Glucocorticoid receptor-dependent immunomodulatory effect of ursodeoxycholic acid on liver lymphocytes in mice

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Abbreviations used in this paper: UDCA, ursodeoxycholic acid; Con-A, concanavalin-A; MNC, mononuclear cells; LPS, lipopolysaccharide; E. coli, Escherichia coli; GR, glucocorticoid receptor; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; FXR, farnesoid X receptor; PXR, pregnane X receptor; i.v., intravenously; CFU, colony-forming units; i.p., intraperitoneally; DMSO, dimethyl sulfoxide; s.c., subcutaneously; FBS, fetal bovine serum; RPMI, Roswell Park Memorial Institute; siRNA, small interfering RNA; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assays; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid.

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Abstract

Although ursodeoxycholic acid (UDCA) has long been used for patients with chronic cholestatic liver diseases, particularly primary biliary cirrhosis, it may modulate the host immune response. This study investigated the effect of UDCA feeding on experimental hepatitis, endotoxin shock, and bacterial infection in mice. C57BL/6 mice were fed a diet supplemented with or without 0.3% (w/v) UDCA for four weeks. UDCA improved hepatocyte injury and survival in concanavalin-A (Con-A)-induced hepatitis by suppressing IFN-\(\gamma\) production by liver mononuclear cells (MNC), especially NK and NKT cells. UDCA also increased survival after lipopolysaccharide (LPS)-challenge; however, it decreased survival of mice following Escherichia coli (E. coli) infection due to the worsening of infection. UDCA-fed mice showed suppressed serum IL-18 levels and production of IL-18 from liver Kupffer cells, which together with IL-12 potently induces IFN-\(\gamma\) production. However, unlike normal mice, exogenous IL-18 pretreatment did not increase the serum IFN-\(\gamma\) levels after E. coli, LPS or Con-A challenge in the UDCA-fed mice. Interestingly, however, glucocorticoid receptor (GR) expression was significantly upregulated in the liver MNC of the UDCA-fed mice but not in their whole liver tissue homogenates. Silencing GR in the liver MNC abrogated the suppressive effect of UDCA on LPS- or Con-A-induced IFN-\(\gamma\) production. Furthermore, RU486, a GR antagonist, restored the serum IFN-\(\gamma\) level in UDCA-fed mice after E. coli, LPS or Con-A challenge. Taken together, these results suggest that IFN-\(\gamma\)-reducing immunomodulatory property of UDCA is mediated by elevated GR in the liver lymphocytes in an IL-12/18 independent manner.
Key words: concanavalin-A, LPS, *Escherichia coli*, IFN-γ, RU486
Introduction

Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid currently used in clinical practice worldwide not only for the dissolution of cholesterol gallstones (2), but also for the treatment of chronic, mainly cholestatic, liver diseases (8, 26, 35), such as primary biliary cirrhosis (PBC) (1, 36), primary sclerosing cholangitis (PSC) (29), and viral hepatitis (33, 43). UDCA has a choleretic action on these cholestatic liver diseases, in particular PBC (8, 14), thereby improving both the biochemical and histological symptoms (1).

Although UDCA may correct the immunological abnormalities observed in PBC and counter the detrimental effects of bile acids, it has been reported to suppress the T cell function in mice (11). Co-culture with UDCA as well as other bile acid fractions decreases LPS-stimulated IFN-γ production by mouse liver mononuclear cells (MNC) (21). This UDCA-induced cellular immune alteration may have a specific effect on patients in various clinical settings. Therefore, it is important to investigate the effect of UDCA treatment on in vivo host immune responses prior to initiating clinical applications.

Recent reports have shown that bile acids are ligands of nuclear receptors, such as farnesoid X receptor (FXR) (28, 34) and pregnane X receptor (PXR) (42, 47). Therefore, bile acid may regulate the host inflammatory responses induced by nuclear receptors (16, 17). Glucocorticoid receptor (GR), the first and most extensively characterized nuclear receptor, crucially regulates host inflammatory and immune responses (7, 13). It would therefore be interesting to elucidate how such in vivo UDCA treatment affects these nuclear receptors and host immune responses.

Clinically, UDCA treatment is administered orally to patients. Bile
acid-supplemented diets have been fed to experimental animals in order to investigate hepatotoxicity of bile acids or their potential therapeutic application, because bile acid components might vary in their pathological and physiological response \textit{in vivo} (3, 49). Therefore, subject mice were fed diets supplemented with 0.3% (w/v) UDCA for 4 weeks. This dose of UDCA (0.3-0.4%) in the diet is reportedly the highest concentration that induces no physiological or pathological alteration in mice under unstimulated conditions (24).

The present study investigated the effect of UDCA treatment on host immunity using these UDCA-fed mice. UDCA treatment markedly suppressed IFN-\(\gamma\) production of the liver MNC in mice with concanavalin-A (Con-A)-induced hepatitis, which is a model of human autoimmune hepatitis (44), and in mice with endotoxin shock as well as \textit{Escherichia coli (E. coli)} infection. The UDCA-induced suppression of cellular immune responses might ameliorate hepatic inflammation, Con-A-induced hepatitis, and endotoxin shock. However, such UDCA-induced immunosuppression may cause a reduction in the cellular immunity-mediated bacterial elimination in the liver. Interestingly, this IFN-\(\gamma\)-reducing immunomodulatory property of UDCA may be mediated by elevated GR in the liver lymphocytes but not in the whole liver tissue homogenates in an IL-12/IL-18 independent manner.
Materials and Methods

This study was conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College.

Mice and diet supplemented with UDCA

Male C57BL/6 mice were used in this study (6 weeks old, 15-18 g, SLC, Shizuoka, Japan). UDCA was kindly provided by Tanabe Mitsubishi Pharma Co. (Osaka, Japan). UDCA-treated mice were fed a diet (CLEA Rodent Diet CE-7, CREA Japan Inc., Tokyo, Japan) supplemented with 0.3% (w/v) UDCA for 4 weeks. Control mice were fed a diet without any supplementation. All mice had free access to both drinking water and the respective diets.

