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LPS-Induced Production of Inflammatory Mediators in the Liver of Postnatal Animals

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LPS-INDUCED PRODUCTION OF INFLAMMATORY MEDIATORS IN THE LIVER OF POSTNATAL ANIMALS

By

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degree of Master of Science in Biology from the
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ABSTRACT

Lipopolysaccharide (LPS) is the primary component of the outer membrane of Gram-negative bacteria and is responsible for the majority of inflammatory effects of infections from Gram-negative bacteria. To gain better understanding of the effects that postnatal age has on the inflammatory response, pups were randomly assigned to be treated with 250 μg/kg of LPS or saline at postnatal day (P) 1, P21, and P70. Two hours post stimulation, the pups were sacrificed and their livers were harvested for total RNA extraction. Relative mRNA levels of inflammatory genes and β-actin were determined using RT-PCR analysis with appropriate rat sense and antisense primers. The specific inflammatory mediators examined were toll-like receptor-4 (TLR4), cluster of differentiation 14 (CD14), myeloid differentiation factor 88 (Myd88), cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and chemokines including macrophage inflammatory protein (MIP)-1β, MIP-2, and monocyte chemotactic protein (MCP)-1. We found that the LPS-induced mRNA expression of the cytokines and chemokines examined appear to be increased as compared to the control pups. Furthermore, we showed that an activation of cytokines and chemokines in the liver exhibited age-dependency in pups treated with LPS at P1, P21, and P70. The pattern shows an increase in relative mRNA expression of cytokines and chemokines as development progresses. Furthermore, we compared the kinetics of cytokine and chemokine induction in P1 and P21 animals. We found that that there was a delayed cytokine and chemokine induction at P1 as compared to P21 pups. Our data suggest that the hepatic innate immunity undergo significant development during early postnatal

development, and the delayed inflammatory response in P1 animals may contribute to increased susceptibility of neonatal animals to infections.

INTRODUCTION

Lipopolysaccharide (LPS) is a glycolipid and the primary component of the outer membrane of Gram-negative bacteria. It is a prime target for recognition by the innate immune system and is responsible for the majority of inflammatory effects of infections from Gram-negative bacteria (Bell et al., 2002). As an endotoxin, it is a potent immune activator which can lead to fatal septic shock syndrome if the inflammatory response is amplified and uncontrolled (Park et al., 2009). LPS is recognized by the innate immune system because it has a typical pathogen-associated molecular pattern that is recognized by toll-like receptor-4 (TLR4) on many cells including monocytes and macrophages (Dantzer, 2004).

LPS is first presented by the LPS-binding protein (LBP) to CD14, a glycosylphosphatidyl inositol (GPI)-anchored monocyte differentiation antigen (Bosshart et al., 2007). After binding to CD14, LPS is delivered to TLR-4 which marks the start of the intracellular signaling cascade (Takeda et al., 2004). This signaling cascade includes adaptor proteins such as myeloid differentiation factor 88 (Myd88) and the phosphorylation and activation of mitogen-activated protein kinases (MAPKs) and ultimately an increased production of inflammatory cytokines & chemokines (Wang et al., 2006).

The MAPK involved in LPS signal transduction in particular is p42/44 MAPK, also known as ERK 1/2. The phosphorylation of this kinase leads to the production of cytokines responsible for pro-inflammatory immune responses, involving namely interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α (Ning et al., 2007).

Cytokines include both pro-inflammatory and anti-inflammatory cytokines. The pro-inflammatory cytokines play an important role in initiating the pro-inflammatory response. IL-1β and TNF-α are of primary importance in the initiation and propagation of the acute phase of the inflammatory response. IL-1β enables an organism to respond quickly to infection by creating a cascade of reactions that leads to inflammation (Bird et. al., 2002). Studies have shown that a minute amount of IL-1β *in vivo* can induce fever, hypotension, and the release of adrenocorticotrophic hormone (Li et. al., 2008).

TNF-α is responsible for a wide range of signaling events within cells, leading to necrosis or apoptosis (Idriss et al., 2000). After LPS stimulation, it has been reported that TNF-α is the first cytokine normally detected at the site of inflammation where as other cytokines are detected later (Luheshi et al., 1997). This cytokine is responsible for many of the systemic effects of infection including septic shock and associated with chronic infections in humans (Dinarello, 2000).

The IL-6-type cytokines include a family of cytokines which consist of IL-6, IL-11, LIF (leukaemia inhibitory factor), OSM (oncostatin M), ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokine. IL-6 is a pleiotropic cytokine engaged in the regulation of inflammatory and immunologic responses (Kishimoto et al., 1997). It is secreted by a broad range of cells including B cells, T cells, fibroblasts, monocytes, and endothelial cells (Benveniste, 1997). IL-6 facilitates the up-regulation of IL-1β, TNF-α, and other acute phase reactants (Chiang et al., 1994) and has both pro- and anti-inflammatory properties which make IL-6 a major contributors in acute-phase and immune responses in organisms (Heinrich et al., 2003). In the liver, IL-6 activated

multiple signaling pathways result in the induction of genes involved in growth, heptocyte-specific metabolic functions, and the acute phase response (Campbell et al., 2001).

Chemokines are chemotactic cytokines known to be critical mediators of inflammatory cell trafficking into sites of injury, modulation of tissue injury, inflammation, and repair (Mühlbauer et al., 2003). Chemokines guide the migration and activation of leukocytes at sites of inflammation or tissue injury (Kim et. al., 2003). Chemokines of interest in this study included macrophage inflammatory protein (MIP)-1β, MIP-2, and monocyte chemotactic protein (MCP)-1. As a member of the CC-chemokine subfamily, MIP-1β is an important mediator of inflammatory reactions with chemotactic and activating properties for several types of leukocytes. Specifically, MIP-1β regulates T-cell trafficking by selectively recruiting activated Th1 subset of cells as well as participate in the recruitment and activation of NK cells, monocytes, macrophages, granulocytes, and dendritic cells (Rapisarda et al, 2002).

Similarly, MIP-2 plays an important role in the chemotactic activity of neutrophils. The primary functions of neutrophils are to recognize, phagocytose, and kill invading microorganisms to protect the host from infections (Cross et al., 2005). It has been noted that an unregulated production of MIP-2 is associated with inflammatory diseases such as arthritis, glomerulonephritis, and sepsis (Kwon et al., 2003).

MCP-1 is produced spontaneously by monocytes and believed to play a significant role in several inflammatory processes such as immunopathological disorders and normal immune response againt microorganisms (Vestergaard et al., 1997). MCP-1

acts as a potent T-lymphocyte and monocyte chemoattractant (Leone et al., 1999) and is involved in monocyte trafficking to sites of infection across endothelial and epithelial cells (Maus et al., 2002).

