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Excessive Inflammation but Decreased Immunological Response Renders Liver Susceptible to Infection in Bile Duct Ligated Mice[1]

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Background/Aims. **Obstructive jaundice (OJ) is associated with increased surgical morbidity and infectious complication. The aim of the current study was to clarify the mechanism of excessive inflammation and susceptibility to infection in OJ.**

Methods. **C57/BL6 mice were subjected to bile duct ligation (BDL) or sham surgery. Expression tumor necrosis factor-alpha, macrophage inflammatory protein-2, monocyte chemoattractant protein-1, inducible protein-10, and interleukin (IL)-10, activation of nuclear factor kappa B, fluorescence activated cell sorter analysis, serum alanine aminotransferase levels, and histology were examined. Survival after lipopolysaccharide (LPS) administration or cecal ligation and puncture 3 or 14 d after surgery was determined. IL-1β and interferon-gamma expression was examined after LPS administration.**

Results. **OJ induced nuclear factor kappa B activation and increased expression of macrophage inflammatory protein-2, which caused significant increases in neutrophil recruitment. Serum alanine aminotransferase levels increased consistent with histological observations in OJ. Mononuclear cells were recruited in the liver after BDL associated with monocyte chemoattractant protein-1 up-regulation. The recruitment of NK and T cells was varied, consistent with IP-10 expression during the time course of OJ. IL-10 expression was significantly up-regulated 14 d after BDL. After LPS administration, the mice at 3 d after BDL and at 3 and 14 d after sham surgery were all still alive, but all mice at 14 d after BDL died. After LPS administration, IL-1β significantly increased in the mice at 14 d after BDL.**

Conclusions. **Immune response such as expression of pro- and anti-inflammatory mediators and recruitment of immune cells may thus differ over the time course of OJ. Prolonged OJ may cause excessive inflammation, thus result in susceptibility to infection.**

Key Words: **endotoxemia; neutrophil; transcriptional factor; inflammation; immune paralysis**

INTRODUCTION

Cholestasis caused by biliary obstruction (OJ) due to cholangiocarcinoma, gallbladder carcinoma, gallstone, and primary sclerosing cholangitis is a high risk factor for surgery and infection, which is associated with complications such as renal insufficiency, liver failure, and multiorgan dysfunction syndrome [1, 2]. Clinical and experimental studies have identified some of the important mediators of the pathogenesis of hepatic injury in OJ. In addition, the chemotactic activity of neutrophils, superoxide production, expression of adhesion molecules, sinusoidal endothelial cell injury, and apoptosis play a pivotal role in organ dysfunction in OJ [3–5].

In the short period of OJ, Kupffer cells are activated, which produce proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and increase phagocytic activity [6]. Neutrophil-chemoattractant CXC chemokines (ELR positive) are subsequently produced, which are associated with recruitment of neutrophils. It is unclear whether mononuclear cells such as monocytes/macrophages and lymphocytes are recruited to the inflammatory sites in OJ. CC chemokine

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such as monocyte chemoattractant protein-1 (MCP-1) accumulates macrophage/monocytes, eosinophils, and basophils [7]. ELR negative CXC chemokine, such as interferon-gamma inducible protein-10 (IP-10), is known to attract NK and T cells [8, 9]. NK and T cells produce interferon-gamma (IFN-γ) in Th1 immune response [10], and IFN-γ subsequently activates function of macrophage/monocytes [11]. In accordance with up-regulations of these proinflammatory mediators, anti-inflammatory cytokines such as interleukin-10 (IL-10) and IL-4 are known to be produced by monocytes/macrophages and lymphocytes [12]. These anti-inflammatory cytokines, in general, are up-regulated in response to inflammation, but excessive expression of these cytokines may lead to immunosuppression. We hypothesized that the expression of chemokines causes accumulation of inflammatory cells such as neutrophils, monocytes/macrophages, and lymphocytes, resulting in hepatic injury in OJ. In addition, the excessive production of anti-inflammatory cytokine in response to inflammation may cause immunosuppression in OJ.

In the present study, we investigated the expression of CXC-, CC-chemokine, and anti-inflammatory cytokine, and the accumulation of inflammatory cells including mononuclear cells by histology and fluorescence activated cell sorter (FACS) analysis during OJ. In addition, we examined the survival after administration of lipopolysaccharide (LPS) or a cecal ligation and puncture (CLP) to determine whether or not OJ causes susceptibility to infection.

MATERIALS AND METHODS

Bile Duct Ligation and LPS Administration Model

Male C57BL/6 mice (Japan SLC, Inc., Hamamatsu, Japan) weighing 20 to 25 g were used in all experiments. This project was approved by Chiba University Animal Care and Use committee and was in compliance with the National Institutes of Health Guidelines. The cholestatic mouse model was used; mice were anesthetized with sodium pentobarbital (60 mg/kg administered intraperitoneally) and the common bile duct was isolated. The common bile duct was ligated at three sites and divided between the proximal double and the distal single ligation. Sham-operated mice underwent the same procedure without bile duct ligation and division (BDL). In addition, mice were anesthetized with sodium pentobarbital, and jaundiced or sham-operated mice were subjected to LPS administration (4 mg/kg *Escherichia coli*; Sigma Aldrich, St. Louis, MO) 3 or 14 d after sham surgery or BDL. In the final experiment, mice were anesthetized with sodium pentobarbital, and jaundiced or sham-operated mice were subjected to a CLP 3 or 14 d after either sham surgery or BDL. To make the CLP model, an incision to the lower quadrant of the abdomen was made, and the cecum was drawn out. The distal one-third was ligated with silk, and two punctures were made on the ligated cecum with a 23-gauge needle. Survival after LPS administration or CLP was examined. In all experiments, mice were sacrificed after the indicated periods, and liver tissue and blood samples were taken for analysis.

