Published in final edited form as:

Hepatology. 2009 January ; 49(1): 215–226. doi:10.1002/hep.22585.

Effect of PolyI:C Co-treatment on Halothane-Induced Liver Injury in Mice

Linling Cheng^{‡,§}, Qiang You^{‡,§}, Hao Yin[‡], Michael Holt[‡], Christopher Franklin[‡], and Cynthia Ju^{‡,&,*}

[‡]Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center.

[&]Integrated Department of Immunology, University of Colorado Health Sciences Center.

Abstract

Drug-induced liver injury (DILI) is a challenging problem in drug development and clinical practice. Patient susceptibility to DILI is multifactorial, making these reactions difficult to predict and prevent. Clinical observations have suggested that concurrent bacterial and viral infections represent an important risk factor in determining patient susceptibility to developing adverse drug reactions, although the underlying mechanism is not clear. In the present study, we employed the viral RNA mimetic (polyinosinic-polycytidylic acid, polyI:C) to emulate viral infection and examined its effect on halothane-induced liver injury. Although pre-treatment of mice with polyI:C attenuated halothane hepatotoxicity due to its inhibitory effect on halothane metabolism, post-treatment significantly exacerbated liver injury with hepatocellular apoptosis being significantly higher than that in mice treated with polyI:C alone or halothane alone. The pan-caspase inhibitor z-VAD-fmk suppressed liver injury induced by polyI:C/post-halothane co-treatment, suggesting that the increased hepatocyte apoptosis contributes to the exacerbation of liver injury. Post-treatment with polyI:C also caused activation of hepatic Kupffer cells and natural killer cells and up-regulated multiple pro-apoptotic factors, including tumor necrosis factor- α , NKG2D and FasL. These factors may play important roles in mediating polyI:C-induced hepatocyte apoptosis. This is the first study to provide evidence that concurrent viral infection can inhibit CYP450 activities and activate the hepatic innate immune system to pro-apoptotic factors. DILI may be attenuated or exacerbated by pathogens depending on the time of infection.

Keywords

Drug; Hepatotoxicity; Kupffer cells; TLRs; NK cells

Introduction

Drug-induced liver injury (DILI) accounts for over 50% of liver failure cases in the US,¹ and is also the most common reason for the withdrawal of FDA-approved drugs from the pharmaceutical market.² The prediction and prevention of these reactions have been a challenge due to their relatively low incidence, the lack of a diagnostic standard and the lack of knowledge of the underlying mechanism. It is also difficult to predict which patients will develop DILI to a given drug because patient susceptibility is likely multifactorial.

*To whom correspondence should be addressed. Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262. Phone: (303) 315-2180. Fax: (303) 315-6281. E-mail: CYNTHIA.JU@UCHSC.EDU.

[§]Indicates equal contribution by authors.

One such risk factor may be bacterial or viral infection concurrent with drug treatment. It has been observed that when ampicillin is given to patients with acute infectious mononucleosis, the risk of developing extensive maculopapular pruritic rash increases markedly (approaching 100%).³⁻⁴ It has also been reported that 57 to 83% of patients with AIDS who received cotrimoxazole developed adverse reactions such as fever, rash, neutropenia, thrombocytopenia and hepatitis,⁵ while the incidence of these reactions are substantially lower in cotrimoxazole-treated patients without HIV infection. A similar increase in the risk of drug-induced hypersensitivity reactions in patients with HIV infection has been noted for several other drugs, including dapsone, carbamazepine, quinolones and penicillins.⁶⁻⁷

In terms of the mechanism of DILI, evidence suggests that the formation of chemically reactive metabolites initiates hepatocyte damage, which in turn, activates the innate immune cells, causing inflammation. Bacterial and viral infection can amplify the inflammatory response by further activation of the innate immune system via Toll-like receptors (TLRs).⁸ TLRs are a family of innate immune receptors that recognize structurally conserved pathogen-associated molecular patterns (PAMPs) of microbial origin.⁹ Such specific microbial products include lipopolysaccharide (LPS), bacterial lipoproteins, peptidoglycan, bacterial DNA and viral nucleic acids. TLRs are broadly expressed on innate immune cells, including macrophages, dendritic cells, neutrophils, and natural killer (NK) cells.¹⁰⁻¹¹ TLR recognition of microbial molecules triggers an inflammatory response, including the production of cytokines, chemokines and adhesion molecules. Polyinosinic-polycytidylic acid (polyI:C) is a viral RNA mimetic that induces immune responses similar to a viral infection.¹² PolyI:C increases the cytotoxic effect by macrophages and NK cells and activates T cells.¹³⁻¹⁵ In the mouse liver, polyI:C treatment causes the recruitment and activation of NK cells, a process dependent on Kupffer cells (KC) release of interleukin (IL)-12.¹⁶ However, the effect of polyI:C in modulating DILI has not been studied.

Halothane causes mild liver injury in approximately 20% of patients,¹⁷ and it leads to severe liver injury in a small percentage of patients, that often develops into fulminant liver failure. It is not known why certain individuals are more susceptible to halothane-induced hepatotoxicity, although several risk factors have been identified, including obesity and the female gender.¹⁸ We have recently developed a mouse model of halothane-induced liver injury,¹⁹ which provides a platform for elucidating susceptibility factors in DILI development. In the present study, we have employed this model to investigate the effect of polyI:C on halothane-induced hepatotoxicity in mice. Although pre-treatment with polyI:C inhibited halothane-induced liver injury, a dramatic augmentation of hepatotoxicity was observed in mice administered with polyI:C after halothane challenge (polyI:C/post-halothane) compared with those treated with polyI:C or halothane alone. The data suggest that post-treatment with polyI:C induced a marked increase in hepatocyte apoptosis through the activation of hepatic KC and NK cells. The findings provide evidence that concurrent infection could decrease or increase patient risk of developing DILI depending on the time of infection.

Materials and Methods

Animal Treatment

Female BALB/cByJ mice (7–10 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and kept in the Center for Laboratory Animal Care at the University of Colorado Health Sciences Center (UCHSC) for one week before treatment. All animal experiments were performed in accordance with guidelines from the UCHSC Institutional Animal Care and Use Committee.

Mice were injected intraperitoneally (i.p.) with polyI:C (GE Healthcare Bio-Science Corp., Piscataway, NJ; 50 µg dissolved in 100 µL of PBS) 12h or 7 days prior to or 6h after i.p. administration of halothane (Halocarbon Labs Inc., Hackensack, NJ; 30 mmole/kg dissolved in 2 mL of olive oil).¹⁹ Control mice were treated with either polyI:C alone or halothane alone.

To deplete NK cells, mice were injected intravenously (i.v.) with anti-AsGM1 polyclonal antibody (100 µL; Wako Chemical USA, Richmond, VA) 2 days prior to halothane treatment.^{20,21} Control mice were administered with the same volume of normal rabbit serum. To deplete hepatic KC, mice were injected i.v. with liposome-entrapped clodronate (liposome/clodronate; Sigma-Aldrich, St. Louis, MO) 2 days prior to halothane treatment.²² Control mice were injected with empty liposomes. For *in vivo* inhibition of caspase activities, mice were injected i.p. with z-VAD-fmk (z-VAD; BACHEM/Peninsula Laboratories Inc., San Carlos, CA; 200 µg dissolved in 1% DMSO) 5h after halothane treatment. Control mice were treated with 1% DMSO. The time course for various polyI:C post-treatments is summarized in the diagram below.