Con-A-induced hepatitis, LPS and E. coli challenge

The mice were intravenously (i.v.) injected with 15 mg/kg of Con-A (Vector Laboratories, Burlingame CA) via the caudal vein (31). The mice received an i.v. injection with 2.5 or 5 mg/kg of LPS (E. coli 0111: B4, Sigma-Aldrich, St. Louis, MO) (18). E. coli strain B (ATCC 11303; Sigma-Aldrich) was grown in a brain-heart infusion broth (Difco Laboratories, Detroit, MI), and 5 × 10^8 colony-forming units (CFU) of E. coli were administered by i.v. injection to the mice (18, 22).

Exogenous IL-18 injection or RU486 pretreatment in E. coli-, LPS- or Con-A-challenged mice

Recombinant IL-18 (10 μg/kg, MBL, Nagoya, Japan) diluted with 0.5 mL PBS or PBS alone (0.5 mL) was intraperitoneally (i.p.) injected into the mice 2 h before E. coli, LPS
or Con-A challenge. RU486 (Mifepristone, 10 or 20 mg/kg, Sigma-Aldrich) diluted
with 50μL dimethyl sulfoxide (DMSO) or DMSO alone (50μL) was also subcutaneously
(s.c.) injected into mice 2 h before the E. coli, LPS or Con-A challenge.

Isolation of liver mononuclear cells (MNC), lymphocytes, and spleen MNC
The liver MNC were obtained as previously described (18, 22, 31). Briefly, after
removing from the mouse, the liver was minced and suspended in Hanks’ balanced salt
solution containing 0.05% collagenase (Wako, Osaka, Japan). The liver specimens were
incubated with shaking for 20 min at 37°C, passed through a 200-gauge stainless steel
mesh, suspended in osmolarity- and pH-adjusted 33% Percoll solution, and centrifuged
at 500 × g for 20 min at room temperature. The red blood cells lysed and the liver MNC
were washed twice in 10% fetal bovine serum (FBS) Roswell Park Memorial Institute
(RPMI)-1640. The liver MNC were cultured in collagen-coated plastic plates in 5%
CO2 at 37°C for 2 h to obtain non-adherent hepatic lymphocytes. The non-adherent
lymphocytes were collected by gentle pipetting (23, 37). Splenocytes were also filtered
through the mesh, treated with a lysing solution, and washed twice.

Cell culture and in vitro Con-A or LPS stimulation
Liver or spleen MNC (5 × 10^5 cells in 200 μL of 10% FBS with RPMI-1640 medium)
were cultured with 5 μg/mL of Con-A in 96-well flat-bottom plates in 5% CO2 at 37°C
for 24 h. The MNC were similarly cultured with 10 μg/mL of LPS for 2 h to measure
TNF or 24 h to measure IL-12, IL-18 or IFN-γ.
Intracellular staining for IFN-γ, GR, perforin or granzyme

Liver MNC were obtained from the mice 12 h after Con-A administration or 2 h after LPS challenge, and were incubated with BD GoldiStop (0.7 μl/ml; BD Pharmingen, San Diego, CA) for 2 h. After incubation with Fc blocker, the cells were stained with a FITC-conjugated anti-mouse NK1.1 mAb (PK136; eBioscience, San Diego, CA), CD4 mAb (RM4-5; eBioscience) or CD8 mAb (53-6.7; eBioscience) and PE-Cy5-conjugated anti-mouse TCR β mAb (H57-597; eBioscience). Subsequently, the cells were incubated with BD Cytofix/Cytoperm solution (BD Pharmingen) at 4°C for 20 min, then washed with BD Perm/Wash solution (BD Pharmingen). Thereafter, the cells were stained with a PE-conjugated anti-mouse IFN-γ mAb (XMG1.2; eBioscience), perforin mAb (eBioOMAK-D; eBioscience), granzyme mAb (NGZB; eBioscience) or isotype control mAb (rat IgG1 for IFN-γ mAb or rat IgG2a for perforin and granzyme mAbs, eBioscience) at 4°C for 30 min. After washing, the cells were analyzed using an EPICS XL instrument (Beckman Coulter). The NK cells were positive for NK1.1 staining but negative for TCR β staining, while the NKT cells were positive for both NK1.1 and TCR β. The CD4+ or CD8+ T cells were positive for CD4 and TCR β or CD8 and TCR β, respectively. After gating these NK, NKT, CD4+ T, or CD8+ T cells, their intracellular intensity of IFN-γ, perforin, or granzyme were evaluated. To evaluate the GR, liver MNC obtained from the control mice (without any challenges) were similarly stained for surface markers and were treated with a fixation and permeabilization kit (eBioscience). Then, the cells were stained with a PE-conjugated polyclonal anti-mouse GR Ab (Bioss, Woburn, MA) or isotype control mAb (rabbit IgG) according to the manufacturer’s recommendations, and were analyzed using the EPICS XL instrument.
**Real-time quantitative PCR**

Total RNA extraction was performed with an RNeasy Mini Kit (QIAGEN, Valencia, CA), following the manufacturer’s instructions, with an additional step where 1 unit/μL of RNase-free DNase was added for 15 min after cell lysis. cDNA was synthesized from total RNA (1 μg) by reverse transcription using a SuperScript III First-Strand synthesis system (Invitrogen, Carlsbad, CA). Quantitative real-time RT-PCR was performed on a LightCycler™ 480 System (Roche Diagnostics, Mannheim, Germany) using SYBR Green PCR reagents. The primers were designed by Takara Bio. Inc. (Tokyo, Japan), FXR: Musmusculus nuclear receptor subfamily 1, group I, member 2 (Nrli2), transcript variant 2, mRNA, PXR: Musmusculus nuclear receptor subfamily 1, group H, member 5 (Nrli5), transcript variant 1, mRNA, and GR: Musmusculus glucocorticoid receptor DNA binding factor 1 (Grlf1), mRNA. The data were normalized by the level of GAPDH expression in each sample and then were averaged.