Myeloperoxidase Assay

Liver myeloperoxidase (MPO) content was assessed by methods described elsewhere [13]. Briefly, liver tissue (50 mg) was homogenized in 2 mL of homogenization buffer (3.4 mM KH_2HPO_4, 16 mM Na_2HPO_4, pH 7.4). After centrifugation for 20 min at 10,000 $\times$ g, 10 volumes of resuspension buffer (43.2 mM KH_2HPO_4, 6.5 mM Na_2HPO_4, 10 mM ethylenediamine tetraacetic acid, 0.5% hexadecyltrimethylammonium, pH 6.0) was added to the pellet and the samples were sonicated for 10 s. After heating for 2 h at 60°C, the supernatant was reacted with 3,3′, 3,5′-tetramethylbenzidine (Sigma Aldrich, St. Louis, MO) and read at 655 nm.

Blood and Tissue Analysis

Blood was obtained by cardiac puncture at the time of sacrifice. Serum samples were analyzed for alanine aminotransferase (ALT) as indices of hepatocellular injury and serum total bilirubin (T-BIL) concentrations, using a diagnostic kit (WAKO Pure Chemical Industries, Osaka, Japan). Quantitative assessment of MCP-1, IP-10, macrophage inflammatory protein-2 (MIP-2), IL-1 β, IL-4, and IL-10 production in the liver was made as described elsewhere [14]. Briefly, liver tissue was homogenized in 10 volumes of homogenization buffer (10 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 0.1 mg/mL soybean trypsin inhibitor, 1.0 mg/mL bovine serum albumin, 0.02% sodium azide, and 0.2 μl/mL protease inhibitor cocktail [1000 $\times$ stock; 1 mg/mL leupeptin, 1 mg/mL aprotinin, and 1 mg/mL pepstatin]). After incubating for 2 h at 4°C, the homogenate was centrifuged at 12,500 g for 10 min. The supernatant was removed and centrifuged again to obtain a clear lysate. Total protein concentration of each sample was measured using a bicinchoninic assay kit (Pierce Chemical Co. Rockford, IL), and samples were dispensed for enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R and D Systems, Inc., Minneapolis, MN, BioSource International, Camarillo, CA). Concentration was calculated per mg total protein.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts of liver tissue were prepared by the method of Deryckere and Gannon [15]. Protein concentrations were determined using the bicinchoninic acid assay with trichloroacetic acid precipitation using bovine serum albumin as a reference standard (Pierce, Rockford, IL). Double-stranded NF-κB consensus oligonucleotide (5′-AGTGAGGGGACTTTCCCAGGC-3′; Promega, Madison, WI) was end-labeled with [γ-^{32}P] ATP (3,000 Ci/mmol at 10 mCi/mL, Amersham, Arlington Heights, IL). Binding reactions containing equal amounts of protein (20 μg) and 35 fmol (~50,000 cpm, Cherenkov counting) of oligonucleotide were performed for 30 min in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM ethylenediamine tetraacetic acid, pH 8.0, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris, pH 7.6, 50 μg/mL poly(Di·dC); Pharmacia, Piscataway, NJ). The reaction volumes were held constant at 15 μL. Reaction products were separated in a 4% polyacrylamide gel and analyzed by autoradiography.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the liver tissue using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA), and single-stranded cDNA was synthesized using the Ready To Go T-primed First-Strand Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The mRNA expressions were determined by quantitative real time PCR using a LightCycler (Roche Diagnostics Ltd., Lewes, United Kingdom). Gene expressions were determined in a final volume of 20 μL using 1 μL of each primer, 0.8 μL $MgCl_2$ (25 mM), 2 μL of the supplied enzyme mix and 10.2 μl H_2O. The enzyme mix contained FastStart Taq DNA polymerase, reaction buffer, dNTP mix, 10 mM $MgCl_2$ and SYBR Green I dye for detection of PCR products. PCR was performed with

TABLE 1

Primer Sequence Used for Quantitative Real-Time PCR Analysis

Gene		Primer sequence
β-actin	Sense	5′-GTGGGCCGCTCTAGGCACCA-3′
	Antisense	5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′
MIP-2	Sense	5′-GAACAAAGGCAAGGCTAACTGA-3′
	Antisense	5′-AACATAACAACATCTGGGCAAT-3′
IP-10	Sense	5′-CCCGGGAATTCATACCATGAACCCAAGTGCTGCC-3′
	Antisense	5′-GTCACGATGAATTCCTTAAGGAGCCCTTTTAGACCT-3′
MCP-1	Sense	5′-CTCACCTGCTGCTACTCATTC-3′
	Antisense	5′-GCATGAGGTGGTTGTGAAAAA-3′
IL-4	Sense	5′-CCTCACAGCAACGAAGAACA-3′
	Antisense	5′-TGCTCTTTAGGCTTTCCAGG-3′
IL-10	Sense	5′-ATAACTGCACCCACTTCCCA-3′
	Antisense	5′-GGTCTTCAGCTTCTCACCCA-3′
TNF-α	Sense	5′-AGCCCACGTAGCAAACCACCAA-3′
	Antisense	5′-ACACCCATTCCCTTCACAGAGCAAT-3′
ICAM-1	Sense	5′-GTCTCGGAAGGGAGCCAAGTA-3′
	Antisense	5′-CGACGCCGCTCAGAAGAA-3′
IFN-γ	Sense	5′-GCT CTG AGA CAA TGA ACG CT-3′
	Antisense	5′-AAA GAG ATA ATC TGG CTC TGC-3′