Assessment of Hepatotoxicity

At 15h and 24h after halothane treatment, mice were anesthetized and blood was collected by retro-orbital puncture. Blood samples were allowed to clot at 4°C before sera were prepared by centrifugation at $10,000 \times g$ for 20 min. Serum alanine transaminase (ALT) levels were measured using a diagnostic assay kit (Teco Diagnostics, Anaheim, CA) following the manufacturer's instruction. At 24h after halothane administration, the animals were sacrificed and the livers removed. Liver sections were fixed in 10% formaldehyde overnight before being transferred into 70% ethanol solution. Paraffin embedded liver sections were mounted onto glass slides and stained with hematoxylin and eosin (H/E; Department of Pathology, UCHSC).

TUNEL Assay

TUNEL assays were performed using TACS™ TdT DAB In Situ Apoptosis Detection Kit (R&D systems, Inc., Minneapolis, MN) following the manufacturer's instruction. Briefly, liver tissue sections were deparaffinized, rehydrated and incubated in 50 µL proteinase K for 15 min at room temperature. After blocking endogenous peroxidase activity using 0.3% H₂O₂ in methanol (v/v), the tissue sections were incubated with terminal deoxynucleotidyl transferase (TdT) for 1h at 37°C. Subsequently, they were incubated with peroxidase conjugated streptavidin for 10 min and with diaminobenzidine (DAB) solution for 5 min at room temperature. The tissue sections were counterstained using Methyl Green Solution (R&D Systems, Inc).

Caspase-3 Activity Assay

Liver tissue samples were homogenized in ice-cold Tris buffer (100 mM, pH 7.5) containing 250 mM sucrose, 2 mM EDTA and a cocktail of protease inhibitors (1:100; Sigma). Caspase-3 activities were measured by using a fluorogenic substrate Ac-DEVD-AMC (Biomol International, L.P., Plymouth Meeting, PA) as previously described.²³ Reactions were performed at 37°C for 1h, and fluorescence intensity was monitored using a Packard FluoroCount plate reader (Packard Instrument, Meriden, CT). Substrate auto-fluorescence was subtracted from each value and specific activities were calculated based on a standard curve of aminomethyl coumarin (Sigma).

Hepatic Leukocyte Isolation and Flow Cytometric Analysis

Hepatic leukocytes were isolated following a previously described method with slight modification.^{24,25} Mice were anesthetized and the liver was perfused *in situ* with Hank's balanced salt solution (HBSS) pre-warmed at 37°C for 5 min. Single cell suspensions were filtered through a 100 µm cell strainer (BD Falcon, Bedford, MA) and centrifuged at 300 × g for 5 min. The pellet was re-suspended in 15 mL of 35% Percoll (Sigma) containing 50 U/mL of heparin (Baxter Healthcare Corporation, Deerfield, IL) and centrifuged at 500 × g for 15 min. The resulting pellet was collected and resuspended in 1.5 mL of red blood cell lysing buffer (Sigma) for 5 min. The cells were then washed in HBSS solution containing 0.6% acid citrate-dextrose (ACD-A, Sigma) and 0.5% BSA. Total viable hepatic leukocytes were counted by trypan blue exclusion.

The NK cell population (DX5⁺CD3⁻) in freshly isolated hepatic leukocytes was identified by staining the cells with phycoerythrin (PE)-conjugated anti-pan-NK cells (anti-DX5, clone DX5; eBioscience, San Diego, CA) and allophycocyanin (APC)-conjugated anti-CD3 (clone 145-2C11; eBioscience) antibodies for 30 min on ice. To prevent non-specific binding, cells were blocked with normal rat serum (Sigma) and anti-mouse FcγR II/III (clone 93; eBioscience) for 5 min at 4°C. In some experiments, the cells were stained with PE-anti-NKG2D (clone CX5; eBioscience) and PE-anti-FasL (clone MFL3; eBioscience) to determine the expression of NKG2D and FasL on hepatic leukocytes. The cells were analyzed on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA). The data were further analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

TNF-α Measurement and Neutralization

Blood was collected and serum samples prepared as described above. Tumor necrosis factor-α (TNF-α) levels were determined by sandwich ELISA using capture and detection antibody pairs according to the manufacturer's instruction (R&D Systems).

In vivo neutralization of TNF-α was accomplished by injecting (i.v.) female BALB/cByJ mice with 200 µg of anti-mouse TNF cV1q antibody (CNTO 2213; kindly provided by Dr. David J. Shealy, Centocor Inc., Radnor, PA) 10h post-halothane challenge (4h post-polyI:C treatment). Control mice were injected i.v. with rat/mouse IgG2a/kappa (CNTO 1322; Centocor Inc.), a direct isotype match of the neutralizing antibody.

RT-PCR Analysis

Total RNA was isolated from 20 mg of frozen liver tissue using RNeasy Mini Kits (Qiagen, Valencia, CA) as instructed by the manufacturer. One µg RNA was reverse transcribed to cDNA at 42°C for 60 min using SuperscriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo (dT) Primers (Invitrogen). The resultant cDNA fragments was amplified for 31 cycles using Platinum Taq polymerase (Invitrogen) and gene specific primers for TNF-α (sense 5'-TTCTGTCTACTGAACTTCGGGGGATCGGTCC-3'; antisense 5'-GTATGAGATAGCAAAATCGGCTGACGGTGTGGG-3'), NKG2D (sense 5'-GCATTGATTTCGTGATCGAAA-3'; antisense 5'-GCCACAGTAGCCCTCTCTTG-3'), FasL (sense 5'-CACAAATCTGTGGCTACCG-3'; antisense 5'-GCCCATATCTGTCCAGTAG-3'), IL-12 (sense 5'-ATGTGTCCTCAGAAGCTAAC-3'; antisense 5'-TCCTAGGATCGGACCCTG-3') and β-actin (sense 5'-TCTTGGGTATGGAATCCTGTGGCA-3'; antisense 5'-ACTCTGCTTGCTGATCCACATCT-3'). All PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. RNA expression levels were determined by normalizing band intensities relative to the levels of β-actin expression using Adobe Photoshop 6.0.

Immunoblot Analysis of TFA-protein adducts

Mice were sacrificed 15h after halothane treatment and liver homogenates prepared as described above. Samples (30 μ g) were resolved on 12% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc., Hercules, CA). Membranes were blocked with 5% (w/v) fat-free milk and probed with a rabbit polyclonal anti-trifluoroacetylchloride (TFA) antisera (1:1000; gift from Dr. Lance Pohl, National Institutes of Health, Bethesda, MD) overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000; Chemicon International Inc., Temecula, CA) for 1h at room temperature. Membranes were then exposed to the ECL Plus Western Blotting Detection System (GE Healthcare Bio-Science Corp.), and data were captured using a Storm 860 system (GE Healthcare Bio-Science Corp.)