**Western blotting analysis**

Liver or spleen MNC were lysed in ice-cold lysis buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 1 mM phenylmethyl sulfonyl fluoride and 150 units/mL of aprotinin. After centrifugation, whole-cell extracts were resolved by SDS-PAGE and proteins were transferred to hydrophobic polyvinylidene difluoride membrane (GE Healthcare, Tokyo, Japan). After blocking with 5% dried milk in TBS and 0.1% Tween-20, membranes were probed with Abs to GR (sc-8992) and GAPDH (sc-20357; Santa Cruz Biotechnology, Santa Cruz, CA), followed by the relevant secondary Abs (Santa Cruz Biotechnology). The protein bands
were visualized by a chemiluminescence detection system (ECL-plus; GE Healthcare).

The signals in the immunoblots were analyzed by an LAS3000 digital imaging system (Fujifilm, Tokyo, Japan).

**Small interfering RNA (siRNA) transfection**

Liver MNC from UDCA-fed mice were transfected using Lipofectamine 2000 (Invitrogen, Tokyo, Japan) according to the manufacturer’s instructions. The pre-designed mouse GR siRNA and control siRNA were purchased from Ambion (Tokyo, Japan). Briefly, cells (2×10⁶) were seeded into 12-well plates in 0.8 mL of media without antibiotics and 0.2 mL of Lipofectamine 2000-siRNA complex. A total of 2 µL of Lipofectamine 2000 in 100µL of Opti-MEM (Gibco) and 80 pmol siRNA in 100 µL of Opti-MEM were mixed together for a final volume of 0.2 mL. The cells were incubated in a 37°C 5%CO₂ incubator for 36h. After transfection, the cells were cultured with or without LPS (10 µg/mL) or Con-A (5 µg/mL) for 24 h, and then the culture medium and cells were collected to perform ELISA and a Western blot analysis, respectively.

**In vivo depletion of NK/NKT cells**

An anti-NK1.1 mAb (PK136; 200 µg/mouse) or PBS was i.v. injected into the control and UDCA-fed mice twice (three and one days before the challenge), after which Con-A was i.v. challenged into the mice. The anti-NK1.1 Ab depleted more than 90% of NK cells and 85% of NKT cells for approximately seven days in UDCA-fed mice similarly to control mice as evaluated by staining with CD122 mAb (TMβ-1, eBioscience, another NK cell marker) and TCR β mAb (eBioscience) or Vβ8 mAb
(F23.1, eBioscience), as previously reported (30, 31). Treatment with the anti-NK1.1 Ab was also administered in the RU486 (GR antagonist)-treated UDCA-fed mice.

Measurement of bile acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and cytokine levels

The mice were sacrificed by exsanguination from the subclavian artery and vein under deep ether anesthesia, and then the liver, lung, kidney, and spleen were removed to produce a homogenized PBS suspension. Bile acid levels of the tissue homogenates were measured by high-performance liquid chromatography according to the method described previously (21). Blood samples were collected from the retro-orbital sinus in mice at indicated time points after Con-A, LPS, or *E. coli* challenge. The serum ALT and AST levels were measured using a FUJI dry-chem system (FUJIFILM, Tokyo, Japan). Cytokines in the sera or culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer’s instructions (with limits in pg/ml): TNF, IL-12, IFN-γ (both from Endogen, Woburn, MA) and IL-18 (MBL, Nagoya, Japan).

Pathological examination

The livers were obtained from the UDCA-fed or control mice 24 h after Con-A, LPS or *E. coli* challenge. Sections were prepared from formalin-fixed, paraffin-embedded tissue samples stained with hematoxylin and eosin using routine procedure.

Statistical analysis
Data are presented as the mean values ± SE. Statistical analyses were performed using an iMac computer (Apple, Cupertino, CA) and the Stat View software package (Abacus Concepts, Berkeley, CA). The survival rates were compared using the Wilcoxon rank test, and any other statistical evaluations were compared using the standard one-way analysis of variance followed by the Bonferroni post hoc test. P< 0.05 was considered to indicate a significant difference.
Results

Total bile acid and its fraction levels in the liver of UDCA-fed mice

UDCA-fed mice showed approximately 25 times higher concentration of total bile acid in the liver homogenates in comparison to that of the control mice (Table 1). UDCA levels in the liver homogenates were more than 100 times higher in the UDCA-fed mice than that of the control mice, although such a large difference was not observed in the levels of other bile acid fractions of the liver homogenates between the UDCA-fed and control mice (Table 1). No bile acids including UDCA were detected in the kidney, lung, or spleen in either the UDCA-fed or control mice (data not shown), thus suggesting that the UDCA levels only increased in the enterohepatic circulation.

The effect of feeding UDCA on the mouse Con-A-induced hepatitis

The UDCA-fed mice showed a significantly higher survival after Con-A administration than that of the control mice (Fig. 1A). These mice also showed significantly lower ALT levels in the sera 12 h after Con-A administration (Fig. 1B). Elevation of serum TNF levels at 1 h after Con-A administration was significantly suppressed in the UDCA-fed mice in comparison to that of control mice, although the serum peak of TNF at 24 h was similar between the UDCA-fed and control mice (Fig. 1C). There was no statistically significant difference in the change in the serum IL-6 or IL-10 level between the UDCA-fed and control mice (data not shown). The elevation of serum IFN-γ after Con-A administration was also significantly suppressed in the UDCA-fed mice (Fig. 1D). The control mice showed massive hepatocyte necrosis and neutrophil infiltration 24 h after Con-A administration, while the UDCA-fed mice did not show such lesions (Fig. 2).
In vitro Con-A-induced IFN-γ production of the liver MNC in UDCA-fed mice

In vitro Con-A-stimulated IFN-γ production by the liver MNC was significantly lower in the UDCA-fed mice than that of the control mice (Fig. 3). However, the spleen MNC of the UDCA-fed mice produced a comparable amount of IFN-γ in response to in vitro Con-A stimulation to those of the control mice (Fig. 3).

