

## Full-length article

## Upregulation of TNF- $\alpha$ and IL-6 mRNA in mouse liver induced by bacille Calmette-Guerin plus lipopolysaccharide

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### Key words

liver injury; lipopolysaccharide binding protein; CD14; tumor necrosis factor- $\alpha$ ; interleukin-6; NF- $\kappa$ B

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### Abstract

**Aim:** To investigate the mechanism of immunological liver injury induced by bacille Calmette-Guerin (BCG) plus lipopolysaccharide (LPS). **Methods:** Mice were injected via the tail vein with 125 mg/kg BCG, and 12 d later, the mice were injected intravenously with different doses of LPS (125, 250, or 375  $\mu$ g/kg). Serum alanine aminotransferase (ALT) activity and liver pathological changes were examined. The expression of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, lipopolysaccharide binding protein (LBP) and CD14 mRNA, and NF- $\kappa$ B and I $\kappa$ B- $\alpha$  protein in mouse liver at different time points after BCG and LPS injection were measured using RT-PCR, immunohistochemistry and Western blotting analysis, respectively. **Results:** The activity of serum ALT in mice treated with BCG and LPS was significantly increased. Different degrees of liver injury, such as inflammatory cell infiltration, spotty necrosis, piecemeal necrosis, even bridging necrosis, could be seen in liver sections from mice after BCG and LPS administration. Furthermore, the levels of TNF- $\alpha$  and IL-6 mRNA in mouse liver were significantly elevated after administration of BCG plus LPS ( $P < 0.05$ ). The levels of LBP and CD14 mRNA in mouse liver were markedly upregulated after treatment with BCG and LPS, and treatment with BCG alone led to an increase in CD14 mRNA in mouse liver. Finally, immunoreactivity for NF- $\kappa$ B p65 was predominantly detected in hepatocyte nuclei from mice treated with BCG plus LPS, compared with the normal group. Protein levels of I $\kappa$ B- $\alpha$  were strikingly decreased by LPS or BCG plus LPS treatment, compared with the normal group or BCG group. **Conclusion:** TNF- $\alpha$  and IL-6 mRNA were partially involved in early immunological liver injury induced by challenge with small doses of LPS after BCG priming. Upregulation of TNF- $\alpha$  and IL-6 mRNA might be related to increases in LBP and CD14 mRNA expression and activation of NF- $\kappa$ B. Furthermore, BCG priming in immunological liver injury may occur via upregulation of CD14 mRNA expression in mononuclear cell infiltration into the liver.

### Introduction

Many liver injury models have been established to study the mechanisms by which injury occurs and to assist in the development of new drugs. It has been reported that severe hepatitis can be induced by injecting small doses of bacte-

rial lipopolysaccharide (LPS) into mice pretreated with bacillus Calmette-Guerin (BCG) or *Corynebacterium parvum*<sup>[1,2]</sup>. BCG priming and LPS challenge in mice causes massive liver injury, which comprises priming and eliciting phases. BCG priming induces mononuclear cell infiltration into the liver lobules and granuloma formation<sup>[3]</sup>. The subsequent LPS

injection elicits acute and massive hepatic injury, with a concomitant release of various cytokines<sup>[4]</sup>.

Inflammatory cytokines are produced after systemic or local inflammation by a wide variety of cells, including monocytes, macrophages, Kupffer cells and endothelial cells. Recent studies have shown that pro-inflammatory mediators, such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$ , are markedly elevated in serum and liver homogenates in immunological liver injury induced by BCG plus LPS<sup>[5,6]</sup>. Furthermore, elimination of Kupffer cells by gadolinium chloride<sup>[7]</sup> and TNF- $\alpha$  antibodies<sup>[8]</sup> are effective in reducing hepatic damage *in vivo*, suggesting that TNF- $\alpha$  secreted by Kupffer cells may play a major role in liver injury. In addition, IL-6 acts as one of the pivotal cytokines in host defense responses to infection and inflammation by activating lymphocytes and inducing hepatic acute phase proteins<sup>[9]</sup>. It has also been shown to play an important role in liver injury<sup>[10]</sup>.

Endotoxin exerts its effects in liver injury via interaction with lipopolysaccharide binding protein (LBP) and CD14<sup>[11]</sup>. LBP in serum can recognize and bind LPS to form LPS-LBP complexes and activate cells through the CD14 receptor on the membranes of these cells, then initiate a process leading to the release of cytokines, prostanoids, and other soluble mediators<sup>[12,13]</sup>.

However, the mechanism of immunological liver injury induced by BCG plus LPS has so far remained elusive. Therefore, the purpose of the present study is to investigate whether TNF- $\alpha$  and IL-6 are involved in immunological liver injury, and, if so, by which processes.

## Materials and methods

**Reagents** Lipopolysaccharide (*Escherichia coli* LPS; serotype 055:B5) was purchased from Sigma (USA). Mycobacterium tuberculosis bacille Calmette-Guerin (BCG) vaccine was purchased from the Institute of Shanghai Biological Products (China). The kit for determining serum alanine aminotransferase (ALT) was purchased from Shanghai Rong-Sheng Biotechnology (China).

