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TLR4KO mice are protected from endothelial overactivation in the absence of Kupffer cells after total hepatic ischemia/reperfusion

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Abstract

Kupffer cells (KC) have been shown to be critical mediators of I/R injury in the murine liver. Utilizing liposomal clodronate (LC) administration, we have shown that KC are protective in models of total hepatic ischemia with bowel congestion. Here, we investigate the role of TLR4 in the damage that occurs after I/R in KC-depleted livers. We injected 8-week old C57BL/10J or C57BL/10ScN (TLR4KO) mice with liposome-encapsulated clodronate 48 hours prior to 35 minutes of warm hepatic ischemia with bowel congestion, followed by either 6 or 24 hours of reperfusion. KC depleted animals had increased mortality as well as a 10-fold increase in transaminase levels that correlated with increases in centrilobular necrosis. These changes were absent in TLR4KO animals. LPS bound extensively to endothelial cells after I/R, and this binding was diminished in TLR4KO animals. In conjunction, there was an upregulation of endothelial cell adhesion molecules in LC-treated animals that was absent in TLR4KO animals. Finally, there was a dramatic increase in proinflammatory cytokine levels in LC-treated animals, and TLR4KO animals were protected against this increase. In conclusion, TLR4 promotes endothelial overactivation after I/R in the absence of KC.

Keywords

Sinusoidal; Adhesion molecules; Lipopolysaccharide; Necrosis

INTRODUCTION

Sentinel pattern recognition receptors (PRRs) are a key component of the innate immune system, especially when it is tasked with responding to the stresses of ischemia/reperfusion (I/R) injury (1). Of these receptors, Toll-like receptors (TLRs) play a central role in the setting of I/R. During liver transplantation, there are long periods of warm and cold ischemia, followed by reperfusion, where TLR ligands, especially TLR4 ligands, are liberated and initiate signaling that results in inflammation(2, 3).

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Multiple TLRs are likely candidates for initiation of inflammation after I/R. TLR2, a the predominant sensor of bacterial peptidoglycan and lipotechoic acid, is one of these, as these bacterial products are likely liberated after bowel congestion. However, multiple studies have failed to show a predominant role for this molecule (4). Additionally, as the predominant receptor for bacterial CpG DNA, TLR9 may also play a role after warm hepatic I/R. However, the role of TLR9 is controversial, as some knockout studies have shown an important role for this molecule, while adoptive transfer models have failed to show changes in ischemic damage (4). TLR4 has been shown in our studies and the studies of others to be predominantly involved in hepatic I/R. TLR4 has been shown to be integral in several models of partial I/R without bowel congestion (5). Specifically, TLR4 has been shown in these models to signal via an MyD88-independent pathway (6), to involve HO-1 (7) and HMGB-1 signaling (8), and to critically involve non-parenchymal cells (9). Additionally, these models have shown that in the absence of bowel congestion, non-LPS ligands are critical to the injury sustained after I/R (3).

However, these models fail to liberate lipopolysaccharide (LPS), one of the key TLR4 ligands (10). During periods of gut hypoperfusion and mesenteric congestion, LPS from the intestinal microflora translocates across the intestinal barrier (11). This translocation occurs during the anhepatic phase of liver transplantation, and we have shown that in certain models of ischemia/reperfusion, this translocated LPS plays a major role in hepatic damage after I/R (2).

Kupffer cells (KC) have been shown to be critically responsible for responding to the I/R insult through the production of proinflammatory cytokines and reactive oxygen intermediates (12). Methods to diminish KC function with gadolinium chloride (GdCl₃) have shown decreases in proinflammatory cytokine production, and overall improvements in animal performance after I/R (13–15). However, other reports have questioned whether GdCl₃ depletes KC or simply impairs their phagocytizing ability (16). Recent studies have embraced the use of liposome encapsulated dichloromethylene biphosphonate (clodronate, LC) to eliminate KC from the hepatic microenvironment via KC apoptosis. Studies in our laboratory have shown that KC play a protective role after I/R. The elimination of KC via LC causes an increase in animal death, increase in necrosis, and inflammatory mediator production. Additionally, there are alterations in IL-10 production in the absence of KC. Reconstitution of IL-10 resulted in a reduction in injury to baseline levels(17).

