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Comparative Analysis of Hepatic and Pulmonary Immune Mediators During Postnatal Development

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**COMPARATIVE ANALYSIS OF HEPATIC AND PULMONARY
IMMUNE MEDIATORS DURING POSTNATAL DEVELOPMENT**

By

MICHELLE MACK

**Submitted in partial fulfillment of the requirement for the
degree of Master of Science in Microbiology from the
Department of Biological Sciences of Seton Hall University
April, 2011**

APPROVED BY:



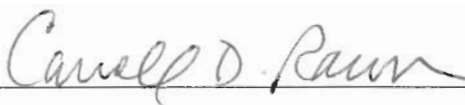
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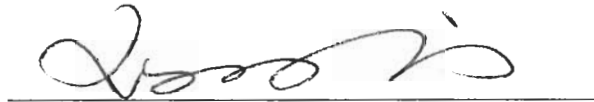
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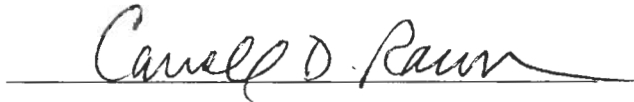
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Abstract

Lipopolysaccharide (LPS) is a major component that is found in the outer membrane of Gram-negative bacteria. It is a potent immunogen that has the ability to induce an uncontrollable inflammatory response that may ultimately lead to septic shock. The toll-like receptor (TLR) 4 signaling pathway when stimulated via LPS is able to activate mitogen activated protein kinases (MAPKs), specifically p38 and p42/44. The activation of the TLR4 signaling pathway is able to induce the release of cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF- α). In this study we used antibody arrays to profile the expression levels of cytokines in the serum of postnatal day (P) 1, P21 and P70 animals. Our antibody array data showed that P1 animals exhibited lower levels of inflammatory cytokines than P21 and P70 animals at 2 h following LPS treatment, suggesting that neonates are relatively immunodeficient. We also compared the expression of key mediators of TLR4 signaling pathway in the lung and liver. Our results showed that the basal protein levels of phosphorylated p42/44 MAPK (P-p42/44 MAPK) and CD14 seemed to be lower in the liver of P1 rats than P21 and P70, yet the basal protein levels of phosphorylated p38 MAPK (P-p38 MAPK) and TLR4 did not appear to be significantly altered in the liver of P1, P21, and P70 rats. In the lung, the basal levels of P-p42/44 MAPK seemed to be lower in P1 rats when compared to that of P21 and P70, yet the basal level of P-p38 MAPK, TLR4, and CD14 did not appear to show any significant difference among P1, P21, and P70 rats. These data suggest that the lower basal level of P-p42/44 MAPKs in both lung and liver is associated with the immune deficiencies in the neonates, and that CD14 expression may be differentially regulated in the lung and liver during postnatal development.

INTRODUCTION

Lipopolysaccharide (LPS), a potent immunogen, is a major component found in the outer membrane of Gram-negative bacteria. An uncontrolled inflammatory response induced by LPS could ultimately lead to septic shock and tissue injury (Kim and Ha, 2010). LPS-binding protein (LBP) acts as a shuttle protein, associates with LPS to form the LBP/LPS complex, and delivers LPS to CD14, a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein that acts as a surface receptor for LPS. CD14 is expressed on many immune cells including monocytes/macrophages (Sweet and Hume, 1996). It aids in the transfer of LPS to the toll-like receptor (TLR)4/MD-2 receptor complex which initiates the intracellular signal transduction cascade ultimately leading to the production of inflammatory cytokines and chemokines (Lu et. al., 2008).

TLR4 utilizes all five adaptor proteins in its signal transduction cascade after LPS recognition. These five adaptor proteins include myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN- β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α and HEAT-Armadillo motifs-containing protein (SARM) (O'Neil and Bowie, 2007). TLR4-mediated signaling is divided into two distinct pathways, a MyD88-dependent pathway responsible for the induction of proinflammatory cytokines and a MyD88-independent pathway that leads to the induction of Type I interferons (Lu et. al., 2008). CD14 has been shown to be necessary for the MyD88-independent pathway whereas the MyD88-dependent pathway signaling is still able to occur when CD14 is absent in macrophages (Jiang et. al., 2005), which suggests that LPS-induced inflammatory response may be at least partially dependent on CD14.

In the MyD88-dependent pathway, MyD88 activates IL-1 receptor-associated kinase-4 (IRAK-4) along with TNF receptor-associated factor 6 (TRAF6), p42/44 mitogen activated protein kinase (MAPK), also called extracellular signal regulated kinase (ERK) 1/2, and p38 MAPK. Upon phosphorylation, p42/44 and p38 MAPKs have been found to activate transcription factors such as activator protein (AP)-1 and nuclear factor- κ B (NF- κ B) (Lu et. al., 2008), increasing the expression of potent inflammatory mediators, specifically tumor necrosis factor alpha (TNF- α), interleukin-8 (IL-8), and interleukin-6 (IL-6) (Palsson-McDermott and O'Neil, 2004). Along with the increased expression of proinflammatory cytokines, the expression of anti-inflammatory cytokines such as interleukin-10 (IL-10) has also been found to be induced by LPS (Palsson-McDermott and O'Neil, 2004). IL-10 is critical in maintaining immune homeostasis. For example, it has been found to inhibit chronic and acute inflammation via decreasing the amount of proinflammatory cytokines produced by activated macrophages (Schaljo et. al., 2009).

It has been shown that by inhibiting the activation of p38 MAPK the release of proinflammatory cytokines such as TNF- α and IL-6 are also inhibited, thereby increasing the survival rate during endotoxin-mediated sepsis in Balb-c mice (O'Sullivan et. al., 2007). Activation of p38 has also been found to be necessary for the production of TNF- α and IL-8 in neutrophils (O'Sullivan et. al., 2007; Lee et. al., 1994). Treatment with a p38 inhibitor, SB-202190, has been found to lead to an increase in macrophage number but a decrease in the amount of cytokines being released (O'Sullivan et. al., 2007). Recent studies have shown that c-Jun N-terminal kinase (JNK), p38, and p42/44 MAPKs work in concert with one another to mediate LPS-induced production of TNF- α in

peritoneal macrophages even though their mechanisms may differ (Dumitru et. al., 2000). For example, p42/44 MAPK plays a role in regulating nucleocytoplasmic transport of TNF- α mRNA in macrophages, p38 MAPK controls stability and translation of TNF- α mRNA (Dumitru et. al., 2000). The release of TNF- α in peritoneal macrophages could be blocked by inhibiting either p38 or p42/44 MAPKs (Dumitru et. al., 2000).