The effect of feeding UDCA on the LPS-challenged mice

UDCA-fed mice showed significantly lower serum peaks of TNF at 1 h and IFN-γ at 6 h after 2.5 mg/kg of LPS challenge than those of the control mice (Figs. 4A, D). However, UDCA treatment did not affect the serum IL-12 levels in the mice after LPS challenge (Fig. 4B). UDCA treatment tended to suppress (but not significant) the elevation of serum IL-18 levels after LPS challenge in mice (Fig. 4C). Although all UDCA-fed and control mice challenged with 2.5 mg/kg of LPS survived, the control mice showed a focal hepatocyte necrosis, while the UDCA-fed mice did not show this pattern (Fig. 2). UDCA-fed mice showed a significantly higher survival rate than that of the control mice following 5 mg/kg of LPS challenge (100% vs. 60% survival, p<0.05, n=10 in each group) and also showed significantly lower serum TNF and IFN-γ levels (data not shown).

The effect of feeding UDCA on the E. coli-challenged mice

Unlike the LPS challenge, UDCA-fed mice showed a significantly lower survival rate after E. coli (5 × 10⁸ CFU/mouse) challenge than that of the control mice (Fig. 5A), and
also showed significantly higher serum ALT and AST levels at 12 h (Figs. 5B, C).

However, UDCA treatment significantly suppressed the serum peaks of TNF at 1 h, IFN-γ at 6 h, and IL-18 at 12-24 h after *E. coli* challenge (Figs. 5D-F), although it did not suppress the peak of IL-12 at 3 h in mice (data not shown). UDCA-fed mice showed a substantial amount of infectious foci and neutrophil accumulation in the liver 24 h after *E. coli* infection, although the control mice did not show such severe lesions (Fig. 2), suggesting that the UDCA-fed mice had obviously increased liver inflammation due to a worsening of the *E. coli* infection.

**In vitro LPS-induced cytokine productions of the liver MNC in UDCA-fed mice**

Liver MNC of the UDCA-fed mice showed significantly lower amounts of IL-18 and IFN-γ production by *in vitro* LPS stimulation in comparison to those of the control mice, although there was no difference in the TNF or IL-12 production of the liver MNC between these mice (Fig. 6). The production of cytokines from spleen MNC of the UDCA-fed mice did not significantly differ from those of control mice (Fig. 6). Despite a significant decrease in the IFN-γ production in response to LPS or Con-A stimulation, the liver MNC of the UDCA-fed mice did not significantly decrease in terms of the proportion of NK, NKT or CD4+ T cells (data not shown), suggesting that there were functional alterations of these IFN-γ-producing cells in response to UDCA.

**Intracellular IFN-γ-staining of the liver MNC in the UDCA-fed mice**

Using the obtained liver MNC, we gated NK1.1 positive but TCR β negative cells as the NK cells (Fig. 7A) and then examined their IFN-γ intensity or GR intensity. We also
gated NK1.1 positive and TCR β positive cells as the NKT cells (Fig. 7A). Similarly, we stained the cells and gated CD4 (or CD8, not shown) positive and TCR β positive cells as the CD4⁺ T cells (or CD8⁺ T cells) (Fig. 7A) and examined their IFN-γ or GR intensity. The UDCA-fed mice exhibited significantly lower percentages of IFN-γ positive cells among the liver NK cells and NKT cells both 12 hours after Con-A administration (and two hours of additional incubation) (Fig. 7B) and two hours after the LPS challenge (and two hours of additional incubation) (Fig. 7C) in comparison to those observed in the control mice, although there were no significant differences in the percentages of IFN-γ-positive CD4⁺ T cells or CD8⁺ T cells after the Con-A and LPS challenges between the UDCA-fed and control mice at the indicated harvesting time points under the following culture conditions (Figs. 7B, C, CD8⁺ T cells are not shown). Both the UDCA-fed and control mice exhibited approximately 28% granzyme-positive cells or 6% perforin-positive cells among the liver NK and NKT cells after the LPS challenge; however, no significant differences in the expressions of these cells were observed between the groups of mice (data not shown). Neither the UDCA-fed nor control mice showed significant positive staining of granzyme or perforin in the liver CD4⁺ or CD8⁺ T cells after the LPS challenge (data not shown). Neither type of mice demonstrated any positive staining of granzyme or perforin in the liver MNC subsets following Con-A administration (data not shown).

**Expression of GR in the liver MNC of UDCA-fed mice**

The whole liver tissue (homogenates) showed no significant difference in the mRNA levels of PXR, FXR or GR between the UDCA-fed and control mice (Fig. 8A). However, UDCA-fed mice showed a significantly higher expression of GR mRNA in...
the liver MNC in comparison to that of the control mice (Fig. 8B). GR mRNA expression was further examined in the non-adherent hepatic lymphocytes. The UDCA-fed mice showed a three-fold higher expression of GR mRNA in the hepatic lymphocytes (Fig. 8C). They also showed a significantly higher protein expression of GR in the liver MNC but not in the whole liver tissue homogenates or spleen MNC (Figs. 8D-F). UDCA-fed mice showed a higher tendency of PXR mRNA expression of the liver MNC in comparison to that of the control mice, although it was not statistically significant (Fig. 8B). No difference was observed in the FXR mRNA levels in the liver MNC between the UDCA-fed and control mice. In addition, there was no significant difference in the mRNA levels of PXR, FXR or GR in the spleen MNC between the UDCA-fed and control mice (data not shown). According to flow cytometry, the percentage of GR-positive cells significantly increased in the liver NK cells, NKT cells and CD4⁺ T cells (but not CD8⁺ T cells) in the UDCA-fed mice in comparison to that observed in the control mice (Fig. 7D, CD8⁺ T cells not shown).