In order to investigate the role of TLR4 in I/R in the absence of KC, we utilized a model of total hepatic ischemia, involving ligation of the portal and arterial blood supplies, contributing to bowel congestion, as occurs during human transplantation. Coupled with this, we depleted KC to more fully investigate the role of other nonparenchymal cells, specifically liver sinusoidal endothelial cells (LSEC), in reperfusion injury.

MATERIALS AND METHODS

Animals

Male, 8-week old inbred C57BL/10J (Control, Jackson Laboratory, Bar Harbor, ME) and C57BL/10ScN (TLR4 deficient, TLR4KO, National Cancer Institute, Frederick, MD) mice were used in all experiments. Mice were housed 3 to 4 per cage in a temperature-controlled room (22–25°C) with a 12-hour light-dark cycle and water and food were available *ad libitum*. All experiments were performed under aseptic conditions, were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (MUSC IACUC), and were in accordance with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the NIH (publication 86-23 revised 1985).

Liposomal clodronate administration

Forty-eight hours prior to ischemia, animals were injected with 1.1 mg/mouse of liposomal clodronate (dichloromethylene biphosphonate, LC) intraperitoneally. LC was provided by Nico van Rooijen (www.clodronateliposomes.org) and was produced as previously described (18).

Total Hepatic Warm Ischemia/Reperfusion

Ischemia/reperfusion was performed as previously described (19) with some modification. Briefly, mice were laparotomized and a sterile pediatric vessel loop was placed around the portal triad and tightened for 35 minutes. After the loop was removed, mice were sutured and allowed to reperfuse for 6 or 24 hours. At sacrifice, blood was collected by sterile cardiac puncture, and whole blood was placed into serum separator tubes and allowed to coagulate for 30 minutes. It was then centrifuged for 5 minutes at 3,200 rpm at room temperature to separate the serum, which was then aliquoted into pyrogen-free, glass vials. Portions of the liver were placed in 10% neutral buffered formalin for histologic analysis and the remaining liver was snap-frozen in liquid nitrogen and stored at -80 degrees Celsius for later analysis.

Serum alanine aminotransferase (ALT)

Serum alanine aminotransferase (ALT) concentrations were measured with a Synchron LX20 system (Beckman Coulter) and expressed as IU/liter (Clinical Laboratory Services, Medical University of South Carolina).

Centrilobular Necrotic Index Grading

Upon sacrifice of the animal, a portion of liver tissue was placed in 10% neutral-buffered formalin for fixation, embedded in paraffin, cut, placed on glass slide, and heat fixed. Hematoxylin and Eosin (H&E) staining was performed. Necrosis grading was quantified from H&E stained slides as previously described on a scale from 0 to 3 with reference to the central vein (20). Briefly, a grade of 0 indicated absent necrosis, 1 indicated individual hepatocyte dropout, 2 indicated small foci of missing hepatocytes up to 2–3 cells across, and 3 indicated confluent foci of hepatocyte dropout greater than 3 cells across. Ten high-powered fields per section were analyzed in relation to the central vein. Necrotic indexing was performed in a blinded fashion.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were incubated with primary antibody (ICAM-1, BD Biosciences #550287; F4/80, abcam #ab6640; E. coli LPS, abcam #ab35654; SE-1, Novus Biologicals #NB110-68095) for 1 hour after antigen retrieval using a heat induced (LPS, ICAM) or enzymatic (F4/80, SE-1) epitope retrieval method. Samples were then washed and incubated with biotinylated secondary antibody (Vector Laboratories). After washing, sections were incubated with Vectastain ABC kit (Vector Laboratories) for an additional hour. Immunoperoxidase staining was performed with the Diaminobenzidine (DAB) substrate kit (Vector Laboratories). The specificity of immunostaining was demonstrated by omission of primary antibody. Sections were counterstained with Harris hematoxylin and examined by light microscopy in a blinded fashion. Where appropriate, positive cells were counted as a ratio of total cells in 10 high-powered fields (HPF) per section.

