (30.8%) had an intrahepatic CPSS.

Paired peripheral and portal plasma samples from nine healthy Beagles were included as control dogs. The median age was 1136 days (range, 497-1660 days), which was significantly greater than dogs with CPSS ($P = 0.036$).

For dogs with CPSS, the median LPS concentration in the portal blood was 0.453 endotoxin units (EU)/mL (range, 0.117-1.418 EU/mL), which was significantly greater than that in the peripheral blood (0.239 EU/mL; range, 0.056-1.410 EU/mL; $P = 0.046$; Fig. 1). For Beagle control dogs, the median LPS concentration in the portal blood was 0.184 EU/mL (range, 0.126-0.565 EU/mL), which was significantly greater than that in the peripheral blood (0.131 EU/mL; range, 0.061-0.187 EU/mL; $P = 0.002$; Fig. 1). The LPS concentrations in the peripheral blood ($P = 0.012$) and portal

blood (P = 0.005) of dogs with CPSS were both significantly greater than for Beagle control dogs (Fig. 1).

qPCR gene expression

Liver samples obtained at the first surgery were available from 49 dogs. The following breeds were included: Yorkshire terrier (*n*=7), Crossbreed (*n*=6), Labrador (*n*=5), Miniature Schnauzer (*n*=5), West Highland white terrier (*n*=5), Cocker spaniel (*n*=4), Jack Russell terrier (*n*=3), Bichon Frise (*n*=2), Golden retriever (*n*=2), Lhasa Apso (*n*=2), Pug (*n*=2), Chihuahua (*n*=1), Hovawart (*n*=1), Irish Setter (*n*=1), Norfolk terrier (*n*=1), Old English sheepdog (*n*=1), Staffordshire bull terrier (*n*=1). The median age was 275 days (range, 97-4374 days). Thirty-eight (77.6%) dogs had an extrahepatic CPSS and 11 (22.4%) had an intrahepatic CPSS. Of the 49 dogs that had surgery, 24 (49%) had complete attenuation and 25 (51%) had partial attenuation. The 25 dogs that had partial attenuation had repeat surgery a median of 110 days post-operatively (range, 69-358 days). At the time of this repeat surgery, liver samples from these 25 dogs were obtained for a second analysis, enabling comparison of results with those from the first liver samples. At second surgery, 20 dogs tolerated complete CPSS attenuation, three dogs had progressed to complete shunt occlusion spontaneously (no flow on portovenography), and two dogs had developed multiple acquired shunts. The liver of all dogs with CPSS at first and second surgery showed characteristic changes consistent with portal hypoperfusion. No additional pathology was noted. Liver samples were acquired from seven Beagle control dogs as controls. The median age of control dogs was 628 days (range, 515-1544 days), which was

278 significantly older than for dogs with CPSS (P = 0.036). The liver of all control dogs
279 was histopathologically unremarkable. The results are summarised in Table 2.
280
281 Relative mRNA expression of IL-1β (P = 0.016) and IL-6 (P = 0.002) were
282 both significantly greater for dogs with CPSS than for Beagle control dogs (Fig. 2).
283 Relative TLR4 mRNA expression was significantly greater in dogs with complete
284 attenuation compared with those with partial attenuation (P = 0.011; Fig. 2). Relative
285 TLR4 mRNA expression significantly increased following partial attenuation (P =
286 0.020; Fig. 2). Relative TLR4 mRNA expression was also compared between dogs
287 with poor portal blood flow and those with good portal blood flow on
288 portovenography (further details below). Relative TLR4 mRNA expression was
289 significantly greater in dogs with good portal blood flow compared to those with poor
290 portal blood flow on both pre-attenuation (P = 0.004) and post-attenuation
291 portovenograms (P = 0.015; Fig. 3, Table 3).
292
293 No significant associations were demonstrated for the relative mRNA
294 expression of TNFα or TLR2.
295
296 *Portovenogram grading*
297 Complete portovenograms were available for 47/49 dogs at first surgery and
298 21/25 dogs at second surgery. Pre-attenuation and post-attenuation portovenogram
299 grades at first surgery were significantly greater for dogs with complete attenuation
300 compared with dogs with partial attenuation (P < 0.001 for each). For dogs treated
301 with partial attenuation, there was a significant increase in both pre-temporary CPSS

attenuation portovenogram grade ($P < 0.001$) and post-temporary CPSS attenuation portovenogram grade ($P = 0.001$) from first to second surgery (Table 4).

