Published in final edited form as:

Hepatology. 2014 March ; 59(3): 1094–1106. doi:10.1002/hep.26748.

Opposing Effects of Prednisolone Treatment on T/NKT Cell- and Hepatotoxin-mediated Hepatitis in Mice

Hyo-Jung Kwon^{*,1,2}, Young-Suk Won^{*,1,3}, Ogyi Park¹, Dechun Feng¹, and Bin Gao¹

¹Laboratory of Liver Diseases, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892, USA

²College of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon, 305-764, South Korea

³Laboratory Animal Resource Center, Korea Research Institute of Bioscience and Biotechnology, Chungbuk, 363-883, South Korea

Abstract

Prednisolone is a corticosteroid that has been used to treat inflammatory liver diseases, such as autoimmune hepatitis and alcoholic hepatitis. However, the results have been controversial, and how prednisolone affects liver disease progression remains unknown. In the current study, we examined the effect of prednisolone treatment on several models of liver injury, including T/NKT cell hepatitis induced by concanavalin A (ConA) and α -galactosylceramide (α -GalCer), and hepatotoxin-mediated hepatitis induced by carbon tetrachloride (CCl₄) and/or ethanol. Prednisolone administration attenuated ConA- and α -GalCer-induced hepatitis and systemic inflammatory responses. Treating mice with prednisolone also suppressed inflammatory responses in a model of hepatotoxin (CCl₄)-induced hepatitis, but surprisingly exacerbated liver injury and delayed liver repair. In addition, administration of prednisolone also enhanced acetaminophen-, ethanol-, or ethanol plus CCl₄-induced liver injury. Immunohistochemical and flow cytometric analyses demonstrated that prednisolone treatment inhibited hepatic macrophage and neutrophil infiltration in CCl₄-induced hepatitis and suppressed their phagocytic activities *in vivo* and *in vitro*. Macrophage and/or neutrophil depletion aggravated CCl₄-induced liver injury and impeded liver regeneration. Finally, conditional disruption of glucocorticoid receptor in macrophages and neutrophils abolished prednisolone-mediated exacerbation of hepatotoxin-induced liver injury.

Conclusion—Prednisolone treatment prevents T/NKT cell hepatitis but exacerbates hepatotoxin-induced liver injury by inhibiting macrophage- and neutrophil-mediated phagocytic and hepatic regenerative functions. These findings may not only increase our understanding of the steroid treatment mechanism but also help us to better manage steroid therapy in liver diseases.

Keywords

α -GalCer; CCl₄; ConA; neutrophils; macrophages

Introduction

Liver inflammation is associated with acute and chronic liver diseases, including alcoholic liver disease, nonalcoholic fatty liver disease, viral hepatitis, and autoimmune hepatitis, which are characterized by the activation and infiltration of inflammatory cells in the

Corresponding author: Bin Gao, M.D., Ph.D., Laboratory of Liver Diseases, NIAAA/NIH, 5625 Fishers Lane, Bethesda, MD 20892. Tel: 301-443-3998, bgao@mail.nih.gov.

*These authors contributed equally to this work.

liver.¹⁻⁷ It is generally believed that liver inflammation is triggered by pathogen infections (e.g., hepatitis viruses) and/or damage-associated molecular patterns (DAMPs) released by damaged hepatocytes.⁶⁻⁸ However, the mechanisms through which DAMPs induce liver inflammation and how such inflammation contributes to the progression of liver disease remain largely unknown.⁶⁻⁸ For example, approximately 95% of heavy drinkers develop fatty liver, but only 20-35% of them progress to alcoholic hepatitis and fibrosis, in which inflammation is thought to play a critical role.^{1, 2, 9} Currently, the mechanisms underlying inflammation development in alcoholic liver injury are not fully understood, and many mechanisms have been proposed,^{1, 2, 9} including the alcohol consumption-associated elevation of hepatic endotoxin levels, hepatocellular damage, elevation of reactive oxygen species (ROS), activation of innate immunity, and activation of Kupffer cells. All of these factors upregulate the expression of pro-inflammatory cytokines and chemokines in the liver, followed by the recruitment of inflammatory cells, such as neutrophils and macrophages.^{1, 2, 9} The general purpose of early neutrophil infiltration is to remove dead or dying cells as a prerequisite for wound repair and regeneration, but neutrophils also cause cell injury, primarily through the formation of ROS and release of proteases.¹⁰⁻¹³ Despite its beneficial effect on liver repair and regeneration, inflammation remains an actively investigated therapeutic target, and immunosuppressive drugs, such as steroids, have been used for the treatment of several types of inflammatory liver diseases. However, the results of steroid therapy for inflammatory liver diseases, especially its results on alcoholic hepatitis, have been controversial.¹⁴⁻²⁰