Confocal Microscopy

Paraffin-embedded sections were incubated as above. In the case of double labeling, incubation was done first with anti-LPS overnight, followed by labeling with Alexa-Fluor

488 Avidin (Invitrogen, Carlsbad, CA). Then, the second primary labeling was done with either SE-1 or F4/80, followed by labeling with Alexa-Fluor 555 Avidin (Invitrogen, Carlsbad, CA). Nuclear staining was obtained by incubation with TO-PRO-III Iodide (Invitrogen, Carlsbad, CA). Samples were coverslipped and visualized on a Leica TCS SP2 AOBS confocal microscope and analyzed with Leica confocal software, version 2.61.

Quantitative real time RT-PCR

Total RNA was extracted from liver samples by RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX). The mRNA coding for TNF, IL-1 β , IL-10, e-selectin, p-selectin, ICAM-1, VCAM-1 and GAPDH were quantified by SYBR Green two-step reverse-transcription (RT) polymerase chain reaction (PCR). Briefly, 1 mg of total RNA from each sample was used for RT with the Transcriptor first strand cDNA synthesis kit (Roche, Indianapolis, IN) to generate first-strand cDNA. PCR reaction mixture was prepared with the use of SYBR Green PCR Master Mix (FastStart SYBR Green Master Mix, Roche, Indianapolis, IN). Table 1 lists the sequences of the primers. Thermal cycling conditions were 10 min at 95°C followed by 50 cycles of 95°C for 15 seconds and 60°C for 30 sec on a Roche LightCycler 480 (Roche, Indianapolis, IN). Each gene expression was normalized to GAPDH mRNA and calculated relative to baseline control using the comparative Ct method.

Statistical Analysis

All values are expressed as mean $\pm$ SEM. An alpha value of 0.05 was established prior to experimentation as the limit for statistical significance. For a single pairwise comparison, a two-tailed *t*-test was used when samples were normally distributed. For multiple comparisons of normally distributed data, a one-way ANOVA with Tukey-Kramer post-hoc analysis was utilized. For histologic analysis and samples that were not normally distributed, a Mann-Whitney U test was utilized. For multiple independent groups, a Kruskal Wallis non-parametric comparison was used, with a Tamhane's test for post-hoc analysis using SPSS® statistical software.

RESULTS

TLR4KO animals are protected from death and hepatocellular damage after LC-treatment and I/R

Pretreatment with LC decreased wild type animal survival following 35 minutes of total hepatic ischemia and 24 hours of reperfusion, while TLR4KO animals showed decreased mortality (~55% versus ~84%, respectively)(Figure 1). Diluent-treated animals showed survival rates of ~95–100%.

Hepatocyte injury was assessed via serum alanine aminotransferase (ALT) levels. After 6 hours of reperfusion, ALT levels increased 10-fold in LC-treated mice subjected to I/R compared to diluent-treated mice subjected to I/R, which only increased 2-fold (Figure 2). Additionally, TLR4KO LC-treated mice showed only a 3-fold increase in ALT. At 24 hours, ALT levels in LC-treated animals remained elevated, where other groups had returned to baseline.

To gain insight into the type of hepatocyte injury, histologic sections were H&E stained and injury was graded on a scale of 0–3, as described previously (20). Large, confluent necrotic foci were noted around central veins in wild-type LC-treated animals after I/R (Figure 3A). These foci were usually accompanied by large amounts of leukocytic infiltration. These foci were absent in both diluent-treated groups, and in TLR4KO LC-treated animals after I/R. Scoring of the necrosis revealed all groups were similar in the absence of I/R (Figure 3B).