The effect of RNA interference targeting GR on the LPS- or Con-A-induced IFN-γ production in the liver MNC of UDCA-fed mice

The liver MNC of the UDCA-fed mice were treated with GR siRNA, and their LPS- or Con-A-induced IFN-γ production was evaluated. When the GR siRNA was used, the increased GR expression of the liver MNC in the UDCA-fed mice was effectively knocked down, and the suppressive effect of UDCA on LPS- or Con-A-induced IFN-γ production was abrogated (Figs. 9A, B), thus suggesting that the increased GR of the liver MNC in UDCA-fed mice is functional.
The effect of exogenous IL-18 injection or RU486 treatment on the IFN-γ response to

\textit{E. coli}, LPS or Con-A challenge in the UDCA-fed mice

The exogenous IL-18 injection of the UDCA-treated mice did not increase the serum IFN-γ levels after the \textit{E. coli}(5 \times 10^8 \text{ CFU/mouse}) or LPS (5 mg/mL) challenge (\textit{E. coli}: Fig. 10A, LPS: IL-18 injection: 7.2 ± 0.2 vs. PBS: 7.5 ± 0.4 ng/mL at 6 h) and did not affect the survival after \textit{E. coli} or LPS challenge (data not shown). IL-18 injection also affected neither the serum IFN-γ levels after the Con-A challenge in the UDCA-treated mice (IL-18 injection: 0.9 ± 0.02 vs. PBS: 0.9 ± 0.05 ng/mL at 6 h) nor survival (data not shown).

In contrast, RU486 treatment (10 mg/kg, s.c.) significantly increased the serum IFN-γ levels after \textit{E. coli} challenge (Fig. 10B); however, it did not significantly improve the survival of UDCA-fed mice (Fig. 10C) and also did not influence the serum IL-18 levels (RU486: 2.9 ± 0.7 vs. DMSO: 2.8 ± 0.2 ng/mL at 12 h). IFN-γ was further enhanced when the UDCA-fed mice received a 20 mg/kg s.c. injection of RU486, while no mice survived after the \textit{E. coli} challenge (Figs. 10B, C). RU486 treatment (10 mg/kg, s.c.) also remarkably increased the serum IFN-γ levels after the LPS (5 mg/mL) challenge in the UDCA-fed mice (RU486: 26.6 ± 4.8 vs. DMSO: 7.5 ± 0.2 ng/mL at 6 h, p<0.01, n=10 in each group), although it impaired their survival (RU486: 30% vs. DMSO: 100%, p<0.01). Similarly, RU486 treatment significantly increased the serum IFN-γ levels at six hours after the Con-A challenge in the UDCA-fed mice (Fig. 10D), although it impaired the survival of the UDCA-fed mice (Fig. 10E). Restoration of IFN-γ production by RU486 may abrogate the beneficial effects of UDCA on murine survival after LPS and/or Con-A challenges.
The effects of NK/NKT cell depletion on the serum IFN-γ levels after the Con-A challenge in the RU486-treated UDCA-fed mice

We first examined the effects of deletion of NK/NKT cells on the serum IFN-γ levels after the Con-A challenge in the UDCA-fed and control mice. In the control mice, depletion of NK/NKT cells moderately (but significantly) decreased the serum IFN-γ levels after the Con-A challenge (NK/NKT depleted: 2.0 ± 0.4 vs. non-depleted: 3.3 ± 0.5 ng/mL at 6 h, p<0.05, n=5 in each group, 40% reduction). Although UDCA treatment decreased the serum IFN-γ levels after the Con-A challenge in both mice with and without NK/NKT cell depletion, there were no differences in the serum IFN-γ levels at six hours between the mice (NK/NKT depleted: 1.1 ± 0.1 vs. non-depleted: 1.0 ± 0.1 ng/mL, n=5 in each group). We then examined the effects of NK/NKT cell depletion on the RU486 (GR antagonist)-treated UDCA-fed mice. Although depletion of NK/NKT cells tended to decrease the serum IFN-γ levels at six hours after the Con-A challenge in the RU486-treated UDCA-fed mice, the NK/NKT cell-depleted UDCA-fed mice continued to produce a substantial amount of IFN-γ after the Con-A challenge under the GR-antagonized conditions (NK/NKT depleted: 4.5 ± 0.5 vs. non-depleted: 5.5 ± 0.6 ng/mL, n=5 in each group). There is a possibility that other IFN-γ-producing cells, such as CD4+ T cells, produce IFN-γ under such conditions in vivo, even if the intracellular staining did not detect a significant level of IFN-γ production (Fig. 7A).
Discussion

UDCA treatment induces the replacement of hydrophobic bile acids that are cytotoxic in the presence of hydrophilic bile acids in the liver (20). This replacement of hydrophobic bile acids by UDCA is thought to exert a hepatoprotective effect. In addition, UDCA has an anti-cholestatic (14), anti-oxidative (25, 40) and anti-apoptotic effects (4). These hepatoprotective effects of UDCA may provide a certain therapeutic efficacy for patients with liver diseases (33, 36). However, one of the most fascinating mechanisms of action that is evoked for UDCA is thought to be its immunomodulating activity (48). UDCA dramatically improves the clinical symptoms of PBC, which is an autoimmune disease that destroys the biliary epithelium (15).