Statistical Analysis

Data are presented as mean $\pm$ SEM. Two-tailed Student's t-test was used to compare two groups. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) with a post-hoc test of significance between individual groups. Differences were considered significant when $p < 0.05$.

Results

PolyI:C Affects Halothane-Induced Liver Injury in Mice

Clinical evidence suggests that concurrent infection and inflammation may be a risk factor in developing DILI.³⁻⁷ In order to reproduce this phenomenon in animal models and to understand the underlying mechanism of the increased risk, we set out to investigate the effect of polyI:C on halothane-induced hepatotoxicity. Female BALB/cByJ mice were used because they were found to be most susceptible to halothane-induced liver injury in our previous studies.¹⁹ Mice were injected i.p. with polyI:C at 12h or 7 days prior to or 6h after halothane treatment. Control mice were treated with polyI:C or halothane alone. Halothane-induced liver injury, evaluated by measuring serum ALT activities, was significantly attenuated by polyI:C given at 12h prior to halothane challenge. However, it did not affect halothane hepatotoxicity when administered 7 days before halothane treatment (Fig. 1A). In contrast, polyI:C administered 6h after halothane challenge significantly worsened halothane-induced hepatic injury, while polyI:C alone did not cause noticeable tissue damage at the dose administered (Fig. 1B). Histological evaluation of liver sections obtained at 24h after halothane treatment revealed much greater levels of hepatocyte necrosis in polyI:C/post-halothane co-treated mice than that in mice treated with halothane alone (Fig. 1C).

Numerous studies have demonstrated that cytochrome (CYP) 450 enzymes are suppressed by interferon inducers, such as polyI:C.²⁶⁻³⁰ CYP450 2E1 is the predominant isoform involved in halothane metabolism. A previous report described significant reductions of mRNA and protein levels of CYP450 2E1 at 6h and 12h, respectively, after polyI:C treatment of rats.²⁷ Consistent with these report, we found that biotransformation of halothane to its reactive intermediate, TFA, was significantly inhibited by treatment with polyI:C at 12h, but not 7 days prior to (data not shown) or 6h after halothane challenge (Fig. 1D). These results suggested that the inhibitory effect of polyI:C on CYP450 is transient, and that the increase in halothane hepatotoxicity caused by polyI:C post-treatment was not due to alterations of CYP450 activities, halothane metabolism to TFA or the formation of TFA-protein adducts.

PolyI:C/Post-Halothane Co-treatment Induces Hepatocyte Apoptosis

To investigate the mechanism by which polyI:C post-treatment potentiates halothane-induced liver injury, hepatocyte apoptosis was examined immunohistochemically using the TUNEL assay. In contrast to very few apoptotic cells detected in the liver of halothane alone treated mice, numerous apoptotic cells were found in liver sections obtained from mice co-treated with polyI:C/post-halothane (Fig. 2A). Furthermore, significantly higher caspase-3 activities were observed in the liver homogenates prepared from polyI:C/post-halothane-treated mice than mice treated with polyI:C alone or halothane alone (Fig. 2B). To examine whether the increased hepatocyte apoptosis contributes to the exacerbation of liver injury observed in polyI:C/post-halothane-treated mice, female BALB/cByJ mice were injected i.p. with a pan-caspase inhibitor, *z*-VAD-fmk (i.p. 200 µg/mouse, dissolved in 1% DMSO), 5h after halothane treatment (1h prior to polyI:C administration). Control mice were treated with 1% DMSO. The data demonstrated that while *z*-VAD-fmk significantly decreased ALT levels in mice co-treated with polyI:C/post-halothane (Fig. 2C), *z*-VAD-fmk did not affect the level of liver injury in mice treated with halothane alone (data not shown). These data suggest that polyI:C induced caspase-dependent hepatocellular apoptosis, thereby causing an aggravation of liver injury initiated by halothane.

To identify candidate apoptotic factors that could mediate polyI:C/post-halothane-induced hepatocyte apoptosis, hepatic mRNA expression levels of TNF- α , NKG2D (a lectin-like stimulatory receptor originally identified on NK cells) and FasL were determined and compared among mice treated with polyI:C, halothane or polyI:C/post-halothane. The mRNA expressions of TNF- α and NKG2D in the liver were induced by both polyI:C alone and polyI:C/post-halothane co-treatment (Fig. 3A and B). In contrast, hepatic mRNA expression levels of FasL were markedly higher in polyI:C/post-halothane-treated mice compared with those in mice treated with either polyI:C alone or halothane alone (Fig. 3A and B). Liver mononuclear cells (LMNC), which include NK, NKT and T cells, are a major cellular source of NKG2D and FasL,²⁰ therefore, these cells were isolated and analyzed by flow cytometry. The expression levels of NKG2D and FasL on LMNC isolated from polyI:C/post-halothane-treated mice were elevated compared with those in mice treated with polyI:C or halothane alone (Fig. 4A). While the hepatic mRNA levels of FasL was dramatically increased in polyI:C/post-halothane co-treated mice, FasL protein only slightly higher than that in mice treated with polyI:C or halothane alone. This result suggests that, aside from LMNC, other cells in the liver, such as KC, may express FasL. Serum levels of TNF- α were also significantly increased in polyI:C/post-halothane treated mice compared with those in halothane alone-treated mice (Fig. 4B). TNF- α production was not detectable in the sera of mice treated with polyI:C alone. These results indicate that TNF- α , NKG2D and FasL may be involved in mediating polyI:C/post-halothane-induced hepatocyte apoptosis.

Hepatic KC and NK Cells Mediate PolyI:C-Induced Increase in Halothane Hepatotoxicity

PolyI:C can activate a variety of innate immune cells, including macrophages and NK cells, ^{11,31-36} and these cells represent major sources of pro-apoptotic factors expression. Therefore, we hypothesized that polyI:C activates hepatic KC and NK cells to produce pro-apoptotic factors, which mediate polyI:C-induced exacerbation of halothane hepatotoxicity. While the number of NK cells (DX5⁺CD3⁻) in halothane-treated mice (4%; Fig. 5A) was similar to that in naïve mice (5%; data not shown), polyI:C and polyI:C/post-halothane treatments resulted in significant increases in the numbers of DX5⁺CD3⁻ NK cells within the liver (Fig. 5A). This is consistent with a published study demonstrating that polyI:C treatment of mice causes recruitment and activation of NK cells within the liver.¹⁶ To determine the role of NK cells in polyI:C/post-halothane-induced liver injury, female BALB/cByJ mice were injected i.v. with anti-AsGM1 antisera to deplete NK cells.^{20,21}

Control mice were injected with normal rabbit serum. Two days later, both groups of mice were co-treated with polyI:C/post-halothane. A nearly complete depletion of hepatic NK cells in anti-AsGM1-treated mice was confirmed by flow cytometric analysis (Fig. 5B). As a result, NKG2D- and FasL-expression on CD3-negative LMNC, which consisted mainly of NK cells, also disappeared (Fig. 5B). Hepatic mRNA expression levels of NKG2D and FasL were also significantly reduced in NK cell-depleted mice compared with control mice, even though TNF- α levels were unchanged (Fig. 5C). These findings suggest that NK cells represent a major cellular source of NKG2D and FasL, but not TNF- α , in the liver. Compared with control mice, serum ALT activities were significantly decreased in NK cell-depleted mice at 24h, but not 15h post halothane treatment (Fig. 6A). NK cell depletion did not affect hepatotoxicity induced by halothane alone (Fig. 6A). These results suggest that polyI:C-induced exacerbation of halothane hepatotoxicity was partially attributable to NK cells.

