Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 537 (2006) 181–189
www.elsevier.com/locate/ejphar

Preventive effects of (5R)-5-hydroxytriptolide on concanavalin A-induced hepatitis

Ru Zhou [a], Wei Tang [a], Yong-Xin Ren [a], Pei-Lan He [a], Yi-Fu Yang [a], Yuan-Chao Li [b], Jian-Ping Zuo [a,*]

[a] Laboratory of Immunopharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, PR China
[b] Laboratory of Chemistry, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, PR China

Received 19 January 2006; received in revised form 7 March 2006; accepted 10 March 2006
Available online 20 March 2006

Abstract

(5R)-5-hydroxytriptolide (LLDT-8) exhibits strong immunosuppressive activities in vitro and in vivo. Here, we investigated the effects of LLDT-8 on concanavalin A-induced hepatitis. Liver damage was evaluated by serum alanine transaminase (ALT) level and liver histology. The effects of LLDT-8 were determined by measurement of serum cytokines, lymphocyte proliferation assay, flow cytometry analysis of splenic T cell percentage and apoptosis, reverse-transcription polymerase chain reaction (RT-PCR) analysis for gene transcriptions. In LLDT-8-treated mice, serum ALT level and histological damage were markedly attenuated. The beneficial effect of LLDT-8 was closely associated with (i) reduction of serum tumor necrosis factor-α, interferon-γ (IFN-γ), interleukin-2, interleukin-12, and interleukin-6 levels; (ii) elimination of activated T cells by increasing proapoptotic genes signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor-1 (IRF-1) expression in spleens; (iii) blockade of mRNA expressions for chemokines (monokine induced by IFN-γ, Mig; IFN-γ-inducible protein-10, IP-10; IFN-inducible T cell-α chemoattractant, I-TAC), vascular adhesion molecule-1 (VCAM-1), and chemokine receptors (C-C chemokine receptor 1, CCR1; C-C chemokine receptor 5, CCR5; C-X-C chemokine receptor 3, CXCR3) in livers. These results suggested the therapeutic potential of LLDT-8 in IFN-γ/STAT1/IRF-1 signaling- and inflammatory cytokines-mediated immune disorders.

Keywords: (5R)-5-hydroxytriptolide; Immunosuppressive; IFN-γ

1. Introduction

T cell-mediated immune responses play an essential role in hepatocellular injury induced by autoimmune hepatitis, viral infection, and hepatotoxins (Bogdanos et al., 2000; Kita et al., 2001; Rehermann and Chisari, 2000). Activated T cells are detected in a variety of human liver diseases. T cell-mediated hepatitis can be induced in rodents by injection of concanavalin A, which rapidly induces clinical and histological evidence of hepatitis, including elevation of transaminase activities within 8–24 h. Liver histology shows massive granulocyte accumulation, T cell infiltration, necrosis, and apoptosis (Tiegs et al., 1992). Effector cells in this model are activated $CD4^+$ T cells, which, together with macrophages, induce hepatocyte apoptosis by direct contact or indirectly through tumor necrosis factor-α (TNF-α) (Gantner et al., 1995; Tiegs et al., 1992).

The induction of concanavalin A-induced hepatitis is associated with the production of various cytokines. In this hepatitis model, TNF-α and interferon-γ (IFN-γ) have direct implications for the induction of liver cell injury, as anti-TNF-α and anti-IFN-γ Abs protect from concanavalin A-induced liver injury (Gantner et al., 1995; Kusters et al., 1996). Mice overexpressing IFN-γ in the liver suffer from chronic hepatitis (Toyonaga et al., 1994).

* Corresponding author. Tel./fax: +86 21 50806701.
E-mail address: jpzuo@mail.shcnc.ac.cn (J.-P. Zuo).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.03.013

IFN-γ has been reported to directly induce cellular apoptosis in cultured hepatocytes in an interferon regulatory factor-1 (IRF-1)-dependent manner (Kano et al., 1999). IFN-$\gamma^{-/-}$ and signal transducer and activator of transcription 1 (STAT1)$^{-/-}$ mice show resistance to concanavalin A-induced liver injury (Hong et al., 2002; Siebler et al., 2003; Streetz et al., 2001). And the detrimental effects of IFN-γ/STAT1 in liver injury is mediated partly through induction of the proapoptotic IRF-1 gene and, consequently, induction of hepatocyte apoptosis and hepatocellular damage (Hong et al., 2002).

The IFN-γ/STAT1/IRF-1 pathway also plays a crucial role in leukocyte infiltration into the liver in concanavalin A-mediated T cell hepatitis model. In addition to induction of apoptosis, IFN-γ also stimulated hepatocytes, sinusoidal endothelial cells, and Kupffer cells partly via a STAT1/IRF-1-dependent mechanism to produce multiple chemokines and adhesive molecules that are responsible for promoting infiltration of leukocytes and, ultimately resulting in hepatitis (Jaruga et al., 2004). Among them, expression of monokine induced by IFN-γ (Mig), IFN-γ-inducible protein-10 (IP-10), IFN-inducible T cell-α chemoattractant (I-TAC), epithelial neutrophil-activating peptide (ENA)-78, and adhesion molecules (ICAM-1 and VCAM-1) was markedly attenuated in IFN-$\gamma^{-/-}$, STAT1$^{-/-}$, and IRF-1$^{-/-}$ mice. Disruption of the IRF-1 gene abolished concanavalin A-induced liver injury and suppressed leukocyte infiltration into the liver (Jaruga et al., 2004). Thus, the IFN-γ/STAT1/IRF-1 pathway might provide one potential target to prevent concanavalin A hepatitis.

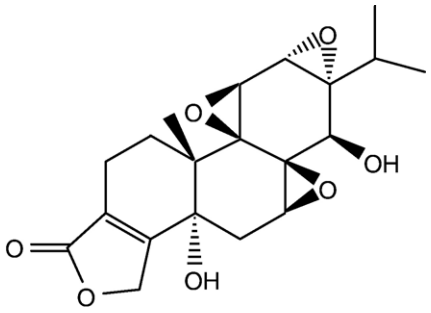
Tripterygium wilfordii Hook F. (TWHF) has been used as a herbal remedy to treat arthritis and other autoimmune inflammatory disorders for several centuries in China. Triptolide is the most active component accounting for the immunosuppressive effects of TWHF (Chen, 2001; Qiu and Kao, 2003). However, the therapeutic potential of triptolide is handicapped by its strong toxicity (Huynh et al., 2000). (5R)-5-hydroxytriptolide (LLDT-8) is a novel analog of triptolide that has shown potent immunosuppressive activities but greatly reduced toxicities in our previous studies (Zhou et al., 2005, 2006). In animal disease models, we found that LLDT-8 potently suppressed bovine type II collagen-induced arthritis in DBA/1 mice (unpublished observations), prevented graft-versus-host disease and prolonged the allogeneic cardiac graft survival in C57BL/6 mice (Tang et al., 2005, 2006). The structure of LLDT-8 is shown in Fig. 1.