All diluent-treated groups showed a 2-fold increase in necrosis at 6 hours, which was maintained at 24 hours. In LC-treated wild-type animals subjected to I/R, there was a 6-fold increase in necrotic index, whereas LC-treated TLR4KO animals had only a 2.5-fold increase. This difference was maintained at 24 hours in wild type LC-treated animals, while LC-treated TLR4KO animals were not different from diluent-treated animals. These results provide evidence that TLR4 deletion in KC depleted animals protects against mortality and increased injury following I/R.

TLR4KO animals show decreased LSEC binding of LPS

To more fully investigate the damage phenotype displayed in LC-treated animals subjected to I/R, we used confocal microscopy to examine liver binding of the gut-derived LPS after I/R. Prior to I/R, there was no significant LPS binding, whereas there was notable SE-1 staining of endothelial cells (Figure 4A). After I/R, LPS was visualized binding extensively to endothelial cells in both diluent-treated wild-type and LC-treated wild-type animals (Figure 4B). There was decreased binding of LPS to endothelial cells in both LC-treated and diluent-treated TLR4KO animals. To confirm that this binding was specific for endothelial cells, we used SE-1, a well-published marker specific for LSECs. There was extensive colocalization of the SE-1 and LPS signals in both groups of wild-type animals. Binding of SE-1 was equally as pronounced in both groups of TLR4KO animals, but the colocalized signal was weaker, due to the decreased endothelial binding of LPS. These results reveal that there is extensive endotoxin binding to endothelial cells after I/R, and this binding is decreased in TLR4KO animals.

LSEC activation is decreased in TLR4KO animals

Due to the extensive LPS interaction with endothelial cells, we investigated endothelial cell activation through observation of endothelial cell adhesion molecules expression. ICAM-1 is an endothelial marker that has been repeatedly studied in the setting of I/R and LPS administration, and is a marker of endothelial activation (21). All of the groups showed mild upregulation at 6 hours of reperfusion (not shown). ICAM-1 expression was markedly elevated in LC-treated wild-type animals at 24 hours (Figure 5A). The diluent-treated wild type animals showed only a very mild increase. Similarly, both diluent-and LC-treated TLR4KO animals showed only a very mild upregulation of ICAM-1. To further investigate endothelial activation, we performed real-time RT-PCR to examine levels of multiple endothelial activation markers and cell adhesion molecules. mRNA levels of e-selectin were unchanged at 6 hours. However, at 24 hours, wild type LC-treated animals showed a 2.5-fold increase, which was not present in either diluent-treated group or in TLR4KO animals. P-selectin was 6-fold upregulated in both wild type groups, but was only 2-fold upregulated in both TLR4KO groups. Wild type levels had returned to TLR4KO levels at 24 hours. ICAM-1 was upregulated in both diluent-treated groups at 6 hours. ICAM-1 was 6-fold elevated in wild type LC-treated animals, but only 2-fold elevated in TLR4KO LC-treated animals. This difference was maintained at 24 hours. Levels of VCAM-1 were unchanged in either diluent-treated group at 6 hours, while wild type LC-treated animals showed a 3-fold increase. TLR4KO LC-treated animals showed no increase. At 24 hours, VCAM-1 levels had decreased in wild type diluent-treated animals, while the remainder of the groups were unchanged over baseline. These results indicate that TLR4KO animals are protected from endothelial activation after LC-treatment and I/R.

Activated dendritic cells (DCs) are similar between all groups

Recent evidence has suggested that DCs are important for anti-inflammatory modulation after ischemia/reperfusion (22). Thus, we sought to analyze the significance of DC activation in our model via immunohistochemical staining with DC-SIGN. Samples showed no difference among any of the groups pre-or post-I/R in terms of the number of activated

DCs present (data not shown). Samples were not quantified due to the paucity of DCs present in any of the samples.