Macrophages express TLR3 that recognizes polyI:C and mediates its stimulatory effects. 11·31·35 PolyI:C-induced activation of NK cells in the liver is dependent on IL-12 production by hepatic KC.¹⁶ To determine whether KC represent another population of hepatic innate immune cells that mediate polyI:C-induced aggravation in halothane hepatotoxicity, KC were depleted by i.v. injection of liposome/clodronate.²² Control mice were injected with empty liposomes. After 2 days, mice were treated with halothane alone or co-treated with polyI:C/post-halothane and liver injury was assessed by measuring serum ALT activities. Although KC depletion did not affect liver injury induced by halothane alone, the absence of KC significantly decreased polyI:C/post-halothane-induced ALT levels, at both 15h and 24h post halothane treatment, approaching those observed in mice treated with halothane alone by 24h (Fig. 6B). KC-depletion also nearly abolished both hepatic mRNA expression and serum levels of TNF- α in mice treated with polyI:C/halothane (Fig. 7A and B). To determine the role of TNF- α in polyI:C-induced aggravation of halothane hepatotoxicity, female BALB/cByJ mice were injected i.v. with an anti-TNF- α neutralizing antibody at 10h after halothane treatment (4h post-polyI:C challenge), while control mice were treated with an isotype control antibody. TNF- α neutralization significantly reduced ALT levels at 24h, but not 15h, post-halothane challenge (Fig. 7C).

The data also demonstrated that liposome/clodronate treatment not only depleted KC, but also significantly inhibited the recruitment of NK cells into the liver (Fig. 8A). Consistent with a previous study demonstrating that the NK cell recruitment and activation induced by polyI:C was dependent on KC stimulation and production of IL-12,¹⁶ we observed that hepatic IL-12 message levels diminished in KC-depleted mice (Fig. 8B). These data may explain the greater suppression of polyI:C/post-halothane-induced ALT levels caused by liposome/clodronate treatment than by NK depletion using anti-AsGM1 antibody. In addition, due to the observation that neutralization of TNF- α only abrogated the role of KC, but not NK cells, the inhibitory effect of TNF- α neutralization on polyI:C/post-halothane-induced liver injury was weaker than that of liposome/clodronate treatment.

Discussion

Clinical observations have suggested that concurrent bacterial and viral infections may increase patient risk of developing drug-induced adverse reactions,³⁻⁷ although the underlying mechanism is not clear. Several studies have demonstrated that co-treatment of rats with LPS and ranitidine, diclofenac, or trovafloxacin could augment hepatotoxicity caused by either LPS or the drug alone.³⁷⁻⁴¹ In these studies, stimulation of the coagulation system and/or increase in the recruitment and activation of neutrophils were observed, which may account for the aggravation in hepatic injury.^{37·38·41} While hepatic innate immune cells are the target of bacterial- and viral-induced activation via signaling through TLRs,

their role in the mechanism of pathogen-induced increases in susceptibility to DILI has not been investigated. The current study examined the effect of polyI:C, a viral RNA mimetic that binds to TLR3, on halothane-induced liver injury in mice.

Our data demonstrated that treatment of BALB/cByJ mice with polyI:C 12h prior to halothane administration inhibited halothane-induced liver injury. It is known that polyI:C-activated innate immune cells, such as dendritic cells, macrophages and NK cells, produce pro-inflammatory cytokines.^{10-11,31-36} These cytokines, including interferons, have been shown to inhibit CYP450 activity.^{26-30,42} Consistent with these reports, we found that TFA-protein adduct formation was significantly decreased by polyI:C pretreatment at 12h (data not shown). This effect was transient as polyI:C treatment at 7 days prior to halothane challenge did not inhibit halothane metabolism and had no effect on halothane-induced liver injury (Fig. 1A). These data suggest that polyI:C can modulate DILI through its inhibitory effect on CYP450 activities. This observation is consistent with findings that pre-treatment of mice with polyI:C significantly decreases acetaminophen (APAP)-induced hepatotoxicity by inhibition of CYP450 2E1.⁴²

In contrast, treatment with polyI:C 6h after halothane challenge dramatically enhanced halothane hepatotoxicity. This is evident by the significantly higher serum ALT activities and increased extent of liver histopathological damage in mice co-treated with polyI:C/post-halothane compared with mice treated with halothane alone (Fig. 1B and C). No obvious liver injury was observed in mice treated with polyI:C alone. A previous study reported that polyI:C caused liver damage,¹⁶ however, the dose required to induce hepatotoxicity is 8-fold higher than that used in the present study. Our results demonstrated a synergistic effect of halothane and polyI:C post-treatment on the induction of liver damage. PolyI:C administered 6h after halothane challenge did not affect halothane metabolism as immunoblot analysis revealed no significant differences in either the patterns or the levels of TFA-protein adduct formation in the livers of halothane alone- and polyI:C/post-halothane-treated mice (Fig. 1D).

We found that both the number of apoptotic hepatocytes and caspase-3 activities in the liver tissue homogenates were increased in polyI:C/post-halothane treated mice compared with those in mice treated with polyI:C or halothane alone (Fig. 2A and B). Furthermore, *in vivo* z-VAD-fmk treatment of polyI:C/post-halothane-challenged mice caused a marked decrease in ALT activities approaching the levels detected in mice treated with halothane alone (Fig. 2C). These data provided strong evidence that the increased hepatocyte apoptosis caused by polyI:C/post-halothane treatment contributed to the exacerbation of liver injury. These data also suggested that hepatocytes were “sensitized” by halothane to become more susceptible to polyI:C-induced apoptosis, as neither polyI:C nor halothane alone caused significant apoptosis. Our hypothesis that halothane “sensitizes” hepatocytes to apoptotic stimuli is supported by previously published studies demonstrating that halothane has a profound inhibitory effect on protein synthesis in hepatocytes.⁴³⁻⁴⁶ Numerous studies have demonstrated that inhibition of protein synthesis directly renders hepatocytes more susceptible to apoptotic stimuli. Mice sensitized by D-galactosamine, an inhibitor of protein synthesis, are highly susceptible to hepatic injury upon exposure to LPS, CpG, polyI:C, superantigen, and whole bacteria, all of which induce the production of the apoptotic mediator, TNF- α .⁴⁷⁻⁵¹ Interestingly, hepatic glutathione (GSH) levels were significantly lower in halothane alone-treated mice (7.8 pmol/ μ g) than in vehicle-treated controls (16.5 pmol/ μ g). Depletion of hepatocellular GSH levels is associated with cytochrome-c release from mitochondria and may dictate cellular susceptibility to apoptotic stimuli.⁵² In this instance, we would expect that APAP, known to deplete hepatic GSH levels, would also “sensitize” hepatocytes to polyI:C-induced injury. However, treating mice with polyI:C post-APAP challenge did not increase APAP-induced liver injury (data not shown). Thus,

GSH depletion is not likely involved in halothane-mediated “sensitization” of hepatocytes to polyI:C-induced apoptosis.