The liver has been shown to be an important organ in recognizing pathogen associated molecular patterns (PAMPs) and ridding toxins, such as LPS, from the body (Scott et. al., 2010). It appears that both nonparenchymal and parenchymal cells play an important role in the liver immune response (Scott et. al., 2010). Liver Kupffer cells are activated by LPS and produce IL-12 (Seki et. al., 2000). IL-12, in turn, is able to activate natural killer (NK) cells that initiate the first line of defense against bacterial infections (Seki et. al., 2000). Stimulation of Kupffer cells with LPS has been shown to induce the production of IL-6 (Gosemann et. al., 2010) and IL-12 (Seki et al, 2000), increase the activation of p38MAPK, and increase the production of TNF- α (Chen et. al., 2005). Hepatocytes have also been shown to express TLR4, CD14, and MyD88 (Scott et. al., 2010) and are able to respond to LPS stimulation (Scott et. al., 2010). These studies suggest that LPS is able to initiate the TLR4 signal transduction cascade and activate MAPKs in the liver, leading to the production of inflammatory cytokines.

In the lung, bronchial epithelial cells are the first line of defense against pathogens and they have been reported to express TLR4 (Basu et al., 2004). Recent findings have shown that the induction of IL-8 by LPS in the lung involves both p38 and p42/44 MAPK pathways, and only the p38 MAPK pathway is involved in the induction of IL-6 (Wu et. al., 2010). These studies suggest that LPS is able to activate TLR4 mediated signaling

pathway in the lung, resulting in the production of proinflammatory cytokines such as IL-6 and IL-8.

Recent studies have shown that MAPK expression changes with aging. For example, it has been reported that the basal level of phosphorylated p42/44 MAPK was higher in the aorta of 6-month old rats when compared to that in 30 and 36-month old rats. In contrast, the basal level of phosphorylated p38 MAPK is the lowest in the aorta of 6-month old rats as compared to that in 30 and 36-months old rats (Rice et. al., 2004). However, studies conducted on adrenalcortical cells of rats subjected to cellular oxidative stress showed that the aged animals exhibit a marked increase in the level of phosphorylated p38, but not phosphorylated p42/44 (Abidi et. al., 2007). A study looking at the effects of age on TLR4 expression showed that the surface expression of TLR4 on murine peritoneal macrophages was not altered with age while there was an age-related decrease in phosphorylated p38 when stimulated by LPS (Boehmer et. al., 2004). Lung alveolar macrophages isolated from neonatal rats have been reported to be functionally immature and secreted less inflammatory mediators such as TNF- α and IL-1 β compared with mature rat lung alveolar macrophages (Bakker et. al., 1998). A similar study has reported that IL-10 expression is minimal in infant rat alveolar macrophages suggesting that the alveolar macrophages in neonates may be functionally different from adults (Lee et. al., 2001).

Our lab has previously found that LPS stimulation for 2 h significantly elevates the levels of both TNF- α and IL-6 in the serum of P1, P21, and P70 rats. We have also previously found that at 2 h following LPS stimulation the expression of TNF- α and IL-1 β mRNA levels were significantly elevated in the lung and liver of postnatal day (P) 1,

P21, and P70 animals. This study looked at the basal level expression of hepatic and pulmonary immune mediators in the TLR4 signaling pathway. The protein expression of TLR4, CD14, p38 MAPK, and p42/44 MAPK was investigated in the liver and lung of P1, P21, and P70 animals treated with saline using Western blot assays. We also examined the cytokines and chemokines present in the serum of P1, P21, and P70 at 2 h following stimulation with saline or LPS.

Materials and Methods

Animals

Adult male and female Sprague-Dawley[®] rats were purchased from Harlan Inc. (Indianapolis, IN). The rats were housed in a temperature and humidity-controlled environment with a 12-hour light/dark cycle and fed a standard rat diet and water *ad libitum*. The rats were allowed to acclimate to the animal facility for at least 7 days prior to beginning any experimentation. Animal studies were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University.

In order for mating to occur, each male rat (250-300g) was housed in the same cage with 1-3 female rats (200-230g) at night, and each female rat was visually inspected for the presence of the vaginal plug the next morning. The day on which a vaginal plug was noted was defined as day 0 of pregnancy, and the female rat was then moved to a separate cage under the aforementioned conditions.

For age-dependency studies, the pups were maintained and received saline at P1, P21, and P70 via i.p. injection. The pups were sacrificed and their liver and lung tissues were collected and stored at -80°C for total protein extraction.

Total Protein Extraction

The liver and lung tissues from each treated animal were finely ground. For every 0.1 g of liver or lung tissue, 150 μ l of lysis buffer containing 50mM HEPES (pH 7.5), 5 mM EDTA, 50 mM NaCl, 10 mM NaPPi, 50 mM NaF, 1% IGEPAL, 2 mM Na₃VO₄, and 1X protease inhibitor cocktail (Roche, Indianapolis, IN) was added. The liver and lung tissues were then homogenized with an eppendorf (EP) tube pestle and sonicated 3 times

with a Branson Sonifier 250 for 15 pulses followed by 10 seconds on ice between each round of sonification. The tissues were then incubated on ice for 30 minutes followed by a 15 minute centrifugation at 14,000g in 4°C. The supernatants were then collected for protein concentration determination and Western blotting.

Protein Concentration Determination

Protein concentration was determined using the bicinchonic acid (BCA) Protein Assay Reagent from Pierce Biotechnology Inc. (Rockford, IL) following the manufacturer's protocol.

Western Blot Assays

Appropriate amounts of lung or liver protein extracts were mixed with an equal volume of 2X sample loading buffer, protein samples were boiled for 5 minutes to detect MAPK; protein samples were heated up to 70°C for 3 minutes to detect CD14 and TLR4. All samples were then centrifuged at 14,000 g for 5 minutes at 4°C. The samples were then resolved using a 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel followed by a transfer onto nitrocellulose membranes. Membranes were then blocked with 5% (w/v) nonfat milk for 2 hours and incubated with primary antibodies that recognize phosphorylated forms of p38 MAPK, p42/44 MAPK, (Cell Signaling Technology, Boston, MA), TLR4, or CD14 and incubated overnight at 4°C. Following four 10-minute washes with tris-buffered saline containing 0.1% Tween-20 (TBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA) for 1

hour at room temperature. Membranes were then washed 4 times with TBST and developed using ECL^{plus} chemiluminescence detection kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) by the addition of ECL reagents directly to membranes and scanned using Storm 860 Molecular Imager (Molecular Dynamics). After scanning, membranes were stripped and reprobed with antibodies for total p38, p42/44 (Cell Signaling, Boston, MA), or β -actin (Sigma, St. Louis, MO).

Cytokine Antibody Array

P1, P21, and P70 rat pups were treated with either saline or 250 μ g/kg LPS (*Salmonella enteric* serovar Typhimurium) Sigma, St. Louis, MO) dissolved in sterile pyrogen-free saline for 2 h (four pups per group) via i.p. injection. Rat blood samples were collected for preparation of serum. Pooled serum samples were incubated with antibody arrays (RayBiotech, Inc.) which allowed simultaneous detection of 19 cytokines and chemokines. Arrays were blocked using 1X blocking buffer at room temperature for 30 minutes and then incubated with 1 ml of serum samples at room temperature for two hours. Three washes were then performed on the arrays with 1X wash buffer at room temperature with 5 minutes per wash. The membranes were then incubated with Biotin-Conjugated anti-cytokines mixture for one hour. The arrays were washed as above followed by incubation with HRP-conjugated streptavidin secondary antibody for one hour. Membranes were then washed three times and developed by ECL^{plus} chemiluminescence detection kit. The membranes were then scanned using Storm 860 Molecular Imager (Molecular Dynamics).