TLR4KO animals showed a decrease in proinflammatory cytokines

To further investigate the inflammatory milieu in the liver following I/R in the presence and absence of KC, we used real-time RT-PCR to examine transcription levels of important hepatic pro-inflammatory cytokines. TNF levels were 5-fold upregulated in wild type LC-treated animals at 6 hours of reperfusion, while TLR4KO LC-treated animals and diluent-treated groups remained unchanged (Figure 6). All groups were similarly unchanged at 24 hours. IL-1b was 10–15-fold upregulated in wild type animals, while TLR4KO animals showed no change at 6 or 24 hours. All groups were similar at 24 hours. IL-6 was 4-fold upregulated at 6 hours and 8-fold upregulated at 24 hours, while the remaining groups were 1–2-fold upregulated at both time points. IL-10 was 4-fold upregulated in wild type diluent-treated animals at 6 hours of reperfusion, while diluent-treated and TLR4KO LC-treated animals showed no increase over baseline. At 24 hours, all groups had returned to baseline. These results indicate that animals deficient in TLR4 did not show an increased production of inflammatory cytokines in the absence of KC.

DISCUSSION

The series of experiments presented here addressed the role of TLR4 in the endothelial activation that occurs in the absence of KC. Work from our lab has shown that despite previous reports (12), KC play a protective role after I/R (17). The damage that occurs seems to be endothelial mediated due to a lack of KC-produced IL-10. We have shown here for the first time that the toll-like receptor system, specifically TLR4, plays the predominant role in endothelial cell activation, thus leading to hepatic damage after I/R in the absence of KC.

We saw significant improvements in survival in TLR4 deficient animals that were depleted of KC as compared to wild-type depleted animals. In conjunction with this, we saw dramatic decreases in the levels of liver damage, as measured by ALT and necrosis, in TLR4KO animals depleted of KC. Corresponding with this, we saw a decrease in endothelial activation in TLR4KO animals, as demonstrated by decreased sinusoidal binding of gut-derived LPS and the down-regulation of endothelial adhesion molecules. Finally, we saw an overall decrease in proinflammatory cytokines after I/R in TLR4KO animals.

Decreased survival in our KC-depleted wild-type animals indicates a role for KC in promoting animal survival after I/R. TLR4KO animals were protective, suggesting a role for TLR4 in KC-depleted damage. The dramatically increased transaminase levels in wild-type LC-treated animals strongly suggest that the liver damage that is occurring is the cause of animal mortality. In the TLR4KO animals, we saw a 10-fold reduction in the transaminase release, which occurred within the first 6 hours of reperfusion. This phenomenon was further exemplified by the fact that there was significant centrilobular necrosis in the LC-treated wild-type animals, whereas the levels in TLR4KO animals were close to diluent-treated levels. This evidence suggests an important role for TLR4 in the damage that occurs after I/R in KC-depleted animals.

LPS has been shown to directly activate endothelial cells, as shown by an up-regulation of p-selectin and ICAM-1 (23, 24). Additionally, ICAM-1 has been shown to be a key molecule in TLR4/LPS mediated injury, as ICAM-1 deficient mice are protected from septic shock (25). Thus, competent endothelial signaling is crucial for the full response to LPS. Our experiments have shown that disrupting TLR4 signaling can also prevent injury after I/R, potentially via endothelial protection. We also saw extensive LPS binding to endothelial

cells. The sinusoidal staining pattern of LPS present after I/R in wild-type animals closely resembled that which we have seen in the literature in terms of TLR4 distribution (26). We saw decreased sinusoidal binding of LPS in TLR4KO animals, indicating that TLR4 is the most likely binding site for the LPS. Since we saw LPS staining as late as 24 hours after reperfusion, it would indicate that endothelial cells can retain LPS and potentially signal for this duration. This is an important finding to note. It has been shown previously that macrophage responses to endotoxin are transient, lasting on the order of minutes to hours. However, endothelial cells have been shown to have transcriptional persistence of cytokines and activation markers for up to 24 hours once stimulated with LPS (27). As major cytokine producers, sustained activation of these nonparenchymal cells could participate in the proinflammatory milieu we saw in KC depleted animals (28). This could help to explain the continued damage that occurred at later time points in our animals. Endothelial cells have also been shown to play a role in hepatic tolerance to LPS, as they can modulate TLR4 activation without altering receptor expression (29). Thus, our results, coupled with findings in the literature, seem to suggest that TLR4 on endothelial cells signals the production of an unchecked inflammatory response in KC-deficient animals.