TNF-α = tumor necrosis factor-alpha; MIP-2 = macrophage inflammatory protein-2; IFN-γ = interferon-γ; MCP-1 = monocyte chemoattractant protein-1; IP-10 = interferon-gamma inducible protein-10; IL-10 = interleukin-10; IL-4 = interleukin-4; ICAM-1 = intercellular adhesion molecule-1.

a 10 min pre-incubation at 95°C followed by 40 cycles of 15 s at 95°C (denaturation), 10 s at 62°C (annealing), and 15 s at 72°C (amplification). PCR products were subjected to melting curve analysis using the LightCycler system to exclude the amplification of unspecific products. The quantification data were analyzed with the LightCycler analysis software as described by Morrison *et al.* [16]. The primers used are shown in Table 1.

Histological Examination

Livers were taken and sections of formalin-fixed paraffin-embedded liver samples were stained with hematoxylin-eosin to assess the accumulation of inflammatory cells and liver injury 3, 7, and 14 d after sham-operation or BDL.

FACS

Lymphomyeloid cells in the liver were prepared as described previously [17]. Briefly, the liver was gently dissociated (on steel screens) and washed once with RPMI medium. The resulting cell population was centrifuged at 500 *g* for 10 min in 33% Percoll (Pharmacia, Piscataway, NJ) containing 100 U/mL heparin. The resulting cell pellet containing lymphomyeloid cells was treated with lyses buffer for 1 min to eliminate small amount of erythrocytes. More than 3×10^6 lymphomyeloid cells/liver were obtained. To examine the cell surface expression of TCR-β and NK 1.1 on hepatic lymphomyeloid cells, the cell preparation (1×10^6 cells) was incubated with fluorescein isothiocynate-conjugated anti-TCR-β and PE-conjugated anti-NK 1.1 monoclonal antibodies (B and D Biosciences, PharMingen, San Jose, CA) for 30 min at 4°C. As controls, fluorescein isothiocynate- and PE-labeled rat isotype IgGs were used. Cells were washed three times with cold phosphate-buffered saline. A fluorescence analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Diego, CA).

Statistical Analysis

All data are expressed as the mean ± SEM. Data were analyzed with a one-way analysis of variance and individual group means were then compared with a Student-Newman-Keuls test, using the SigmaStat software program. Differences were considered significant when $P < 0.05$.

TABLE 2

Serum T-BIL and ALT levels and Neutrophil Accumulation Defined by MPO content

		OJ group (time after bile duct ligation)			
Sham group		POD 1	POD 3	POD 7	POD 14
T-BIL (mg/dL)	0.8 ± 0.2	2.1 ± 0.5*	5.6 ± 1.2*	14.7 ± 3.9*	22.4 ± 3.5*
ALT (IU/L)	9.3 ± 1.8	116.1 ± 26.6*	114.1 ± 16.9*	143.9 ± 12.2*	156.8 ± 12.7*
MPO (OD)	0.05 ± 0.02	0.06 ± 0.01	0.09 ± 0.03	0.23 ± 0.05*	0.39 ± 0.08*

Note. Values are expressed as mean ± SEM ($n = 5$).

OJ = obstructive jaundice; BDL = bile duct ligation; T-BIL = total bilirubin; ALT = alanine aminotransferase; MPO = myeloperoxidase; POD = postoperative day; IU = international unit; OD = optical density.

* $P < 0.05$ compared with the Sham group.

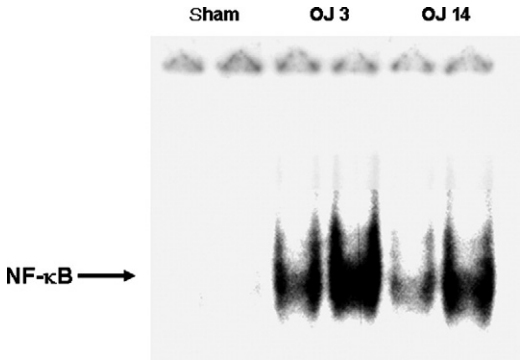

FIG. 1. NF-κB activation in whole liver homogenates during OJ. Nuclear extracts from liver tissue were subjected to EMSA. Results are representative of three separate time course experiments. Mice underwent either a sham operation or BDL.

RESULTS

Serum Bilirubin Levels, Liver injury, and Hepatic Neutrophil Accumulation

We assessed serum levels of T-BIL after BDL, and T-BIL levels were elevated the first day after BDL and remained elevated for up to 14 d after BDL. To determine whether hepatic injury was increased after BDL, we assessed serum levels of ALT. Serum ALT levels were increased from 1 to 14 d after BDL. Neutrophil accumulation was assessed by measuring the tissue MPO content and the MPO content was significantly increased from 7 to 14 d after BDL (Table 2).

Activation of Nuclear Factor Kappa B (NF-κB) during OJ, TNF-α, and Intercellular Adhesion Molecule-1 (ICAM-1) Expression

Time course of NF-κB activation in liver nuclear extracts during OJ was determined by EMSA. The nuclear translocation of NF-κB increased 3 d after BDL and was also activated 14 d after BDL in comparison to the sham-operated controls (Fig. 1). To determine whether the signaling of NF-κB is transcribed, we measured TNF-α expression. TNF-α mRNA expression was significantly increased the first day after BDL, and remained elevated up to 14 d after BDL (Table 3). Since ICAM-1 expression is known to be induced by TNF-α [18], we examined ICAM-1 expression. Hepatic ICAM-1 mRNA expression was significantly increased the first day after BDL, and remained elevated up to 14 d after BDL (Table 3).