Emerging evidence suggests that the liver is an important part of the body's innate immune response to pathogens and plays a critical role in the processing and clearance of LPS. The liver is composed of hepatocytes that carry out the metabolic and detoxifying needs of the body. Seventy percent of liver cells are composed of hepatocytes (Gao et al., 2008); the remaining cells are made up of nonparenchymal cells, namely Kupffer cells. There are additional nonparenchymal cells which include sinusoidal endothelial cells, stellate cells, and lymphocytes (Malik et al., 2002).

Hepatocytes play a key role in controlling systemic innate immunity. During an acute phase inflammatory response, pro-inflammatory cytokines can stimulate hepatocytes to produce high levels of complements and pattern-recognition receptors (Gao et al. 2008). Kupffer cells are macrophages and are the first to respond to the presence of LPS. Pro-inflammatory mediators, such as cytokines and chemokines, released by Kupffer cells may in turn propagate signals to hepatocytes (Tukov et al., 2006).

There have been studies reporting that age plays a factor on the response of the immune system. Reported studies have shown that there is a diminished induction of IL-1β, IL-6, and TNF-α in cord blood as compared to adult peripheral blood monocytes (Förster-Waldl et al, 2005). Additionally, the polymorphonuclear leukocytes (PMNs) from preterm infants have been reported to exhibit a much reduced antibacterial activity

than those from adult (Henneke et al., 2003). More recently, studies from our laboratory showed that LPS-induced phosphorylation of p42/44 MAPK in the liver is age-dependent with p42/44 MAPK being significantly activated at postnatal (P) day 21 and P70 but not at P1 compared to their saline controls (Surriga, 2009).

The present study was designed to analyze the effects of age on LPS-induced hepatic inflammatory response. For this study, rat pups at the ages of P1 day, P21, and P70 were chosen. P1 is newborn, only being one day out of the mother's womb. P21 rat pups are at the age in which the pups are weaned off from the mother and P70 is the age in which the rat is considered a young adult. The rats were treated with LPS or saline at P1, P21, and P70. At two hours following stimulation, liver tissues were collected and processed to examine the relative mRNA levels of cytokines and chemokines, namely IL-1β, IL-6, TNF-α, and MIP-1β, MIP-2, MCP-1 respectively, as well as the upstream inflammatory mediators such as TLR4, CD14, and Myd88.

MATERIALS & METHODS

Animals

Adult male and female Sprague-Dawley rats were purchased from Harlan Inc. (Indianapolis, IN). For at least one week prior to the experiment, the rats were held in a temperature- and humidity- controlled animal facility with a 12-hour light/dark cycle. The rats were fed a standard rat diet and water *ad libitum*. With the approval of the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, the animal studies were conducted.

For mating purposes, 1-3 female rats (200-300 g) were housed overnight with one male rat (250-300 g). Each of the female rats that were exposed to the male rats was visually inspected for the presence of a vaginal plug. The female rats were moved to a separate cage in the aforementioned conditions once the vaginal plug was noted. The presence of a vaginal plug was designated as gestational day 0, and the day the rat pups were born was designated as postnatal day 0.

For age-dependency studies, the pups were kept in the above-mentioned conditions. The pups were allowed to mature until postnatal days (P) 1, 21, or 70. Each was randomly assigned to receive one intraperitoneal (i.p.) injection of saline or 250 μg/kg LPS (*Salmonella enteric* serovar Typhimuriuml Sigma, St. Louis, MO). The pups were sacrificed two hours post saline or LPS stimulation and their liver tissues were collected and stored at -80°C for total RNA extraction.

For time-course studies, the pups were allowed to mature in above-mentioned conditions until either P1 or P21 after birth. At designated age, rat pups were treated via

i.p. injection with 250 μg/kg LPS for 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h (four pups per group). Following treatment, the pups were then sacrificed and their liver tissues were harvested and stored at -80°C for total RNA extraction.

Total RNA extraction

Total RNA was isolated from individual dissected liver tissues collected from each treated animal using TRIzol reagent (Invitrogen, Grand Island, NY). The liver tissues were homogenized with 1000μL TRIzol reagent using a power homogenizer (Polytron). 200 μL of chloroform was added for each 1000 μL of TRIzol reagent, and the samples were then mixed and centrifuged at 14,000g at 4°C for 15 minutes. After centrifugation and rinsing with 70% ethanol, the total RNA samples were dissolved in 50μL RNase-free water and stored at -80°C.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

cDNA was synthesized from 2μg of total RNA using oligo $(dT)_{12-18}$ primer and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Grand Island, NY). After cDNA synthesis, PCR amplification was carried out using appropriate sense and antisense primers for rat β-actin, TNF-α, IL-1β, IL-6, MIP-1β, MIP-2, MCP-1, TLR-4, CD14, and Myd88 (synthesized by Fisher Scientific, Springfield, NJ). The final volume was 50μL consisting of 2μL of cDNA, 1x PCR buffer, 0.2μL of each sense and antisense primer, 0.2mM dNTPs, and I unit of Taq DNA polymerase. The sense and antisense primers for rat β-actin were: 5'-AGC-CAT-GTA-CGT-AGC-CAT-CC-3' and

5'- CTC-TCA-GCT-GTG-GTG-GTG-AA-3' respectively; the sense and antisense primers for rat TNF-α were: 5'-GAG-GTC-AAC-CTG-CCC-AAG-TA-3' and 5'-CGT-GTG-TTT-CTG-AGC-ATC-G-3', respectively; the sense and antisense primers for rat IL-1β were: 5'-AGT-CTG-CAC-AGT-TCC-CCA-AC-3' and 5'-AGA-CCT-GAC-TTG-GCA-GAG-GA-3', respectively; the sense and antisense primers for rat IL-6 were: 5'-TGT-GCA-ATG-GCA-ATT-CTG-AT-3' and 5'-AAC-GGA-ACT-CCA-GAA-GAC-CA-3', respectively; the sense and antisense primers for rat MIP-1β were: 5'-CTC-TCT-CCT-CCT-GCT-TGT-GG-3' and 5'-CAC-AGA-TTT-GCC-TGC-CTT-TT-3', respectively; the sense and antisense primers for rat MIP-2 were: 5'-CTG-GAT-CGT-ACC-TGA-TGT-GCC-3' and 5'-CAG-TGT-GGA-GGT-GGT-GTA-GTC-3', respectively; the sense and antisense primers for rat MCP-1 were: 5′-TGC-TGC-TAC-TCA-TTC-ACT-GGC-AA-3′ and 5′-GTT-TCT-GAT-CTC-ACT-TGG-TTC-TGG-3′, respectively; the sense and antisense primers for rat TLR-4 were: 5′-TGC-TCA-GAC-ATG-GCA-GTT-TC-3′ and 5′-TCA-AGG-CTT-TTC-CAT-CCA-AC-3′, respectively; the sense and antisense primers for rat CD14 were: 5′-GTG-CTC-CTG-CCC-AGT-GAA-AGA-3′ and 5′-GAT-CTG-TCT-GAC-AAC-CCT-GAG-T-3′, respectively; and the sense and antisense primers for rat Myd88 were: 5'-AGA-ACA-GAC-AGA-CTA-TCG-GCT-3' and 5'-CGG-CGA-CAC-CTT-TTC-TCA-AT-3, respectively (Lasala and Zhou, 2007, Surriga et al., 2009, Chen et al., 2009, and Velayudham et al., 2006). The optimum number of cycles was 30 cycles for TLR-4, 29 cycles for MIP-1β and MIP-2, 28 cycles for IL-6 and MCP-1, 27 cycles for IL-1β, 26 cycles for TNF-α and CD14, 25 cycles for Myd88, and 21 cycles for β-actin.