A role for DCs would be suggested in light of recent (22) and previous (30) evidence implicating their role in modulating the anti-inflammatory milieu after I/R through the production of IL-10. This is a controversial role, in light of other investigations suggesting that TLR4 ligation on DCs is essential for ischemic injury in models of sterile inflammation (31). We did not find a role for activated DCs in our model, as IHC staining failed to reveal significant numbers of these cells either before or after I/R. We would expect that clodronate would impact the numbers of these cells, as all phagocytic cells would likely be affected by clodronate, but we see little infiltration in our untreated samples as well. While this does not obviate a role for DCs in total hepatic I/R, initially our investigations suggest little role for them in our model.

LPS has long been implicated in the clinical damage that occurs after I/R, as early patient morbidity after liver transplantation correlates with increased endotoxin levels (32, 33). TLR4 has been shown to have numerous other endogenous ligands, including heat shock proteins 70 (34) and 72 (35), HO-1 (7), as well as fibrinogen, hyaluronic acid, and heparan sulfate. Other models of I/R have shown that LPS is not an important player in TLR4 signaling after I/R. Specifically, studies have utilized recombinant small molecule inactivators of LPS to show that endogenous ligands are likely playing a large role post-I/R (3). However, these reports used a partial model of ischemia with no bowel congestion rather than a model of total hepatic ischemia. We believe that a model of total ischemia with bowel congestion is more clinically relevant to liver transplantation. Even when patients were put on venous bypass during transplantation, portal congestion was not significantly influenced, thus implicating endotoxin as a major ligand at reperfusion (36). In steatotic models of I/R, we have already shown that this is the case, as removal with a monoclonal antibody to endotoxin significantly improved outcomes post I/R (2).

In conclusion, we have shown that TLR4 plays a major role in the damage that occurs after I/R in animals that have been depleted of KC. TLR4KO animals had improved survival, less liver damage, decreased proinflammatory cytokines, and lowered levels of endothelial activation. This, along with our confocal data showing LPS binding to endothelial cells, implicates TLR4 on endothelial cells as a major inciting pathway for I/R. Additionally, we believe that it is LPS that is the important ligand here. Further research is necessary to elucidate the role of TLR4 on endothelial cells as a therapeutic target for clinical improvements post transplantation.

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Nonstandard abbreviations

TLR4KO	Toll-like receptor 4 knockout
I/R	Ischemia/Reperfusion
KC	Kupffer Cell
LC	Liposomal Clodronate
LSECs	Liver sinusoidal endothelial cells
ALT	Alanine Aminotransferase
IHC	Immunohistochemistry
ICAM	Intracellular adhesion molecule

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Figure 1. TLR4KO animals are protected from death after LC-treatment and I/R

TLR4KO LC-treated animals had an improved survival rate as compared to WT LC-treated animals. This mortality rate was not different from diluent-treated groups. * $p < 0.05$. $n = 8-10$ /group.

Figure 2. Liver damage is decreased in TLR4KO LC-treated animals

TLR4KO LC-treated animals were protected from liver damage as compared to wild-type LC-treated animals, which showed a 10-fold increase in ALT levels at 6 hours. All groups showed a significant increase at 6 hours, with KO LC-treated animals being elevated above diluent treated groups. Diluent-treated groups had returned to baseline at 24 hours, while wild type LC-treated animals remained significantly elevated. Means with different lettered subscripts within each group are significantly different from each other, $P < 0.05$. Data are expressed as mean $\pm$ SEM; $n=8-10$.