Expression of ELR Positive CXC Chemokine in OJ

To determine whether the ELR positive CXC chemokine (MIP-2) was increased after BDL, mRNA expression as well as hepatic levels of MIP-2 were assessed by real-time PCR and ELISA. The expression of MIP-2 mRNA was significantly increased the first day after BDL, and continued to increase up to 14 d after BDL (Table 3). Hepatic MIP-2 levels were elevated 3 d after BDL, and remained elevated up to 14 d after BDL (Fig. 2A).

Expression of ELR Negative CXC Chemokine (IP-10) and CC Chemokine (MCP-1) in OJ

To determine whether IP-10 and MCP-1 increased after BDL, the mRNA expression as well as hepatic levels

TABLE 3

Real-Time PCR Analysis of Liver Tissue in Sham-Operated and Bile Duct Ligated (OJ) Mice

		Baseline	POD 1	POD 3	POD 7	POD 14
TNF-α	Sham	1.00 ± 0.34	1.02 ± 0.47	1.62 ± 0.29	1.11 ± 0.32	1.52 ± 0.28
	OJ	1.01 ± 0.23	3.74 ± 1.01*	4.47 ± 0.64*	6.17 ± 1.01*	6.67 ± 1.46*
ICAM-1	Sham	1.00 ± 0.23	1.22 ± 0.16	0.87 ± 0.11	0.84 ± 0.16	0.98 ± 0.07
	OJ	1.01 ± 0.10	4.33 ± 0.64*	4.14 ± 0.90*	2.94 ± 0.31*	5.19 ± 0.63*
MIP-2	Sham	1.00 ± 0.09	1.29 ± 0.10	1.20 ± 0.11	1.00 ± 0.08	1.03 ± 0.07
	OJ	1.03 ± 0.15	3.17 ± 0.53*	4.83 ± 0.74*	5.66 ± 0.56*	4.27 ± 0.71*
MCP-1	Sham	1.00 ± 0.17	1.92 ± 0.22	2.02 ± 0.25	1.96 ± 0.36	1.20 ± 0.17
	OJ	1.05 ± 0.04	9.26 ± 1.98*	10.8 ± 2.66*	9.94 ± 0.88*	15.4 ± 1.72*
IP-10	Sham	1.00 ± 0.21	1.77 ± 0.37	1.41 ± 0.39	0.91 ± 0.32	1.04 ± 0.24
	OJ	1.01 ± 0.21	9.06 ± 1.26*	10.7 ± 1.86*	4.97 ± 1.18*	2.64 ± 0.71
IL-10	Sham	1.00 ± 0.11	0.91 ± 0.16	0.95 ± 0.11	1.08 ± 0.11	0.92 ± 0.10
	OJ	1.00 ± 0.08	0.87 ± 0.16	1.13 ± 0.17	1.81 ± 0.49*	1.94 ± 0.26*
IL-4	Sham	1.00 ± 0.13	0.94 ± 0.15	0.98 ± 0.11	1.00 ± 0.10	0.92 ± 0.13
	OJ	1.00 ± 0.17	0.90 ± 0.14	1.05 ± 0.16	1.38 ± 0.45	1.40 ± 0.24*

Note. Values are expressed as mean ± SEM ($n = 5$).

OJ = obstructive jaundice; POD = postoperative day; TNF-α = tumor necrosis factor-α; ICAM-1 = intercellular adhesion molecule-1; MIP-2 = macrophage inflammatory protein-2; MCP-1 = macrophage chemoattractant protein-1; IP-10 = interferon-gamma inducible protein-10; IL = interleukin.

* $P < 0.05$ compared with the Sham group. Real-time PCR was used to analyze liver tissue for the expression of genes encoding markers of inflammation. In each individual sample, the expression level of each gene was first normalized with that of β-actin, and then the relative differences between groups were expressed as relative increases, setting sham-operated controls as 1.0.

of IP-10 and MCP-1 were assessed by real-time PCR and ELISA. The hepatic IP-10 mRNA expression significantly increased the first day after BDL, but decreased 7 d after BDL, and was not significant relative to sham-operated controls 14 d after BDL (Table 3). Hepatic IP-10 levels were increased 3 d after BDL relative to sham-operative controls, but were not significantly increased 14 d after BDL (Fig. 2B). The hepatic MCP-1 mRNA expression significantly increased the first day after BDL, and remained elevated and constant up to 14 d after BDL (Table 3). Hepatic MCP-1 levels were increased 3 and 14 d after BDL (Fig. 2C).