**Animals and treatments** Male 8- to 10-week-old BALB/c mice, weighing 20–24 g, were provided by Anhui Anke Biotechnology Co. All animals were housed under standard laboratory conditions. All experiments were approved by the Ethics Review Committee for Animal Experimentation of the Institute of Clinical Pharmacology, Anhui Medical University. The animals were allowed free access to food and water at all time and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50% $\pm$ 5%) environment for 1 week before use.

**Establishment of immunological liver injury model** For experiment 1, mice were randomly divided into 6 equal groups: (1) normal control group (N): mice were injected via the tail vein with saline at the same time as the BL groups (see below); (2) BCG group (B): mice were injected with saline 12 d after injection via the tail vein with BCG (125 mg/kg); (3) LPS group (L1): mice were injected with LPS (125  $\mu$ g/kg) 12 d after injection via the tail vein with saline; (4) BCG/LPS groups (BL1, BL2, BL3): mice were injected with LPS (125, 250, or 375  $\mu$ g/kg, respectively) 12 d after injection via the tail vein with 125 mg/kg doses of BCG (approximately  $5 \times 10^7$  viable units per mouse) in saline. The mice were killed 12 h after LPS. Blood and liver samples were collected for biochemical and pathological examinations, respectively.

For experiment 2, mice were randomly divided into 6 equal groups: normal control group (N), BCG group (B), LPS group (L1), and the groups treated with BCG plus three different doses of LPS (BL1, BL2, BL3). Mice from the N, L1, BL1, BL2, and BL3 groups were treated in the same way as those in experiment 1. Mice in these groups were decapitated at 0.5, 1, 3, 6, or 12 h after LPS treatment. Mice in the B group were injected via the tail vein with a 125 mg/kg dose of BCG (approximately  $5 \times 10^7$  viable units per mouse) and decapitated at either d 11, 13, or 15 after BCG treatment (3 mice per time point). Liver samples were collected for isolation of total RNA and protein, respectively.

**Measurement of serum ALT** Serum ALT activity was measured with a commercially available kit.

**Histopathological analysis** Liver samples from the same liver lobes were fixed in 10% formalin immediately and then embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) and were examined for inflammatory cell accumulation and liver damage by light microscopy.

**Isolation of total RNA and semiquantitative RT-PCR for measurement of mRNA** Fifty milligrams of liver tissue was collected from each mouse. Total RNA was extracted using TRIzol reagent (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China), according to the manufacturer's instructions. The integrity and concentration of RNA were determined by measuring absorbance at 260 nm, after which the RNA was electrophoretically separated on agarose gels. Total RNA was stored at -80 °C. For the reverse transcription-polymerase chain reaction (RT-PCR) [One-step RNA PCR kit (AMV); TaKaRa, Japan], 1  $\mu$ g of total RNA from each sample was resuspended in a 25  $\mu$ L final volume of reaction buffer, which contained 10 $\times$  one-step RNA PCR buffer, 25 mmol/L MgCl<sub>2</sub>, 10 mmol/L dNTP mixture, 20 U RNase inhibitor, 2.5 U AMV RTase XL, 2.5 U AMV-Optimized Taq, 20  $\mu$ mol/L forward primer, 20  $\mu$ mol/L reverse

primer, and RNase-free dH<sub>2</sub>O. PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed in each individual sample as an internal control. The following primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China), according to sequences previously described by others<sup>[14,15]</sup>: GAPDH forward primer 5'-GAG GGG CCATCC ACA GTC TTC-3' and reverse primer 5'-CAT CAC CAT CTT CCA GGA GCG-3'; TNF- $\alpha$  forward primer 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3' and reverse primer 5'-ACA TTC GAG GCT CCA GTG AAT TCG G-3'; IL-6 forward primer 5'-CTG GTG ACA ACC ACG GCC TTC CCTA-3' and reverse primer 5'-ATG CTT AGG CAT AAC GCA CTA GGT T-3'; LBP forward primer 5'-GGC TCT GCA GAG AGAG GCT GTA CAA-3' and reverse primer 5'-TAG TTA AGG AAT GCC TGG AAC AGG-3'; CD14, forward primer 5'-ACA TCT TGA ACC TCC GCA AC-3' and reverse primer 5'-AGG GTT CCT ATC CAG CCT GT-3'. The sizes of the amplified PCR products were 340 bp for GAPDH, 307 bp for TNF- $\alpha$ , 600 bp for IL-6, 1147 bp for LBP and 500 bp for CD14, respectively. The number of cycles and annealing temperature were optimized for each primer pair. For GAPDH, amplification was initiated by 2 min of denaturation at 94 °C for 1 cycle, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. For TNF- $\alpha$  and IL-6, amplification was initiated by 2 min of denaturation at 94 °C for 1 cycle, followed by 28 and 35 cycles at 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min. After the last cycle of amplification, samples were incubated for 5 min at 72 °C. For LBP and CD14, amplification was initiated by 2 min of denaturation at 94 °C for 1 cycle, followed by 27 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min. After the last cycle of amplification, samples were incubated for 5 min at 72 °C. The amplified RT-PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma, USA) for 45 min. A 100 bp DNA ladder marker was used as a molecular marker. Agarose gels were stained with 0.5 mg/mL ethidium bromide (Sigma, USA) TAE buffer. The gel bands were examined by using a Tanon gel image system (China).