Enzyme-linked immunosorbent assay (ELISA)

The serum levels of IL-6 and TNF- α were measured using ELISA kits purchased from Invitrogen (Grand Island, NY), and ELISA assays were performed following manufacturer's protocols.

Statistical Analysis

Data were presented as means \pm SE and analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post-tests. Results with $p < 0.05$ were considered statistically significant.

RESULTS

Antibody array measurement of LPS-induced inflammatory mediators in the serum

In order to examine protein levels of multiple cytokines and chemokines in the serum of P1, P21, and P70 animals following treatment with LPS, P1, P21, and P70 animals were treated with saline or 0.25 mg/kg LPS via i.p. injection and sacrificed 2 h later. Blood samples were collected and serum was prepared. Pooled serum samples were incubated with antibody arrays, which allowed simultaneous detection of 19 cytokines and chemokines. The antibody array measurements were then confirmed using ELISA.

LPS stimulation significantly elevated the serum levels of cytokine-induced neutrophil chemoattractant (CINC)-3, interleukin (IL)-10, and tumor necrosis factor (TNF)- α in P1, P21, and P70 animals (Figs. 1A, 1B, 1D, 1F). The serum levels of CINC-3 and TNF- α in P1 animals were significantly lower than that in P21 and P70 animals (Figs. 1A, 1B, 1F). LPS-induced IL-10 level in the serum of P1 animals was not significantly different from that in P21 and P70 animals (Figs. 1A, 1D). The serum levels of IL-6 and macrophage chemoattractant protein (MCP)-1 were significantly elevated in P21 and P70 animals following LPS stimulation while LPS treatment did not appear to significantly elevate the serum levels of IL-6 and MCP-1 in P1 animals (Figs. 1A, 1C, 1E). The measurements obtained from antibody arrays were also confirmed by ELISA results (Fig. 2).

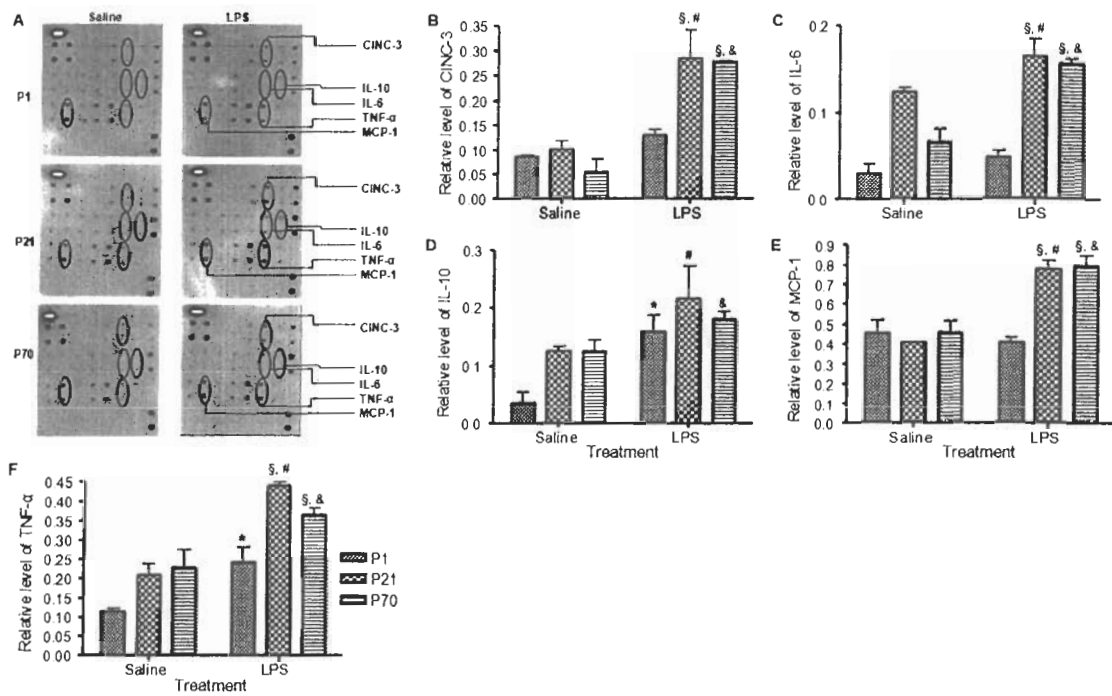


Figure 1. Antibody array measurement of cytokines and chemokines in the serum of P1, P21, and P70 animals following saline or LPS stimulation. Animals were treated via i.p. injection of saline or 0.25mg/kg LPS for 2 h. Serum samples were prepared and used for determination of cytokine and chemokine levels using antibody arrays. (A) Antibody array images of cytokines and chemokines (n=2). (B-F) Graphical representations of relative levels of CINC-3, IL-6, IL-10, MCP-1, and TNF- α respectively. Legend: * vs. P1 saline, # vs. P21 saline, & vs. P70 saline, § vs. P1 LPS.

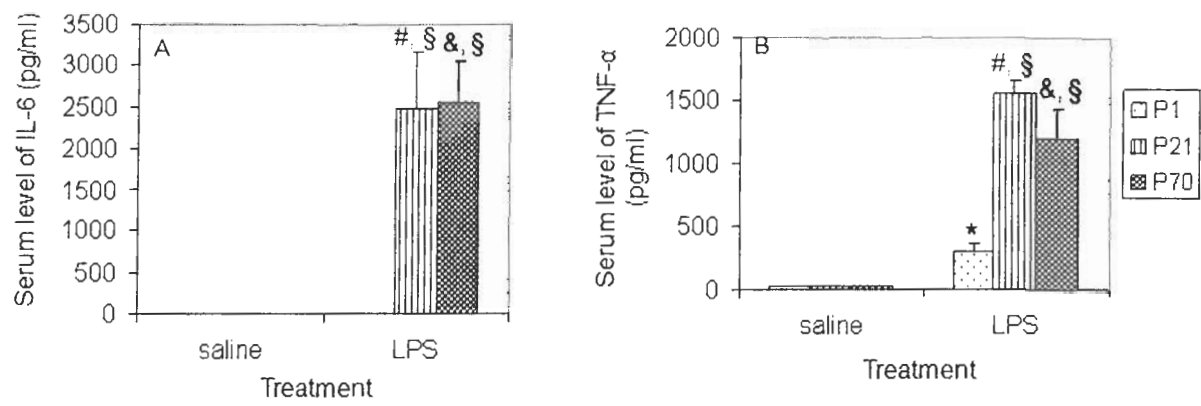


Figure 2. ELISA results of IL-6 and TNF- α in the serum of P1, P21, and P70 animals following saline or LPS stimulation. Animals were treated via i.p. injection of saline or 0.25mg/kg LPS for 2 h. IL-6 and TNF- α levels in the serum samples of these animals were then determined by ELISA. Legend: * vs. P1 saline, # vs. P21 saline, & vs. P70 saline, § vs. P1 LPS.