Serum IL-6 concentration

Serum samples taken before and at 24 and 48 h post-surgery from 22 dogs with CPSS were analysed. The following breeds were included: Yorkshire terrier (*n*=4), Norfolk terrier (*n*=3), West Highland white terrier (*n*=3), Jack Russell terrier (*n*=2), Miniature Schnauzer (*n*=2), Shih Tzu (*n*=2), British bulldog (*n*=1), Crossbreed (*n*=1), Golden retriever (*n*=1), Labrador (*n*=1), Miniature Dachshund (*n*=1), Shetland sheepdog (*n*=1). The median age was 334.5 days (range, 114-2282 days). Nineteen (86.4%) dogs had an extrahepatic CPSS and three (13.6%) had an intrahepatic CPSS. Twelve dogs (54.5%) had complete attenuation and 10 (45.5%) had partial attenuation.

Serum samples from seven healthy Beagles and five dogs undergoing abdominal surgery were included as controls. The following breeds were included in the abdominal surgery controls: Crossbreed (*n*=2), Golden retriever (*n*=1), Labrador (*n*=1), Shar Pei (*n*=1). The dogs were undergoing abdominal surgery for the investigation or treatment of insulinoma, adrenal carcinoma, splenic carcinoma, phaeochromocytoma and extrahepatic biliary tract obstruction. The median age of control dogs was 925 days (range, 526-4204 days); they were significantly older than dogs with CPSS ($P < 0.001$).

There was no significant difference in pre-operative IL-6 concentrations between dogs with CPSS (median, 0 pg/mL; range, 0-876.75 pg/mL) and control dogs

(median, 0 pg/mL; range, 0-40.66 pg/mL). The median IL-6 concentration at 24 h in dogs with CPSS was 34.461 pg/mL (range, 0-483.726 pg/mL) and at 48 h was 8.137 pg/mL (range, 0-683.925 pg/mL; Fig. 4). In dogs with CPSS, there was a significant difference in the concentration of IL-6 at the different time points ($P < 0.001$). Pair-wise comparison of this data set confirmed that IL-6 at 24 h post-surgery was significantly greater than pre-surgery ($P < 0.001$).

Discussion

We measured the LPS concentration in peripheral and portal blood samples from dogs with CPSS and healthy Beagle control dogs. In normal animals and humans, LPS from the gut is transported to the liver by the portal system and is cleared by the Kupffer cells (Bradfield, 1974; Zlydaszyk and Moon, 1976; Jacob et al., 1977). Therefore, LPS is routinely detected in the portal circulation, but only a small amount is found in peripheral venous blood (Prytz et al., 1976; Jacob et al., 1977). Portal LPS concentrations are significantly greater than peripheral concentrations in both healthy humans and those with liver disease (Tachiyama et al., 1988; Lumsden et al., 1988; Benten et al., 2011; Sanada et al., 2012). However, LPS is increased in both the peripheral and portal blood of humans with liver cirrhosis due to increased absorption from the gut and decreased hepatic clearance (Lumsden et al., 1988; Tachiyama et al., 1988; Lin et al., 1995; Kaser et al., 2002). This increase is thought to be due to either the shunting of blood past the liver via multiple acquired shunts (MAS) or impaired LPS clearance due to liver pathology (Kaser et al., 2002).

Our study demonstrated that portal LPS concentration was significantly greater than peripheral concentration in dogs with CPSS and Beagle control dogs.

Peripheral and portal LPS concentrations were also significantly greater in dogs with CPSS compared to Beagle control dogs. These findings are consistent with those in humans. It is unsurprising that LPS concentrations were significantly greater in dogs with CPSS, as hepatic clearance is reduced as a consequence of shunting, as in humans with MAS. However, in people with liver cirrhosis there is also increased absorption of LPS from the gut, which is presumably not the case in dogs with CPSS. Thus, the main reason for the increase in LPS is likely to be decreased hepatic clearance. A previous study measured LPS in the peripheral and portal blood of 10 dogs with CPSS compared with five control dogs using an LAL assay (Peterson et al., 1991). Contrary to our study, there were no significant differences in LPS concentrations between peripheral and portal samples or between CPSS and control dogs. The reason for this discrepancy could be due to differences in experimental methodology or to variables in the dogs studied. Another study measured LPS concentration in the portal vein, hepatic vein and caudal vena cava of 10 dogs with experimentally created MAS and six control dogs using an LAL assay (Howe et al., 1997). In agreement with our study, LPS concentration was significantly greater in all vessels for dogs with MAS compared with control dogs.