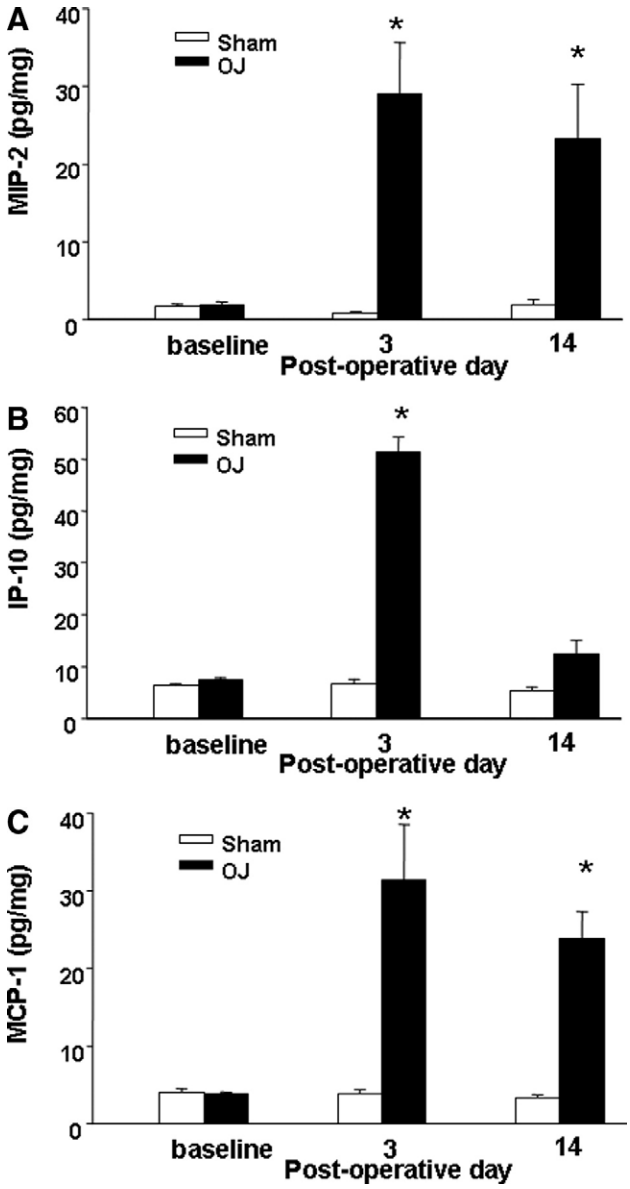

FIG. 2. Protein expression was analyzed by tissue ELISA. (A) The hepatic protein expression of MIP-2 at 3 and 14 d after BDL. (B) The hepatic expression of IP-10 at 3 and 14 d after BDL. (C) The hepatic protein expression of MCP-1 at 3 and 14 d after BDL. Mice underwent either a sham operation (Sham) or BDL (OJ). Data are mean ± SEM. *$P < 0.05$ *versus* sham-operated controls. For all groups, $n = 5$.

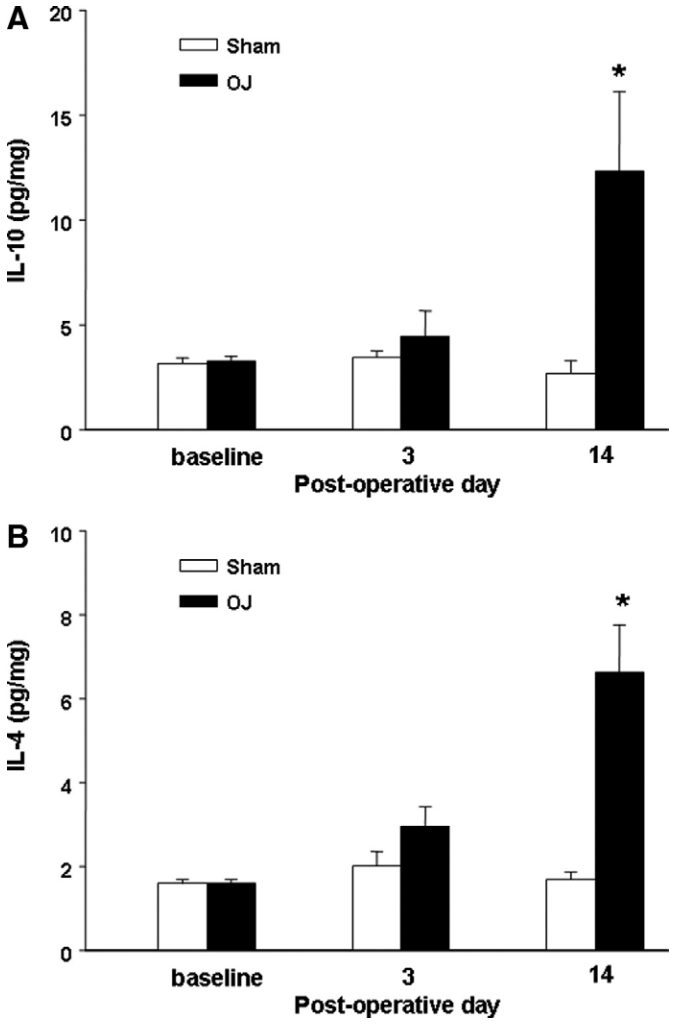

FIG. 3. Protein expression was analyzed by tissue ELISA. (A) The hepatic protein expression of IL-10 at 3 and 14 d after BDL. (B) The hepatic expression of IL-4 at 3 and 14 d after BDL. Mice underwent either a sham operation (Sham) or BDL (OJ). Data are the mean ± SEM. * $P < 0.05$ *versus* sham-operated controls. For all groups, $n = 5$.