No report of triptolide on hepatitis is shown. The present study was to assess the immunomodulatory effect of LLDT-8 in a murine model of concanavalin A-induced, T-cell dependent hepatitis. The in vivo and in vitro results demonstrated that administration of LLDT-8 significantly ameliorated liver injury through regulating IFN-γ/STAT1/IRF-1 signaling and inhibiting inflammatory cytokines production.

2. Materials and methods

2.1. Mice

Female BALB/c mice (6–8 weeks old, 20–22 g) were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences. The animals were housed in specific pathogen-free conditions. All mice were allowed to acclimatize in our facility for 1 week before any experiments were started. All experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica.



Formula: $C_{20}H_{24}O_7$

Molecular weight: 376

Fig. 1. Chemical structure of (5R)-5-hydroxytriptolide (LLDT-8).

2.2. Induction of concanavalin A-induced hepatitis and drug administration

Concanavalin A (Sigma, St. Louis, USA) was dissolved in pathogen-free saline and filtered through 0.45 μm filter (Minipore). Mice liver injury was induced by injection into the tail vein at the indicated doses of concanavalin A (in 0.2 ml saline). (5R)-5-hydroxytriptolide (LLDT-8) was synthesized from triptolide that was separated from TWHF. LLDT-8 is the white amorphous powder with 99% purity by reverse phase high performance liquid chromatography. Stock solution of LLDT-8 was prepared in dimethyl sulphoxide (DMSO, Sigma) and diluted in pathogen-free saline to the doses required. LLDT-8 was administered intraperitoneally (i.p., in 0.2 ml solvent) once daily on days −3, −2, −1 and 1 h before concanavalin A injection on day 0, or where indicated.

2.3. Liver transaminase activity

Sera from individual mice were obtained 24 h after concanavalin A injection. Alanine transaminase (ALT) activity was measured by the standard photometric method with a commercial kit (Rongsheng, Shanghai, China).

2.4. Quantification of serum cytokine

Sera from individual mice were collected 1, 3, 8, and 24 h after concanavalin A injection and stored at −20 °C. TNF-α, interleukin-2 (IL-2), IFN-γ, IL-12p40, IL-12p70, and IL-6 concentrations were determined by ELISA (BD PharMingen, San Diego, CA, USA).

2.5. Cell preparation

Mice were sacrificed and their spleens were removed aseptically. A single spleen cell suspension was prepared and cell debris and clumps were removed. Erythrocytes were lysed with Tris-buffered ammonium chloride. Mononuclear cells were washed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 mg/ml).

2.6. Proliferation assay

Splenocytes (5×10^6 cells/ml) were cultured in triplicate with medium alone, or stimulated with concanavalin A (5 μg/ml) for 24 h in 96-well plates at 37 °C incubator with 5% CO_2. Cells were pulsed with 0.25 μCi of [^{3}H]-thymidine for 8 h and harvested onto glass fiber filters. The incorporated radioactivity was then counted using a Beta Scintillation Counter (MicroBeta Trilux, PerkinElmer Life Sciences, Boston, MA).

2.7. Flow cytometry analysis

Spleens were harvested 24 h after concanavalin A injection. To analyze the T cell percentage, spleen cells were stained with FITC conjugated anti-mouse CD3 mAb (PharMingen), and analyzed on a fluorescence activated cell sort (FACS) Calibur (Becton Dickinson, CA). To determine apoptosis, spleen cells were stained with PI/Annexin V and analyzed by flow cytometry.

2.8. Concanavalin A-induced splenocyte apoptosis in vitro

Spleen cells from naive BALB/c mice were preactivated with 5 μg/ml concanavalin A for 24 h. Then cells were washed and treated with or without 100 nM LLDT-8 in the presence of 5 μg/ml concanavalin A. 48 h later, cells were collected and stained with PI/Annexin V and analyzed by flow cytometry.

2.9. Reverse-transcription polymerase chain reaction (RT-PCR) analysis

B cell-depleted population was prepared by immunomagnetic negative selection (Yang et al., 2002). Briefly, spleen cells were incubated with magnetic particles bound to goat anti-mouse Ig (Qiagen, Valencia, CA), followed by removing cell-bound magnetic particles with a rare earth magnet (Polysciences, Inc). B cell-depleted spleen cells or liver homogenates were lysed using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated, reverse transcribed, and PCR amplified using specific primers. RT-PCR products were visualized by electrophoresis through 1% agarose gels containing ethidium bromide.

Gene-specific primers were as follows:

STAT1: (sense) 5′-CACGCTGCCTATGATGTCTC-3′, (anti-sense) 5′-ACGCTTGCTTTTCCGTATGT-3′. The cDNA was amplified by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min.

IRF-1: (sense) 5′-TTAGCCCGGACACTTTCTCTGATGG-3′, (anti-sense) 5′-GTCCCCTCGAGGGCTGTCAATCTC-T-3′. The cDNA was amplified by 30 cycles of denaturation at 94 °C for 45 s, annealing at 61 °C for 45 s, and extension at 72 °C for 90 s.

Mig: (sense) 5′-ATGAAGTCCGCTGTTCTTTTCC-3′, (anti-sense) 5′-TTATGTAGTCTTCCTTGAACGAC-3′. The cDNA was amplified by 30 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 30 s, and extension at 72 °C for 90 s.

IP-10: (sense) 5′-CCTATCCTGCCCACGTGTTG-3′, (anti-sense) 5′-CGCACCTCCACATAGCTTACA-3′. The cDNA was amplified by 30 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 30 s, and extension at 72 °C for 90 s.

I-TAC: (sense) 5′-GAACAGGAAGGTCACAGCCA-TAGC-3′, (anti-sense) 5′-ATGAGGCGAGCTTGCTTG-GATCTG-3′. The cDNA was amplified by 35 cycles of denaturation at 94 °C for 30 s, annealing at 66 °C for 30 s, and extension at 72 °C for 45 s.

VCAM-1: (sense) 5′-CAAGGGTGACCAGCTCATGA-3′, (anti-sense) 5′-TGTGCAGCCACCTGAGATCC-3′. The cDNA was amplified by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min.

CCR1: (sense) 5′-TTTTAAGGCCCAGTGGGAGTT-CACTCACCG-3′, (anti-sense) 5′-TGGTATAGCCACATG-CCTTTGAAACAGCTGC-3′. The cDNA was amplified by 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min.

CCR5: (sense) 5′-TACCAGATCTCAGAAAGAAGGTTT-TCATTA-3′, (anti-sense) 5′-GCGTTTGACCATGTGTTTT-CGGAAGAACACT-3′. The cDNA was amplified by 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min.

CXCR3: (sense) 5′-GCTAGATGCCTCGGACTTTG-3′, (anti-sense) 5′- GCTGATCGTAGTTGGCTGATA-3′. The cDNA was amplified by 40 cycles of denaturation at 94 °C for 2 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min.