For the amplification of rat MIP-2, the PCR reaction was heated to 94°C for 5 minutes, and cycled through a 30 second denaturation step at 94°C, a 30 second annealing step at 58°, and a 30 second extension step at 72°C (29 cycles for MIP-2) followed by a 7 minute extension step at 72°C. The annealing temperatures were as followed: MIP-2 at 58°C; TNF-α, IL-1β, IL-6, MIP-1β, MCP-1, TLR-4 and Myd88 at 57°; and β-actin at 56°C. Through electrophoresis, the PCR products were then separated on a 2.0% agarose gel. Using a UVP GelDocIt™ imaging system (UVP, Upland, CA), the gel images were documented and digitized using Vision Works™ LS software (UVP, Upland, CA). The relative intensities for the genes amplified were normalized to β-actin in the same sample. Expression levels of cytokine and chemokine mRNA were determined using RT-PCR analysis.

Immunohistochemistry

The embedded liver tissues were cut into 10-μm sections on a microtome (Leitz) and transferred onto slides. Once on the slides, the liver sections were then dewaxed and rehydrated through xylenes (5 min, three times), 100% ethanol (5 min, twice), 95% ethanol (5 min), 70% ethanol (5 min), 25% ethanol (5 min), and TBS (5 min). After rehydration, the liver sections were heated for 3 min in 10 mM sodium citrate (pH 6.0) in a microwave and cooled down for 20 minutes at room temperature. The liver sections were then rinsed with TBS and incubated with 0.3% H_2O_2 in methanol for 30 minutes at room temperature. Subsequently, the sections were blocked with 5% normal host serum in TBS containing 0.2% Triton X-100, incubated with antibodies for phosphorylated

p42/44 MAPK (Cell Signaling Technologies) overnight at 4°C, and detected with the ECTASTAIN Elite ABC Kit (Vector Laboratories, Budingame, CA) following manufacturer's instructions (Surriga, 2009).

Statistical analysis

The intensity of the RT-PCR data was normalized to β-actin of the same sample using Vision Works™ LS software (UVP, Upland, CA). All data were presented as means± SE. A two-way analysis of variance (ANOVA) for repeated measures was used to analyze the data. Results with $p < 0.05$ were considered statistically significant.

RESULTS

LPS-induced activation of cytokines in P1, P21, and P70 rat liver

To examine how age effects the LPS-induced activation of cytokines, pups were randomly assigned to receive one i.p. injection of 250 μg/kg of LPS or saline at postnatal day (P) 1, P21, and P70. Two hours post stimulation, the pups were sacrificed and their livers were harvested for total RNA extraction. Expression levels of cytokine mRNA were determined using RT-PCR analysis with appropriate sense and anti-sense primers.

Analysis of the expression levels showed an increase of TNF-α and IL-1β in LPS-treated pups at P1, P21, and P70 as compared to their saline controls (Figs. 1 and 2). Additionally, there appears to be an increase in the expression of these cytokines at P21 and P70 as compared to P1 following LPS stimulation (Figs. 1 and 2). The expression level of IL-6 was increased in P21 and P70 pups following LPS stimulation, however, the mRNA expression of IL-6 was hardly detectable by RT-PCR (Fig. 3).

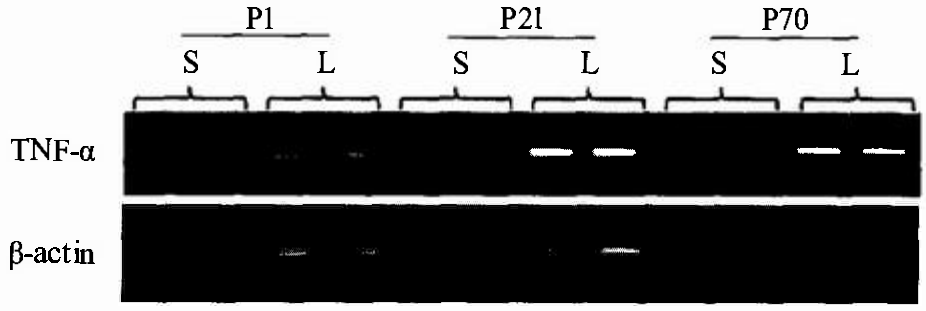

Figure 1. Representative RT-PCR of TNF-α expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

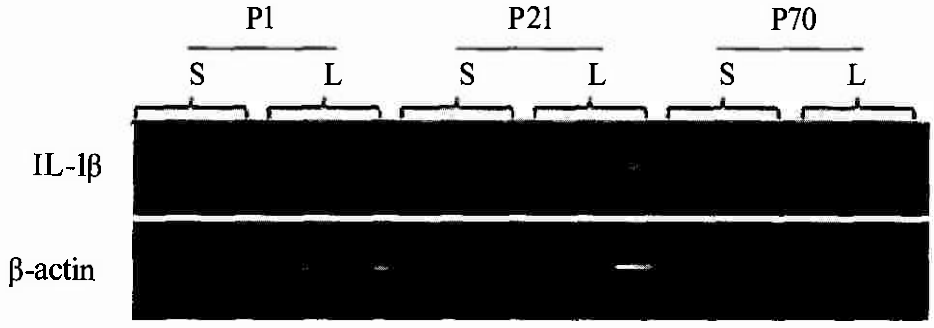

Figure 2. Representative RT-PCR of IL-1β expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