Bile duct-ligated mice show obstructive jaundice and markedly increased levels of bile acids, particularly secondary bile acids, in the liver (21). These mice had a severely impaired host defense against *E. coli* infection, because their host immune response mediated by IFN-γ was markedly impaired, which is crucial for the host to eliminate invading *E. coli* (22). Bile acid fractions including UDCA dramatically suppressed LPS (derived from *E. coli*)-induced IFN-γ production by liver MNC, thus suggesting that increased bile acids in the liver decrease bacterial elimination (21).

UDCA-fed mice showed remarkably higher levels of UDCA in the liver but not other organs (Table 1). Tissue inflammatory responses to Con-A stimulation may be markedly suppressed in the liver of UDCA-fed mice by suppressing IFN-γ that is a potent proinflammatory cytokine thereby causing severe inflammatory tissue reactions (39). Several investigators have reported that UDCA treatment down-regulates Con-A-stimulated blood lymphocyte activity in humans (27, 48) and that a single oral administration of UDCA protects against Con-A-induced liver injury in mice (19).
Although the anti-inflammatory activity of UDCA may significantly improve aseptic inflammatory tissue damage in the liver, such as PBC and experimental Con-A-induced hepatitis, it may attenuate cellular immune response-mediated (IFN-\(\gamma\)-mediated) elimination of bacteria. UDCA treatment suppressed the LPS-induced IFN-\(\gamma\) production by the mouse liver MNC (Figs. 6, 7). This suppressive effect of UDCA may reduce the LPS-induced shock and multi-organ dysfunction including the liver, thereby increasing mouse survival after LPS challenge. UDCA-fed mice also reduced the proinflammatory cytokine responses to \(E. \text{coli}\) (Figs. 5B-D), which in turn may render mice susceptible to \(E. \text{coli}\) infection. It is quite different from the LPS challenge model.

IL-18 induces a potent IFN-\(\gamma\) production from NK/NKT cells in the presence of IL-12 (32). UDCA treatment suppressed serum IL-18 elevation in \(E. \text{coli}\)-challenged mice (Fig. 5E) and decreased in vitro LPS-stimulated IL-18 production in the liver MNC (Fig. 6). Exogenous IL-18 was injected into the UDCA-fed mice before the \(E. \text{coli}\) challenge to replace the insufficient production of IL-18, because IL-18 potently induce IFN-\(\gamma\) in normal and immunocompromised burn-injured mice after an \(E. \text{coli}\) infection, which improves the survival of burn-injured mice from infection (5, 22).

However, exogenous IL-18 injection neither affected serum IFN-\(\gamma\) levels nor survival after \(E. \text{coli}\) challenge in the UDCA-fed mice (Fig. 10A). UDCA-induced IFN-\(\gamma\) regulation thus may be independent of the IL-12/IL-18 pathway.

Nuclear receptors are closely involved in the homeostasis of bile acids (17). Although FXR and PXR were originally identified as orphan receptors, primary bile acid chenodeoxycholic acid (CDCA), secondary bile acid deoxycholic acid (DCA) and
Lithocholic acid (LCA) are physiological ligands for FXR (28, 34). LCA and 3-keto LCA are also ligands for PXR (42, 47). GR, which belongs to the steroid/thyroid-hormone receptor superfamily, is a ligand receptor of endogenous glucocorticoids and its activation strongly suppresses the inflammatory responses (6). The anti-inflammatory properties of UDCA appear to be similar to the effects of glucocorticoids (46), and UDCA reportedly activates GR in hepatocytes in a dose-dependent manner, although UDCA might not directly bind GR (46). Silencing GR also abrogated the suppressive effect of UDCA on IFN-γ production in the liver MNC (Fig. 9). However, UDCA-mediated GR activation is ligand-independent, but may facilitate the translocation of GR to the nucleus by dissociating GR from its cytosolic chaperone, heat shock protein 90 (41). Although UDCA treatment affected neither the mRNA level of FXR, PXR, nor GR in the whole liver tissue homogenates (Fig. 8A), it significantly upregulated GR expression in the mouse liver MNC (Figs. 7, 8B-E). PXR mRNA expression tended to be upregulated in the liver MNC of UDCA-fed mice, but FXR mRNA was not changed. UDCA might activate PXR (38) but not FXR (28), although the direct binding of UDCA to PXR has not been confirmed. Considering that proinflammatory cytokines such as TNF and IFN-γ are mostly produced in the Kupffer cells, liver NK cells, and NKT cells but not hepatocytes (12, 39), liver lymphocytes may play crucial roles in the immunomodulatory action of UDCA. UDCA may improve liver diseases including PBC by this immune mechanism. RU486, a GR antagonist, markedly restored the IFN-γ response to E. coli, LPS or Con-A in the UDCA-fed mice (Fig. 10B, D), thus suggesting that UDCA suppresses the IFN-γ response via activating GR. The inhibition of GR using RU486, as well as knockdown of GR using siRNA, also reportedly attenuated the inhibitory effect of
However, antagonizing GR by RU486 did not improve the survival of UDCA-fed mice following *E. coli* infection; moreover, a 20 mg/kg s.c. injection of RU486 was lethal for these mice (Fig. 10C). RU486 also impaired survival after the LPS and Con-A challenges in the UDCA-fed mice. Endogenous glucocorticoid might thus play a crucial role in the host defense to maintain homeostasis against various harmful stresses including bacterial infection, because the adrenal insufficiency renders the host extremely susceptible to sepsis and septic shock (9). Glucocorticoid might have many important actions on the host homeostasis, such as regulation of the cardiovascular system, neuroendocrine system and glucose metabolism (6). RU486 may inhibit beneficial host responses exerted by endogenous glucocorticoid in the UDCA-fed mice. In contrast to UDCA, which is located and acts exclusively in the liver and intestine, glucocorticoid acts everywhere in the host.