Expression of Anti-Inflammatory Cytokine in OJ

To determine whether IL-10 and IL-4 expressions were increased after BDL, mRNA expression as well as hepatic levels of IL-10 and IL-4 were assessed by real-time PCR and ELISA. Hepatic IL-10 mRNA expression was not increased until 3 d after BDL, but was increased 7 and 14 d after BDL relative to sham-operated controls (Table 3). Hepatic IL-10 levels were increased at 14 d after BDL (Fig. 3A). The increase in the hepatic IL-4 mRNA expression 14 d after BDL was statistically significant compared with that of sham-operated controls (Table 3). He-

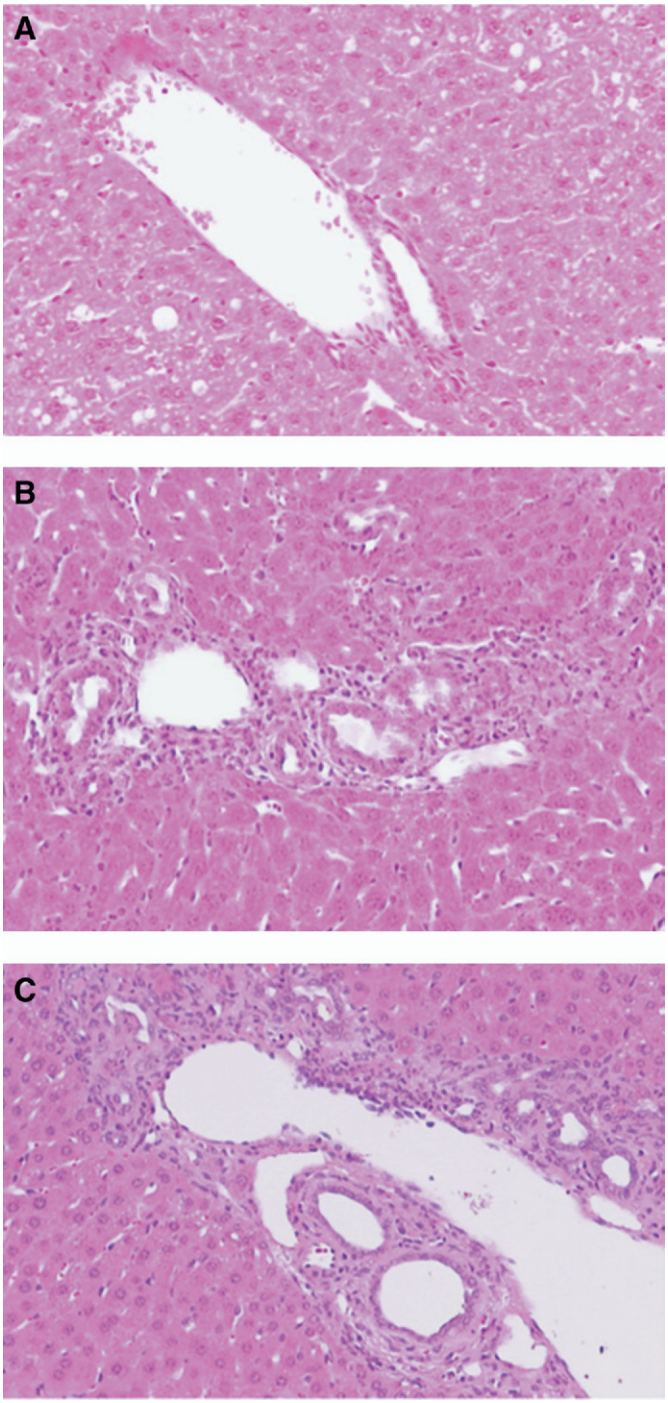

FIG. 4. Liver histology was examined to assess accumulation of inflammatory cells (Hematoxylin-Eosin staining). (A) Liver tissue obtained after the sham operation. (B) Liver tissue obtained 7 d after BDL. (C) Liver tissue obtained 14 d after BDL. (original magnification, 200×). (Color version of figure is available online.)

patic IL-4 levels were increased 14 d after BDL relative to sham-operated controls (Fig. 3B).

Histological Examination in OJ

To assess whether the hepatic accumulation of neutrophils and mononuclear cells increased, we examined histology of hematoxylin and eosin staining. Histological observations showed that neutrophil recruitment was faintly increased 7 d after BDL, while it more strongly increased 14 d after BDL. Mononuclear cell accumulation also increased after BDL, but the increase was less relative to neutrophil accumulation (Fig. 4). Focal necrosis was evident 14 d after BDL. Both neutrophils and mononuclear cells were mainly recruited in the periportal area and sinusoids.

FACS Analysis in OJ

To determine whether NK and T cell accumulation after BDL is increased, a FACS analysis was performed. Three days after BDL, NK and T cells were significantly accumulated in the liver compared with sham-operated controls. The number of NK cells returned to baseline levels and T cells were statistically