Hepatocytes express TLR3,^{53,54} and polyI:C, through engaging TLR3, can activate the caspase cascade and cause apoptosis of hepatocytes.⁵⁵ Therefore, it is possible that polyI:C directly targets hepatocytes and stimulates apoptotic death, particularly when the cells are “sensitized” to undergo apoptosis due to halothane pre-treatment. Given the strong stimulatory effect of polyI:C on the innate immune system, polyI:C may alternatively induce hepatocyte apoptosis through the activation of hepatic innate immune cells to produce pro-apoptotic factors. NK cells represent a major target of polyI:C, as treatment of mice with polyI:C causes recruitment and activation of hepatic NK cells.^{16,56,57} A recent study demonstrated that dimethyl sulfoxide (DMSO), an organic solvent often used to facilitate the dissolution of pharmacological compounds, could activate hepatic NK and NKT cells and up-regulate their expression of cytotoxic effector molecules, such as interferon- γ and granzyme B (Masson et al., Accepted Article Online: Hepatology, 2008). We found that PolyI:C also up-regulates the expression of several pro-apoptotic molecules, including FasL and NKG2D on hepatic NK cells, rendering these cells more susceptible to the induction of apoptosis.^{16,20,21,58} The ligands for NKG2D include Rae-1 (retinoic acid early inducible transcript 1) and Mult-1 (mouse UL16-binding protein-like transcript 1), cellular expression of which is induced under various stress conditions.^{59,60} A recent study demonstrated that the expression of Rae-1 and Mult-1 in hepatocytes were markedly increased upon concanavalin-A (Con A) stimulation in hepatitis B virus transgenic mice, and that NKG2D recognition of Rae-1 or Mult-1 contributed to the sensitivity of these mice to Con A-induced liver injury.⁶¹ We found that the hepatic mRNA expression levels of Rae-1 and Mult-1 were increased by halothane and polyI:C/post-halothane treatments (data not shown), suggesting the involvement of NKG2D in mediating the cytotoxicity of NK cells.

We witnessed a significantly higher number of NK cells in the livers of mice treated with polyI:C or polyI:C/post-halothane than in halothane alone-treated mice (Fig. 5A). This observation is consistent with previous reports on polyI:C-mediated recruitment and activation of NK cells.¹⁶ Our results demonstrate that administration of anti-AsGM1 antibody selectively depleted NK cells. However, this depletion resulted in only a small, but significant, decrease in ALT levels at 24h after halothane treatment and no change in ALT activities at 15h (Fig. 6A). While anti-AsGM1 antibody administration significantly reduced hepatic message levels of NKG2D and FasL, TNF- α expression, which was also up-regulated in polyI:C/post-halothane-treated mice, remain unchanged (Fig. 5C). Therefore, it is possible that TNF- α , perhaps in conjunction with NKG2D and FasL, is involved in mediating polyI:C-induced hepatocyte apoptosis. In this regard, both hepatic mRNA and serum levels of TNF- α were significantly lower in KC-depleted mice compared with control mice upon polyI:C/post-halothane co-treatment (Fig. 7A and B), suggesting that KC are a predominant source of TNF- α in the liver. Furthermore, depletion of KC by liposome/clodronate suppressed polyI:C/post-halothane-induced liver injury to the levels approaching that observed in mice treated with halothane alone (Fig. 6B). Administration of anti-TNF- α neutralizing antibody also significantly reduced ALT levels in mice treated with polyI:C/post-halothane (Fig. 7C), however, the degree of reduction was much less than that deserved in response to KC depletion. Our data revealed that polyI:C-induced recruitment of NK cells was significantly inhibited when KC were depleted (Fig. 8A), perhaps due to the lack of IL-12 production in the absence of KC (Fig. 8B). We postulate that the strongest inhibition of polyI:C/post-halothane-induced liver injury observed in liposome/clodronate-treated mice was due to depletion of both KC and NK cells, resulting in the reduction of multiple pro-apoptotic factors. These results suggest that the combination of TNF- α , NKG2D and FasL are necessary in mediating polyI:C-induced hepatocyte apoptosis and liver injury.

In summary, our studies demonstrated that in comparison with mice treated with halothane alone, co-treatment of mice with polyI:C/post-halothane caused a profound increase in tissue damage. The exacerbation of liver injury correlated with enhanced hepatocyte apoptosis, which appeared to be a synergistic effect of the co-administration of polyI:C/post-halothane. Our findings also support a model in which polyI:C induced activation of hepatic KC and NK cells enhances the expression of multiple pro-apoptotic factors, including TNF- α , NKG2D and FasL, that mediate polyI:C-induced hepatocyte apoptosis. This is the first study to provide evidence that concurrent infection, through the activation of the hepatic innate immune system and subsequent inflammation, increases patient risk of developing DILI.

Abbreviations

DILI	drug-induced liver injury
TLRs	toll-like receptors
PAMPs	pathogen-associated molecular patterns
LPS	lipopolysaccharide
NK	natural killer
polyI:C	polyinosinic-polycytidylic acid
KC	Kupffer cells
IL	interleukin
i.p.	intraperitoneally
i.v.	intravenously
liposome/clodronate	liposome-entrapped clodronate
ALT	alanine transaminase
H/E	hematoxylin and eosin
PE	phycoerythrin
APC	allophycocyanin
TNF-α	tumor necrosis factor- α
TFA	trifluoroacetylchloride
CYP	cytochrome
LMNC	liver mononuclear cells
APAP	acetaminophen
GSH	glutathione
Rae-1	retinoic acid early inducible transcript 1
Mult-1	mouse UL16-binding protein-like transcript 1
Con A	concanavalin-A

Acknowledgments

We thank Dr. Lance Pohl (NIH, Bethesda, MD) for the generous gift of anti-TFA antisera.

Financial Support: U.S. National Institutes of Health grant RO1 ES012914 (to C.J.).