In our study, and in the two canine studies mentioned above, an LAL assay was used to measure plasma LPS concentration. The LAL assay has been commonly used to measure plasma LPS in people with liver disease (Jacob et al., 1977; Lumsden et al., 1988; Tachiyama et al., 1988; Lin et al., 1995; Benten et al., 2011). Many variables can affect assay results and there is considerable variation in methodology between studies, which can affect the sensitivity and reliability of the LAL assay (Novitsky, 1994; Hurley, 1995). The endotoxin activity assay (EAA) is a more recent

technique for measuring endotoxin and uses chemiluminescence of neutrophil activity (Romaschin et al., 1998; Marshall et al., 2002). It has been used in both humans and dogs and is considered to be more accurate than the LAL method (Marshall et al., 2002; Kjelgaard-Hansen et al., 2008; Sanada et al., 2011). It is possible that inclusion of the EAA test in our methodology might have improved the reliability of our results, but this test was not available to us for logistical and financial reasons.

Our findings demonstrated that LPS concentration was increased in the portal blood of dogs with CPSS, most likely due to decreased hepatic clearance, suggesting that reduced delivery of portal blood to the liver is accompanied by reduced LPS delivery to the liver. This is consistent with the concept that portal delivery of LPS to the liver is a potential trigger for the hepatic response to attenuation. Surgical CPSS attenuation increases portal blood flow and hence increases LPS delivery to the liver. Following CPSS attenuation, normalisation of LPS concentrations is expected due to increased hepatic clearance. However, follow-up samples were not available so this was not assessed.

We have demonstrated that there was a significant increase in serum IL-6 at 24 h following CPSS attenuation. IL-6 is a key mediator of the initial stages of hepatic regeneration and rapid increases in hepatic and serum IL-6 are observed following PH in rodents (Cressman et al., 1996; Sakamoto et al., 1999; Iwai et al., 2001; Aldeguer et al., 2002). Increased portal blood flow results in stimulation of Kupffer cells and release of IL-6, an initiator of regeneration (Decker, 1990; Meijer et al., 2000; Abshagen et al., 2007; Riehle et al., 2008). The increase in IL-6 in dogs with CPSS following attenuation could, at least in part, be due to increased hepatic

blood flow and IL-6 release as part of liver regeneration. However, there is another possible explanation for the increase in IL-6. Abdominal surgery in humans is associated with an inflammatory response, resulting in increased IL-6 in the peripheral and portal blood (Cruickshank et al., 1990; Di Padova et al., 1991; Baigrie et al., 1992; Glaser et al., 1995; Biffl et al., 1996; Kimura et al., 1998). Logically, the increase in serum IL-6 following CPSS surgery might also be due to surgical trauma. A control group of dogs undergoing laparotomy for reasons unrelated to CPSS attenuation with pre- and post-operative serum samples would have provided more information on the specificity of post-operative increase in IL-6.

Significant post-operative increases in serum IL-6 are seen in humans undergoing PH for tumour resection and in individuals donating or receiving liver transplants (Kimura et al., 1998, 1999; Hu et al., 1999; Asakura et al., 2000; Chijiiwa et al., 2002; Slotwinski et al., 2002). One study demonstrated a significant increase in serum IL-6 at 24, 72 and 168 h post-hepatectomy for liver donation, but there was no significant increase post-hepatectomy for benign neoplasia (Slotwinski et al., 2002). The study concluded that in humans with normal livers, IL-6 increased following partial hepatectomy, consistent with hepatic regeneration. This is similar to the findings of our study, although IL-6 rapidly returned to normal, whereas it remained increased in people following hepatectomy. In other studies of abdominal surgery in people, IL-6 levels increase rapidly to peak at 4-48 h post-operatively, but can remain increased for 48-72 h (Di Padova et al., 1991; Baigrie et al., 1992; Biffl et al., 1996). The reason for these discrepancies is unclear, but could be related to differences in the nature of the surgery and therefore the hepatic response, species differences, differences in methodology and assay sensitivity. However, pre-existing liver disease

is associated with increased IL-6 and this could affect post-operative changes (Hu et al., 1999; Slotwinski et al., 2002). Similarly, dogs with liver disease have increased serum IL-6 concentrations compared with healthy dogs (Neumann et al., 2012). In a recent study, plasma IL-6 concentrations were increased in dogs with CPSS (Kilpatrick et al., 2014). Our study did not find a significant difference in serum IL-6 concentrations between CPSS and control dogs. However, it is possible that differences in the populations and methodology could be responsible for this discrepancy. Additionally, our study had a relatively small number of control dogs, perhaps resulting in a type II statistical error.