Figure 3. TLR4KO LC-treated animals have less necrosis at 24 hours

(A) Significant centrilobular necrosis is present at 24 hours in LC-treated wild-type animals. Additionally, there is significant leukocytic infiltration in and around areas of necrosis. This necrosis is absent in similarly treated TLR4KO animals. Neither TLR4 phenotype showed significant necrosis in diluent-treated groups. (B) Grading of necrosis revealed significant necrosis the wild type diluent-treated group at 6 hours. Additionally, both TLR4KO groups showed significantly increased necrosis at 6 hours over baseline. However, wild type LC-treated animals showed dramatically increased necrosis as compared with their TLR4KO counterparts. This trend was continued at 24 hours. Means with different lettered subscripts within each group are significantly different from each other, $P < 0.05$. Data are expressed as mean $\pm$ SEM; $n=8-10$.

Figure 4. TLR4KO animals have decreased endothelial binding of LPS

(A) At baseline prior to I/R, there was no binding of LPS to endothelial cells. An endothelial marker, SE-1, is present in all samples. (B) After I/R, there was significant LPS translocation in both wild-type groups. However, there were significantly lower levels of LPS translocated and bound to endothelial cells in TLR4KO animals, regardless of their LC-treatment group.

Figure 5. TLR4KO animals are protected from endothelial cell activation

(A) ICAM-1 levels are dramatically upregulated in wild type LC-treated animals after I/R. Diluent-treated wild type animals have only low levels of activation. Similarly, both diluent- and LC-treated TLR4KO animals show only slight ICAM-1 activation at 24 hours after I/R. (B) To quantify levels of endothelial cell adhesion molecule upregulation, we used qRT-PCR. Levels of e-selectin were similar among all groups at 6 hours. However, wild type LC-treated animals showed significant upregulation at 24 hours. Levels of p-selectin were significantly blunted in both TLR4KO groups versus corresponding wild type groups at 6 hours. At 24 hours, these levels had returned to baseline. Levels of ICAM-1 mRNA were upregulated at 6 and 24 hours in LC-treated wild type animals versus all other groups, which were not elevated above baseline. Levels of VCAM-1 were upregulated at 6 hours in LC-treated wild type animals versus other groups. At 24 hours, all groups had returned to baseline levels except wild type diluent-treated animals, which showed a significant decrease in VCAM-1 levels at 24 hours. Means with different lettered subscripts within each group are significantly different from each other, $P < 0.05$. Data are expressed as mean $\pm$ SEM; $n=8-10$

Figure 6. TLR4KO alters cytokine production after LC-treatment and I/R

Levels of TNF were significantly upregulated at 6 hours in wild type LC-treated animals versus other 6 hour groups. This upregulation had returned to diluent-treated, baseline levels at 24 hours. TLR4KO LC-treated animals showed no upregulation over diluent-treated groups. IL-1 β levels in wild type animals were significantly higher than TLR4KO groups at 6 hours. At 24 hours, these increases had returned to near baseline levels. Levels of IL-6 were upregulated in wild type LC-treated animals at 6 hours, and continued to increase at 24 hours. No other groups showed upregulation at either timepoint. Finally, IL-10 production was significantly blunted in wild type LC-treated animals versus wild type diluent-treated animals. Similarly, there was no upregulation in IL-10 production in any of the TLR4KO groups. Means with different lettered subscripts within each group are significantly different from each other, $P < 0.05$. Data are expressed as mean $\pm$ SEM; $n=8-10$.

TABLE 1

Sequences of the primers for SYBR Green real-time RT-PCR

Target	Forward primers (5'-3')	Reverse primers(5'-3')
e-selectin	AGCTACCCATGGAACACGAC	CGCAAGTTCTCCAGCTGTT
p-selectin	ATGCCTGGCTACTGGACACT	CTTCATCGCACATGAACTGG
ICAM-1	GGCTGGCATTGTTCTCTAA	TTCAGAGGCAGGAAACAGG
VCAM-1	CCCAAACAGAGGCAGAGTGT	CAGGATTTTGGGAGCTGGTA
IL-1 β	CAACCAACAAGTGATATTCTCC	GATCCACACTCTCCAGCTGCA
IL-6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
IL-10	ACAGGAGAAGGGACGCCAT	GAAGCCCTACAGACGAGCTCA
TNF	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA