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

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Takigawa T, Miyazaki H, Kinoshita M, Kawarabayashi N, Nishiyama K, Hatsuse K, Ono S, Saitoh D, Seki S, Yamamoto J. Glucocorticoid receptor-dependent immunomodulatory effect of ursodeoxycholic acid on liver lymphocytes in mice. Am J Physiol Gastrointest Liver Physiol 305: G427–G438, 2013. First published July 18, 2013; doi:10.1152/ajpgi.00205.2012—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% (wt/vol) UDCA for 4 wk. UDCA improved hepatocyte injury and survival in concanavalin-A (Con-A)-induced hepatitis by suppressing IFN-γ production by liver mononuclear cells (MNC), especially NK and NKT cells. UDCA also increased survival after lipopolysaccharide (LPS)-challenge; however, it increased mortality of mice following Escherichia 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 potentiously induce IFN-γ production. However, unlike normal mice, exogenous IL-18 pretreatment did not increase the serum IFN-γ 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-γ production. Furthermore, RU486, a GR antagonist, restored the serum IFN-γ level in UDCA-fed mice after E. coli, LPS, or Con-A challenge. Taken together, these results suggest that IFN-γ-reducing immunomodulatory property of UDCA is mediated by elevated GR in the liver lymphocytes in an IL-12/IL-18-independent manner.

Conclusions: Our results suggest that UDCA treatment alters the expression of hepatic immunomodulatory cytokines and the expression of the immunomodulatory cytokine receptor GR, in mice under unstimulated conditions (24). It would therefore be interesting to elucidate how such in vivo UDCA treatment affects these nuclear receptors and host immune responses.

Clinical implications: UDCA treatment is administered orally to patients. Bile acid-supplemented diets have been fed to experimental animals to investigate hepatotoxicity of bile acids or their potential therapeutic application, because bile acid components might vary in their pathological and physiological response in vivo (1, 49). Therefore, subject mice were fed diets supplemented with 0.3% (wt/vol) UDCA for 4 wk. 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 in these UDCA-fed mice. UDCA treatment markedly suppressed IFN-γ 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 Escherichia 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-γ-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

Data are expressed as means ± SD. Unless otherwise stated, the statistic used was the Student t-test. Significance was accepted at a value of P < 0.05.
Mice and diet supplemented with UDCA. Male C57BL/6 mice were used in this study (6 wk old, 15–18 g, SLC, Shizuoka, Japan). UDCA was kindly provided by Tanabe Mitsubishi Pharma (Osaka, Japan). UDCA-treated mice were fed a diet (CLEA Rodent Diet CE-7, CREA Japan, Tokyo, Japan) supplemented with 0.3% (wt/vol) UDCA for 4 wk. Control mice were fed a diet without any supplementation. All mice had free access to both drinking water and the respective diets.

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

<table>
<thead>
<tr>
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<th>UDCA Group</th>
<th>Control Group</th>
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<tr>
<td>Total bile acid, µmol/l</td>
<td>513.3 ± 205.0*</td>
<td>19.5 ± 3.1</td>
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<tr>
<td>UDCA, µmol/l</td>
<td>502.3 ± 202.0*</td>
<td>3.8 ± 0.9</td>
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<tr>
<td>CA, µmol/l</td>
<td>0.3 ± 0.3</td>
<td>2.7 ± 0.6</td>
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<tr>
<td>CDCA, µmol/l</td>
<td>5.1 ± 2.4</td>
<td>2.4 ± 0.1</td>
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<tr>
<td>DCA, µmol/l</td>
<td>2.8 ± 0.9</td>
<td>9.7 ± 1.8</td>
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<tr>
<td>LCA, µmol/l</td>
<td>2.5 ± 0.5</td>
<td>1.0 ± 0.8</td>
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Data represent means ± SE of 5 mice in each group; *P < 0.01 vs. control.

UDCA, ursodeoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid.