HPRT: (sense) 5′-GTTGGATACAGGCCAGACTTTGTT-G-3′, (anti-sense) 5′-GAGGGTAGGCTGGCCTATAGGCT-3′. The cDNA was amplified by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min.

2.10. Histological examination

Mice were sacrificed 24 h after concanavalin A injection and their livers were removed, fixed in 10% formalin, and embedded in paraffin. Five μm sections were stained with hematoxylin–eosin (H&E), and examined by two pathologists that were blinded to the experiment.

2.11. Statistical analysis

Data are expressed as mean ± S.E.M. of indicated experiments. Dunnett's multiple comparison test was used to

determine variances between groups where appropriate. p value <0.05 was considered significant.

3. Results

3.1. LLDT-8 effectively attenuated concanavalin A-induced liver damage

Concanavalin A can be toxic to animals when given orally or parenterally (Tyan, 1974). In this study, concanavalin A induced liver damage in a dose-dependent manner (data not shown). Concanavalin A-treated mice receiving solvent alone served as vehicle control. Death consistently occurred 8–24 h after challenging each mouse with 30 mg/kg concanavalin A by injection into the tail vein, and the survival rate was only about 40% at 24 h (Fig. 2A). Administration of LLDT-8 significantly improved the survival of hepatitis mice. Pretreatment with 0.5, 1 or 2 mg/kg LLDT-8 (by i.p.) four times (on days −3, −2, −1 and 1 h before concanavalin A injection) significantly increased the survival rates to 83%, 86% and 100%, respectively. The LD_{50} of acute toxicity for LLDT-8 is 9.3 mg/kg (i.p.) (Zhou et al., 2005). At 0.5, 1, and 2 mg/kg, LLDT-8 does not affect the mobility, body weight, spleen weight, and thymus weight of normal mice (data not shown). Due to the high mortality when mice were injected with 30 mg/kg concanavalin A, dose of 20 mg/kg concanavalin A per mouse was used in subsequent experiments according to our preliminary study.

Liver injury can be manifested by a significant elevation of serum ALT level (Shirin et al., 1998). Intravenous administration of concanavalin A resulted in a time-dependent and dose-dependent increase in serum ALT level (data not shown). Pretreatment with 0.5, 1 or 2 mg/kg LLDT-8 four times significantly decreased the serum ALT level that was determined 24 h after concanavalin A injection (Fig. 2B), and pretreatment with 2 mg/kg LLDT-8 twice (on day −1 and 1 h before concanavalin A injection) also remarkably reduced the ALT level (952 versus 2304 U/l, $p<0.05$). We also investigated the hepatoprotective effect of LLDT-8 after the concanavalin A injection, and a single dose of LLDT-8 at 2 mg/kg (1 h after concanavalin A injection) effectively inhibited the ALT increase in sera (533 versus 2304 U/l, $p<0.05$).

Mice were pretreated with 0.5, 1 or 2 mg/kg LLDT-8 four times and sacrificed at 24 h after concanavalin A injection. Livers were harvested for histological examination. In vehicle-treated hepatitis mice, massive necrosis was observed in livers at 24 h after concanavalin A injection (Fig. 2C, panel i). The size and number of necrotic lesions were significantly reduced by LLDT-8 treatment. Marked alleviation of multifocal hepatocellular necrosis was observed in 0.5 mg/kg LLDT-8-treated hepatitis mice (Fig. 2C, panel ii). And this liver damage was abrogated in hepatitis mice treated with 1 mg/kg (Fig. 2C, panel iii) or 2 mg/kg LLDT-8 (Fig. 2C, panel iv).

3.2. LLDT-8 decreased the serum cytokine levels

In response to concanavalin A, there is a release of large quantities of macrophage- and T cell-derived cytokines, including

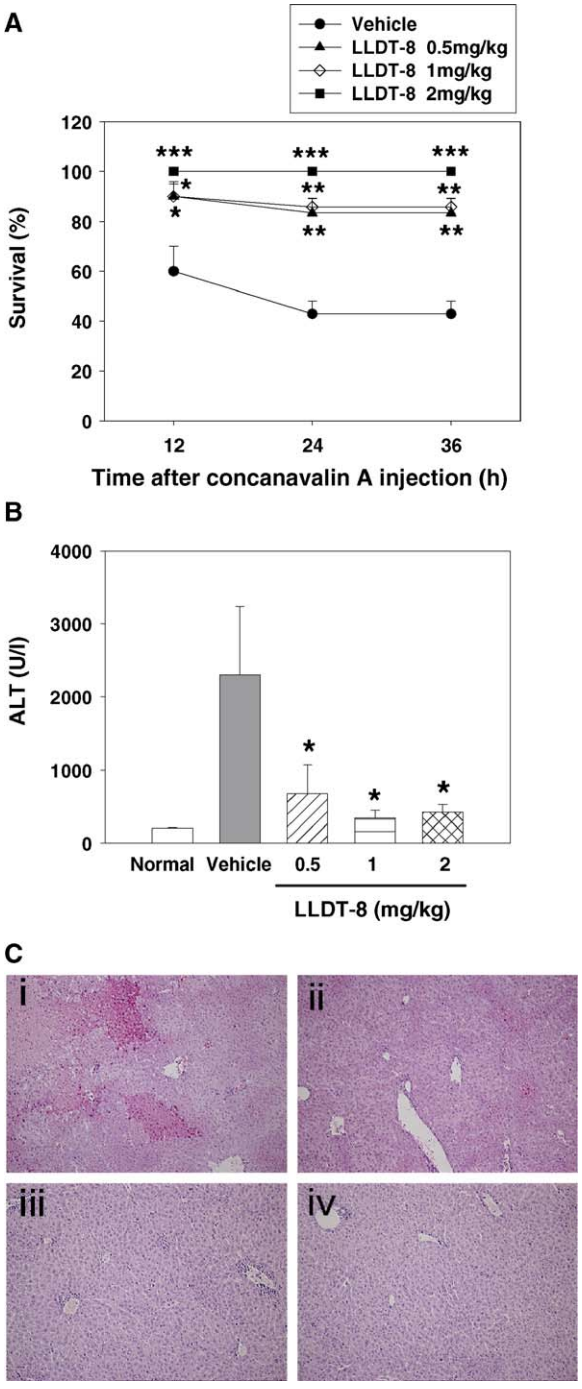


Fig. 2. Effect of LLDT-8 on concanavalin A-induced hepatitis. BALB/c mice were intraperitoneally administered with vehicle, or 0.5, 1, and 2 mg/kg LLDT-8 on days −3, −2, −1, and 1 h before i.v. injection with concanavalin A. (A) LLDT-8 improved the survival of hepatitis mice. Each mouse was i.v. treated with 30 mg/kg concanavalin A. Survival rate was assessed at the indicated times after concanavalin A injection ($n=10$ mice/group). (B) LLDT-8 reduced the ALT levels. Each mouse was i.v. treated with 20 mg/kg concanavalin A. Serum ALT activity was determined 24 h after concanavalin A injection. Data are expressed as mean ± S.E.M. from individual mice ($n=10$ mice/group). *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus vehicle-treated mice. Three separate experiments are performed that give similar results. (C) Histological examination of liver sections. Livers were harvested at 24 h following concanavalin A (20 mg/kg) injection and stained with H&E (original magnification 10×). (i) Vehicle-treated hepatitis mice; hepatitis mice pretreated with (ii) 0.5 mg/kg LLDT-8, (iii) 1 mg/kg LLDT-8, and (iv) 2 mg/kg LLDT-8. Vehicle control represents mice suffering from concanavalin A-induced hepatitis treated only with solvent.