**Immunohistochemical analysis** Liver tissue fixed in 10% formaldehyde was prepared by paraffin embedding and cut into 4  $\mu$ m sections. Immunohistochemical staining was performed using a DAB kit (Beijing Zhongshan Golden Bridge Biotechnology, China). Briefly, tissue sections were deparaffinized in xylene and rehydrated through a graded alcohol series to water. Endogenous peroxidase activity was quenched with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 5 min. Slides were incubated with a 1:300 dilution of anti-NF- $\kappa$ Bp65 antibodies (Santa Cruz, USA) overnight at 4 °C. Antibody binding was localized by incubation with goat anti-rabbit IgG antibody-

HRP (Power Rision Two-step Histostaining Reagent) for 25 min. Staining was developed with DAB. Slides were counterstained with hematoxylin, and tissue sections were mounted.

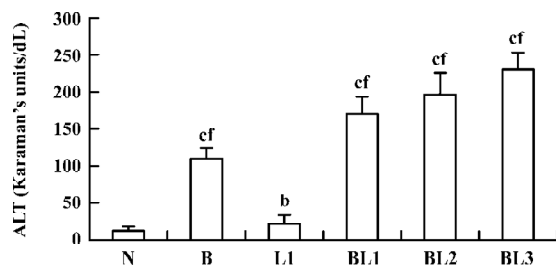
**Western blot analysis** Western blotting was carried out using the protocol for molecular cloning. Briefly, frozen tissues were lysed in lysis buffer. The lysates were centrifuged at 4000 $\times$ g, and supernatants were immediately stored at -70 °C until use. Protein concentration was determined using Lowery method, and 50  $\mu$ g of protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Non-specific binding was blocked by preincubation of the nitrocellulose in Tris-buffered saline containing 5% non-fat milk for 1 h. The nitrocellulose was incubated overnight at 4 °C with polyclonal anti-I $\kappa$ B- $\alpha$  antibodies (Santa Cruz Biotech, USA). Bound primary antibody was detected using a peroxidase-conjugated secondary antibody (Boshide, China) and enhanced chemiluminescence reagents (Pierce, USA).

**Statistical analysis** The TNF- $\alpha$ , IL-6, LBP, and CD14 mRNA levels were normalized against the GAPDH mRNA level in the same sample. Quantified data from analysis of serum ALT and the RT-PCR assay are expressed as mean $\pm$ SEM at each point. ANOVA and the Student-Neumann-Keuls *post hoc* test were used to determine the statistical significance of differences between the treated animals and the controls.

## Results

**Effect of BCG and/or LPS on the activity of serum ALT in mice** When mice were injected with BCG, LPS, or BCG plus LPS, serum ALT activity, indicating hepatic cell damage, was significantly increased compared with normal controls. BCG markedly elevated serum ALT compared with LPS. The degree of elevation of serum ALT activity was positively correlated with the dose of LPS after BCG priming (Figure 1).

**Effect of BCG and/or LPS on histological changes in mouse liver** In the normal control group, there was no pathological abnormality. Morphology of the liver parenchyma was normal and hepatocytes were arranged around the central vein. No congestion and inflammation were observed in the sinusoids (Figure 2A). In the BCG group, however, there was obvious pathological abnormality. Infiltration of the liver lobules by inflammatory cells in the form of smaller granulomas was observed in the liver sections (Figure 2B). In the BCG plus LPS group, there was still more severe pathological abnormality. Spotty necrosis, piecemeal necrosis, even bridging necrosis could be seen, and inflammatory cells were arranged around the necrotic tissue. Congestion in the