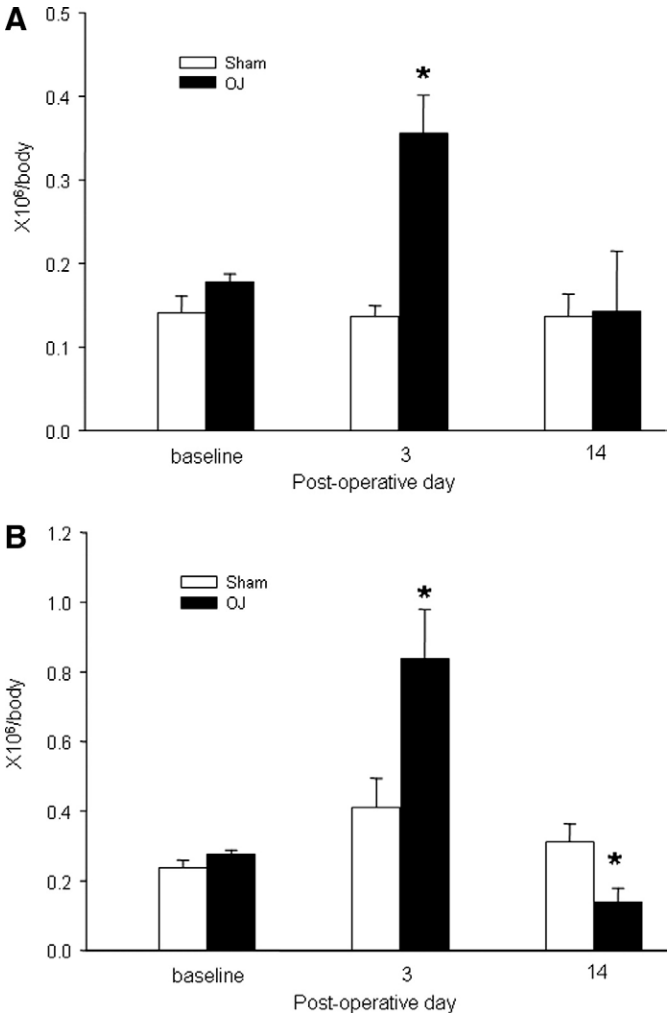

FIG. 5. Lymphocyte recruitment was analyzed by FACS. (A) NK cell accumulation at 3 and 14 d after BDL. (B) T cell accumulation at 3 and 14 d after BDL. Mice underwent either a sham operation (Sham) or BDL (OJ). Data are the mean ± SEM. *$P < 0.05$ *versus* sham-operated controls.

TABLE 4

Survival after LPS Administration (hours)

Sham (POD 3)	>24, >24, >24, >24, >24, >24
OJ (POD 3)	>24, >24, >24, >24, >24, >24
Sham (POD 14)	>24, >24, >24, >24, >24, >24
OJ (POD 14)	1, 2, 7, 11, 12, 12,

OJ = obstructive jaundice; POD = postoperative day.

diminished in the liver relative to sham-operated controls 14 d after BDL (Fig. 5). The total number of lymphocytes 14 d after BDL was equivalent to sham-operated controls (data not shown).

Survival After LPS Administration or CLP in OJ

To determine the susceptibility to infection in OJ, mice were subjected to LPS administration (4 mg/kg) or a CLP. Sham-operated mice undergoing LPS administration at 3 or 14 d after sham surgery were all alive longer than 24 h after LPS administration. Mice receiving LPS administration 3 d after BDL were all alive, but all mice receiving LPS administration 14 d after BDL died within 12 h after LPS administration (Table 4). In addition, mice were subjected to CLP. The survival rate of sham-operated mice undergoing CLP at 3 or 14 d after sham surgery was 80% at 7 dafter CLP. The survival rate of mice undergoing CLP 3 d after BDL was 60% at 7 d after CLP. All mice undergoing CLP 14 d after BDL died within 7 d after CLP (Table 5).

IFN-γ and IL-1β Expression after LPS Administration in OJ

To determine whether IFN-γ expression was increased after LPS administration in OJ, mRNA expression of IFN-γ was assessed by real-time PCR. The mRNA expression of IFN-γ in mice 3 and 14 d after BDL was not significantly increased. The mRNA expression of IFN-γ 3 h after LPS administration in the jaundiced mice 14 d after BDL was lower than that of sham-operated controls and jaundiced mice 3 d after BDL at 3 h after LPS administration (Table 6). To determine whether the IL-1β expression increased after LPS administration in OJ, hepatic levels of IL-1β were assessed by ELISA. The hepatic expression of IL-1β in mice 14 d after BDL significantly increased in comparison to the other mice at 3 h after LPS administration (Fig. 6).

TABLE 5

Survival after Cecal Ligation and Puncture (days)

Sham (POD 3)	4, >7, >7, >7, >7
OJ (POD 3)	3.5, 4, >7, >7, >7
Sham (POD 14)	5, >7, >7, >7, >7
OJ (POD 14)	1.5, 2, 2, 3, 6

OJ = obstructive jaundice; POD = postoperative day.

TABLE 6

Real-Time PCR Analysis of Hepatic IFN-γ mRNA Expression in Sham-Operated and Bile Duct Ligated (OJ) Mice 3 Hours after LPS Administration

	POD 3	POD 14
Sham + saline	1.00 ± 0.10	1.01 ± 0.12
Sham + LPS	1.85 ± 0.14*	2.08 ± 0.28*
OJ + saline	1.00 ± 0.19	1.06 ± 0.42
OJ + LPS	1.98 ± 0.23*	1.34 ± 0.31**

Notes. Values are expressed as mean ± SEM (n = 5). Real-time PCR was used to analyze hepatic IFN-γ mRNA expression. In each individual sample, the expression level of each gene was first normalized with that of β-actin, and then the relative differences between groups were expressed as relative increases, setting sham-operated controls as 1.0.

OJ = obstructive jaundice; POD = time after surgery; IFN = interferon.

* $P < 0.05$ compared with saline treatment group.