TNF-α, IL-2 and IFN-γ, but with different kinetics according to our preliminary studies and reports by others (Cao et al., 1998; Okamoto et al., 1998). In LLDT-8-treated mice, serum TNF-α was significantly lower as compared with vehicle-treated mice (Fig. 3A). The serum level of IL-2 in LLDT-8-treated mice was approximately only half of that in vehicle control mice (Fig. 3B). Serum level of IFN-γ was markedly reduced in LLDT-8-treated mice (Fig. 3C), and the suppressive effect was stronger at 24 h (1508 versus 4375 pg/ml, $p<0.01$). The production of IL-12p70, IL-12p40, and IL-6 were also significantly reduced in LLDT-8-treated mice compared with vehicle control (Fig. 3D–F).

3.3. LLDT-8 significantly impaired lymphocyte proliferation

We further investigated the proliferation response of lymphocytes after in vivo LLDT-8 treatment. The spontaneous proliferation of splenocytes was dramatically inhibited in LLDT-8-treated mice compared with vehicle control (at doses of 0.5, 1, and 2 mg/kg, LLDT-8 inhibited proliferation by 36%, 85%, and 78%, respectively, Fig. 4). When stimulated with concanavalin A ex vivo, splenocytes from LLDT-8-treated mice at the doses of 0.5, 1, and 2 mg/kg showed the capability of proliferation as 80%, 27%, and 21% compared with vehicle control, respectively (Fig. 4).

3.4. LLDT-8 promoted concanavalin A-activated splenic T cells to undergo apoptosis

To test whether the inhibitory effect of LLDT-8 was associated with the reduction of T cell percentage in the spleens, FACS analysis was done. Compared with vehicle control mice, treatment with LLDT-8 significantly reduced the T cell percentage (34 ± 3% in hepatitis control mice versus 21 ± 1% in LLDT-8 treated mice, Fig. 5A).

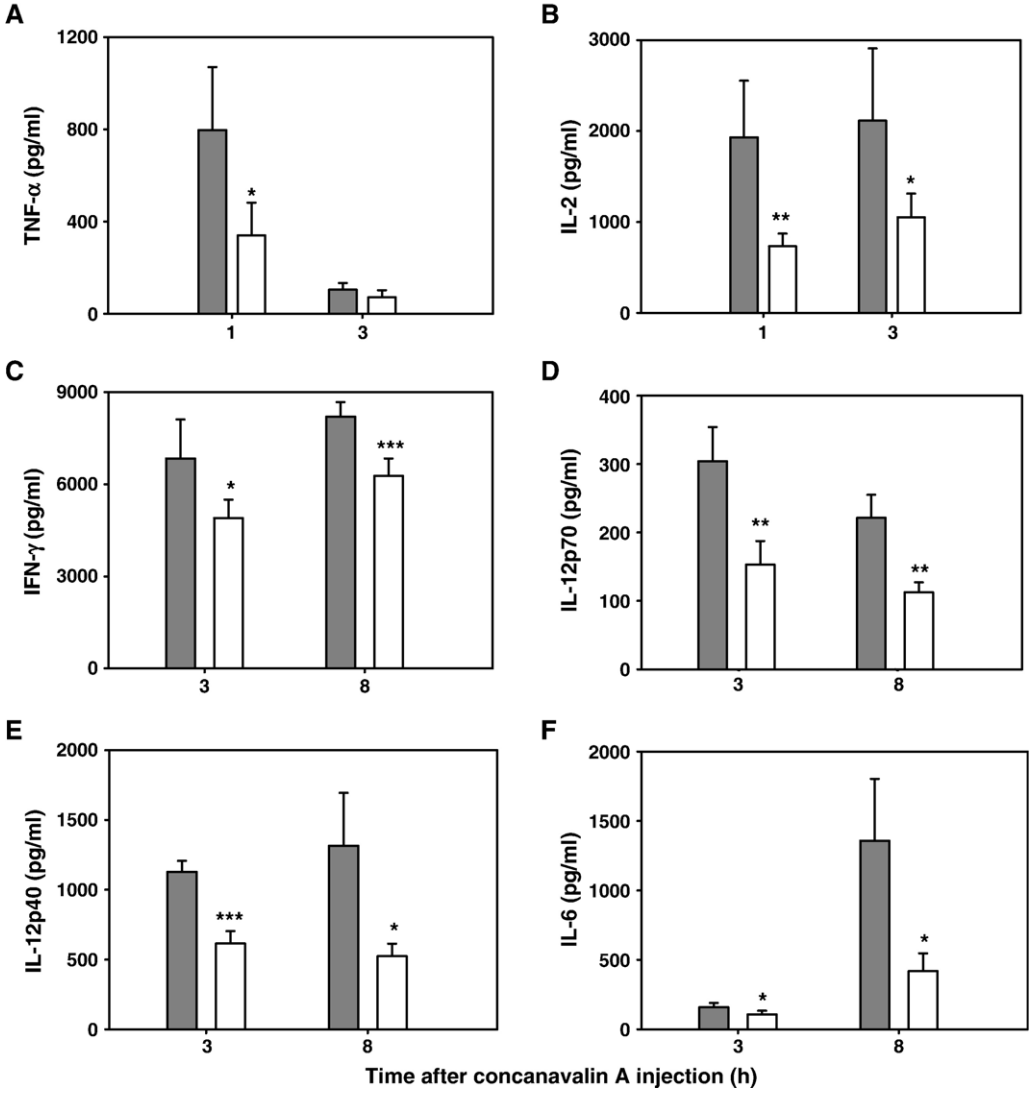


Fig. 3. Effect of LLDT-8 on serum cytokines in hepatitis mice. BALB/c mice were treated with vehicle or 2 mg/kg LLDT-8 on days −3, −2, −1, and 1 h prior to concanavalin A (20 mg/kg) injection. Serum (A) TNF-α, (B) IL-2, (C) IFN-γ, (D) IL-12p70, (E) IL-12p40, and (F) IL-6 were determined by ELISA. Data are expressed as mean ± S.E.M. from individual mice ($n=6$ mice/group). *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus vehicle-treated mice. Three separate experiments are performed that give similar results. The gray bar represented hepatitis mice treated with vehicle alone and the white bar represented hepatitis mice pretreated with LLDT-8.