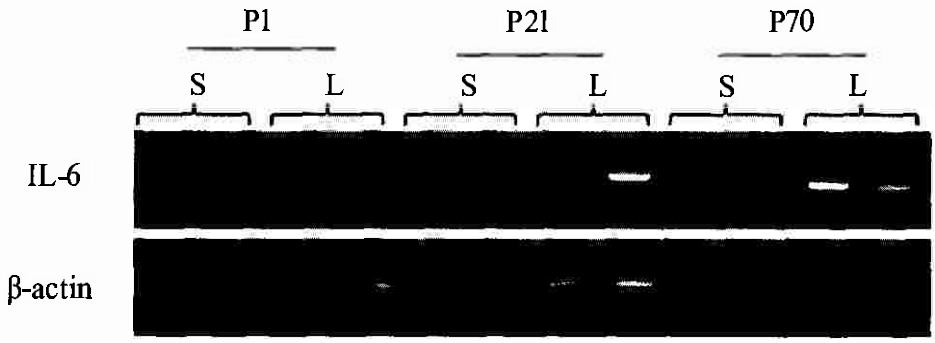

Figure 3. Representative RT-PCR of IL-6 expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

LPS-induced activation of chemokines in P1, P21, and P70 rat liver

Analysis of the expression levels showed an increase of MIP-1β, MIP-2, and MCP-1 in LPS-treated pups at P1, P21, and P70 as compared to their saline controls (Figs. 4, 5, and 6). Additionally, there appears to be an increase in the expression of MIP-1β and MCP-1 at P21 and P70 as compared to P1 following LPS stimulation (Figs. 4 and 6).

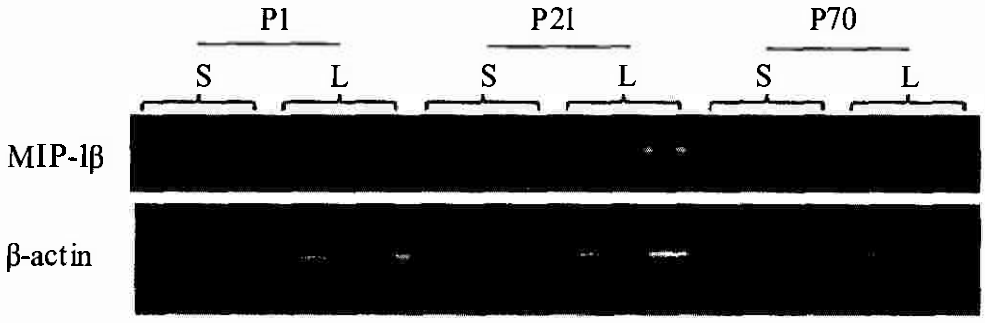

Figure 4. Representative RT-PCR of MIP-1β expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

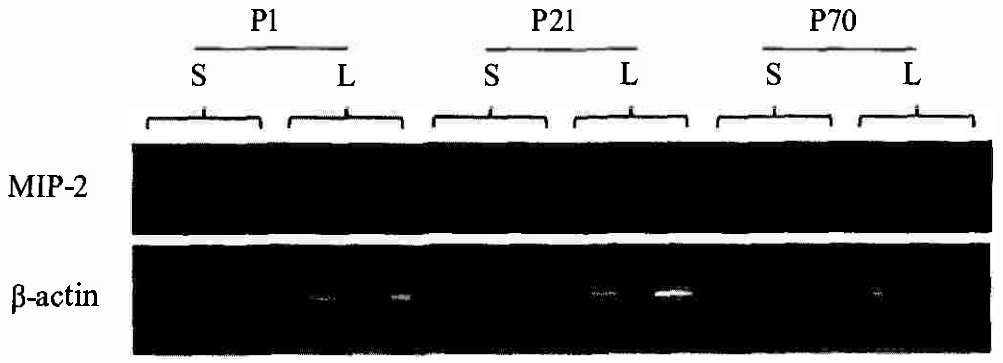

Figure 5. Representative RT-PCR of MIP-2 expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

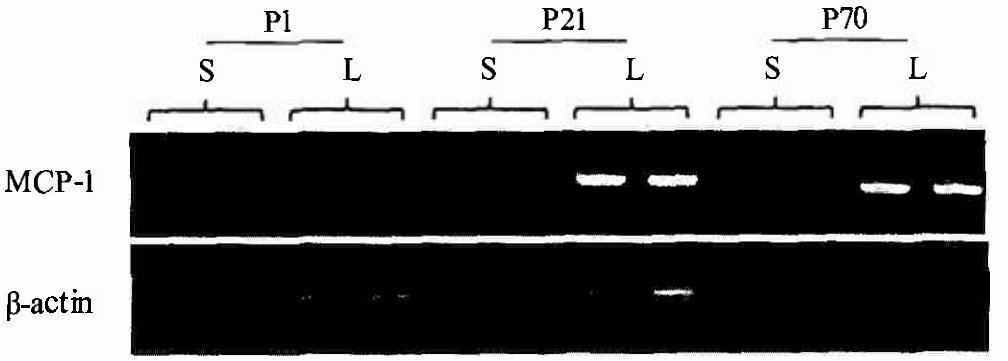

Figure 6. Representative RT-PCR of MCP-1 expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

LPS-induced activation of TLR4, CD14, and Myd88 in P1, P21, and P70 rat liver

We also examined the mRNA expression of TLR4, CD14, and Myd88 using RT-PCR analysis with appropriate sense and anti-sense primers.

TLR4 mRNA expression seemed to be decreased in LPS-treated pups at P21 and P70 as compared to their saline controls. Additionally the basal level of TLR4 expression appeared to be increased at P21 and P70 liver compared to P1 liver (Fig. 7). The expression levels showed an increase in LPS-treated pups at the ages studied for CD14. Within the LPS-treated pups, there appears to be an increase in the level of expression in P21 and P70 pups as compared to P1 pups after LPS stimulation (Fig. 8). The expression levels of Myd88 increase in the LPS treated pups at P1, P21, and P70 compared to their saline controls. Additionally, the expression levels of Myd88 appear to be increased at P21 and P70 as compared to P1 after LPS stimulation (Fig. 9).