We measured the expression of IL-1β, IL-6 and TNFα mRNA in liver tissue. The expression of both IL-1β and IL-6 mRNA in liver tissue were significantly greater in dogs with CPSS compared to Beagle control dogs. IL-1β, IL-6 and TNFα are inflammatory cytokines that are released by Kupffer cells in response to stimulation by LPS (Shirahama et al., 1988; Decker et al., 1989; Busam et al., 1990). IL-6 and TNFα are initiators of regeneration and IL-1β inhibits regeneration (Boulton et al., 1997; Fausto et al., 2006b; Riehle et al., 2011). Therefore, it seems incongruous that both IL-6 and TNFα are increased in an underdeveloped CPSS liver. As mentioned above, abdominal surgery initiates an inflammatory response accompanied by increases in serum IL-6 and IL-1β; increases in IL-1β precede those for IL-6 (Baigrie et al., 1992; Glaser et al., 1995; Kimura et al., 1998). It is possible that liver biopsy would result in similar increases in IL-1β and IL-6 in the traumatised tissue. Therefore, the increased cytokine expression in CPSS liver tissue could be due to acute release following surgical trauma. As control liver tissue was obtained post-mortem, there might not have been similar increases in cytokine expression.

452 However, this potential explanation is conjecture and remains unproven. Several
453 studies have shown that dogs with CPSS, and in particular those with hepatic
454 encephalopathy, have evidence of generalised inflammation with increased serum IL-
455 6, plasma C-reactive protein (CRP) and systemic inflammatory response syndrome
456 scores (Gow et al., 2012; Kilpatrick et al., 2014; Tivers et al., 2014c, 2015). It is
457 possible that more generalised increases in IL-1β and IL-6 as a result of pre-existing
458 inflammation could be responsible for the increased hepatic expression of these
459 cytokines. It is also possible that differences in IL-1β and IL-6 between the CPSS and
460 control dogs could have been due to differences in breed and age between the two
461 groups. However, if this were the case, a physiological reason for this difference is
462 unclear.
463
464 We measured the hepatic mRNA expression of TLR2 and TLR4. TLR4
465 mRNA expression was significantly increased in dogs with CPSS tolerating complete
466 attenuation compared to those which tolerated only partial attenuation. Dogs with
467 well-developed intrahepatic portal vasculature on portovenography had significantly
468 increased TLR4 mRNA expression. Additionally, TLR4 mRNA expression
469 significantly increased following partial attenuation. In contrast, no significant
470 differences were identified for TLR2. This finding might be because TLR2 plays a
471 major role in detection of Gram-positive bacteria, recognising components of the cell
472 wall including peptidoglycan, lipoteichoic acid and lipoproteins (Yoshimura et al.,
473 1999). Gram-positive bacteria are not the predominant component of typical gut
474 flora. The absence of a significant difference in TLR2 mRNA expression in
475 conjunction with a significant increase in TLR4 mRNA expression increases the

likelihood that these data reflect a specific interaction between gut flora pathogen-associated molecular patterns and the hepatic response to CPSS attenuation.

TLR4 is expressed by Kupffer cells and binds LPS, enabling circulatory clearance of LPS (Freudenberg et al., 1982; Fenton and Golenbock, 1998). LPS is very important for normal hepatic regeneration (Cornell, 1985a, b, 1990; Gao et al., 1999). Kupffer cell release of IL-6 and TNFα in response to LPS is implicated in the initiation of hepatic regeneration (Fausto, 2006a). Increased expression of TLR4 mRNA suggests increased LPS binding capacity in dogs with CPSS and good portal blood flow and in those able to tolerate complete attenuation. As partial attenuation increases portal blood flow, increased TLR4 mRNA is therefore consistent with increased LPS delivery. This provides further evidence that TLR4 and blood flow are important in the hepatic response to surgery. These findings demonstrate that TLR4 mRNA expression is linked with portal blood flow and in the response to surgical attenuation in dogs with CPSS. We have previously shown that the hepatic expression of hepatocyte growth factor (HGF) and methionine adenosyltransferase 2 A, which are both markers of hepatocyte replication, are significantly increased following partial CPSS attenuation (Tivers et al., 2014a). We have also shown that vascular endothelial growth factor receptor 2 (VEGFR2) is significantly associated with the degree of portal blood flow and significantly increases following partial CPSS attenuation (Tivers et al., 2014b). In addition, these studies also demonstrated that there were significant increases in HGF and VEGF immediately following CPSS surgery (Tivers et al., 2014a, b). These data suggest that both hepatic regeneration, in the form of hepatocyte replication and angiogenesis, are associated with CPSS attenuation. The findings of the current study are in broad agreement with these

findings and support the concept that activation of Kupffer cells via TLR4 binding of LPS could be involved in this process. Further work is needed to explore this concept.

There are a number of limitations to the current study that must be taken into account. The number of dogs included in the study was relatively small, particularly for the measurement of plasma LPS and in the control groups for the other experiments. Larger group size might have allowed further statistically significant findings to be identified. Nevertheless, we were able to identify a number of findings that were both biologically relevant and statistically significant. In addition, the experimental Beagles used as control dogs were significantly older than the dogs with CPSS. Consequently, it is possible that the differences detected in LPS concentration and cytokine mRNA expression could have been related to breed or age rather than to CPSS.

Conclusions

Our results have demonstrated that portal LPS is increased in dogs with CPSS, consistent with decreased hepatic clearance due to shunting. In addition, hepatic TLR4 mRNA expression was significantly associated with portal blood flow and significantly increased following CPSS attenuation. This suggests that LPS binding capacity via TLR4 is linked to blood flow and the degree of portal development. This provides supporting evidence for the concept that LPS triggers liver regeneration via Kupffer cell binding and signalling following CPSS attenuation. Further investigations are warranted to explore this mechanism in more depth.

Conflict of interest statement

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

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Table 1

Table showing details of reference gene and gene of interest primer pairs for quantitative PCR.

Gene	Primer sequences	PCR amplicon length (bp)	Genbank accession number	Primer sequence reference
HMBS	Forward: TCACCATCGGAGCCATCT Reverse: GTTCCCACCACGCTCTTCT	112	XM546491	Peters et al., 2007
RPL13A	Forward: GCCGGAAGGTTGTAGTCGT Reverse: GGAGGAAGGCCAGGTAATTC	87	AJ388525	Peters et al., 2007
RPL32	Forward: TGGTTACAGGAGCAACAAGAAA Reverse: GCACATCAGCAGCACTTCA	100	XM_848016	Peters et al., 2007
RPS18	Forward: TGCTCATGTGGTATTGAGGAA Reverse: TCTTATACTGGCGTGGATTCTG	116	XM_532106	Peters et al., 2007
IL-1β	Forward: TCTCCCACCAGCTCTGTAACAA Reverse: GCAGGGCTTCTTCAGCTTCTC	80	Z70047	Wang et al., 2007
IL-6	Forward: TCCTGGTGATGGCTACTGCTT Reverse: GACTATTTGAAGTGGCATCATCCTT	78	U12234	Wang et al., 2007
TNFα	Forward: GAGCCGACGTGCCAATG Reverse: CAACCCATCTGACGGCACTA	79	Z70046	Wang et al., 2007
TLR2	Forward: AGTGGCCAGAAAAGCTGAAA Reverse: ATCCAGTTGCTCCTTCGAGA	263	NM001005264	House et al., 2008
TLR4	Forward: CAAAATCCCCAACAACATCC Reverse: TGGTTTAGGCCCTGATATGC	171	NM001002950	House et al., 2008

bp, base pairs; HMBS, hydroxymethyl-bilane synthase; RP, ribosomal protein; TNF, tumour necrosis factor; TLR, toll-like receptor

Table 2

Relative mRNA expression of cytokines and toll-like receptors (normalised with respect to four liver specific reference genes) in liver biopsies from 49 dogs with congenital portosystemic shunts (CPSS) and seven Beagle control dogs. Results are presented as median and range.

Gene	Control compared to CPSS			Complete attenuation compared to partial attenuation			Before and after partial attenuation (*n*=25)		
	Control [a] (*n*=7)	CPSS (*n*=49)	*P*	Partial (*n*=25)	Complete (*n*=24)	*P*	Before partial attenuation	After partial attenuation	*P*
IL-1β	3.351 (2.361-5.723)	11.172 (1.654-919.494)	0.016[b]	7.817 (1.654-197.141)	11.849 (2.826-919.494)	0.052	7.817 (1.654-197.141)	8.981 (1.975-169.281)	0.800
IL-6	2.569 (1.288-3.463)	9.473 (1.581-332.589)	0.002[b]	8.310 (1.914-332.589)	13.044 (1.581-229.601)	0.421	8.310 (1.914-332.589)	5.543 (1.548-207.752)	0.155
TNFα	2.554 (2.158-4.402)	2.818 (1.509-11.364)	0.772	2.736 (1.509-5.917)	2.833 (1.544-11.364)	0.601	2.736 (1.509-5.917)	2.748 (1.326-14.032)	0.174
TLR2	2.041 (1.700-3.525)	2.241 (1.396-9.904)	0.298	2.204 (1.396-5.046)	2.242 (1.602-9.904)	0.401	2.204 (1.396-5.046)	2.372 (1.189-12.289)	0.148
TLR4	4.340 (4.005-7.049)	5.067 (1.581-20.575)	0.396	4.337 (1.581-14.505)	6.189 (3.183-20.575)	0.011[b]	4.337 (1.581-14.505)	6.065 (1.627-11.617)	0.020[b]

IL, interleukin; TNF, tumour necrosis factor; TLR, toll-like receptor

[a] Beagle dogs

[b] Statistically significant value ($P \leq 0.05$)

Table 3

Relative mRNA expression of toll-like receptor 4 (TLR4), normalised with respect to four liver specific reference genes, in liver biopsies from 47 dogs with congenital portosystemic shunts as related to portal blood flow on pre-attenuation and post-attenuation portovenogram, at first surgery. Results are presented as median and range.

Gene	Pre-attenuation portal blood flow			Post-attenuation portal blood flow		
	Poor (35 dogs)	Good (12 dogs)	*P*	Poor (12 dogs)	Good (35 dogs)	*P*
TLR4	4.607 (1.581-14.505)	7.638 (3.423-20.575)	0.004[a]	4.271 (1.581-8.060)	5.513 (2.188-20.575)	0.01 [a]

[a] Statistically significant value ($P \leq 0.05$)

Table 4

Portovenogram grade before and after temporary congenital portosystemic shunt attenuation in 21 dogs at first and second surgery. This group of dogs all had a partial attenuation at the first surgery. There was a significant increase in portovenogram grade for both pre-attenuation and post-attenuation from first to second surgery ($P < 0.001$ and $P = 0.001$, respectively).

Timing of assessment	*n* (%) dogs for each portovenogram grade			
	Grade 1	Grade 2	Grade 3	Grade 4
1st Surgery Pre-attenuation	18 (85.7)	3 (14.3)	0 (0)	0 (0)
1st Surgery Post-attenuation	0 (0)	12 (57.1)	9 (42.9)	0 (0)
2nd Surgery Pre-attenuation	2 (9.5)	8 (38.1)	6 (28.6)	5 (23.8)
2nd Surgery Post-attenuation	0 (0)	4 (19.0)	6 (28.6)	10 (52.4)

Figure legends

Fig. 1. Measurement of lipopolysaccharide (LPS) concentration (endotoxin units [EU]/mL) in peripheral and portal plasma from 13 dogs with congenital portosystemic shunts (CPSS) and nine healthy Beagle control dogs using a limulus amebocyte lysate (LAL) assay. Statistical significance is highlighted with the corresponding *P* value. (A) Peripheral and portal LPS concentration in dogs with CPSS. (B) Peripheral and portal LPS concentrations in Beagle control dogs. (C) Peripheral LPS concentration in Beagle control dogs compared with dogs with CPSS. (D) Portal LPS concentration in Beagle control dogs compared with dogs with CPSS.