Basal levels of TLR4 in P1, P21, and P70 rat liver

The basal levels of TLR4 in the liver of P1, P21, and P70 were examined using Western blot analysis with a specific antibody that recognizes TLR4. The basal level of TLR4 in P1 liver did not appear to be significantly different from that in P21 and P70 liver (Figure 3).

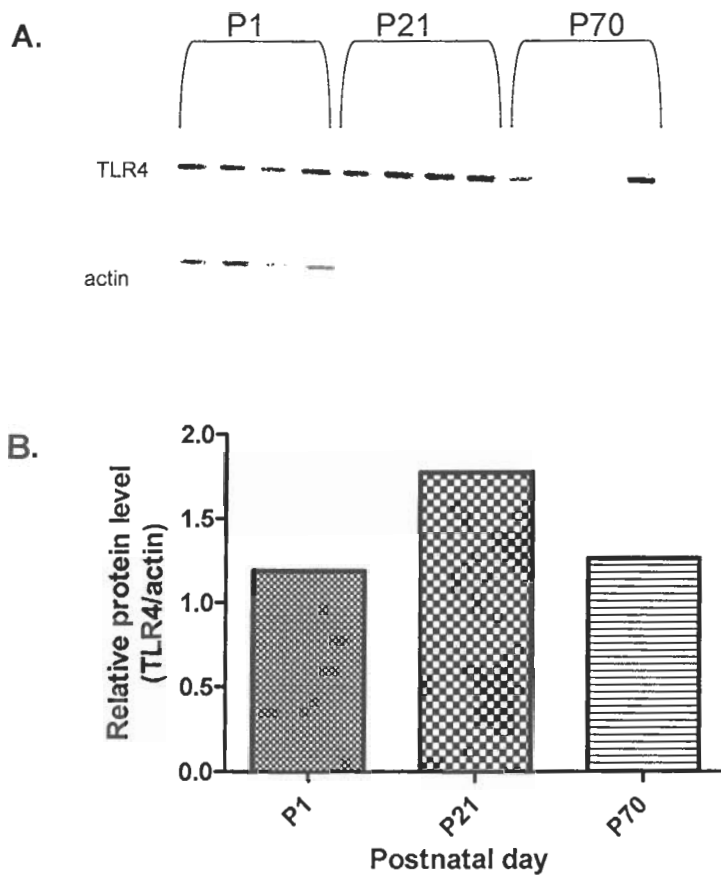


Figure 3. Age-dependent production of TLR4 in the liver of P1, P21, and P70 animals following saline treatment. Pups were treated via i.p. injection of saline for 2 h. TLR4 level was then determined by western blot. (A) Western blot image of TLR4 in the liver of P1, P21 and P70 animals along with actin control (n=4). (B) Preliminary quantitation of TLR4 protein expression level normalized to actin. Values represented the means of four samples in each group.

Basal levels of CD14 in P1, P21, and P70 rat liver

The basal level of CD14 in the liver of P1, P21, and P70 was also examined using Western blot analysis with a specific antibody that recognizes CD14. The basal level of CD14 protein in the liver of P1 rats appeared to be at a much lower level than that in the liver of P21 and P70 rats (Figure 4).

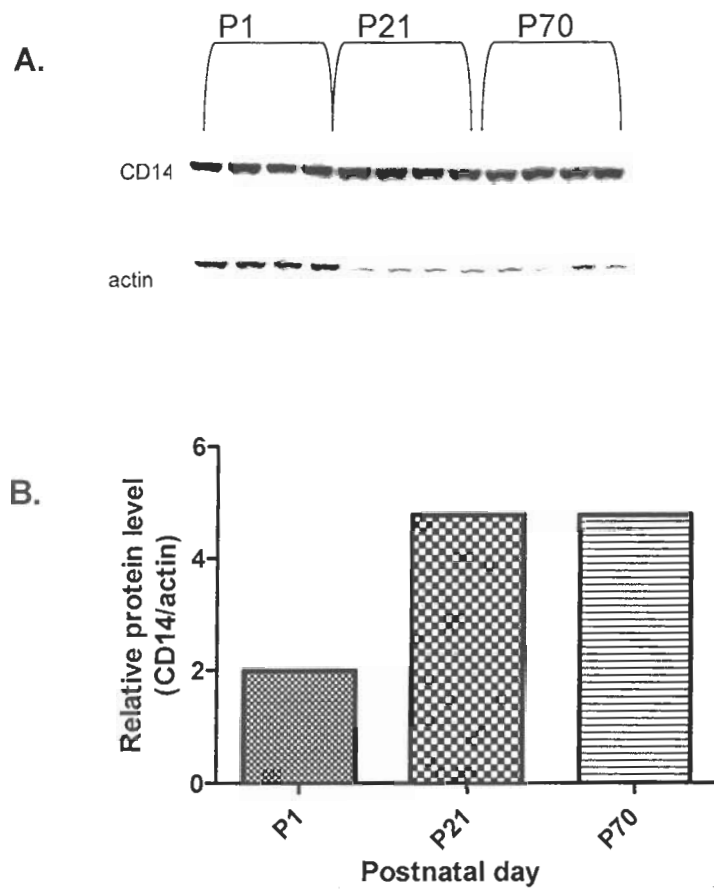


Figure 4. Age-dependent production of CD14 in the liver of P1, P21, and P70 animals following saline treatment. Pups were treated via i.p. injection of saline for 2 h. CD14 levels were then determined by Western blot. (A) Western blot image of CD14 in the liver of P1, P21, and P70 animals along with actin control (n=4). (B) Preliminary quantitation of CD14 normalized to actin. Values represented the means of four samples in each group.

Basal levels of TLR4 in P1, P21, and P70 rat lung

The basal levels of TLR4 in the lung of P1, P21, and P70 were examined using Western blot analysis with a specific antibody that recognizes TLR4. The basal level of TLR4 in P1 lung did not seem to be significantly different from that in the lung of P21 and P70 animals (Figure 5).