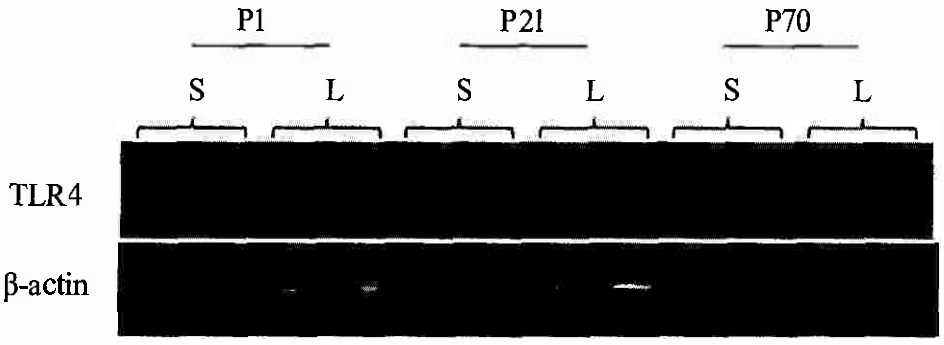

Figure 7. Representative RT-PCR of TLR4 expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

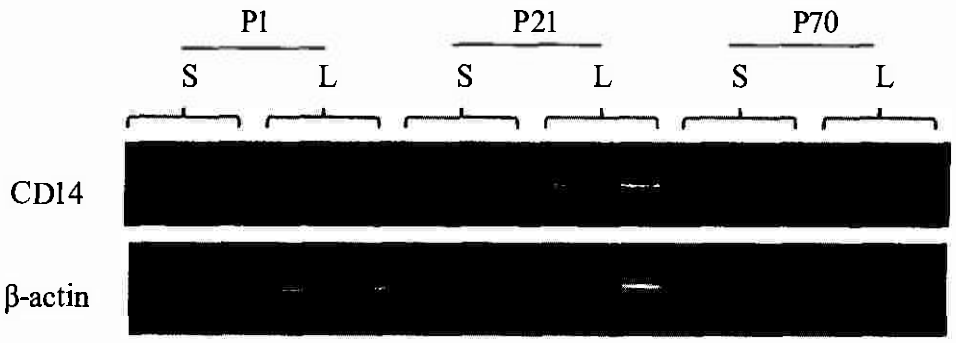

Figure 8. Representative RT-PCR of CD14 expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

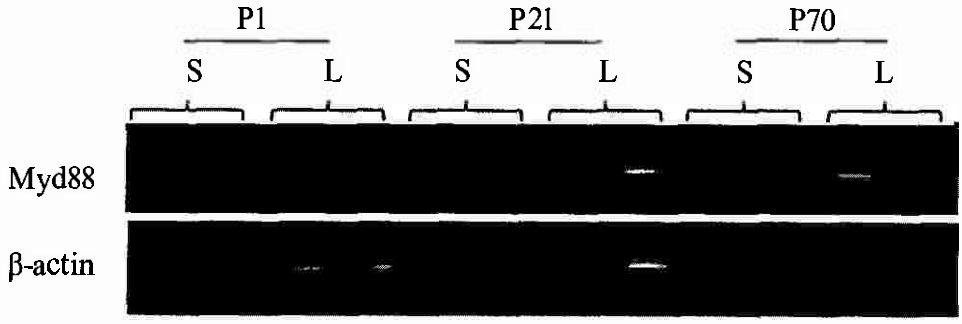

Figure 9. Representative RT-PCR of Myd88 expression in P1, P21, and P70 animals at 2 h following stimulation of 250 μg/kg LPS (L) or saline (S).

Effects of LPS stimulation on MAPK phosphorylation in P1, P21, and P70 rat liver

Immunohistochemistry was used to investigate the localization of MAPK immunoreactivity. Although quantitation of immunohistochemical signal in each individual cell was not performed, the number of cells immmunoreactive to p-p42/44 in LPS-treated pups were increased compared to those pups that were saline treated, which is consistent with the cytokine and chemokine data. Furthermore, there was an increase in the number of cells immunoreactive to p-p42/44 in P21 and P70 as compared to P1 amongst the LPS-treated pups (Figures 10A, 10B, 10C, 10D, 10E, and 10F).

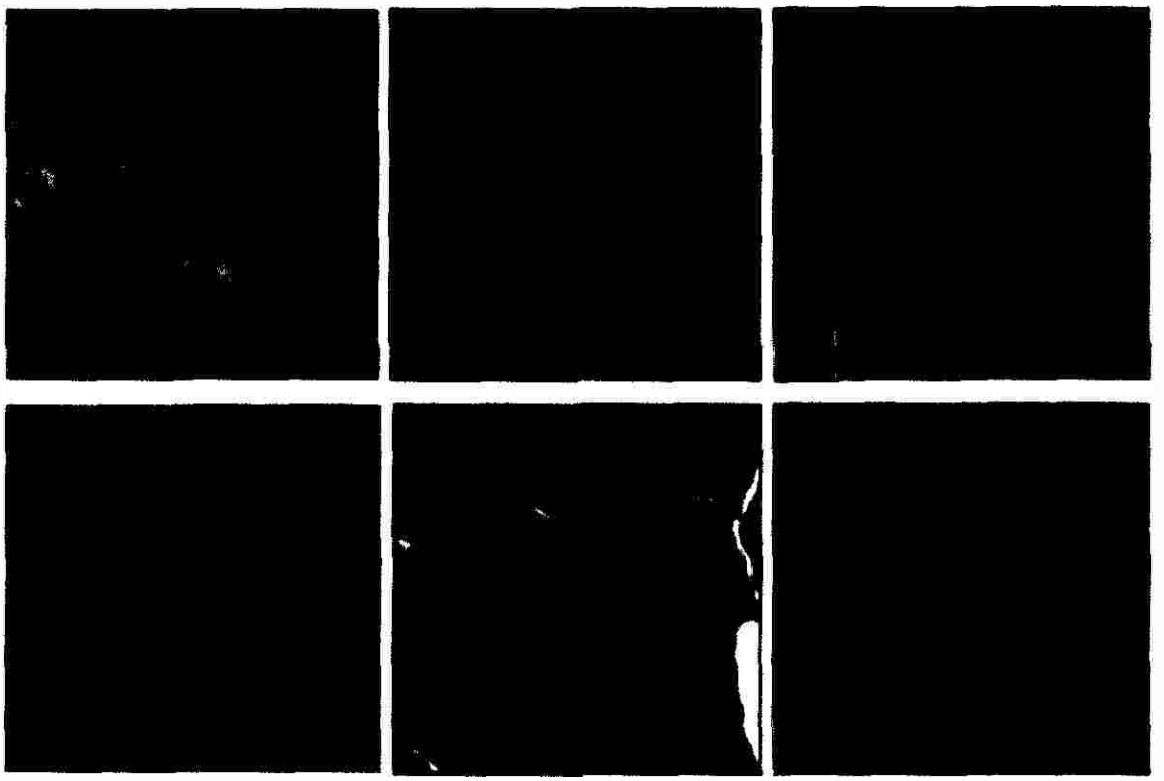

Figure 10. Representative images ofp-p42/44 MAPK immunohistochemistry. Pups were treated for 2 hours with either 250 μ/kg LPS or saline at P1, P21, and P70. (A) P1 Saline. (B) P21 Saline. (C) P70 Saline. (D) P1 LPS. (E) P21 LPS. (F) P70 LPS, Bar: 50 μm

Time-course of cytokine induction in P1 rats

Rat pups were treated at P1 with one dose of 250 μg/kg of LPS via i.p. injection for 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h to assess the time-course of relative mRNA levels in P1 rat liver. The rats were then sacrificed and their liver tissues harvested for preparation of total RNA. RT-PCR was conducted with appropriate sense and anti-sense primers to evaluate the levels of relative mRNA expression in inflammatory genes.