Reference List

1. Ostapowicz G, Fontana RJ, Schiodt FV, et al. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann Intern Med.* 2002; 137:947–954. [PubMed: 12484709]
2. Temple RJ, Himmel MH. Safety of newly approved drugs: implications for prescribing. *JAMA.* 2002; 287:2273–2275. [PubMed: 11980528]
3. McKenzie H, Parratt D, White RG. IgM and IgG antibody levels to ampicillin in patients with infectious mononucleosis. *Clin Exp Immunol.* 1976; 26:214–221. [PubMed: 991453]
4. Pullen H, Wright N, Murdoch JM. Hypersensitivity reactions to antibacterial drugs in infectious mononucleosis. *Lancet.* 1967; 2:1176–1178. [PubMed: 4168380]
5. Jaffe HS, Abrams DI, Ammann AJ, Lewis BJ, Golden JA. Complications of co-trimoxazole in treatment of AIDS-associated *Pneumocystis carinii* pneumonia in homosexual men. *Lancet.* 1983; 2:1109–1111. [PubMed: 6138645]
6. Ackerman Z, Levy M. Hypersensitivity reactions to drugs in acquired immunodeficiency syndrome. *Postgrad Med J.* 1987; 63:55–56. [PubMed: 2890152]
7. Coopman SA, Johnson RA, Platt R, Stern RS. Cutaneous disease and drug reactions in HIV infection. *N Engl J Med.* 1993; 328:1670–1674. [PubMed: 8487826]
8. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* 2004; 4:499–511. [PubMed: 15229469]
9. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature.* 1997; 388:394–397. [PubMed: 9237759]
10. Muzio M, Bosisio D, Polentarutti N, et al. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol.* 2000; 164:5998–6004. [PubMed: 10820283]
11. Visintin A, Mazzoni A, Spitzer JH, et al. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J Immunol.* 2001; 166:249–255. [PubMed: 11123299]
12. Wang M, Ellison CA, Gartner JG, HayGlass KT. Natural killer cell depletion fails to influence initial CD4 T cell commitment in vivo in exogenous antigen-stimulated cytokine and antibody responses. *J Immunol.* 1998; 160:1098–1105. [PubMed: 9570522]
13. Pfeffer LM, Dinarello CA, Herberman RB, et al. Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. *Cancer Res.* 1998; 58:2489–2499. [PubMed: 9635566]
14. Sun S, Zhang X, Tough DF, Sprent J. Type I interferon-mediated stimulation of T cells by CpG DNA. *J Exp Med.* 1998; 188:2335–2342. [PubMed: 9858519]
15. Marrack P, Kappler J, Mitchell T. Type I interferons keep activated T cells alive. *J Exp Med.* 1999; 189:521–530. [PubMed: 9927514]
16. Dong Z, Wei H, Sun R, et al. Involvement of natural killer cells in PolyI:C-induced liver injury. *J Hepatol.* 2004; 41:966–973. [PubMed: 15582130]
17. Wright R, Eade OE, Chisholm M, et al. Controlled prospective study of the effect on liver function of multiple exposures to halothane. *Lancet.* 1975; 1:817–820. [PubMed: 48053]
18. Cousins MJ, Plummer JL, Hall PD. Risk factors for halothane hepatitis. *Aust N Z J Surg.* 1989; 59:5–14. [PubMed: 2643940]
19. You Q, Cheng L, Reilly TP, Wegmann D, Ju C. Role of neutrophils in a mouse model of halothane-induced liver injury. *Hepatology.* 2006; 44:1421–1431. [PubMed: 17133481]
20. Radaeva S, Sun R, Jaruga B, et al. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. *Gastroenterology.* 2006; 130:435–452. [PubMed: 16472598]
21. Wang J, Sun R, Wei H, et al. Poly I:C prevents T cell-mediated hepatitis via an NK-dependent mechanism. *J Hepatol.* 2006; 44:446–454. [PubMed: 16310275]
22. Ju C, Reilly TP, Bourdi M, et al. Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol.* 2002; 15:1504–1513. [PubMed: 12482232]

23. Franklin CC, Krejsa CM, Pierce RH, et al. Caspase-3-Dependent Cleavage of the Glutamate-L-Cysteine Ligase Catalytic Subunit during Apoptotic Cell Death. *Am J Pathol.* 2002; 160:1887–1894. [PubMed: 12000740]
24. Liu ZX, Govindarajan S, Kaplowitz N. Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. *Gastroenterology.* 2004; 127:1760–1774. [PubMed: 15578514]
25. Yin H, Cheng L, Langenbach R, Ju C. Prostaglandin I(2) and E(2) mediate the protective effects of cyclooxygenase-2 in a mouse model of immune-mediated liver injury. *Hepatology.* 2007; 45:159–169. [PubMed: 17187424]
26. Delaporte E, Renton KW. Cytochrome P4501A1 and cytochrome P4501A2 are downregulated at both transcriptional and post-transcriptional levels by conditions resulting in interferon-alpha/beta induction. *Life Sci.* 1997; 60:787–796. [PubMed: 9064483]
27. Sakai H, Okamoto T, Kikkawa Y. Suppression of hepatic drug metabolism by the interferon inducer, polyribinosinic acid:polyribocytidylic acid. *J Pharmacol Exp Ther.* 1992; 263:381–386. [PubMed: 1403800]
28. Knickle LC, Spencer DF, Renton KW. The suppression of hepatic cytochrome P4504A mRNA mediated by the interferon inducer polyinosinic acid:polycytidylic acid. *Biochem Pharmacol.* 1992; 44:604–608. [PubMed: 1380810]
29. Renton KW, Mannering GJ. Depression of the hepatic cytochrome P-450 mono-oxygenase system by administered tilorone (2,7-bis(2-(diethylamino)ethoxy)fluoren-9-one dihydrochloride). *Drug Metab Dispos.* 1976; 4:223–231. [PubMed: 6226]
30. Leeson GA, Biedenbach SA, Chan KY, Gibson JP, Wright GJ. Decrease in the activity of the drug-metabolizing enzymes of rat liver following the administration of tilorone hydrochloride. *Drug Metab Dispos.* 1976; 4:232–238. [PubMed: 6227]
31. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature.* 2001; 413:732–738. [PubMed: 11607032]
32. Chen Y, Sun R, Jiang W, Wei H, Tian Z. Liver-specific HBsAg transgenic mice are over-sensitive to Poly(I:C)-induced liver injury in NK cell- and IFN-gamma-dependent manner. *J Hepatol.* 2007 Aug; 47(2):183–190. Epub 2007 Mar. 2007;47:183-190. [PubMed: 17448568]
33. Jeong WI, Park O, Gao B. Abrogation of the antifibrotic effects of natural killer cells/interferon-gamma contributes to alcohol acceleration of liver fibrosis. *Gastroenterology.* 2008; 134:248–258. [PubMed: 18166357]
34. Matsumoto M, Seya T. TLR3: Interferon induction by double-stranded RNA including poly(I:C). *Adv Drug Deliv Rev.* 2008; 60:805–812. [PubMed: 18262679]
35. Pruett SB, Schwab C, Zheng Q, Fan R. Suppression of innate immunity by acute ethanol administration: a global perspective and a new mechanism beginning with inhibition of signaling through TLR3. *J Immunol.* 2004; 173:2715–2724. [PubMed: 15294990]
36. Sivori S, Falco M, Carlomagno S, et al. Heterogeneity of TLR3 mRNA transcripts and responsiveness to poly (I:C) in human NK cells derived from different donors. *Int Immunol.* 2007; 19:1341–1348. [PubMed: 17962643]
37. Deng X, Stachlewitz RF, Liguori MJ, et al. Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. *J Pharmacol Exp Ther.* 2006; 319:1191–1199. [PubMed: 16990511]
38. Luyendyk JP, Maddox JF, Green CD, Ganey PE, Roth RA. Role of hepatic fibrin in idiosyncrasy-like liver injury from lipopolysaccharide-ranitidine coexposure in rats. *Hepatology.* 2004; 40:1342–1351. [PubMed: 15565632]
39. Luyendyk JP, Shaw PJ, Green CD, et al. Coagulation-mediated hypoxia and neutrophil-dependent hepatic injury in rats given lipopolysaccharide and ranitidine. *J Pharmacol Exp Ther.* 2005; 314:1023–1031. [PubMed: 15933155]
40. Tukov FF, Luyendyk JP, Ganey PE, Roth RA. The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. *Toxicol Sci.* 2007; 100:267–280. [PubMed: 17698507]