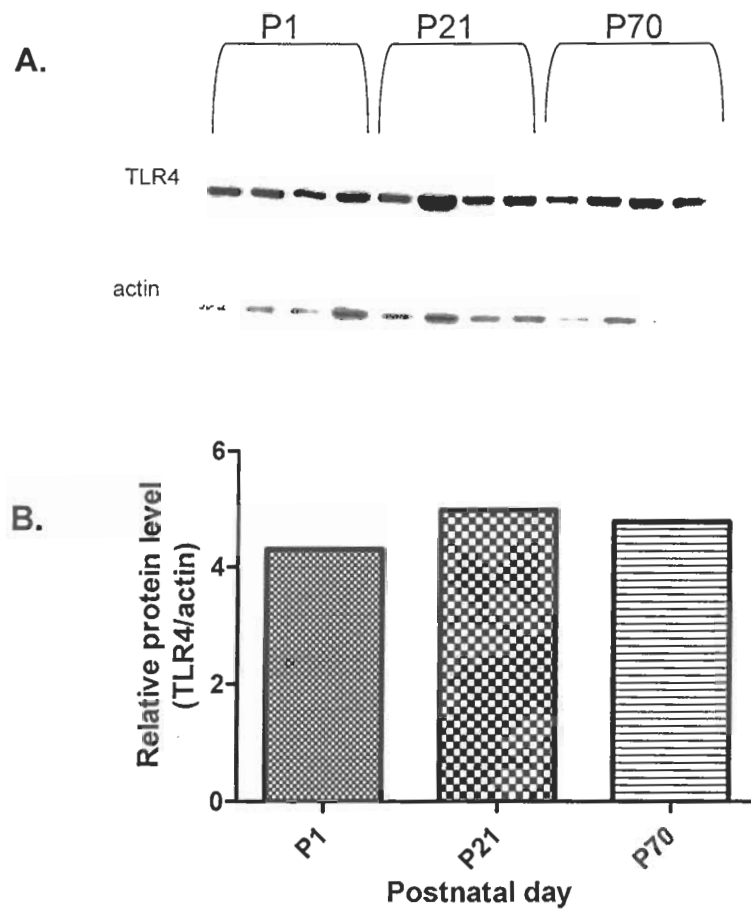


Figure 5. Age-dependent production of TLR4 in the lung of P1, P21, and P70 animals following saline stimulation. Pups were treated via i.p. injection of saline for 2 h. TLR4 levels were then determined by Western blot. (A) Western blot image of TLR4 in the lung of P1, P21, and P70 animals along with actin control (n=4). (B) Preliminary quantitation of TLR4 normalized to actin. Values represented the means of four samples in each group.

Basal levels of CD14 in P1, P21, and P70 rat lung

The basal levels of CD14 in the lung of P1, P21, and P70 were examined using Western blot analysis with a specific antibody that recognizes CD14. The basal level of CD14 in P1 lung did not seem to be significantly different from that in the lung of P21 and P70 animals (Figure 6).

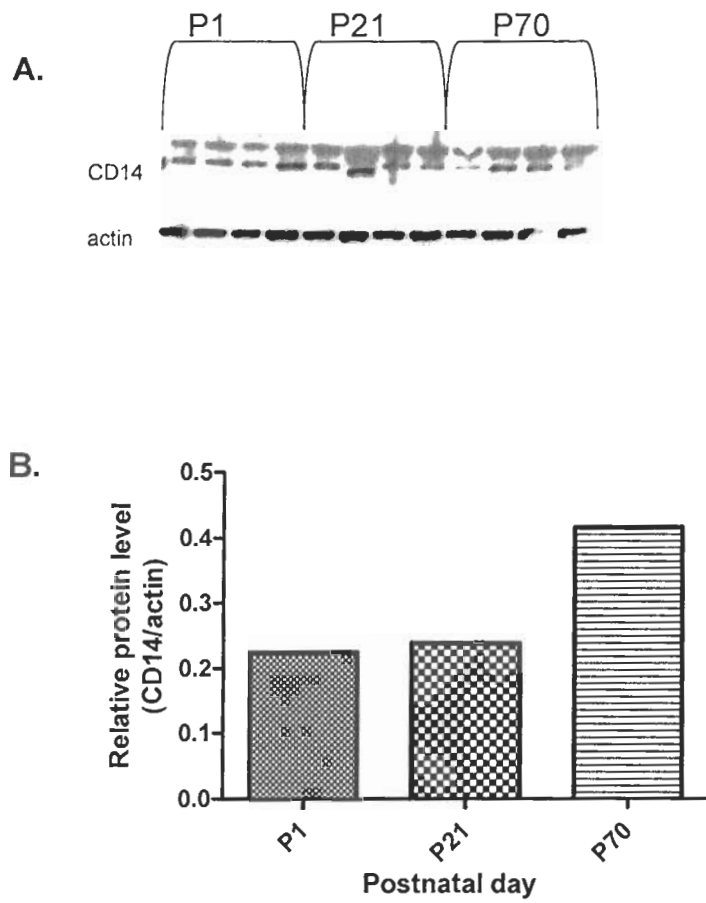


Figure 6. Age-dependent production of CD14 in the lung of P1, P21, and P70 animals following saline treatment. Pups were treated via i.p. injection of saline for 2 h. CD14 level was then determined by western blot. (A) Western blot image of CD14 in the lung of P1, P21 and P70 animals along with actin control (n=4). (B) Preliminary quantitation of CD14 normalized to actin. Values represented the means of four samples in each group.

Basal level of p42/44 MAPK in P1, P21, and P70 rat liver

The basal levels of phosphorylated and total p42/44 MAPK in the liver of P1, P21, and P70 were examined using Western blot analysis with specific antibodies that recognize p-p42/44 and total p42/44 respectively.

The basal levels of phosphorylated p42 and phosphorylated p44 MAPK appeared to be lower in the liver of P1 rats when compared to their levels in the liver of P21 and P70 rats (Fig 7), yet the basal levels of total p42 and total p44 MAPK did not seem to be different in the liver of P1, P21, and P70 rats by preliminary quantitation.