Fig. 1. Effect of ursodeoxycholic acid (UDCA) on concanavalin-A (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 alanine aminotransferase (ALT) at 12 h (B), TNF (C), and IFN-γ (D) levels.
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 IgG3, for perforin and granzyme MAbs, eBioscience) at 4°C for 30 min. After washing, the cells were analyzed with an EPICS XL instrument (Beckman Coulter). The NK cells were positive for NK1.1 staining but negative for TCR β staining, whereas 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, we evaluated their intracellular intensity of IFN-γ, perforin, or granzyme. 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 with the EPICS XL instrument.

**Real-time quantitative PCR.** Total RNA extraction was performed with an RNeasy Mini Kit (QIAGEN, Valencia, CA), by 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) with SYBR Green PCR reagents. The primers were designed by Takara Bio (Tokyo, Japan). FXR: Mus musculus nuclear receptor subfamily 1, group I, member 2 (Nr1i2), transcript variant 2, mRNA; PXR: Mus musculus nuclear receptor subfamily 1, group H, member 5 (Nr1i5), transcript variant 1, mRNA; GR: Mus musculus 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).

**siRNA transfection.** Liver MNC from UDCA-fed mice were transfected with Lipofectamine 2000 (Invitrogen, Tokyo, Japan) according to the manufacturer’s instructions. The predesigned mouse GR small interfering RNA (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 medium without antibiotics and 0.2 ml of Lipofectamine 2000-siRNA complex. A total of 2 μl of Lipo-
fectamine 2000 in 100 μl 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 36 h. 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 iv injected into the control and UDCA-fed mice twice (3 and 1 days before the challenge), after which Con-A was iv challenged into the mice. The anti-NK1.1 Ab depleted more than 90% of NK cells and 85% of NKT cells for approximately 7 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 (18, 22, 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, ALT, AST, and cytokine levels. The mice were euthanized by exsanguination from the subclavian artery and vein under lethal anesthesia using pentobarbital sodium, 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 alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by use of a FUJI dry-chem system (FUJIFILM, Tokyo, Japan). Cytokines in the sera or culture supernatants were measured by 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 by routine procedure.

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

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**Fig. 3.** Con-A-induced IFN-γ production from the liver and spleen mononuclear cells (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.** 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.
RESULTS

Total bile acid and its fraction levels in the liver of UDCA-fed mice. UDCA-fed mice showed ~25 times higher concentration of total bile acid in the liver homogenates compared with 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.

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 compared with 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, whereas 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

Fig. 5. 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), aspartate aminotransferase (AST) at 12 h (C), TNF (D), IL-18 (E), and IFN-γ (F) levels.
amount of IFN-γ in response to in vitro Con-A stimulation to those of the control mice (Fig. 3).

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 (Fig. 4, A and 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, whereas 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).

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 (Fig. 5, B and 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 (Fig. 5, D–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 compared with 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⁺ T cells (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 h after Con-A administration (and 2 h of additional incubation) (Fig. 7B) and 2 h after the LPS challenge (and 2 h of additional incubation) (Fig. 7C) compared with 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 (Fig. 7, B and C, CD8⁺ T cells are not shown). Both the UDCA-fed and control mice exhibited ~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 compared with that of the control mice (Fig. 8B). GR mRNA expression was further
examined in the nonadherent hepatic lymphocytes. The UDCA-fed mice showed a threefold 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. 8, D–F). UDCA-fed mice showed a higher tendency of PXR mRNA expression of the liver MNC compared with 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 compared with that observed in the control mice (Fig. 7D, CD8+ T cells not shown).

**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. 9, A and B), thus suggesting that the increased GR of the liver MNC in UDCA-fed mice is functional.

**Effect of exogenous IL-18 injection or RU486 treatment on the IFN-γ response to 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 E. coli (5 × 10^8 CFU/mouse) or LPS (5 mg/ml) challenge (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 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 sc) significantly increased the serum IFN-γ levels after *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 after *E. coli* challenge (Fig. 10A). RU486 treatment (10 mg/kg sc) 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 6 h 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.

**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. nondepleted: 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 deple-
cells, such as CD4/H9253 each group). There is a possibility that other IFN-production bile acids that are cytotoxic in the presence of hydrophilic bile/H9253 a significant level of IFN-production conditions in vivo, even if the intracellular staining did not detect.