41. Waring JF, Liguori MJ, Luyendyk JP, et al. Microarray analysis of lipopolysaccharide potentiation of trovafloxacin-induced liver injury in rats suggests a role for proinflammatory chemokines and neutrophils. *J Pharmacol Exp Ther.* 2006; 316:1080–1087. [PubMed: 16299187]
42. Kalabis GM, Wells PG. Biphasic modulation of acetaminophen bioactivation and hepatotoxicity by pretreatment with the interferon inducer polyinosinic-polycytidylic acid. *J Pharmacol Exp Ther.* 1990; 255:1408–1419. [PubMed: 2262910]
43. Kvolik S, Glavas-Obrovac L, Bares V, Karner I. Effects of inhalation anesthetics halothane, sevoflurane, and isoflurane on human cell lines. *Life Sci.* 2005; 77:2369–2383. [PubMed: 15993426]
44. Palmer LK, Rannels SL, Kimball SR, Jefferson LS, Keil RL. Inhibition of mammalian translation initiation by volatile anesthetics. *Am J Physiol Endocrinol Metab.* 2006; 290:E1267–E1275. [PubMed: 16434554]
45. Ghantous HN, Fernando JL, Keith RL, Gandolfi AJ, Brendel K. Effects of halothane and other volatile anaesthetics on protein synthesis and secretion in guinea pig liver slices. *Br J Anaesth.* 1992; 68:172–177. [PubMed: 1540460]
46. Ghantous H, Fernando J, Gandolfi AJ, Brendel K. Inhibition of protein synthesis and secretion by volatile anesthetics in guinea pig liver slices. *Adv Exp Med Biol.* 1991; 283:725–730. [PubMed: 2069047]
47. Dejager L, Libert C. Tumor necrosis factor alpha mediates the lethal hepatotoxic effects of poly(I:C) in D-galactosamine-sensitized mice. *Cytokine.* 2008; 42:55–61. [PubMed: 18331798]
48. Sparwasser T, Miethke T, Lipford G, et al. Bacterial DNA causes septic shock. *Nature.* 1997; 386:336–337. [PubMed: 9121548]
49. Freudenberg MA, Galanos C. Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-galactosamine-treated mice. *Infect Immun.* 1991; 59:2110–2115. [PubMed: 2037372]
50. Nagaki M, Muto Y, Ohnishi H, et al. Hepatic injury and lethal shock in galactosamine-sensitized mice induced by the superantigen staphylococcal enterotoxin B. *Gastroenterology.* 1994; 106:450–458. [PubMed: 8299910]
51. Mignon A, Rouquet N, Fabre M, et al. LPS challenge in D-galactosamine-sensitized mice accounts for caspase-dependent fulminant hepatitis, not for septic shock. *Am J Respir Crit Care Med.* 1999; 159:1308–1315. [PubMed: 10194182]
52. Ghibelli L, Coppola S, Fanelli C, et al. Glutathione depletion causes cytochrome c release even in the absence of cell commitment to apoptosis. *FASEB J.* 1999; 13:2031–2036. [PubMed: 10544186]
53. Liu S, Gallo DJ, Green AM, et al. Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. *Infect Immun.* 2002; 70:3433–3442. [PubMed: 12065483]
54. Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. *Biol Pharm Bull.* 2005; 28:886–892. [PubMed: 15863899]
55. Khvalevsky E, Rivkin L, Rachmilewitz J, Galun E, Giladi H. TLR3 signaling in a hepatoma cell line is skewed towards apoptosis. *J Cell Biochem.* 2007; 100:1301–1312. [PubMed: 17243100]
56. Chen Y, Wei H, Sun R, Tian Z. Impaired function of hepatic natural killer cells from murine chronic HBsAg carriers. *Int Immunopharmacol.* 2005; 5:1839–1852. [PubMed: 16275620]
57. Sun R, Park O, Horiguchi N, et al. STAT1 contributes to dsRNA inhibition of liver regeneration after partial hepatectomy in mice. *Hepatology.* 2006 Oct; 44(4):955–966. 2006;44:955-966. [PubMed: 17006930]
58. Jeong WI, Park O, Radaeva S, Gao B. STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. *Hepatology.* 2006 Dec; 44(6):1441–1451. 2006;44:1441-1451. [PubMed: 17133483]
59. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol.* 2003; 3:781–790. [PubMed: 14523385]
60. Cerwenka A, Lanier LL. Ligands for natural killer cell receptors: redundancy or specificity. *Immunol Rev.* 2001; 181:158–169. [PubMed: 11513137]

61. Chen Y, Wei H, Sun R, et al. Increased susceptibility to liver injury in hepatitis B virus transgenic mice involves NKG2D-ligand interaction and natural killer cells. *Hepatology*. 2007; 46:706–715. [PubMed: 17626270]

Figure 1. PolyI:C co-treatment affects halothane-induced liver injury in mice

Female BALB/cByJ mice were treated with 50 μ g polyI:C/mouse (i.p.) 12h or 7 days prior to or 6h after halothane challenge (30 mmole/halothane/kg, i.p.). Control mice were treated with either polyI:C alone or halothane alone. (A and B) Serum ALT levels were measured at 15h and 24h after halothane administration. Results shown represent mean $\pm$ SEM of 8 mice per group, and individual samples were assayed in triplicates. (*) $p < 0.05$ compared with halothane alone-treated mice, (#) $p < 0.05$ compared with polyI:C alone-treated mice. (C and D) Mice were treated with halothane alone or polyI:C/post-halothane as described above. (C) Livers were excised at 24h after halothane treatment and paraffin-embedded sections were prepared. Photomicrographs (200 $\times$, final magnification) of H/E stained liver sections are shown. (D) Livers were harvested at 15h after halothane administration and tissue homogenates (30 μ g/lane) were immunoblotted with anti-TFA polyclonal antibodies (1:1000 dilution). Molecular mass markers (kDa) are indicated on the right.