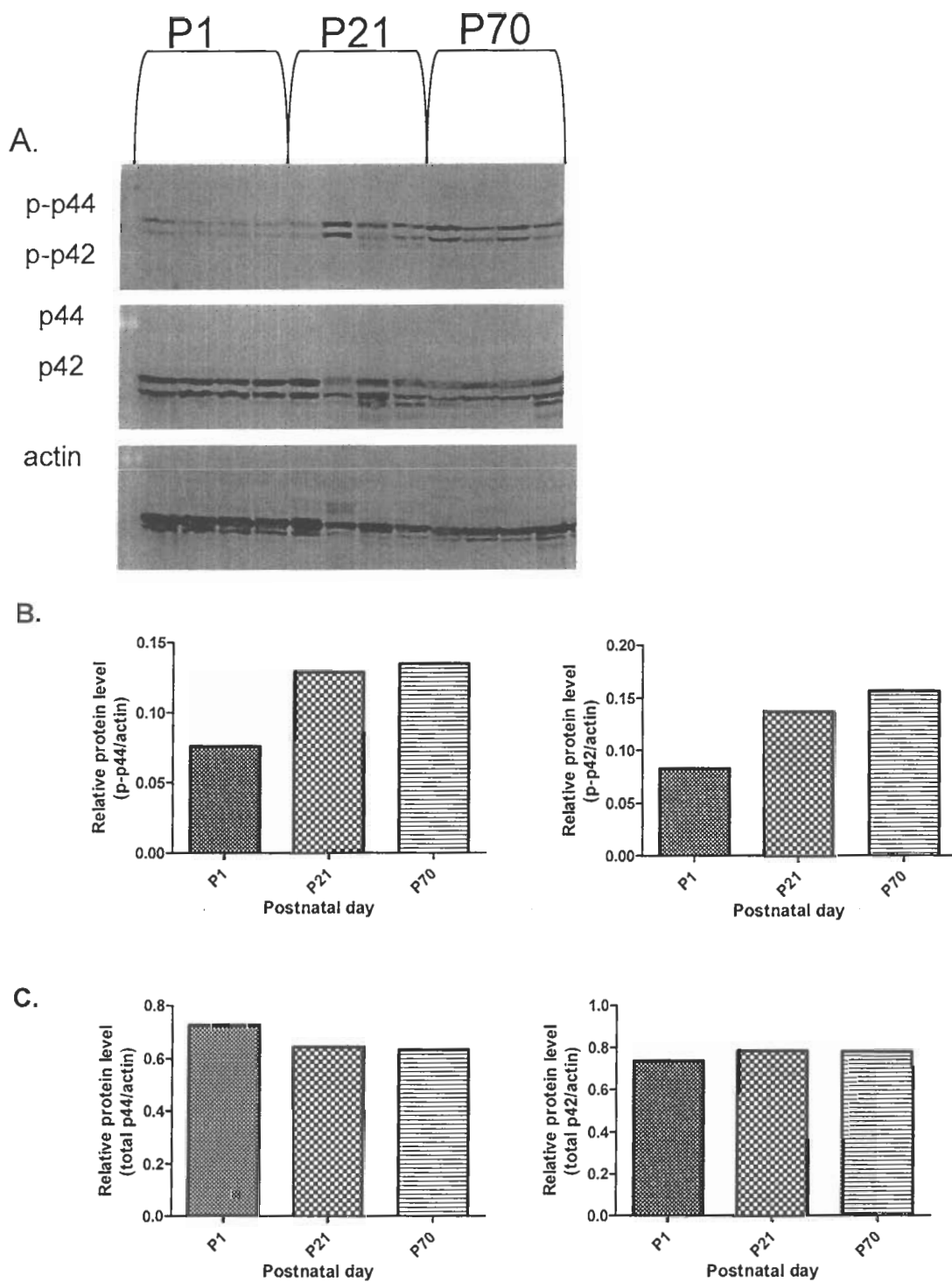


Figure 7. Age-dependent basal expression of p-p42/44 MAPK and total p42/44 MAPK in the liver of P1, P21, and P70 animals following saline treatment. Pups were treated via i.p. injection of saline for 2 h. Phosphorylated and total p42/44 MAPK levels were then determined by western blot. (A) Western blot image of p-p42/44 MAPK and total p42/44 MAPK in the liver of P1, P21, and P70 animals along with actin control (n=4). (B and C) Preliminary quantitation of p-p42/44 and total p42/44 MAPK normalized to actin. Values represented the means of four samples in each group.

Basal level of p38 MAPK in P1, P21, and P70 rat liver

The basal levels of phosphorylated and total p38 MAPK in the liver of P1, P21, and P70 were examined using Western blot analysis with specific antibodies that recognize p-p38 and total p38 respectively.

The basal levels of phosphorylated p38 MAPK did not appear to differ in the liver of P1, P21, or P70 rats by preliminary quantitation of p-p38/actin (Fig. 8). The basal levels of total p38 MAPK did not appear to differ in the liver of P1, P21, or P70 rats by preliminary quantitation of p38/actin either (Fig. 8)

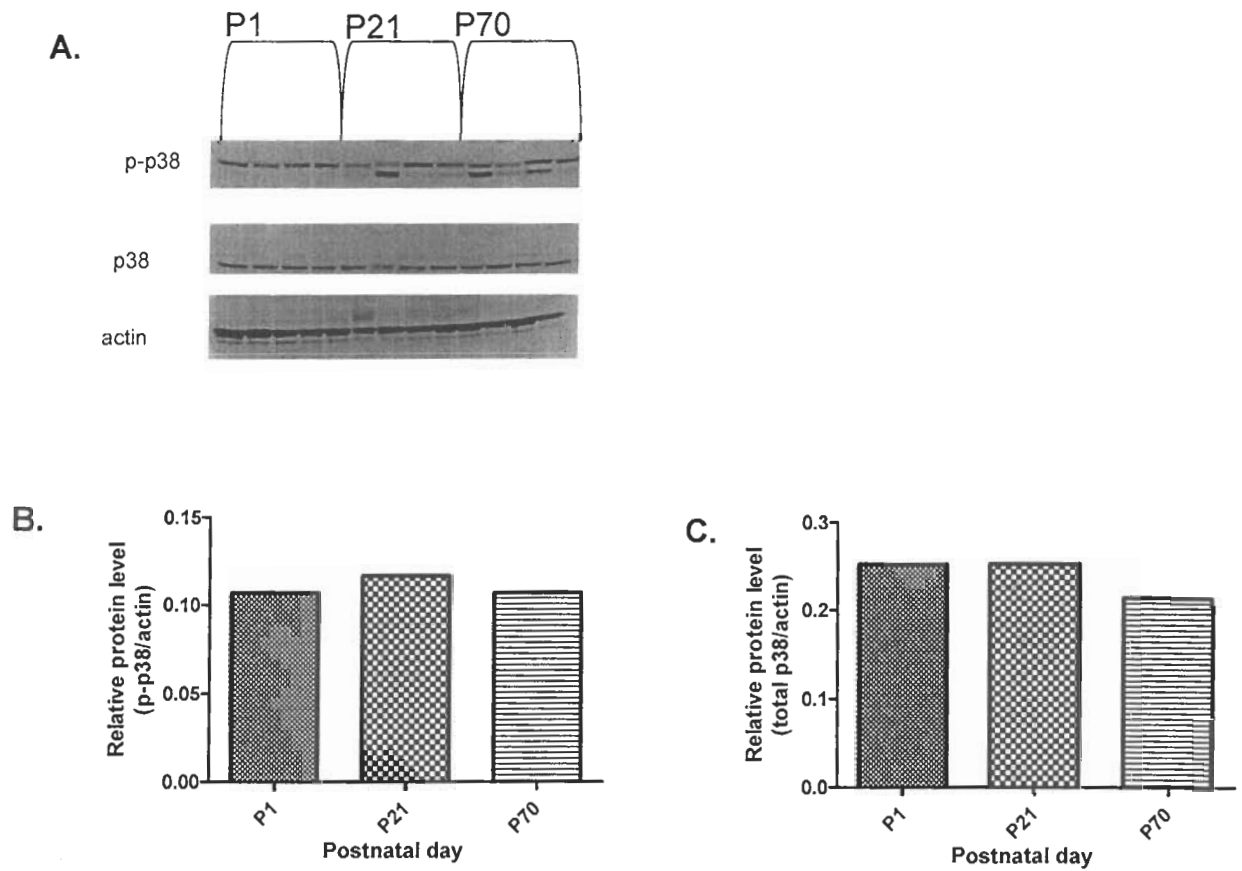
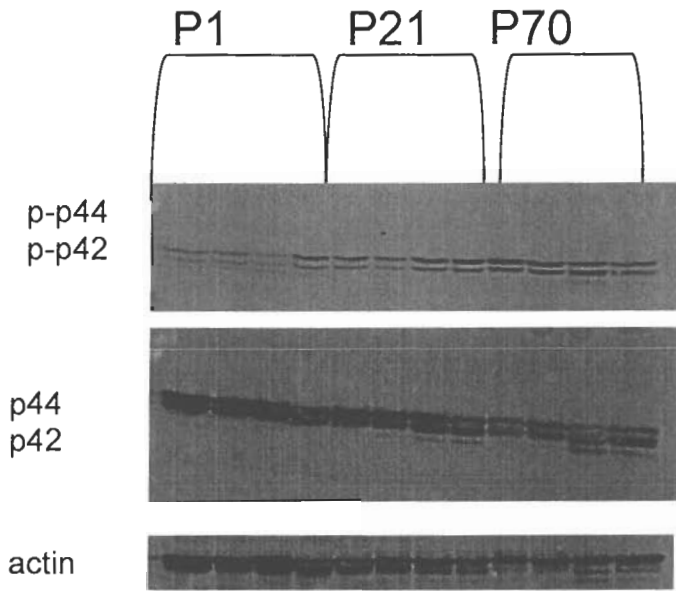