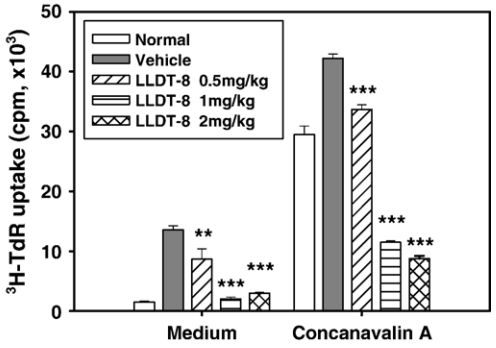


Fig. 4. Effect of LLDT-8 on lymphocyte proliferation in hepatitis mice. BALB/c mice were treated with vehicle or various doses of LLDT-8 on days −3, −2, −1, and 1 h prior to concanavalin A injection. Twenty-four hours after concanavalin A challenge, spleen cells (5×10^6 cells/ml) were cultured in triplicate without or with concanavalin A (5 μg/ml) for 24 h and pulsed with [^{3}H]-thymidine. The incorporated radioactivity was measured. Data are mean±S.D. of triplicate cultures. Three separate experiments are performed that give similar results. $^{**}p<0.01$, $^{***}p<0.001$ versus vehicle-treated mice.

To confirm whether the reduction of T cells was caused by apoptosis, splenocytes from hepatitis mice with and without LLDT-8 treatment were directly stained by Annexin V and PI. The results showed that the percentage of typical apoptotic (Annexin V$^+$ and PI$^+$) splenocytes in LLDT-8-treated hepatitis mice was higher than that in control mice (Fig. 5B). When splenocytes from naive mice were activated with concanavalin A in the presence of 100 nM LLDT-8, the similar promoting effect was observed (Fig. 5C). STAT1 and IRF-1 are proapoptotic genes in some inflammatory diseases (Jaruga et al., 2004). Even though serum level of IFN-γ in LLDT-8-treated mice was lower than vehicle control, the IFN-γ-triggered STAT1 and IRF-1 mRNA expressions in splenocytes were enhanced in the presence of LLDT-8 treatment (Fig. 5D).

3.5. LLDT-8 inhibited the expression of IFN-γ-regulated chemokines and chemokine receptors in liver

The activation of proapoptotic IFN-γ/STAT1/IRF-1 pathway can lead to liver damage. The induction of STAT1 and IRF-1 expressions in liver was significantly blocked by LLDT-8 treatment at 3 h and 24 h following concanavalin A injection (Fig. 6A).

To understand the mechanisms underlying the effects of LLDT-8 in leukocyte infiltration, expressions of a variety of chemokines and adhesion molecules were compared between vehicle and LLDT-8-treated mice. These molecules were tightly regulated by IFN-γ (Jaruga et al., 2004). Data were summarized in Fig. 6A. Concanavalin A injection significantly induced expression of Mig, IP-10, I-TAC, and VCAM-1 in the livers of vehicle-treated mice, dramatically elevated at 3 h after injection, and declined at 24 h. However, these molecules expressions were markedly suppressed in LLDT-8-treated mice.

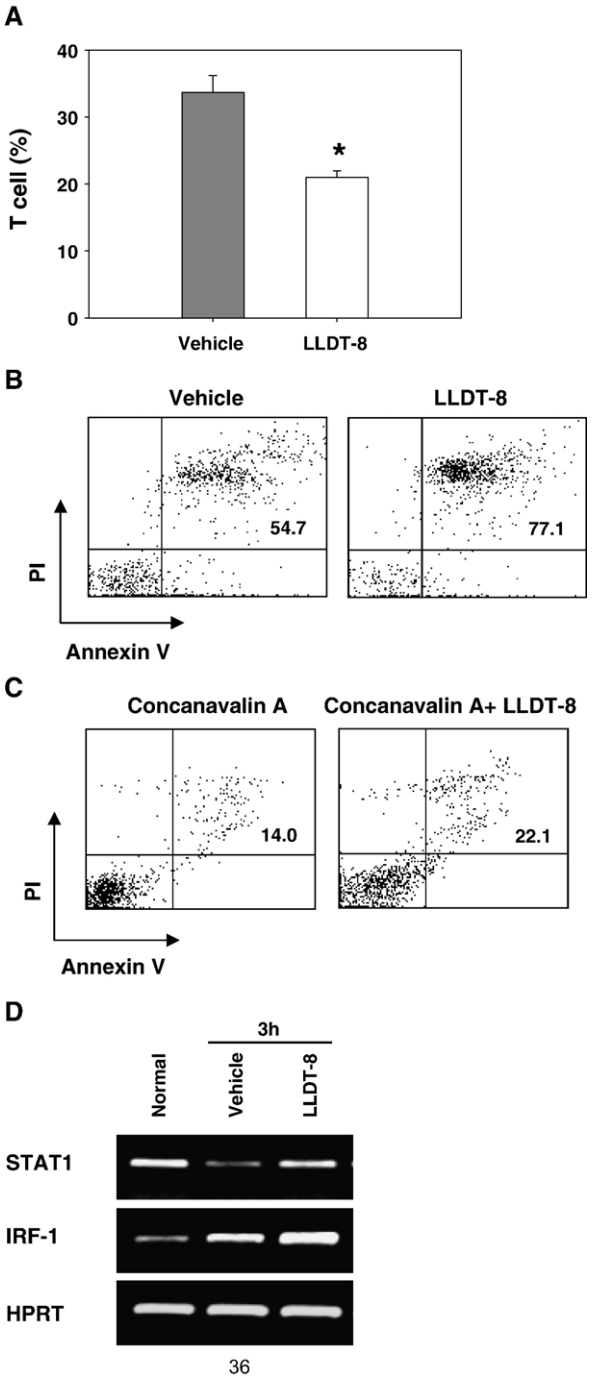


Fig. 5. Effect of LLDT-8 on splenic T lymphocyte apoptosis. BALB/c mice were treated with vehicle or 2 mg/kg LLDT-8 on days −3, −2, −1, and 1 h prior to concanavalin A injection. (A) LLDT-8 reduced T cell percentage. Twenty-four hours after concanavalin A challenge, spleen cells were stained with FITC-conjugated anti-mouse CD3 mAb and analyzed by flow cytometry. Data are expressed as mean±S.E.M. from three individual experiments. $^*p<0.05$ versus vehicle-treated mice. (B) LLDT-8 enhanced T lymphocyte apoptosis in vivo. Twenty-four hours after concanavalin A challenge, spleen cells were stained with PI/Annexin V and analyzed by flow cytometry. (C) LLDT-8 increased the apoptosis of T lymphocyte in vitro. Spleen cells from naive mice were preactivated with 5 μg/ml concanavalin A for 24 h. Then cells were washed and treated without or with 100 nM LLDT-8 in the presence of 5 μg/ml concanavalin A. 48 h later, cells were stained with PI/Annexin V and analyzed by flow cytometry. (D) Three hours after concanavalin A challenge, B cell-depleted spleen cells were lysed, and total RNA was isolated and analyzed for mRNA expression with RT-PCR. HPRT was done as internal control. Three independent experiments are performed that give similar results. HPRT, hypoxanthine-guanine phosphoribosyltransferase.