Figure 2. PolyI:C/post-halothane co-treatment resulted in increased hepatocyte apoptosis (A) Female BALB/cByJ mice were treated with halothane or polyI:C/post-halothane as described above. Livers were excised at 15h after halothane treatment and liver homogenates and paraffin-embedded sections were prepared. Photomicrographs (200 $\times$, final magnification) of TUNEL assay staining on tissue sections obtained from halothane alone- and polyI:C/post-halothane-treated mice are shown. (B) Caspase-3 activities were measured in liver homogenates obtained from polyI:C-, halothane- and polyI:C/post-halothane-treated mice. Results shown represent mean $\pm$ SEM of at least 5 mice per group, and individual samples were assayed in duplicates. (*) $p < 0.05$ compared with halothane alone-treated mice, (#) $p < 0.05$ compared with polyI:C alone-treated mice. (C) Mice treated with polyI:C/

post-halothane were divided into two groups. Five hours after halothane treatment, one group was injected with z-VAD-fmk (200 µg dissolved in 1% DMSO, i.p.) and the other injected with 1% DMSO (i.p.). Serum ALT levels were examined at 15h and 24h after halothane administration. Results shown represent mean ± SEM of 5 mice per group, and individual samples were assayed in triplicates. (*) $p < 0.05$ compared with 1% DMSO-treated control mice.

Figure 3. PolyI:C/post-halothane co-treatment up-regulated the hepatic mRNA expression of pro-apoptotic mediators

Female BALB/cByJ mice were treated with polyI:C, halothane or polyI:C/post-halothane as described above. Fifteen hours after halothane treatment, livers were removed for mRNA extraction. Message levels of TNF- α , NKG2D and FasL were determined by RT-PCR. (A) Results shown are representative of mice from various treatment groups. (B) Relative mRNA expression levels of various pro-apoptotic mediators. Results shown represent mean $\pm$ SEM of 7 mice per group. (*) $p < 0.05$ compared with halothane alone-treated mice. (#) $p < 0.05$ compared with polyI:C alone-treated mice.

Figure 4. Protein expression levels of pro-apoptotic mediators were up-regulated in polyI:C/post-halothane-treated mice

Female BALB/cByJ mice were treated with polyI:C, halothane or polyI:C/post-halothane as described above. Blood was collected and LMNC isolated at 15h after halothane treatment. (A) Freshly isolated LMNC were stained with anti-NKG2D or anti-FasL antibody and analyzed by flow cytometry. The results are shown as histograms labeled with fluorescence intensity on the x-axis and cell number on the y-axis. Data depicted are representative of at least 4 mice per treatment group, including polyI:C (P), halothane (H) and polyI:C/post-halothane (P/H). (B) Serum levels of TNF- α were measured by ELISA. Results shown represent mean $\pm$ SEM of at least 10 mice per group, and individual samples were assayed in triplicates. ND indicates non-detectable. (*) $p < 0.05$ compared with halothane alone-treated mice. (#) $p < 0.05$ compared with polyI:C alone-treated mice.

Figure 5. NK cells represented a major source of NKG2D and FasL in the liver of mice treated with polyI:C/post-halothane

(A) Female BALB/cByJ mice were treated with polyI:C, halothane or polyI:C/post-halothane as described above. Freshly isolated LMNC were obtained at 15h after halothane treatment. Cells were stained with anti-DX5 and anti-CD3, as well as 7-AAD, and analyzed by flow cytometry. Live cells (7-AAD-negative) were gated and their expression of DX5 and CD3 are demonstrated in dot plots. Data shown are representative of 4 mice per group. (B) To deplete NK cells, mice were treated (i.v.) with 100 μ L anti-AsGM1 antibody 2 days prior to polyI:C/post-halothane treatment. Control mice were administered normal rabbit serum. Liver tissues were removed for RNA extraction and LMNC isolation at 15h after halothane treatment. LMNC were stained with anti-CD3 and 7-AAD, along with either anti-DX5, anti-NKG2D or anti-FasL, and analyzed by flow cytometry. DX5 and CD3 expression are demonstrated in dot plots. NKG2D and FasL expression on gated CD3-negative cells are shown in histograms labeled with fluorescence intensity on the x-axis and cell number on the y-axis. Data shown are representative of 8 mice from anti-AsGM1-treated group (solid line) and 7 mice from normal rabbit serum-treated group (dashed line). (C) Hepatic mRNA expression levels of TNF- α , NKG2D and FasL were determined by RT-PCR. Results shown are representative of 4 mice from each group.

Figure 6. Depletion of KC or NK cells mitigated polyI:C/post-halothane-induced liver injury
 Female BALB/cByJ mice were treated with halothane alone or polyI:C/post-halothane as described above. (A) To deplete NK cells, mice were injected i.v. with anti-AsGM1 antibody 2 days prior to halothane administration. Control mice were treated with normal rabbit serum. (B) To deplete KC, mice were injected i.v. with liposome/clodronate (Lipo-Cld) 2 days prior to halothane challenge. Control mice were treated with empty liposomes (Lipo-PBS). Blood was collected at 15h and 24h after halothane treatment for measurement of serum ALT levels. Results shown represent mean $\pm$ SEM of at least 9 mice per group. (*) $p < 0.05$ compared with normal rabbit serum-treated (A) or Lipo-PBS-treated mice (B).

Figure 7. PolyI:C-induced exacerbation of halothane hepatotoxicity was partially attributable to TNF- α production by hepatic KC

Female BALB/cByJ mice were treated with polyI:C/post-halothane as described above. To deplete KC, mice were injected i.v. with liposome/clodronate (Lipo-Cld) 2 days prior to halothane challenge. Control mice were treated with empty liposomes (Lipo-PBS). (A) Livers were removed at 10h after halothane treatment for RNA extraction, and message levels of TNF- α were measured by RT-PCR. Results shown are representative of mice from each group. (B) Serum levels of TNF- α were measured at 10h after halothane treatment by ELISA. Results shown represent mean $\pm$ SEM of 5 mice per group. (*) $p < 0.05$ compared with Lipo-PBS-treated mice. (C) To neutralize TNF- α mice were injected i.v. with anti-mouse TNF cV1q antibody (200 μ g/mouse) at 10h post-halothane challenge. Control mice were treated with the isotype control antibody. Blood was collected at 15h and 24h after halothane treatment for measurement of serum ALT levels. Results shown represent mean $\pm$ SEM of at least 12 mice per group. (*) $p < 0.05$ compared with isotype control antibody-treated mice.

Figure 8. Depletion of KC resulted in decrease of hepatic NK cells and diminished hepatic IL-12 message expression

KC were depleted in female BALB/cByJ mice by i.v. injection with liposome/clodronate (Lipo-Cld). Control mice were treated with empty liposomes (Lipo-PBS). After 2 days, mice were treated with polyI:C/post-halothane as described above. (A) Freshly isolated LMNC were obtained at 15h after halothane treatment from Lipo-PBS- and Lipo-Cld-treated mice. The cells were stained with anti-DX5 and anti-CD3, as well as 7-AAD, and analyzed by flow cytometry. Live cells (7-AAD-negative) were gated and their expression of DX5 and CD3 are demonstrated in dot plots. Data shown are representative of 4 mice per group. (B) Mice were sacrificed 10h after halothane challenge and livers removed for RNA extraction. Hepatic message levels of IL-12 p40 were measured by RT-PCR. Results shown are representative of 4 mice from each group.