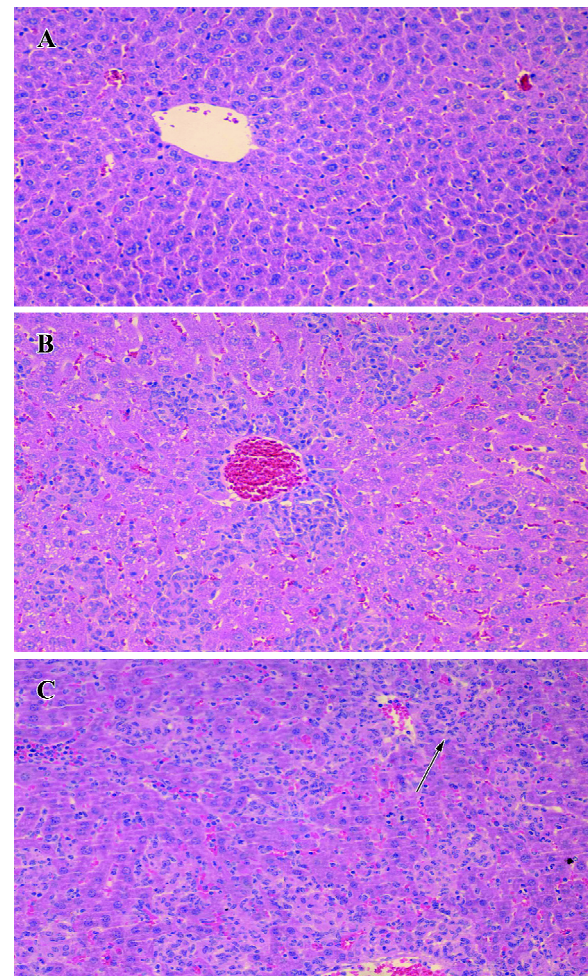


**Figure 1.** Serum ALT activity in BALB/c mice treated with BCG, LPS, or BCG plus LPS. Mice received injections of various doses of LPS (125, 250, or 375  $\mu\text{g}/\text{kg}$ ) or saline (N, B) 12 d after iv injection of BCG (125 mg/kg) or saline (N). Activities of serum ALT were determined 12 h after LPS injection. Each point represents the mean $\pm$ SD for 10 mice. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs normal control group. <sup>f</sup> $P<0.01$  vs LPS group. N, normal control group; B, BCG group; L1, LPS (125  $\mu\text{g}/\text{kg}$ ) group; BL1, BCG plus LPS (125  $\mu\text{g}/\text{kg}$ ) group; BL2, BCG plus LPS (250  $\mu\text{g}/\text{kg}$ ) group; BL3, BCG plus LPS (375  $\mu\text{g}/\text{kg}$ ) group.

liver sinusoids was significant, with scattered infiltration of inflammatory cells (Figure 2C).

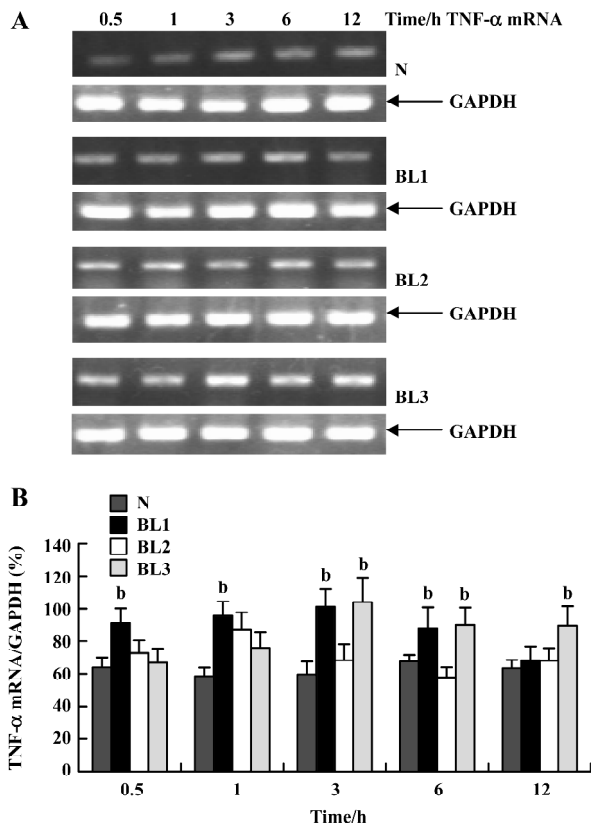
**Effect of BCG and/or LPS on TNF- $\alpha$  and IL-6 mRNA in mouse liver** To explore the mechanism of immunological liver injury induced by BCG and LPS, we examined the expression of TNF- $\alpha$  and IL-6 mRNA in mouse liver at different time points after BCG or LPS treatment. There was no significant difference in the levels of TNF- $\alpha$  or IL-6 mRNA in the BCG group and the normal control group (data not shown). However, treatment with both BCG and LPS led to significant increases in TNF- $\alpha$  and IL-6 mRNA levels, as compared with the normal control and BCG groups. For TNF- $\alpha$  mRNA, lower doses of LPS significantly increased its expression at 0.5 h ( $P<0.05$ ), which then peaked at 3 h, and decreased at 12 h after LPS administration. The medium dose of LPS significantly upregulated TNF- $\alpha$  mRNA levels at 1 h after LPS administration, whereas a higher dose of LPS significantly increased levels at 3 h ( $P<0.05$ ), which were maintained until 12 h after LPS administration (Figure 3). For IL-6 mRNA, lower doses of LPS increased its expression at 1 h, which peaked at 3 h after LPS administration, whereas in the higher and medium groups, its expression was markedly elevated at 0.5 h, also peaked at 3 h, then gradually decreased (Figure 4).

**Effect of BCG and/or LPS on LBP and CD14 mRNA in mouse liver** To determine the mechanism of elevation of TNF- $\alpha$  and IL-6 mRNA expression in mouse liver induced by BCG and/or LPS, we examined the expression of LBP and CD14 mRNA in mouse liver after BCG and/or LPS treatment. There was no significant difference in the levels of LBP mRNA between the BCG group and the normal control group



**Figure 2.** Light photomicrographs of liver tissue histology slides. BALB/c mice received injections of various doses of LPS or saline (N, B) 12 d after iv injections of BCG or saline (N). (A) Liver samples were taken from the normal control group at the same time as the BCG plus LPS group. (B) Liver samples were taken from mice treated with BCG only at the same time as the BCG plus LPS group. (C) Liver samples were taken from mice 12 h after LPS treatment (after being primed with 250  $\mu\text{g}/\text{kg}$  BCG). Hematoxylin-eosin stain.  $\times 200$ .

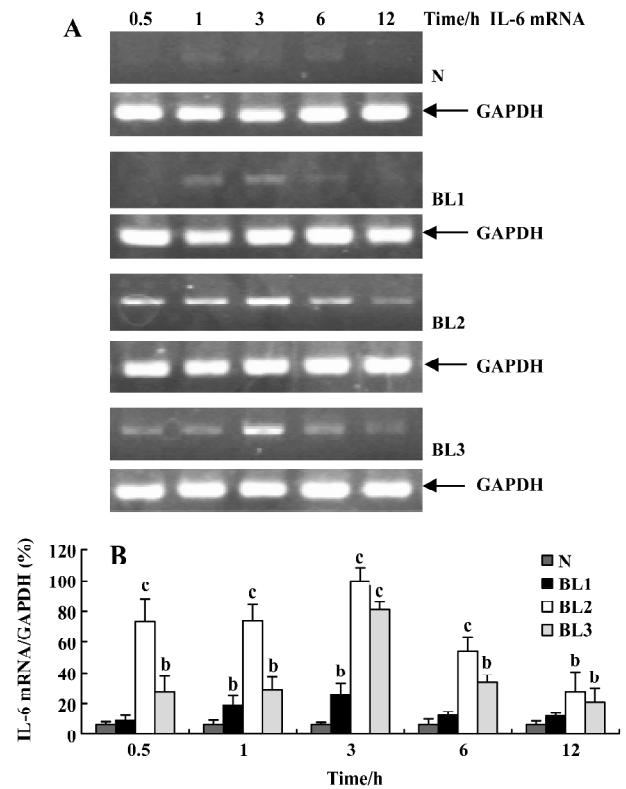
(data not shown). However, the level of CD14 mRNA was significantly increased in the BCG group compared with the normal control group (Figure 5). Furthermore, treatment with both BCG and LPS led to a marked increase in LBP mRNA levels, as compared with the normal control group ( $P<0.01$ ). Lower and medium doses of LPS significantly increased LBP mRNA levels at 1 h ( $P<0.01$ ), which then peaked at 12 h after administration of LPS. A higher dose of LPS markedly increased LBP mRNA levels at 0.5 h ( $P<0.01$ ), which peaked at 6 h (Figure 6) after administration of LPS. The expression of CD14 mRNA in mouse liver treated with BCG plus LPS in all



**Figure 3.** Expression of TNF- $\alpha$  mRNA in mouse liver after administration of LPS after BCG priming. Mice were injected intravenously with LPS (125, 250, or 375  $\mu\text{g}/\text{kg}$ ) or saline (N) 12 d after treatment with BCG (125 mg/kg) or saline (N). (A) Livers were collected at 0.5, 1, 3, 6, or 12 h after LPS injection. Total RNA extracted from 3 mouse livers was pooled for each treatment. TNF- $\alpha$  mRNA levels were determined using RT-PCR. (B) Semiquantitative analysis of TNF- $\alpha$  mRNA was performed on 3 individual mouse liver RNA samples at each point. TNF- $\alpha$  mRNA levels were normalized against the GAPDH mRNA level in the same sample. <sup>b</sup> $P < 0.05$  vs normal control group.

dose groups was significantly upregulated compared with the normal control group (Figure 7A, 7B). Administration of BCG and LPS also led to significant elevation of CD14 mRNA, compared with BCG alone or LPS alone (Figure 7C, 7D).

**Effect of BCG and/or LPS on NF- $\kappa\text{B}$  p65 and I $\kappa\text{B}$ - $\alpha$  in mouse liver** The expression and localization of NF- $\kappa\text{B}$  p65 in livers of mice were studied to study further its mechanism. Immunohistochemical studies demonstrated that hepatocyte nuclei in normal control mice were weakly immunoreactive for the p65 subunit of NF- $\kappa\text{B}$ , and that what immunoreactivity was present was localized mainly to the cytoplasm (Figure 8A). In contrast, immunoreactivity for NF- $\kappa\text{B}$  p65 was predominantly detected in hepatocyte nuclei for mice treated with BCG plus LPS (Figure 8B). It has been well documented



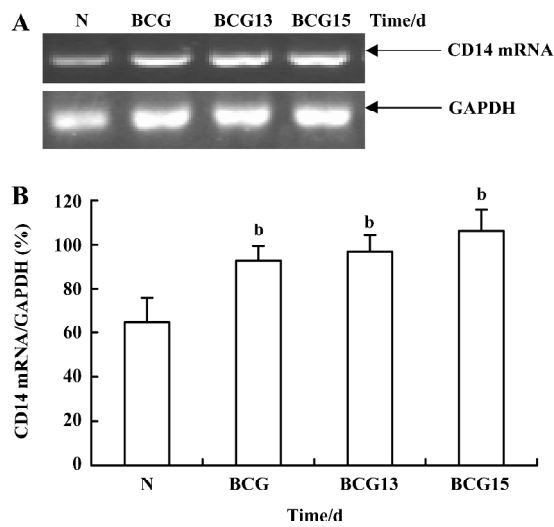
**Figure 4.** Expression of IL-6 mRNA in mouse liver after treatment with BCG plus LPS. Mice were injected intravenously with LPS (125, 250, or 375  $\mu\text{g}/\text{kg}$ ) or saline (N) 12 d after BCG (125 mg/kg) or saline (N) treatment. Livers were collected at 0.5, 1, 3, 6, or 12 h after LPS injection. (A) Total RNA extracted from 3 mouse livers was pooled for each treatment. IL-6 mRNA levels were determined using RT-PCR. (B) Semiquantitative analysis of IL-6 mRNA was performed on 3 individual mouse liver RNA samples at each point. IL-6 mRNA levels were normalized against the GAPDH mRNA level in the same sample. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs normal control group.

that activation of NF- $\kappa\text{B}$  correlates with rapid proteolytic degradation of I $\kappa\text{B}$ - $\alpha$ , so we assessed (Figure 9).

## Discussion

A major goal of the present study was to explore the mechanism of immunological liver injury induced by treatment with BCG plus LPS. Our results showed that serum ALT levels in mice treated with BCG and LPS were significantly increased and that severe pathological changes were seen in the liver when compared with the normal control group. Having determined that the liver injury model was successful, we next examined the effect of BCG and LPS on the expression of cytokines.

Many previous studies have indicated that TNF- $\alpha$  is a critical factor in immune-mediated hepatitis<sup>[16]</sup>. Endotoxemia

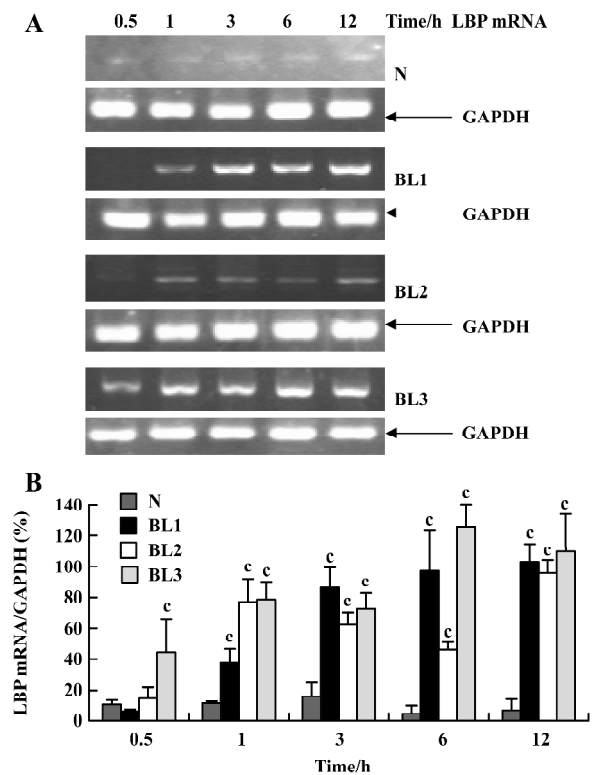


**Figure 5.** Expression of CD14 mRNA in the livers of normal control and BCG treatment mice. Mice were injected intravenously with BCG (125 mg/kg) or saline (N) once. Livers were collected at d 10, 13, or 15 after BCG treatment. Livers were excised and total RNA was extracted. Total RNA extracted from 3 mouse livers was pooled for each treatment. (A) CD14 mRNA levels were determined using RT-PCR. Semiquantitative analysis of CD14 mRNA was performed for 3 individual mouse liver RNA samples at each time point. (B) CD14 mRNA levels were normalized against the GAPDH mRNA level in the same sample. <sup>b</sup>*P*<0.05 vs normal control group.

causes liver injury in which TNF- $\alpha$  plays a significant role by inducing hepatic apoptosis<sup>[17,18]</sup>. In our present study, TNF- $\alpha$  expression in mouse liver was increased. However, the increase in TNF- $\alpha$  expression was negatively related to the dose of LPS at 0.5 h and 1 h after administration. In the medium dose group, there was no elevation of TNF- $\alpha$  expression between 3 h and 12 h after administration of LPS.

IL-6 is a multifunctional cytokine produced by many different cell types, including monocytes/macrophages, fibroblasts and vascular endothelial cells<sup>[19]</sup>. Experimental studies have demonstrated that it has a protective role against fulminating hepatic failure through inhibiting hepatocyte apoptosis<sup>[20]</sup>. Other studies have indicated that IL-6 inhibits TNF- $\alpha$  production both *in vivo* and *in vitro*<sup>[21,22]</sup>. In the present study, IL-6 mRNA expression in mouse liver after BCG plus LPS treatment was markedly upregulated, especially in the medium dose group, which may have been related to a lower level of TNF- $\alpha$  being present.

*In vitro* studies have shown that TNF- $\alpha$  produced by Kupffer cells in response to low concentrations of LPS is mediated via LBP and CD14<sup>[23]</sup>. LBP is an acute-phase protein produced predominantly by hepatocytes<sup>[24]</sup>. It has been found that LBP plays a major role in activating monocytes

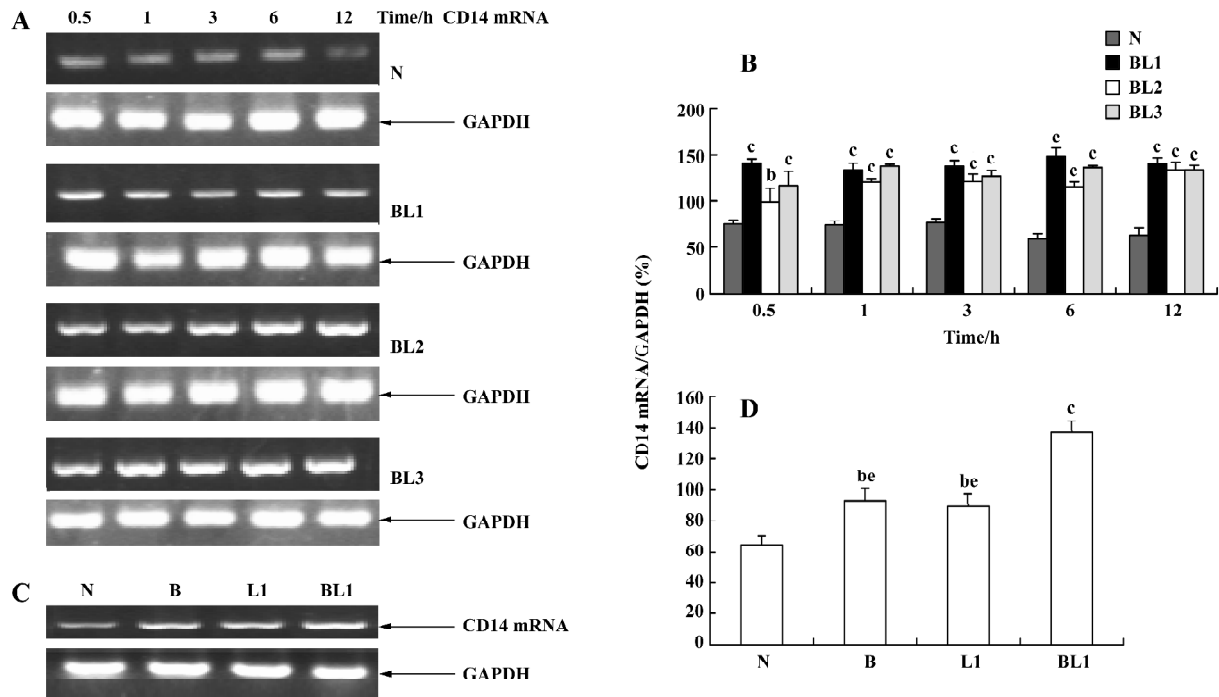


**Figure 6.** Expression of LBP mRNA in mouse liver after BCG plus LPS treatment. Mice were injected intravenously with LPS (125, 250, or 375  $\mu$ g/kg) or saline (N) 12 d after BCG (125 mg/kg) or saline (N) treatment. Livers were collected at 0.5, 1, 3, 6, or 12 h after LPS injection. Total RNA extracted from 3 mouse livers was pooled for each treatment. (A) LBP mRNA levels were determined using RT-PCR. Semiquantitative analysis of LBP mRNA was performed on 3 individual mouse liver RNA samples at each time point. (B) LBP mRNA levels were normalized against the GAPDH mRNA level in the same sample. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs normal control group.

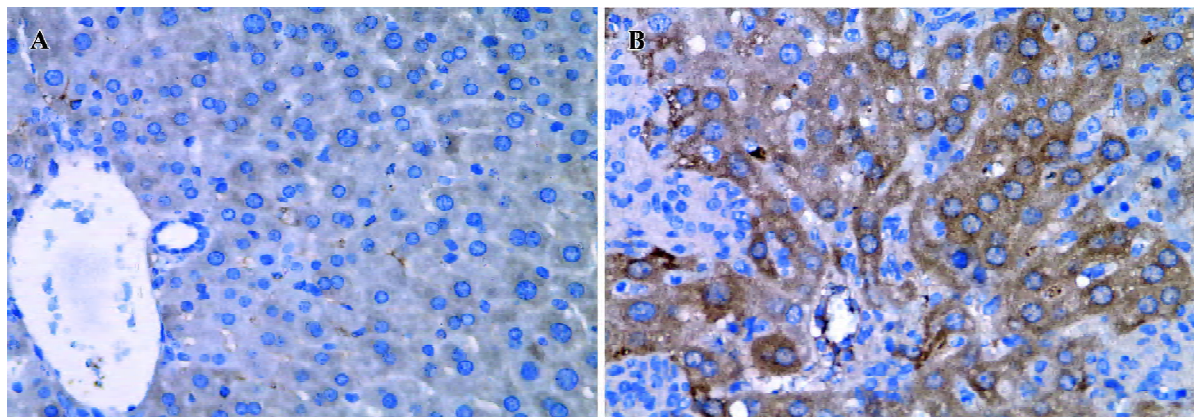
and Kupffer cells<sup>[25]</sup> and modulating liver injury<sup>[26]</sup>. Previous studies have shown that injection of LPS leads to higher LBP mRNA levels in hepatocytes<sup>[27]</sup>, as regulated by IL-1 $\beta$ , TNF- $\alpha$ , and IL-6<sup>[28]</sup>. In our present study, similar to the results of previous studies<sup>[29]</sup>, baseline levels of LBP mRNA were detectable in the normal control group. LBP mRNA levels in mouse liver after BCG plus LPS treatment were significantly increased. There were 2 peaks in both of the groups with the medium and higher doses of LPS. The first peak was at 1 h and the second at 12 h and 6 h after administration for the medium and higher dose groups, respectively.

Recent studies have shown that Kupffer cells from CD14 knockout mice produce significantly less TNF- $\alpha$  in response to the same amount of LPS, supporting a critical role for CD14 in Kupffer cell responses to LPS<sup>[23]</sup>. *In vivo* studies show that high expression levels of the CD14 gene can be





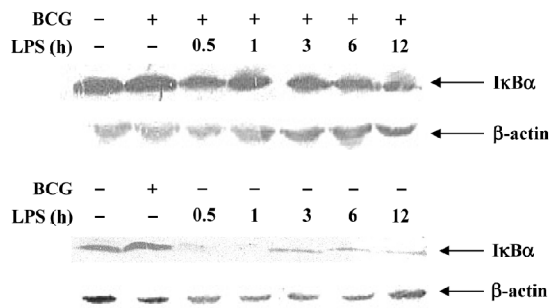
**Figure 7.** Expression of CD14 mRNA in mouse liver after BCG and LPS treatment. Mice were injected intravenously with LPS (125, 250, or 375  $\mu\text{g}/\text{kg}$ ) or saline (N, B) 12 d after BCG (125  $\text{mg}/\text{kg}$ ) or saline (N, L1) treatment. Livers were collected at 0.5, 1, 3, 6, or 12 h after LPS injection. Total RNA extracted from 3 mouse livers was pooled for each treatment. (A) CD14 mRNA levels were determined using RT-PCR. Semiquantitative analysis of CD14 mRNA was performed for 3 individual mouse liver RNA samples at each time point. (B) CD14 mRNA levels were normalized against the GAPDH mRNA level in the same sample. Livers were collected 0.5 h after LPS administration. Total RNA extracted from 3 mouse livers was pooled for each treatment. (C) CD14 mRNA levels were determined using RT-PCR. Semiquantitative analysis of CD14 mRNA was performed on 3 individual mouse liver RNA samples at each point. (D) CD14 mRNA levels were normalized against the GAPDH mRNA level in the same sample. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs normal control group. <sup>e</sup> $P < 0.05$  vs BL1 group.



**Figure 8.** Expression and location of NF- $\kappa$ B p65 in mouse liver after BCG and LPS treatment, as detected by immunohistochemical methods ( $\times 400$ ). (A) Normal mouse liver; (B) Injured mouse liver (induced with BCG plus LPS).

induced posthepatectomy<sup>[30]</sup>. *In vitro*, upregulation of CD14 expression in Kupffer cells can be induced by LPS<sup>[31]</sup>. Consistent with the results of previous studies, our results show that administration of BCG and LPS leads to upregulation of

CD14 mRNA in mouse liver. In the medium dose group, the increase in CD14 mRNA was lower than that observed in the other groups. Interestingly, the mRNA levels 3 h after BCG plus LPS administration in the medium dose group were



**Figure 9.** IkB- $\alpha$  expression levels as detected by Western blotting. Mice were injected intravenously with LPS (125  $\mu$ g/kg) or saline (N, B) 12 d after BCG (125 mg/kg) or saline (N, LPS groups). Livers were collected at 0.5, 1, 3, 6, or 12 h after LPS injection.

notable as follows: (1) TNF- $\alpha$ , LBP, and CD14 mRNA levels were lower; (2) IL-6 mRNA levels were the highest of all groups. Production of TNF- $\alpha$  is known to be mediated via LBP and CD14. IL-6 can inhibit TNF- $\alpha$ . Therefore, the lower level of TNF- $\alpha$  mRNA in mouse liver may be related to the lower expression of LBP and CD14 mRNA and the higher level of IL-6 mRNA. More importantly, we found that there was significant upregulation of CD14 mRNA expression in mice treated with BCG alone compared with normal controls. This suggests that BCG alone may induce an increase in CD14 mRNA in mouse liver 10 d later, a value chosen according to the findings of previous studies<sup>[1,4,5]</sup>. Accordingly, we presume that the BCG infection set up granulomatous hepatitis, with the LPS dosing leading to activation of *in situ* macrophages, and subsequent inflammation with disruption of the granulomas.

In summary, our results indicate that BCG and LPS administration leads to a significant increase in IL-6 mRNA levels in mouse liver. These may be related to an increase in LBP and CD14 mRNA expression and activation of NF- $\kappa$ B. In addition, BCG may induce mononuclear cell infiltration into the liver, and those cells may express CD14 mRNA, resulting in upregulation of CD14 mRNA in mouse liver.

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