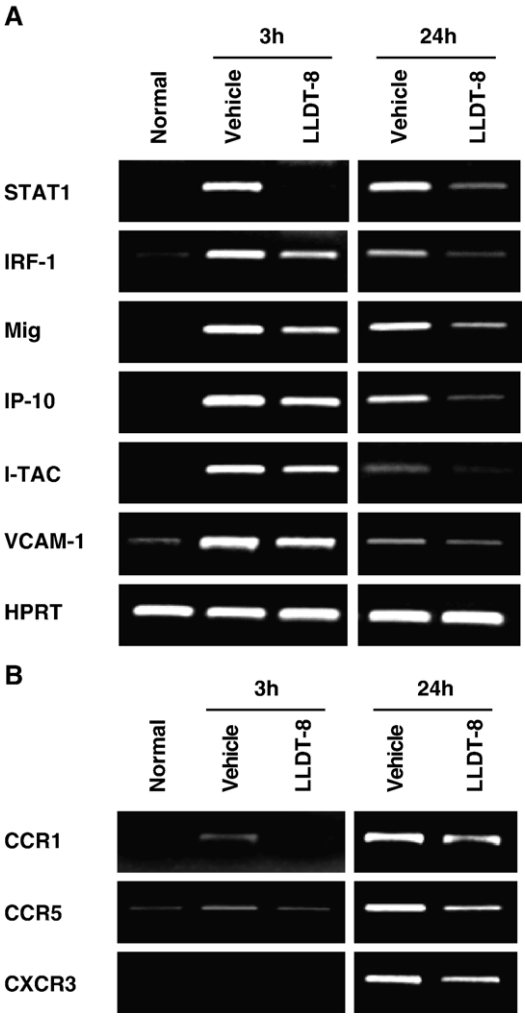


Fig. 6. Effect of LLDT-8 on chemokines, adhesion molecule, and chemokine receptors expression in hepatitis liver. BALB/c mice were treated with vehicle or 2 mg/kg LLDT-8 on days −3, −2, −1, and 1 h prior to concanavalin A injection. 3 h and 24 h after concanavalin A challenge, total RNA was isolated from liver homogenates and analyzed with RT-PCR for mRNA expression of (A) chemokines and adhesion molecule, or (B) chemokine receptors. HPRT was done as internal control. Three independent experiments are performed that give similar results.

CCR5 is present on most Th1 cells, while CXCR3 is present on both Th1 and Th2 cells. Both CCR5 and CXCR3 are involved in Th1 cell recruitment to sites of inflammation (Qin et al., 1998). In this study, chemokine receptors CCR1, CCR5, and CXCR3 expressions were increased in concanavalin A hepatitis mice liver, showing a dramatic increase at 24 h after concanavalin A injection (Fig. 6B). Their expressions were greatly inhibited in hepatitis mice receiving LLDT-8 treatment.

4. Discussion

In the present study, we report that LLDT-8 ameliorated concanavalin A-induced mice hepatitis. LLDT-8 improved hepatitis mice survival; LLDT-8 efficiently reduced serum ALT level and liver damage; further studies showed that LLDT-8 decreased serum cytokines levels, including TNF-α, IFN-γ, IL-2, IL-12, and IL-6; LLDT-8 increased proapoptotic STAT1 and IRF-1 gene expression and promoted activated T cell apoptosis that probably accounted for the elimination of T cells in spleen; LLDT-8 inhibited chemokines and adhesion molecule expressions in IFN-γ/STAT1/IRF-1 pathway, leading to the reduced lymphocyte infiltration into liver; LLDT-8 also suppressed chemokine receptors expressions. Thus, LLDT-8 might prevent concanavalin A hepatitis via its regulatory effect on IFN-γ/STAT1/IRF-1 signaling and inhibition of inflammatory cytokines production.

Concanavalin A-induced hepatitis is a T cell-dependent severe liver injury model that is ideal for studying human immune-mediated liver disease (Tiegs et al., 1992). Here we showed that pretreatment with LLDT-8 efficiently blocked concanavalin A-dependent mortality. Even when administered to mice 1 h after concanavalin A injection, LLDT-8 still significantly reduced the serum ALT level.

Infiltration of activated lymphocytes into the liver has been reported to result in increased concentrations of several cytokines that have important effects on the degree of liver damage. Previous studies have shown that concanavalin A-induced hepatitis requires TNF-α, IFN-γ and IL-2 (Gantner et al., 1995; Tagawa et al., 1997). TNF-α and IFN-γ have a direct cytotoxic effect on hepatocytes in vitro (Morita et al., 1995), and TNF-α is known to trigger apoptosis and/or necrosis of hepatocytes in vivo (Leist et al., 1994). IFN-γ enhances the ability of monocytes and macrophages to produce TNF-α, as well as acting synergistically with TNF-α in this model. In our study, LLDT-8 greatly reduced circulating TNF-α level 1 h after concanavalin A injection. IL-2 is a representative factor that reflects T cell function. LLDT-8 significantly inhibited IL-2 production 3 h after concanavalin A treatment. It was interesting, however, that LLDT-8 did not have a dramatically inhibitory effect on IFN-γ at its maximal level (i.e., 8 h after concanavalin A injection), but remarkably suppressed its level at 24 h. IL-12 is involved in IFN-γ production by natural killer cells and T cells, and promotes Th1-cellular immunity (Trinchieri, 1995). Administration of LLDT-8 led to a reduction of IL-12 level. Serum IL-6 level was significantly elevated by concanavalin A and consistent with hepatocyte injury (Shirin et al., 1999). It was also reduced by LLDT-8. The inhibitory effects of LLDT-8 on circulating cytokines were consistent with our in vitro results (Zhou et al., 2005). Taken together, TNF-α and IFN-γ seemed to be the key cytokines accounting for the suppressive activity of LLDT-8.

Spleen cell proliferation was significantly inhibited by LLDT-8, and the percentage of T cells in spleen was markedly decreased. It has been proposed that in concanavalin A-induced hepatitis, liver-infiltrating T lymphocytes are recruited from the spleen and migrate to the liver, where either activated macrophages or T cells may directly cause hepatocyte death (Gantner et al., 1995). The method by which activated T cells undergo apoptosis, called activation induced cell death, may be crucial for mice to recover from this disease (Russell, 1995). The percentage of apoptotic splenocytes (mainly T lymphocytes) was higher in LLDT-8-treated hepatitis mice than in hepatitis mice treated with vehicle alone. The ability of LLDT-

8 to promote activated T cell to undergo apoptosis was also confirmed in vitro. STAT1 and IRF-1 can function as proapoptotic factors in some inflammation circumstances. RT-PCR results showed the increased expression of STAT1 and IRF-1 mRNA in spleen of hepatitis mice with LLDT-8 treatment, providing the basis for its effect on apoptosis. $CD4^+$ T cells represent the major population of liver-infiltrating T cells (Shirin et al., 1998), and activated T cells progressively destroy liver cells. LLDT-8 selectively eliminated activated T cells and inhibited T cell proliferation in spleen that reduced the number of liver infiltrating T cells.