** $P < 0.05$ compared with sham + LPS group and OJ3 + LPS group.

DISCUSSION

Both postoperative hepatic failure and susceptibility to infection in patients with OJ remain an important clinical issue [19, 20]. Experimental studies have reported some of important mechanisms leading to hepatic failure in OJ. Kupffer cells play a role in the up-regulation of proinflammatory mediators in OJ [21–24]. Jiang *et al.* demonstrated that an exaggerated oxidative response by circulating neutrophils is implicated in liver failure in OJ [25]. Gujral *et al.* demonstrated that activated neutrophils play a role in liver

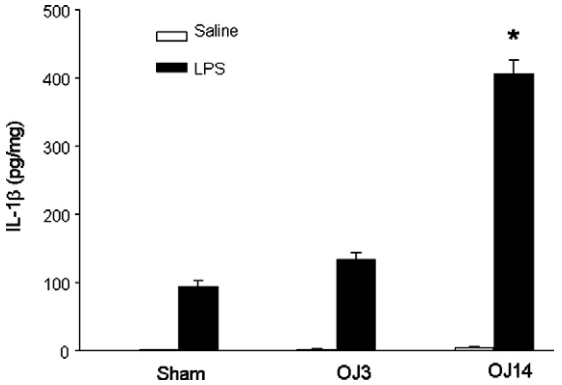

FIG. 6. The hepatic protein expression of IL-1β was analyzed by ELISA. (A) The hepatic protein expression of IL-1β after LPS administration in mice at 3 and 14 d after surgery. Mice underwent either a sham operation (Sham) or BDL (OJ). Data are the mean ± SEM. * $P < 0.05$ *versus* sham-operated controls and jaundiced mice 3 d after BDL at 3 h after LPS administration. For all groups, n = 5.

injury in the short period of OJ (within 5 d after BDL) [26]. However, most of these previously reported findings were investigated at only one time point after BDL. Furthermore, Cherqui *et al.* demonstrated that major liver resections without preoperative biliary drainage are safe in most patients with OJ [27]. It is therefore considered important to investigate whether the immune response differs over the time course of OJ or not, which may be associated with postoperative complication. The current study examined the time course of expression of pro-, anti-inflammatory cytokines, and chemokines during OJ. The up-regulation of neutrophil-chemoattractant CXC chemokine (MIP-2) significantly increased over a prolonged period of OJ, which is strikingly associated with the increase in neutrophil accumulation and was demonstrated by the data on MPO content and histological observations. Thereafter, activated and accumulated neutrophils appear to adhere to vascular endothelial cells associated with ICAM-1 expression, and extravasate into the hepatic tissue, thus resulting in respiratory burst and subsequent liver injury [28].

The link between cytokines, chemokines, and adhesion molecules involved in the development of cholestatic liver injury is their transcriptional regulation. Each of these mediators is controlled, at least in part, by the transcription factor, NF-κB [29], and we demonstrated that NF-κB is activated 3 and 14 d after BDL (Fig. 1). This finding is consistent with other studies demonstrating that NF-κB is activated 5 d after BDL [30]. While the current study assessed NF-κB activation in whole liver extracts, which are largely representative of hepatocytes, it remains to be determined whether OJ induces NF-κB activation in Kupffer cells or hepatocytes. Canbay *et al.* demonstrated that cholestatic liver injury is deteriorated by Kupffer cell engulfment of apoptotic bodies which promotes inflammation and fibrogenesis [31], suggesting that the initial cellular origin of NF-κB activation may be Kupffer cells. However, there still remains concern whether or not NF-κB is activated in hepatocytes during OJ.

The recruitment of mononuclear cells in OJ has not been well elucidated. The current data showed that mononuclear cells are recruited in the liver beginning 3 d after BDL, which is strikingly associated with an increase in expression of MCP-1. Mononuclear cells are known to be expressing β2 integrins like neutrophils [32] and ICAM-1 expression was increased during OJ, suggesting that mononuclear cells also play a role in liver injury in OJ, at least, by sinusoidal disturbance due to capillary plugging. As a consequence, our findings showed that inflammatory cells such as neutrophils and mononuclear cells recruited in the cholestatic liver, which is associated with an increase in expression of several proinflammatory cytokines and chemokines. This observation was supported by the study of Ito *et al.* by intravital microscopy [33].

In the current study, we investigated the time course of the accumulation of lymphocytes during OJ. The number of NK and T cells in the liver was increased 3 d after BDL, but returned to baseline levels 14 d after BDL, which was correlated with hepatic IP-10 expression. NK and T cells are known to produce IFN-γ and IP-10 induces IFN-γ expression [8–10]. IFN-γ has been known to stimulate macrophage phagocytic function [11]. Moreover, the number of mononuclear cells 14 d after BDL was increased, suggesting that non-NK and non-T cells were likely to be accumulated in the liver. Bogdan *et al.* revealed that a primary cellular target of IL-10 is macrophages [34] and IL-10 is known to shut down IFN-γ expression which is produced by NK and T cells [35]. In the current study, the IL-10 expression significantly increased in the long-standing OJ (14 d after BDL), thus suggesting that an increase in mononuclear cell recruitment might contribute to IL-10 expression in response to excessive inflammation. We previously reported that jaundiced patients exhibit an anti-inflammatory response that potentially modulates host defense and is susceptible to infection [36]. Others also reported that IL-10 is responsible for impaired bacterial clearance in OJ [37]. In the current study, after LPS administration, IL-1β expression significantly increased in the mice 14 d after BDL (prolonged OJ), but the IFN-γ expression after LPS administration in mice at 14 d after BDL was lower than that of other mice after LPS administration. This observation is consistent with the findings of Kennedy *et al.* demonstrating an increased secretion of proinflammatory cytokines after LPS administration in the prolonged OJ [38]. The current results also showed that mice in the short period of OJ are tolerant of LPS and CLP; on the other hand, mice in the prolonged OJ are sensitized to LPS and CLP. Taken together, it seems likely that mice with a prolonged OJ are therefore susceptible to infection.

In conclusion, our data suggested that the immune response, such as the expression of pro- and anti-inflammatory mediators and the recruitment of immune cells, including neutrophils and lymphocytes, may differ during the time course of OJ. As a result, long-standing OJ may cause excessive inflammation and susceptibility to infection. The present data may need further investigation of the mechanism of cholestatic liver injury.

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