Fig. 9. Effect of GR small interfering RNA (siRNA) on 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 5 individual experiments. IB, immunoblot.

tion, there were no differences in the serum IFN-γ levels at 6 h between the mice (NK/NKT depleted: 1.1 ± 0.1 vs. nondepleted: 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 6 h 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. nondepleted: 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 by UDCA. UDCA is thought to exert a hepatoprotective effect. In addition, UDCA has an anticholestatic (14), antioxidative (25, 40), and antiapoptotic effects (2). 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 downregulates 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-γ-mediated) elimination of bacteria. UDCA treatment suppressed the LPS-induced IFN-γ production by the mouse liver MNC (Figs. 6 and 7). This suppressive effect of UDCA may reduce the LPS-induced shock and multiorgan dysfunction including the liver, thereby increasing mouse survival after LPS challenge. UDCA-fed mice also reduced the proinflammatory cytokine responses to E. coli (Fig. 5, B–D), which in turn may render mice susceptible to E. coli infection. It is quite different from the LPS challenge model.

IL-18 induces a potent IFN-γ production from NK/NKT cells in the presence of IL-12 (32). UDCA treatment suppressed serum IL-18 elevation in E. 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. coli challenge to replace the insufficient production of IL-18, because IL-18 potently induce IFN-γ in normal and immunocompromised burn-injured mice after an E. coli infection, which improves the survival of burn-injured mice from infection (3, 22). However, exogenous IL-18 injection affected neither serum IFN-γ levels nor survival after E. coli challenge in the UDCA-fed mice (Fig. 10A). UDCA-induced IFN-γ 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 chenodeoxy-
cholic 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 (4). 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). RU486 (10 or 20 mg/kg) or DMSO was injected subcutaneously into the UDCA-fed or control mice 2 h before E. coli (B and C) or Con-A (D and E) challenge, and their serum IFN-γ levels were examined (A). RU486 (10 or 20 mg/kg) or DMSO was injected subcutaneously into the UDCA-fed or control mice 2 h before E. coli (B and C) or Con-A (D and E) challenge, and their serum IFN-γ levels (B and D) and survival rates (C and E) were examined.

Fig. 10. Effect of intraperitoneal (ip) injection of exogenous IL-18 on UDCA-fed mice challenged with E. coli (A), and the effect of subcutaneous (sc) injection with RU486 on UDCA-fed mice challenged with E. coli (B and C) or Con-A (D and E). Recombinant IL-18 (10 μg/kg) or PBS was injected ip 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 sc into the UDCA-fed or control mice 2 h before E. coli (B and C) or Con-A (D and E) challenge, and their serum IFN-γ levels (B and D) and survival rates (C and E) were examined.

RU486, a GR antagonist, markedly restored the IFN-γ response to E. coli, LPS, or Con-A in the UDCA-fed mice (Fig. 10, B and D), thus suggesting that UDCA suppresses the IFN-γ response via activating GR. The inhibition of GR with RU486, as well as knockdown of GR with siRNA, also reportedly attenuated the inhibitory effect of UDCA on the DCA-induced Golgi fragmentation in a colon cancer cell line (8). However, antagonizing GR by RU486 did not improve the survival of UDCA-fed mice following E. coli infection; moreover, a 20 mg/kg sc injection of RU486 was lethal for these mice (Fig. 10C). RU486 also impaired survival after the LPS and Con-A

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 (10, 39), liver lymphocytes may play crucial roles in the immunomodulatory action of UDCA. UDCA may improve liver diseases including PBC by this immune mechanism.
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 (7). Glucocorticoid might have many important actions on the host homeostasis, such as regulation of the cardiovascular system, neuroendocrine system, and glucose metabolism (4). 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. (19) 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.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS
T.T., H.M., and M.K. conception and design of research; T.T., H.M., M.K., T.T., T.T., H.M., and M.K. prepared figures; M.K. and S.S. interpreted results of experiments; H.M. and M.K. prepared manuscript; M.K. and S.S. edited and revised manuscript; M.K. and J.Y. approved final version of manuscript.

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Effect of UDCA on liver lymphocytes in mice