Concanavalin A stimulates natural killer T cells and other cells to produce IFN-γ, which then targets hepatocytes, sinusoidal endothelial cells, and Kupffer cells via activation of STAT1, STAT3, and IRF-1. IRF-1 binds to IRF-1 response elements in the promoter regions of genes to stimulate genes transcription (Kroger et al., 2002). IRF-1 response elements have been identified in the promoters of VCAM-1, ICAM-1, Mig, IP-10, and I-TAC (Hamilton et al., 2002; Lechleitner et al., 1998; Nazar et al., 1997; Ohmori et al., 1997). Chemokines produced at the sites of inflammation play an essential role in the recruitment of particular cell types that infiltrate and participate in the pathologic lesions (Moser and Loetscher, 2001). Activation of STAT1 and IRF-1 stimulates expression of VCAM-1, ICAM-1, Mig, ENA-78, I-TAC, and IP-10, which along with other chemokines, attracts neutrophils and eosinophils into the liver, resulting in hepatitis (Jaruga et al., 2004). Here LLDT-8 potently blocked IFN-γ/STAT1/IRF-1 pathway in the liver. In the presence of LLDT-8 treatment, the mRNA levels of STAT1 and IRF-1 were significantly lower than those in vehicle-treated mice. Since Mig, IP-10, I-TAC, and VCAM-1 were tightly regulated by IFN-γ/STAT1/IRF-1 pathway, their expressions were causally and markedly inhibited when hepatitis mice were administered with LLDT-8. Collectively, the reduced expression of chemokines may lead to the decreased attraction of inflammatory cells to infiltrate into liver.

Hepatitis injury develops with infiltration and/or proliferation of T cells and macrophages (Gantner et al., 1995; Tiegs et al., 1992). In the LLDT-8-treated hepatitis mice, the expressions of CCR1, CCR5, and CXCR3 were reduced. These chemokine receptors are mainly expressed on lymphocytes, especially on Th1 cells. Therefore, LLDT-8 might block lymphocytes infiltrating into liver.

Inducible nitric oxide synthase (iNOS)-mediated nitric oxide (NO) production is critical for concanavalin A-induced liver injury (Sass et al., 2001), and LLDT-8 has been demonstrated to effectively reduce NO production and iNOS expression by inhibiting IFN-γ-triggered IRF-1 expression and LPS-triggered MAPK phosphorylation and NF-κB activation in macrophages (Zhou et al., 2006). This data suggested that the NO production pathway may also contribute to the beneficial action of LLDT-8 in liver.

In the present study, the expression and production of most molecules were regulated by IFN-γ, and it is reported that these molecules expression is abolished in IFN-$\gamma^{-/-}$ mice (Hong et al., 2002; Jaruga et al., 2004). And in our previous studies, LLDT-8 displayed a stronger inhibitory effect on IFN-γ production and IFN-γ signaling (Tang et al., 2005; Zhou et al., 2005, 2006). Thus, we concluded that LLDT-8 prevented concanavalin A hepatitis by regulating IFN-γ-induced gene expression via an IRF-1-dependent mechanism, depending on the microenvironment (spleen/liver).

In conclusion, our results demonstrated the preventive effects of LLDT-8 on concanavalin A-induced liver hepatitis. The outlined mechanism would be beneficial in understanding the immunosuppressive property of LLDT-8.

Acknowledgements

We thank the research group of Dr. Jin Ren for providing valuable technical assistance with histology. This work was supported by the grant from The Knowledge Innovation Program of Chinese Academy of Sciences (No. KSCX2-SW-202).

References

Bogdanos, D.P., Mieli-Vergani, G., Vergani, D., 2000. Virus, liver and autoimmunity. Dig. Liver Dis. 32, 440–446.

Cao, Q., Batey, R., Pang, G., Russell, A., Clancy, R., 1998. Il-6, ifn-gamma and tnf-alpha production by liver-associated t cells and acute liver injury in rats administered concanavalin A. Immunol. Cell Biol. 76, 542–549.

Chen, B.J., 2001. Triptolide, a novel immunosuppressive and anti-inflammatory agent purified from a chinese herb *tripterygium wilfordii* hook F. Leuk. Lymphoma 42, 253–265.

Gantner, F., Leist, M., Lohse, A.W., Germann, P.G., Tiegs, G., 1995. Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. Hepatology 21, 90–98.

Hamilton, N.H., Banyer, J.L., Hapel, A.J., Mahalingam, S., Ramsay, A.J., Ramshaw, I.A., Thomson, S.A., 2002. IFN-gamma regulates murine interferon-inducible T cell alpha chemokine (i-tac) expression in dendritic cell lines and during experimental autoimmune encephalomyelitis (eae). Scand. J. Immunol. 55, 171–177.

Hong, F., Jaruga, B., Kim, W.H., Radaeva, S., El-Assal, O.N., Tian, Z., Nguyen, V.A., Gao, B., 2002. Opposing roles of stat1 and stat3 in t cell-mediated hepatitis: Regulation by socs. J. Clin. Invest. 110, 1503–1513.

Huynh, P.N., Hikim, A.P., Wang, C., Stefonovic, K., Lue, Y.H., Leung, A., Atienza, V., Baravarian, S., Reutrakul, V., Swerdloff, R.S., 2000. Long-term effects of triptolide on spermatogenesis, epididymal sperm function, and fertility in male rats. J. Androl. 21, 689–699.

Jaruga, B., Hong, F., Kim, W.H., Gao, B., 2004. Ifn-gamma/stat1 acts as a proinflammatory signal in t cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1. Am. J. Physiol. Gastrointest. Liver Physiol. 287, G1044–G1052.

Kano, A., Haruyama, T., Akaike, T., Watanabe, Y., 1999. IRF-1 is an essential mediator in ifn-gamma-induced cell cycle arrest and apoptosis of primary cultured hepatocytes. Biochem. Biophys. Res. Commun. 257, 672–677.

Kita, H., Mackay, I.R., Van De Water, J., Gershwin, M.E., 2001. The lymphoid liver: considerations on pathways to autoimmune injury. Gastroenterology 120, 1485–1501.

Kroger, A., Koster, M., Schroeder, K., Hauser, H., Mueller, P.P., 2002. Activities of IRF-1. J. Interferon. Cytokine Res. 22, 5–14.

Kusters, S., Gantner, F., Kunstle, G., Tiegs, G., 1996. Interferon gamma plays a critical role in t cell-dependent liver injury in mice initiated by concanavalin A. Gastroenterology 111, 462–471.

Lechleitner, S., Gille, J., Johnson, D.R., Petzelbauer, P., 1998. Interferon enhances tumor necrosis factor-induced vascular cell adhesion molecule 1 (cd106) expression in human endothelial cells by an interferon-related factor 1-dependent pathway. J. Exp. Med. 187, 2023–2030.

Leist, M., Gantner, F., Bohlinger, I., Germann, P.G., Tiegs, G., Wendel, A., 1994. Murine hepatocyte apoptosis induced in vitro and in vivo by tnf-alpha requires transcriptional arrest. J. Immunol. 153, 1778–1788.

Morita, M., Watanabe, Y., Akaike, T., 1995. Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes. Hepatology 21, 1585–1593.

Moser, B., Loetscher, P., 2001. Lymphocyte traffic control by chemokines. Nat. Immunol. 2, 123–128.

Nazar, A.S., Cheng, G., Shin, H.S., Brothers, P.N., Dhib-Jalbut, S., Shin, M.L., Vanguri, P., 1997. Induction of IP-10 chemokine promoter by measles virus: comparison with interferon-gamma shows the use of the same response element but with differential DNA–protein binding profiles. J. Neuroimmunol. 77, 116–127.

Ohmori, Y., Schreiber, R.D., Hamilton, T.A., 1997. Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappab. J. Biol. Chem. 272, 14899–14907.

Okamoto, T., Nakano, Y., Asakura, W., Kobayashi, T., Tsuzuike, N., Hara, K., 1998. Expression of cytokine mRNA in extrahepatic organs in a mouse concanavalin A-hepatitis model. Jpn. J. Pharmacol. 77, 219–225.

Qin, S., Rottman, J.B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., Koch, A.E., Moser, B., Mackay, C.R., 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of t cells associated with certain inflammatory reactions. J. Clin. Invest. 101, 746–754.

Qiu, D., Kao, P.N., 2003. Immunosuppressive and anti-inflammatory mechanisms of triptolide, the principal active diterpenoid from the chinese medicinal herb *tripterygium wilfordii* hook. F. Drugs R D 4, 1–18.

Rehermann, B., Chisari, F.V., 2000. Cell mediated immune response to the hepatitis c virus. Curr. Top. Microbiol. Immunol. 242, 299–325.

Russell, J.H., 1995. Activation-induced death of mature T cells in the regulation of immune responses. Curr. Opin. Immunol. 7, 382–388.

Sass, G., Koerber, K., Bang, R., Guehring, H., Tiegs, G., 2001. Inducible nitric oxide synthase is critical for immune-mediated liver injury in mice. J. Clin. Invest. 107, 439–447.

Shirin, H., Bruck, R., Aeed, H., Frenkel, D., Kenet, G., Zaidel, L., Avni, Y., Halpern, Z., Hershkoviz, R., 1998. Pentoxifylline prevents concanavalin a-induced hepatitis by reducing tumor necrosis factor alpha levels and inhibiting adhesion of t lymphocytes to extracellular matrix. J. Hepatol. 29, 60–67.

Shirin, H., Dotan, I., Papa, M., Maaravi, Y., Aeed, H., Zaidel, L., Matas, Z., Bruck, R., Moss, S.F., Halpern, Z., Oren, R., 1999. Inhibition of concanavalin a-induced acute t cell dependent hepatic damage in mice by hypothyroidism. Liver 19, 206–211.

Siebler, J., Wirtz, S., Klein, S., Protschka, M., Blessing, M., Galle, P.R., Neurath, M.F., 2003. A key pathogenic role for the stat1/t-bet signaling pathway in t-cell-mediated liver inflammation. Hepatology 38, 1573–1580.

Streetz, K., Fregien, B., Plumpe, J., Korber, K., Kubicka, S., Sass, G., Bischoff, S.C., Manns, M.P., Tiegs, G., Trautwein, C., 2001. Dissection of the intracellular pathways in hepatocytes suggests a role for jun kinase and IFN regulatory factor-1 in con a-induced liver failure. J. Immunol. 167, 514–523.

Tagawa, Y., Sekikawa, K., Iwakura, Y., 1997. Suppression of concanavalin a-induced hepatitis in IFN-gamma(−/−) mice, but not in TNF-alpha(−/−) mice: role for ifn-gamma in activating apoptosis of hepatocytes. J. Immunol. 159, 1418–1428.

Tang, W., Yang, Y., Zhang, F., Li, Y.C., Zhou, R., Wang, J.X., Zhu, Y.N., Li, X. Y., Yang, Y.F., Zuo, J.P., 2005. Prevention of graft-versus-host disease by a novel immunosuppressant, (5R)-5-hydroxytriptolide (lldt-8), through expansion of regulatory t cells. Int. Immunopharmacol. 5, 1904–1913.

Tang, W., Zhou, R., Yang, Y., Li, Y.C., Wang, J.X., Yang, Y.F., Zuo, J.P., 2006. Suppression of (5R)-5-hydroxytriptolide (LLDT-8) on allograft rejection in full MHC-mismatched mouse cardiac transplantation. Transplantation 81 (6) (March 27).

Tiegs, G., Hentschel, J., Wendel, A., 1992. A T cell-dependent experimental liver injury in mice inducible by concanavalin a. J. Clin. Invest. 90, 196–203.

Toyonaga, T., Hino, O., Sugai, S., Wakasugi, S., Abe, K., Shichiri, M., Yamamura, K., 1994. Chronic active hepatitis in transgenic mice expressing interferon-gamma in the liver. Proc. Natl. Acad. Sci. U. S. A. 91, 614–618.

Trinchieri, G., 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. 13, 251–276.

Tyan, M.L., 1974. In vivo toxicity of concanavalin a. Proc. Soc. Exp. Biol. Med. 146, 1163–1165.

Yang, Y.F., Mukai, T., Gao, P., Yamaguchi, N., Ono, S., Iwaki, H., Obika, S., Imanishi, T., Tsujimura, T., Hamaoka, T., Fujiwara, H., 2002. A non-peptide CCR5 antagonist inhibits collagen-induced arthritis by modulating t cell migration without affecting anti-collagen t cell responses. Eur. J. Immunol. 32, 2124–2132.

Zhou, R., Zhang, F., He, P.L., Zhou, W.L., Wu, Q.L., Xu, J.Y., Zhou, Y., Tang, W., Li, X.Y., Yang, Y.F., Li, Y.C., Zuo, J.P., 2005. 5r)-5-hydroxytriptolide (LLDT-8), a novel triptolide analog mediates immunosuppressive effects in vitro and in vivo. Int. Immunopharmacol. 5, 1895–1903.

Zhou, R., Zheng, S.X., Tang, W., He, P.L., Li, X.Y., Yang, Y.F., Li, Y.C., Geng, J.G., Zuo, J.P., 2006. Inhibition of inducible nitric-oxide synthase expression by (5R)-5-hydroxytriptolide (LLDT-8) in interferon-{gamma} and bacterial lipopolysaccharide stimulated macrophages. J. Pharmacol. Exp. Ther. 316, 121–128.