1L-1β mRNA expression was increased at 0.5 h, elevated at 2 h and 6 h, and slightly decreased at 24 h following LPS stimulation (Fig. 11). The relative mRNA level of1L-6 increased and peaked at 6 h, and returned to basal levels at 24 h (Fig. 12).

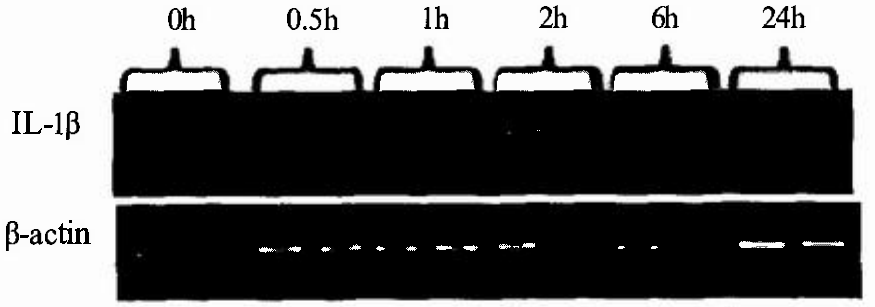

Figure 11. A time-course representative RT-PCR of IL-1β expression of LPS-induced (250μg/kg) P1 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h.

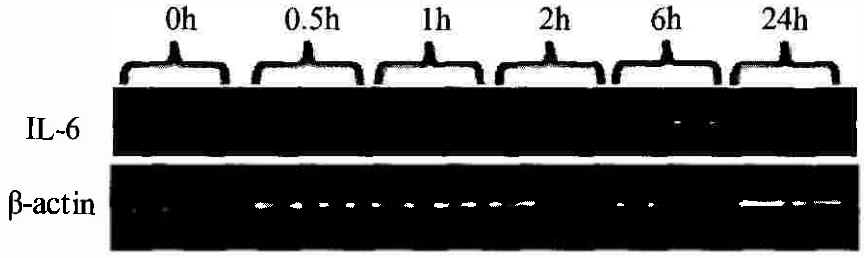

Figure 12. A time-course representative RT-PCR of IL-6 expression of LPS-induced (250μg/kg) P1 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h.

Time-course of chemokine induction in P1 rats

Rat pups were treated in the aforementioned conditions. MIP-1β mRNA expression was significantly increased and peaked at 6 h and remained elevated at 24 h following LPS stimulation (Fig. 13). The relative mRNA level of MCP-1 showed similar results with an increase and peak at 6 h and remained elevated at 24 h (Fig. 14).

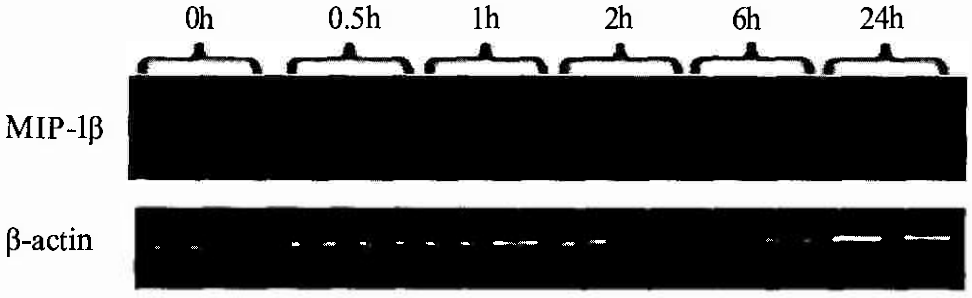

Figure 13. A time-course representative RT-PCR of MIP-1β expression of LPS-induced (250μg/kg) P1 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h.

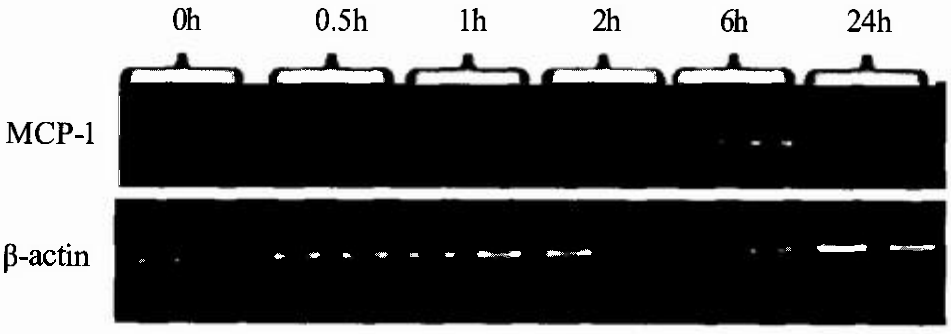

Figure 14. A time-course representative RT-PCR of MCP-1 expression of LPS-induced (250μg/kg) P1 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h.

Time-course of cytokine induction in P21 rats

Rat pups were treated at P21 with one dose of 250 μg/kg of LPS via i.p. injection for 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h to assess the time-course of relative mRNA levels in P21 rat liver. The rats were then sacrificed and their liver tissues harvested for preparation of total RNA. RT-PCR was conducted with appropriate sense and anti-sense primers to evaluate the levels of relative mRNA expression of inflammatory genes.

TNF-α mRNA expression was significantly increased at 0.5 h, peaked at 1 h, remained elevated at 2 h, and returned to basal levels 6 h following LPS stimulation (Figs. 15A and 15B). The relative mRNA level of IL-1β showed a significant increase at 0.5 h, significantly elevated at 1 h and 2 h, remained elevated at 6 h, and returned to basal levels at 24 h (Figs. 16A and 16B). IL-6 mRNA expression was significantly increased at 1 h, peaked at 2 h, and returned to basal levels at 6 h following LPS stimulation (Figs. 17A and 17B).