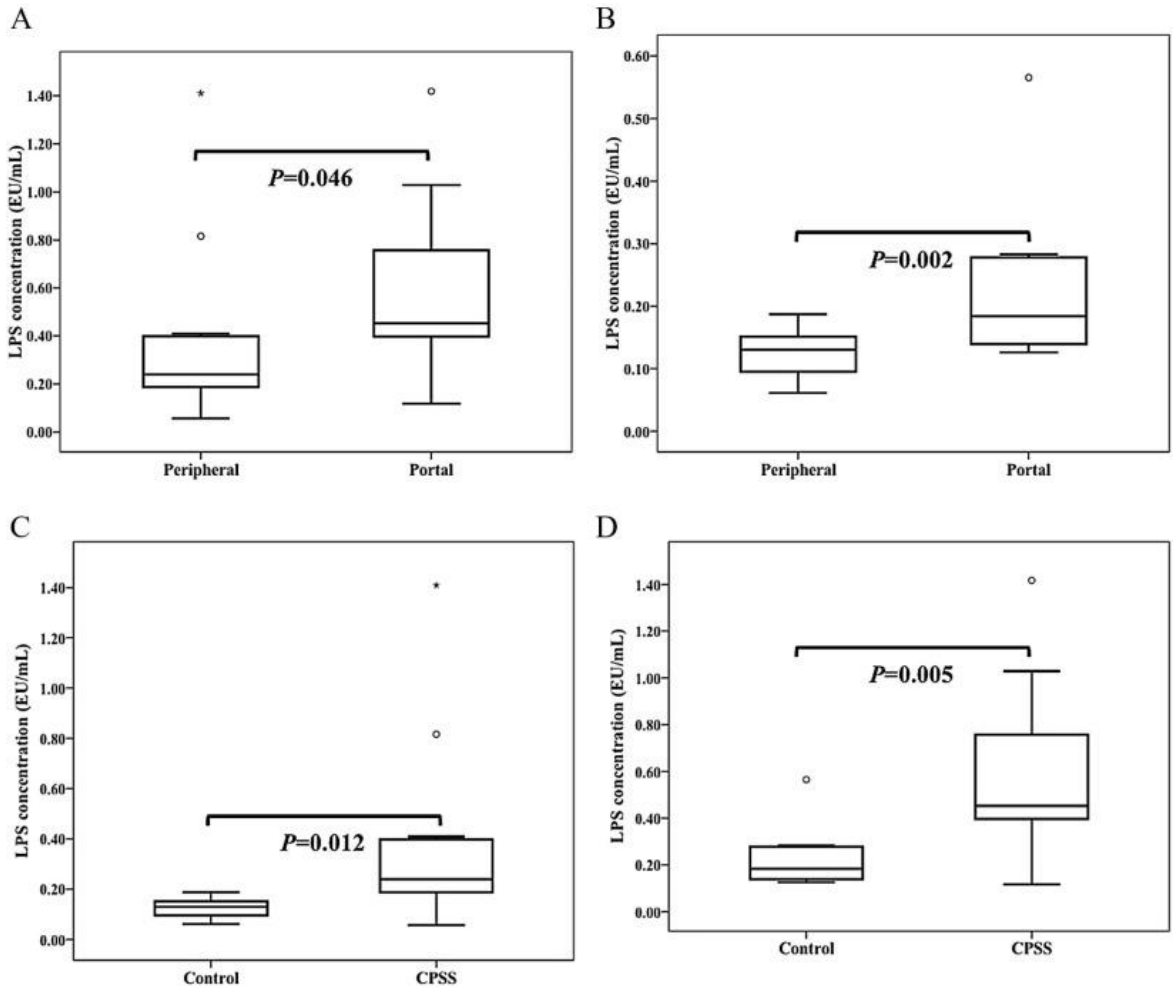

Fig. 2. Relative cytokine and toll-like receptor mRNA expression (normalised with respect to four liver specific reference genes) in liver biopsies from 49 dogs with congenital portosystemic shunts (CPSS) and seven Beagle control dogs. The graphs show statistically significant findings for the five genes assessed. Statistical significance is highlighted with the corresponding *P* value. (A) Interleukin 1 beta (IL-1β) mRNA expression in Beagle control dogs compared with dogs with CPSS. (B) Interleukin 6 (IL-6) mRNA expression in Beagle control dogs compared with dogs with CPSS. (C) Toll-like receptor 4 (TLR4) mRNA expression in dogs with CPSS tolerating a partial attenuation compared with those tolerating a complete attenuation. (D) TLR4 mRNA expression in dogs with CPSS at first surgery compared with second surgery.