Figure 8. Age-dependent basal activation of total and phosphorylated p38 MAPK in the liver of P1, P21, and P70 animals following saline treatment. Pups were treated via i.p. injection of saline for 2 h. Phosphorylated and total p38 MAPK levels were then determined by Western blot. (A) Western blot image of total and p-p38 MAPK in the liver of P1, P21, and P70 animals along with actin control (n=4). (B and C) Preliminary quantitation of p-p38 and total p38 MAPK normalized to actin. Values represented the means of four samples in each group.

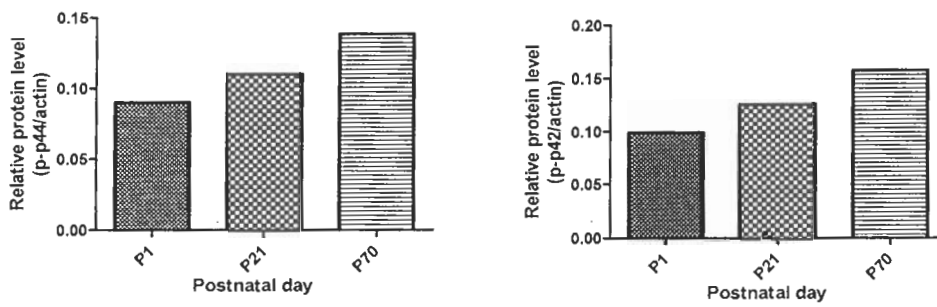
Basal level of p42/44 MAPK in P1, P21, and P70 rat lung

The basal levels of phosphorylated and total p42/44 MAPK in the lung of P1, P21, and P70 were examined using Western blot analysis with specific antibodies that recognize both p-p42/44 and total p42/44 respectively. The basal levels of phosphorylated p42 and phosphorylated p44 MAPK appeared to be lower in the lung of P1 rats when compared to their basal levels in the lung of P21 and P70 rats, yet the basal levels of total p42/44 MAPK did not seem to be altered in the lung of P1, P21, or P70 rats by preliminary quantitation (Fig 9).

A.



B.



C.

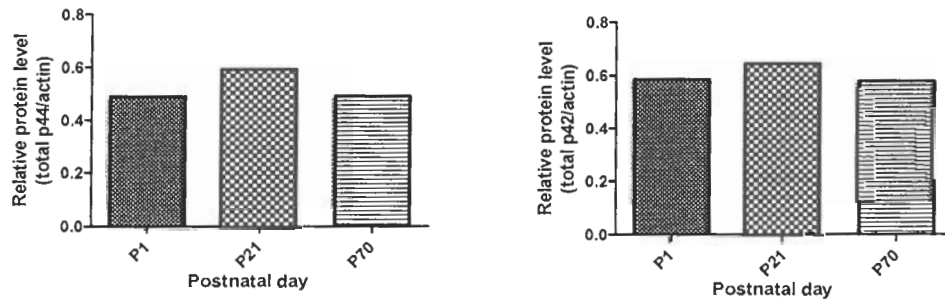


Figure 9. Age-dependent basal activation of total and phosphorylated p42/44 MAPK in the lung of P1, P21, and P70 animals following saline stimulation. Pups were treated via i.p. injection of saline for 2 h. Phosphorylated and total p42/44 MAPK levels were then determined by Western blot. (A) Western blot image of total and p-p42/44 MAPK in the lung of P1, P21, and P70 animals along with actin control (n=4). (B and C) Preliminary quantitation of p-p42/44 and total p42/44 MAPK normalized to actin. Values represented the means of four samples in each group.

Basal level of p38 MAPK in P1, P21, and P70 rat lung

The basal levels of phosphorylated and total p38 MAPK in the lung of P1, P21, and P70 were examined. Western blot analysis was used to look at phosphorylated and total p38 MAPK levels by probing with specific antibodies that recognize p-p38 and total p38 respectively.

The basal levels of phosphorylated p38 MAPK did not appear to differ in the lung of P1, P21, or P70 rats by preliminary quantitation of p-p38/actin (Fig 10). Also, the basal levels of total p38 MAPK did not appear to differ in the lung of P1, P21, or P70 rats by preliminary quantitation of p38/actin.

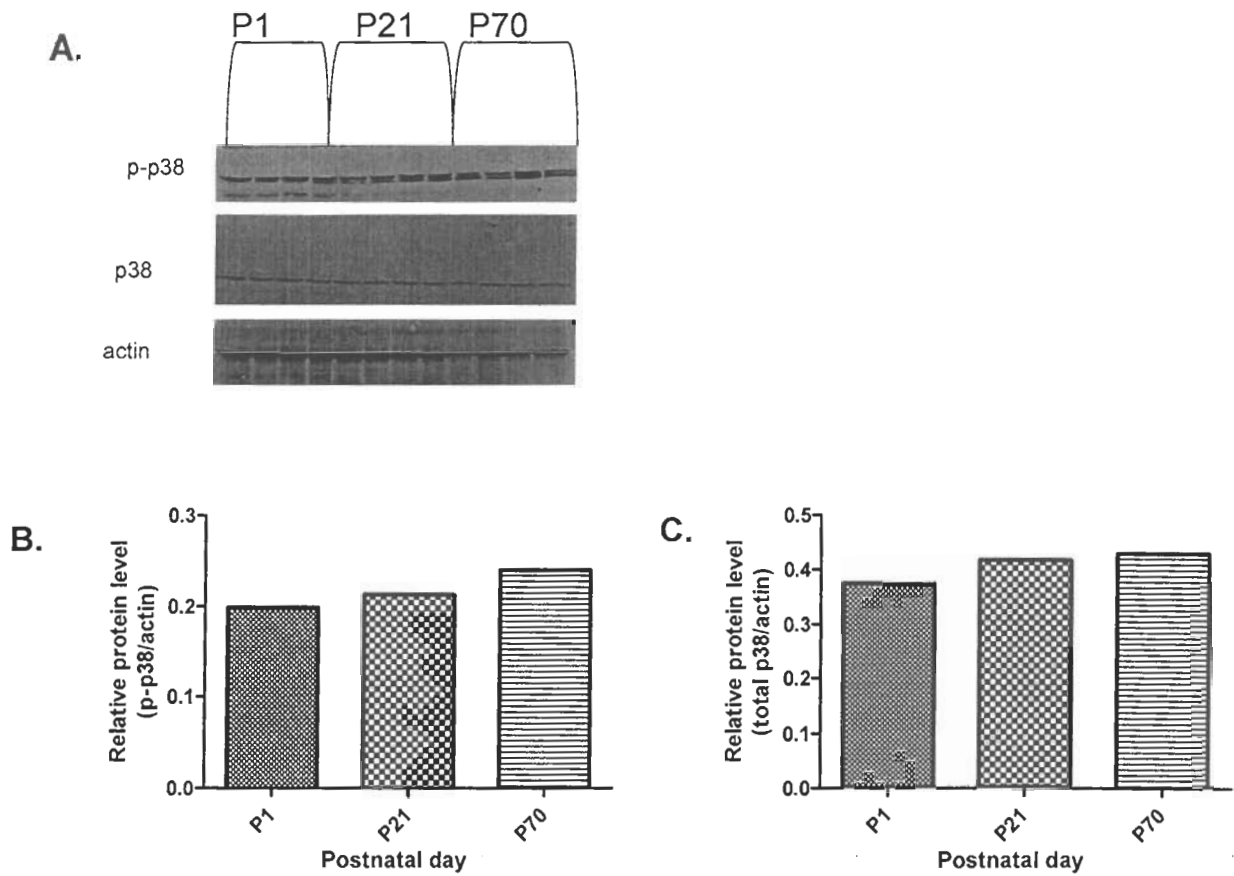


Figure 10. Age-dependent basal activation of total and phosphorylated p38 MAPK in the lung of P1, P21, and P70 animals following saline stimulation. Pups were treated via i.p. injection of saline for 2 h. Phosphorylated and total p38 MAPK levels were then determined by Western blot. (A) Western blot image of total and p-p38 MAPK in the lung of P1, P21, and P70 animals along with actin control (n=4). (B and C) Preliminary quantitation of p-p38 and total p38 MAPK normalized to actin. Values represented the means of four samples in each group.

Discussion

Antibody array measurement and ELISA studies showed that P1 animals exhibited lower levels of inflammatory cytokines in the serum following LPS treatment than P21 and P70 animals further supporting the hypothesis that the immune response undergoes significant development during early postnatal period. This study also explored the protein levels of CD14, TLR4, and MAPKs in the liver and lung during postnatal development in P1, P21, and P70 animals. Western blot results showed that the basal levels of both P-p42/44 MAPK and CD14 seemed to be lower in the liver of P1 rats than P21 and P70, suggesting that p42/44 MAPK and CD14 may play a key role in the postnatal development of hepatic innate immune function. In contrast, the basal level of CD14 in the lung of P1 animals did not seem to be significantly different from that in the lung of P21 and P70 animals even though the basal levels of p42/44 MAPK in the lung of P1 animals seemed to be lower than that of P21 and P70 animals. These studies suggest that CD14 expression is differentially regulated in the lung and liver during postnatal development.

A recent study has shown that upon LPS stimulation toll like receptor genes could be upregulated by both p38 and p42/44 MAPK signaling pathways in mouse macrophages (An et. al., 2002). It has been previously shown that Toll-like receptors are important proteins involved in the recognition of bacterial products such as LPS by immune cells (Poltorak et. al., 1998; Aliprantis et. al., 1999; Underhill et. al., 1999). This could be suggestive that macrophages with an increased expression of TLRs might be able to respond to invading bacteria more quickly and possibly enhance the immune response by the release of immunomodulatory cytokines than those with decreased TLR expression (An et. al., 2002). Furthermore, it has been shown that not only is the

activation of p42/44 MAPK crucial to the release of cytokines via the TLR4 signaling pathway in nasopharynx epithelial cells but also the expression of both TLR4 and CD14 has been shown to be important modulators for cytokine expression (Yang et. al., 2006). Additionally, previous studies have shown that leptin, a proinflammatory cytokine, is able to increase TNF- α production by activating the p38 MAPK pathway in LPS stimulated kupffer cells (Shen et. al., 2005). It has also been reported that IL-6 is dependent on p42/44 activation (Yang et. al., 2006) and TNF- α expression was increased following p38 MAPK activation. (Shen et. al., 2005). Taken together, these studies suggest that different cytokines are regulated by different MAPK signaling pathways, and that the decreased levels of p-p42/44 in the liver and lung of P1 animals may contribute to decreased innate immune function in neonatal liver and lung.

Future studies could be done to look at the activation of transcription factors involved in the TLR4 signaling pathway. The transcription factors involved in TLR4 signaling pathway that would be of interest include AP-1, NF- κ B, and IRF5 (Lu et. al., 2008). In addition, it would also be interesting to look at whether inhibiting single TLRs such as TLR4 could block the inflammatory response while maintaining host defenses (O'Neill, 2006).

In summary, we showed that P1 animals exhibit lower levels of inflammatory cytokines in the serum following LPS treatment than P21 and P70 animals, suggesting that neonates are relatively immunodeficient. We also examined the expression of hepatic and pulmonary immune mediators in the TLR4 signaling pathway. Our results showed that the basal protein levels of both P-p42/44 MAPK and CD14 seemed to be lower in the liver of P1 rats than P21 and P70, yet the basal protein levels of both P-p38

MAPK and TLR4 did not seem to be altered in the liver of P1, P21, and P70 rats. In the lung, the basal levels of P-p42/44 MAPK seem to be lower in P1 rats when compared to that of P21 and P70, yet the basal levels of P-p38 MAPK, TLR4, and CD14 did not appear to be altered in the lung of P1, P21, and P70 rats. These data suggest that while the lower basal level of p42/44 MAPKs in both lung and liver correlated with the immune deficiencies in the neonates, and that CD14 expression may be differentially regulated in the lung and liver during postnatal development.

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