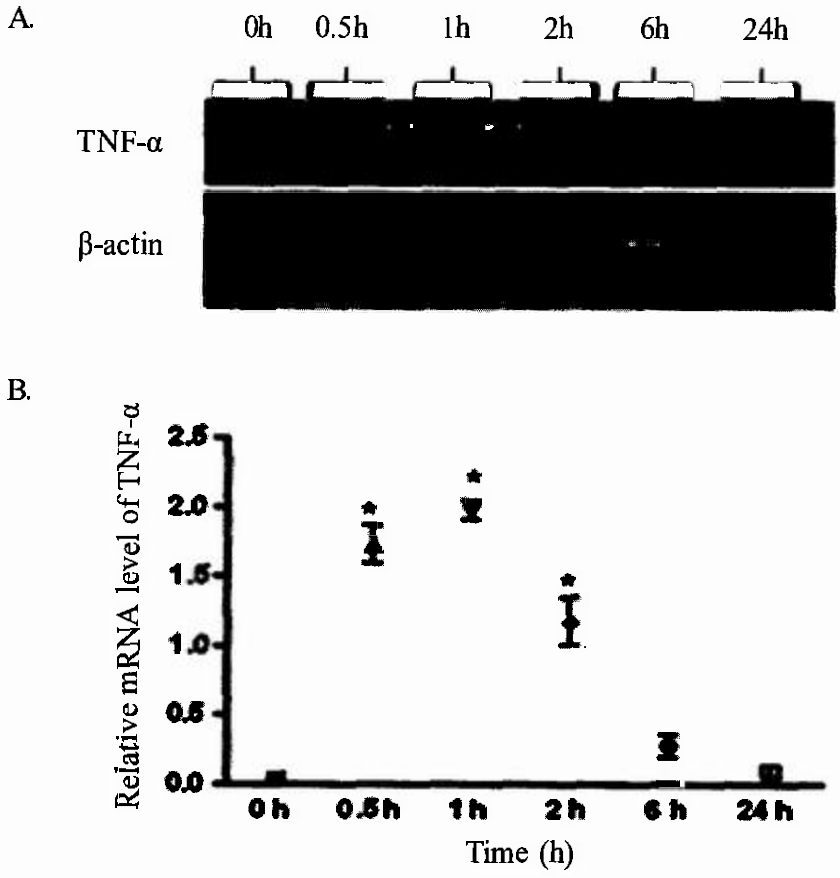

Figure 15. (A) Representative RT-PCR of TNF-α expression of LPS-induced (250μg/kg) P21 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h. (B) Quantitation of TNF-α normalized to β-actin. * represent significant difference as compared to basal levels. Values are means± SE, $p < 0.05$.

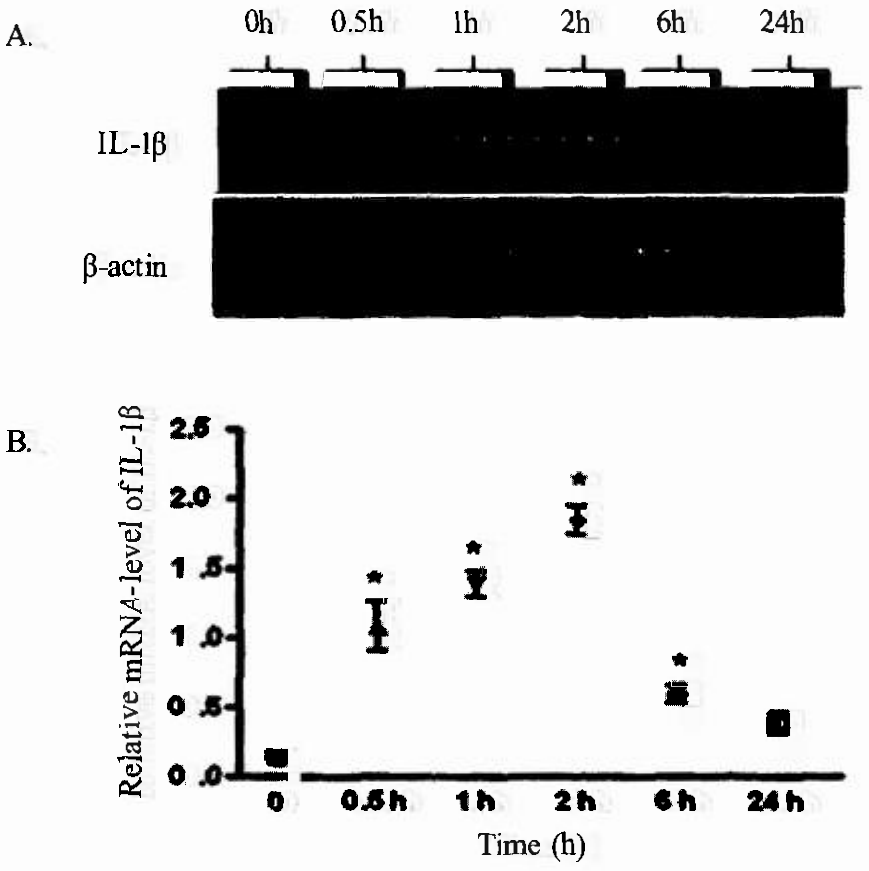

Figure 16. (A) Representative RT-PCR of IL-1β expression of LPS-induced (250μg/kg) P21 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h. (B) Quantitation of TNF-α normalized to β-actin. * represent significant difference as compared to basal levels. Values are means± SE, $p < 0.05$.