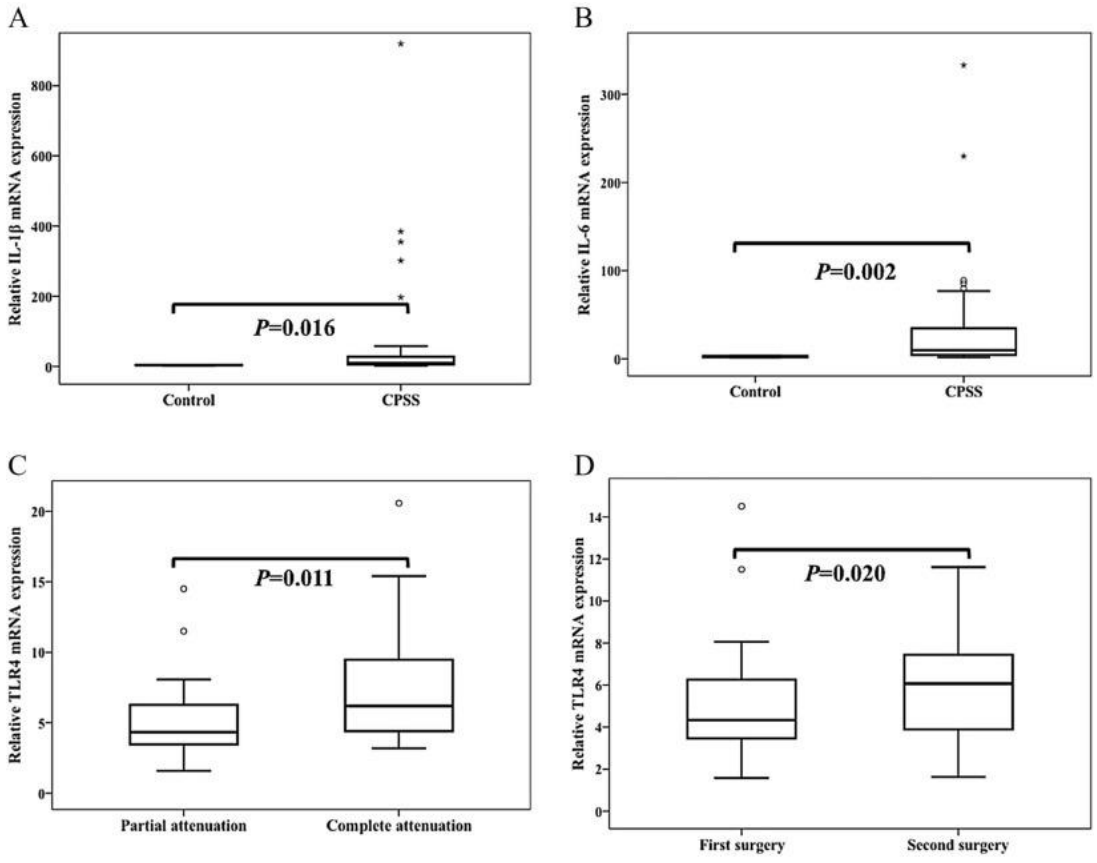

Fig. 3. Relative mRNA expression of toll-like receptor 4 (TLR4), normalised with respect to four liver specific reference genes, in liver biopsies from 47 dogs with congenital portosystemic shunts (CPSS) as related to portal blood flow on pre-attenuation and post-attenuation portovenogram. Portovenogram grades of 1 and 2 were considered poor portal blood flow and portovenogram grades of 3 and 4 were considered good portal blood flow. Statistical significance is highlighted with the corresponding *P* value. (A) TLR4 mRNA expression in dogs with CPSS with poor portal blood flow compared with dogs with CPSS with good portal blood flow on pre-attenuation portovenogram. (B) TLR4 mRNA expression in dogs with CPSS with poor portal blood flow compared with dogs with CPSS with good portal blood flow on post-attenuation portovenogram.

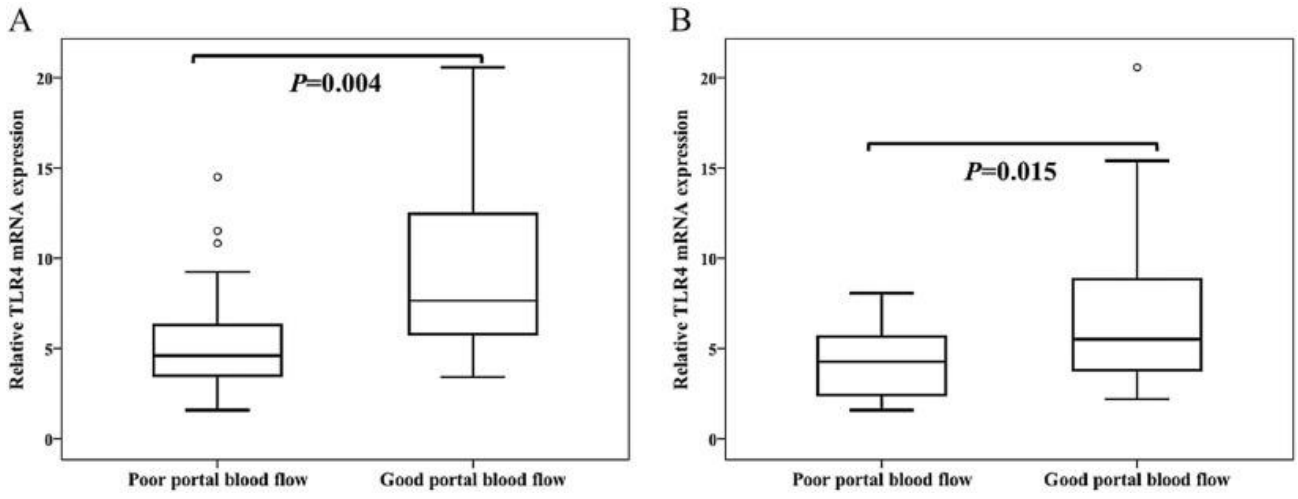

Fig. 4. Serum interlukin 6 (IL-6) concentration in 22 dogs with congenital portosystemic shunts pre-surgery and at 24 and 48 h post-surgery. IL-6 concentration was measured using a canine IL-6 ELISA kit. There was a significant difference in the concentration of IL-6 at the different time points ($P < 0.001$). Pair-wise comparison of this data set confirmed that IL-6 at 24 h post-surgery was significantly greater than pre-surgery ($P < 0.001$).