Ishizaki et al. demonstrated that the UDCA-treated mice tended to exhibit increased IL-10 levels in the liver homogenates after Con-A injection, although no clear changes in the plasma IL-10 level was observed after the UDCA treatment. We also did not observe any significant difference in the serum IL-10 levels between the UDCA-fed and control mice after Con-A injection. IL-10 is a representative anti-inflammatory cytokine and inhibits proinflammatory cytokine-induced tissue damage. Antagonizing GR by RU486 reportedly inhibits the IL-10 production in blood lymphocytes (45). The UDCA-induced hepatoprotective effects may also involve the secretion of IL-10 in the liver via the upregulation of GR. Further studies are required to confirm whether this is the case.
Collectively, UDCA may exert an immune-suppressive effect by reducing IFN-γ production by liver lymphocytes, such as NK and NKT cells and presumably also CD4⁺ T cells, in an IL-12/IL-18 independent but GR dependent manner, which may therefore be an important immunological mechanism regarding the effect of UDCA on cholestatic liver diseases.
References


39. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, and Hiraide H. The liver as a crucial organ in the first line of host defense: the roles of


46. **Weitzel C, Stark D, Kullmann F, Scholmerich J, Holstege A, and Falk W.** Ursodeoxycholic acid induced activation of the glucocorticoid receptor in primary rat

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Figure legends

**Fig. 1. The effect of UDCA on Con-A-induced hepatitis in mice.** The UDCA-fed mice and control mice were intravenously administered Con-A (15 mg/kg) to examine the survival (A), serum ALT at 12 h (B), TNF (C), and IFN-γ (D) levels.

**Fig. 2. Histological examinations of the mouse livers after Con-A administration and LPS or *E. coli* challenge.** The UDCA-fed mice and control mice were intravenously injected with Con-A (15 mg/kg), LPS (2.5 mg/kg) or *E. coli* (5 × 10⁸ CFU/mouse), and 24 h after the injection, the livers were obtained from the mice and stained with hematoxylin and eosin (×400). The arrows show that there was massive hepatocyte necrosis in the Con-A-administered control mice, focal necrosis in the LPS-challenged control mice, and severe infectious foci in the *E. coli*-challenged UDCA-fed mice. Representative data are shown from five mice in each group.

**Fig. 3. Con-A-induced IFN-γ production from the liver and spleen MNC.** The liver and spleen MNC were obtained from the UDCA-fed and control mice (n=5 in each group) and were cultured with or without Con-A (5 μg/mL) for 24 h to measure IFN-γ levels in the supernatants.

**Fig. 4. The effect of UDCA on LPS-challenged mice.** The UDCA-fed mice and control mice were intravenously challenged with LPS (2.5 mg/kg) to examine the serum TNF (A), IL-12 (B), IL-18 (C), and IFN-γ (D) levels.

**Fig. 5. The effect of UDCA on *E. coli*-challenged mice.** The UDCA-fed mice and control mice were intravenously challenged with *E. coli* (5 × 10⁸ CFU/mouse) to examine the survival (A), serum ALT at 12 h (B), AST at 12 h (C), TNF (D), IL-18 (E), and IFN-γ (F) levels.
Fig. 6. LPS-induced cytokine production from the liver and spleen MNC. Liver and spleen MNC were obtained from the UDCA-fed and control mice (n=5 in each group) and were cultured with or without LPS (10 μg/mL) for 2 h to measure TNF or 24 h to measure other cytokine levels in the supernatants.

Fig. 7. IFN-γ-positive cells in the liver MNC after the Con-A (B) or LPS (C) challenge and GR expression-positive cells in the liver MNC (D) in the UDCA-fed mice. We gated NK1.1 positive but TCR β negative cells as the NK cells, and NK1.1 positive and TCR β positive cells as the NKT cells (A) to examine their IFN-γ intensity (B, C) or GR intensity (D). Similarly, we also stained the cells and gated CD4 positive and TCR β positive cells as the CD4+ T cells (A) and examined their IFN-γ (B, C) or GR (D) intensity. Liver MNC were obtained from the UDCA-fed or control mice 12 h after Con-A administration, followed by incubation for 2 h (B), or were obtained at 2 h after LPS challenge, followed by incubation for 2 h (C), to examine the IFN-γ expression. Liver MNC were also obtained from the UDCA-fed and control mice to examine the GR expression (D). The data from UDCA-fed mice are shown by a solid line, data from control mice are shown with a dotted line, and the isotype is shown by a shaded area. Representative data are shown from five mice in each group, and the percentages of IFN-γ- and GR-positive cells are shown as the mean ± SE.

Fig. 8. Expression of nuclear receptor mRNA and GR protein levels in the liver or spleen MNC and tissue homogenates. Liver homogenates (A), MNC (B), or hepatic lymphocytes (C) were obtained from the UDCA-fed and control mice to examine mRNA levels of GR, FXR, and PXR by the real-time quantitative PCR assay. Protein expression of GR in the liver tissue homogenates (D), liver MNC (E) and spleen MNC
Fig. 9. The effect of GR siRNA on the LPS (A)- or Con-A (B)-induced IFN-γ production in the liver MNC of UDCA-fed mice. The liver MNC of the UDCA-fed mice were treated with GR siRNA for 36 h. Subsequently, the cells were cultured with or without LPS (10 µg/mL) (A), or cultured with or without Con-A (10 µg/mL) (B) for 24 h, and then the culture medium and cells were collected to examine the IFN-γ production and GR expression. The data shown are from five individual experiments.

Fig. 10. The effect of i.p. injection of exogenous IL-18 on UDCA-fed mice challenged with *E. coli* (A), and the effect of s.c. injection with RU486 on UDCA-fed mice challenged with *E. coli* (B, C) or Con-A (D, E). Recombinant IL-18 (10 µg/kg) or PBS was injected i.p. into the UDCA-fed or control mice 2 h before *E. coli* (5 × 10⁸ CFU/mouse) challenge, and their serum IFN-γ levels were examined (A). RU486 (10 or 20 mg/kg) or DMSO was injected s.c. into the UDCA-fed or control mice 2 h before *E. coli* (B, C) or Con-A (D, E) challenge, and their serum IFN-γ levels (B, D) and survival rates (C, E) were examined.
Fig. 1

A. Survival rates (%)

B. Serum ALT at 12 h (IU/mL)

C. Serum TNF (ng/mL)

D. Serum IFN-γ (ng/mL)

Mean ± SE  † p<0.05 vs Control
Fig. 2

UDCA Control

Con-A

LPS

E. coli
Fig. 3

IFN-γ levels in supernatants (ng/mL)

<table>
<thead>
<tr>
<th></th>
<th>Liver MNC</th>
<th>Spleen MNC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con-A (-)</td>
<td>UDCA</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Mean ± SE  * p<0.01, † p<0.05 vs Con-A (-)
**Fig. 4**

A. Serum TNF

B. Serum IL-12

C. Serum IL-18

D. Serum IFN-γ

Mean ± SE  † p<0.05 vs Control

Control (n=10)  UDCA (n=10)
Fig. 5

A

Survival rates (%)

100
80
60
40
20
0

1 3 5 7 day

Control (n=15)

UDCA (n=15) †

B

Serum ALT at 12 h (IU/mL)

720
600
500
400
300
200
100
0

UDCA (n=15)

Control (n=15)

C

Serum AST at 12 h (IU/mL)

600
500
400
300
200
100
0

UDCA (n=15)

Control (n=15)

D

Serum TNF (ng/mL)

0.8
0.6
0.4
0.2
0

0 1 3 6 12 24 48 72 h

UDCA (n=15)

Control (n=15)

E

Serum IL-18 (ng/mL)

5
4
3
2
1
0

0 1 3 6 12 24 48 72 h

UDCA (n=15)

Control (n=15)

F

Serum IFN-γ (ng/mL)

12
10
8
6
4
2
0

0 1 3 6 12 24 48 72 h

UDCA (n=15)

Control (n=15)

* p<0.01, † p<0.05 vs Control

Mean ± SE
Liver Spleen

![Graphs showing cytokine levels in UDCA and control groups in liver and spleen.](#)

**Fig. 6**

- **TNF (ng/mL)**
  - Liver: UDCA group shows a higher TNF level compared to the control group.
  - Spleen: Similar pattern observed.

- **IL-12 (ng/mL)**
  - Liver: UDCA group shows a higher IL-12 level compared to the control group.
  - Spleen: Similar pattern observed.

- **IL-18 (ng/mL)**
  - Liver: UDCA group shows a higher IL-18 level compared to the control group.
  - Spleen: Similar pattern observed.

- **IFN-γ (ng/mL)**
  - Liver: UDCA group shows a higher IFN-γ level compared to the control group.
  - Spleen: Similar pattern observed.

**Mean ± SE**

† p<0.05 vs Control
Fig. 7

A

NK cells

NKT cells

TCR β

CD4+ T cells

B

NK cells

NKT cells

CD4+ T cells

Con-A

Percentage of positive cells

Control 27 ± 2%

UDCA 11 ± 1% *

isotype

Control 35 ± 2%

UDCA 12 ± 1% *

Con-A

Percentage of positive cells

Control 4 ± 0.4%

UDCA 2 ± 0.4%

C

IFN-γ

LPS

Percentage of positive cells

Control 15 ± 1%

UDCA 7 ± 1% *

isotype

Control 31 ± 1%

UDCA 8 ± 1% *

D

IFN-γ

GR

Percentage of positive cells

Control 11 ± 1%

UDCA 25 ± 2% *

isotype

Control 12 ± 1%

UDCA 25 ± 1% *

Control 9 ± 1%

UDCA 14 ± 1%

* p<0.01, † p<0.05 vs Control
Mean ± SE  * p<0.01 vs Control  Gapdh was used as an internal control.
A

![Graph A](image)

*Fig. 9*

<table>
<thead>
<tr>
<th>IFN-γ (ng/mL)</th>
<th>in vitro LPS</th>
<th>siRNA control</th>
<th>siRNA GR</th>
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</thead>
<tbody>
<tr>
<td>0.25</td>
<td>(-) (+)</td>
<td>(-) (+)</td>
<td>IB: GR</td>
</tr>
</tbody>
</table>

B

![Graph B](image)

Mean ± SE

* p<0.01 vs others, † p<0.05 vs Con-A (-) siRNA control
Fig. 10

A

Serum IFN-γ (ng/mL) vs Time (h)

B

Serum IFN-γ (ng/mL) vs Time (h)

C

Survival rates (%) vs Time (day)

D

Serum IFN-γ at 6 h (ng/mL)

E

Survival rates (%) vs Time (day)

* p<0.01, † p<0.05 vs others, ‡ p<0.05 vs Control+PBS

** p<0.01 vs DMSO

Mean ± SE
Table 1. Total bile acid and bile acid fraction levels in the liver of UDCA-fed and control mice

<table>
<thead>
<tr>
<th></th>
<th>UDCA group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bile acid (μmol/L)</td>
<td>513.3 ± 205.0 *</td>
<td>19.5 ± 3.1</td>
</tr>
<tr>
<td>UDCA (μmol/L)</td>
<td>502.3 ± 202.0 *</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>CA (μmol/L)</td>
<td>0.3 ± 0.3</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>CDCA (μmol/L)</td>
<td>5.1 ± 2.4</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>DCA (μmol/L)</td>
<td>2.8 ± 0.9</td>
<td>9.7 ± 1.8</td>
</tr>
<tr>
<td>LCA (μmol/L)</td>
<td>2.5 ± 0.5</td>
<td>1.0 ± 0.8</td>
</tr>
</tbody>
</table>

Data represent the mean ± SE of five mice in each group, *p<0.01 vs. control.