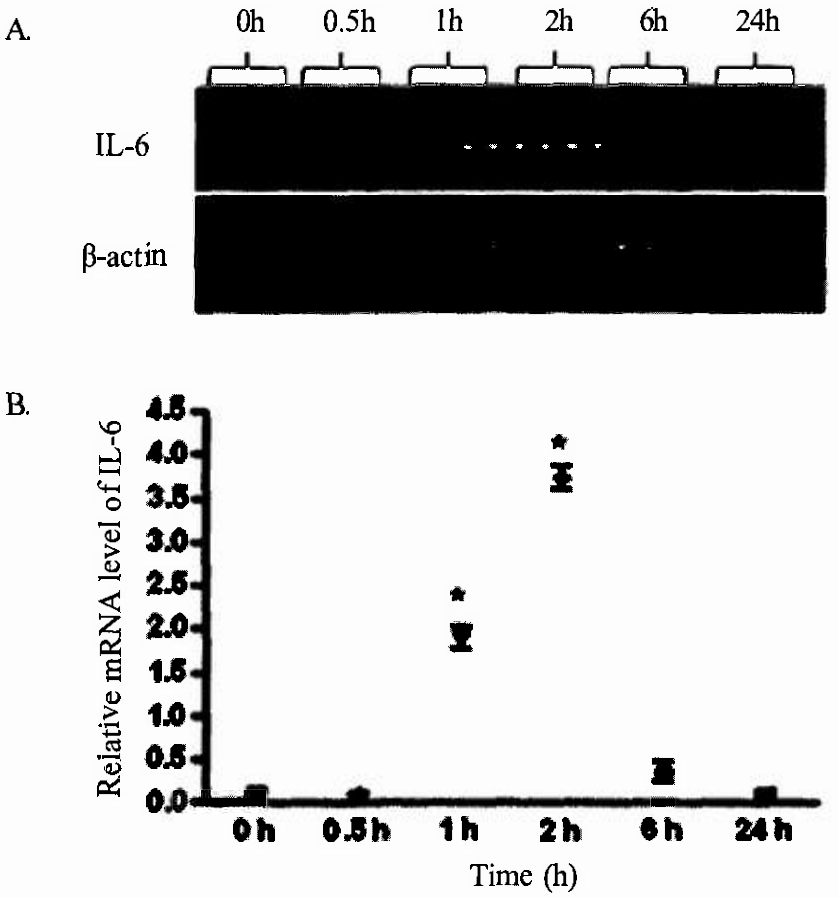

Figure 17. (A) Representative RT-PCR of IL-6 expression of LPS-induced(250μg/kg) P21 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h. (B) Quantitation of TNF-α normalized to β-actin. * represent significant difference as compared to basal levels. Values are means± SE, $p < 0.05$.

Time-course of chemokine induction in P21 rats

Rat pups were treated in the aforementioned conditions. MIP-1β mRNA expression was increased at 0.5 h, elevated at 1 h and 2 h, remained elevated at 6 h, and returned to basal levels at 24 h following LPS stimulation (Fig. 18). The relative mRNA level of MCP-1 appear to be increased at 0.5 h, elevated at 1 h, 2 h, and 6 h, and returned to basal levels at 24 h (Fig. 19).

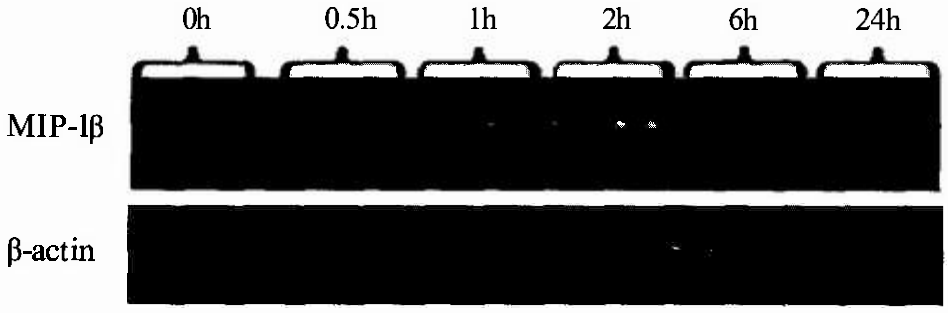

Figure 18. A time-course representative RT-PCR of MIP-1β expression of LPS-induced (250μg/kg) P21 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h.

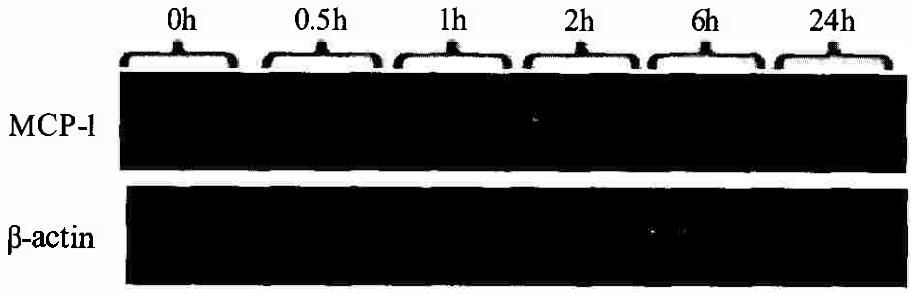

Figure 19. A time-course representative RT-PCR of MCP-1 expression of LPS-induced (250μg/kg) P21 pups at 0 hour (h), 0.5 h, 1 h, 2 h, 6 h, and 24 h.

DISCUSSION

This study investigated the effects of age on LPS-induced hepatic inflammatory response. The primary findings of this investigation showed that an activation of cytokines and chemokines in the liver exhibited age-dependency in pups treated with LPS at postnatal (P) day 1, P21, and P70. The pattern shows an increase in relative mRNA expression of cytokines and chemokines as development progresses.

Among the LPS stimulated pups, there was a significant increase of relative mRNA levels in P70 pups as compared to LPS stimulated P1 pups for the cytokines and chemokines studied. There was also a significant increase of relative mRNA levels among the LPS-treated of the P21 pups as compared to P1 pups for the cytokines and chemokines studied using RT-PCR analysis.

We also found that there was a decrease in the expression of TLR4 in LPS-treated among the P21 and P70 pups as compared to their saline controls. In contrast, the relative mRNA level of CD14 was increased in LPS-treated animals as compared to their saline controls. In addition, amongst the LPS stimulated pups, there was an increase in the expression of CD14 from P1 to P70.

Consistently, phosphorylation of p42/44 MAPK in the liver has been reported to be increased in P21 and P70 animals as compared to their saline controls (Surriga, 2009), and confirmed in our immunohistochemical studies.

Similar studies have reported that human neonates are highly susceptible to infections by bacteria, fungi, and viruses. As a result, infection remains the single most common killer in early life (Marodi, 2006). It has been duly noted that deficiencies of

innate immunity contribute to the impaired neonatal host defense. With an impaired host defense, it has been reported that polymorphonuclear leukocytes from preterm infants exhibit a much reduced antibacterial activity than those from adult (Henneke et al., 2003). This results in an inhibited immune response, similar to what was reported with P1 pups in this present study. Additional studies have shown there is a diminished induction of IL-1β, IL-6, and TNF-α in cord blood as compared to adult peripheral blood monocytes (Förster-Waldl et al, 2005). Likewise, due to the lack of cytokines present in P1 pups, there was a significantly lower expression of mRNA levels of cytokines as compared to the older P21 and P70 pups.

In conclusion, we found that there was a delayed cytokine and chemokine induction at P1 as compared to P21 and P70 pups. This data suggest that the hepatic innate immunity undergo significant development during early postnatal development, and the delayed inflammatory response in P1 animals may contribute to increased susceptibility of neonatal animals to bacterial infections. It would be worthwhile to undergo additional studies to further investigate specific ages in which there is a definitive increase in cytokine and chemokine induction.

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