Glucocorticoids (GCs) are steroid hormones that are synthesized in the adrenal cortex and play a wide variety of important functions in the body, including the modulation of metabolism, regulation of stress responses, and suppression of inflammation. The actions of GCs are mediated by binding to the GC receptor (GR) and subsequently inhibiting the activity of transcription factors, which control the expression of proinflammatory cytokines, chemokines, and adhesion molecules.²¹ Because of its broad anti-inflammatory effects, synthetic GCs, such as prednisolone, have been widely used to treat inflammatory liver diseases that are caused by an overactive immune system. Prednisolone treatment has been shown to ameliorate symptoms and improve biochemical and histologic abnormalities in many types of liver diseases, including autoimmune hepatitis,⁵ cirrhotic patients with septic shock,²² and liver transplantation.²³ Steroids have also been used for the treatment of alcoholic hepatitis for many years, but the results have been mixed.¹⁵⁻²⁰ Surprisingly, although steroids have been used to treat inflammatory liver diseases for more than five decades,²⁴ the mechanism through which prednisolone affects liver disease pathogenesis has not been explored. The aim of the present study was to examine the effect of prednisolone on liver injury in different experimental mouse models of inflammatory liver injury, including concanavalin A (ConA)-induced T cell hepatitis,²⁵ α -galactosylceramide (α -GalCer)-induced NKT cell hepatitis,²⁶ hepatotoxin CCl₄- or acetaminophen (APAP)-induced hepatitis,^{27, 28} chronic plus binge ethanol feeding model,^{29, 30} ethanol plus CCl₄-induced liver fibrosis.³¹ Our results demonstrated that prednisolone treatment prevented ConA- and α -GalCer-induced acute T/NKT cell hepatitis but exacerbated CCl₄-, acetaminophen-, ethanol-, or ethanol plus CCl₄-induced liver injury. Subsequent studies suggested that prednisolone treatment worsened CCl₄-induced hepatocellular damage by inhibiting macrophage- and neutrophil-mediated phagocytic and regenerative functions.

Materials and Methods

Mice

C57BL/6N mice were purchased from the NCI (Frederick, MD). Myeloid-specific glucocorticoid receptor knockout (LysCre⁺GR^{flox/flox})(mGR KO) mice were kindly provided by Dr. Louis J. Muglia (University of Cincinnati).³² LysCre^{-/-} GR^{flox/flox} mice

were used as littermate wild-type (WT) controls. All animal experiments were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee.

Mouse models

For the acute liver injury model, 8- to 10-week old male mice were injected with ConA (20 mg/kg body weight, i.v.) (Sigma-Aldrich, St Louis, MO), α -GalCer (100 mg/kg body weight, i.p.) (Enzo Life Science, Farmingdale, NY), or CCl₄ (0.2 ml/kg body weight, i.p.) (Sigma-Aldrich). Subsequently, prednisolone (10 mg/kg body weight in 5% ethanol, i.p.; Sigma-Aldrich) or vehicle (5% ethanol) was administered to mice, as described in supporting Fig. 1. In some experiments, mice were injected i.p. with 50 mg/kg body weight 5-bromo-2'-deoxy-uridine (BrdU; Sigma-Aldrich) 2h before sacrifice for the examination of liver regeneration.

The chronic plus binge ethanol model was described previously.²⁹ For ethanol plus CCl₄-induced liver fibrosis model, mice were fed Liber-DeCarli liquid diet for 8 weeks, and CCl₄ (0.1 mg/kg) were administered twice a week during the 8-week feeding. Prednisolone was injected i.p. daily for the last two weeks. For acetaminophen (APAP)-induced liver injury, mice were fasting for 24h and subsequently injected with APAP (200 mg/kg, i.p.), and prednisolone or vehicle was administered as described in the figure legends.

Other materials and methods are included in supporting document.

Results

Co-treatment with prednisolone ameliorates acute T/NKT cell hepatitis induced by ConA or α -GalCer

To investigate the role of prednisolone in experimental T cell hepatitis, mice were co-treated with ConA plus prednisolone or vehicle (supporting Fig. 1A). Treatment of mice with prednisolone alone did not induce elevation of serum ALT and no obvious liver necrosis was observed in mice after prednisolone treatment alone (supporting Fig. 2). ConA injection induced an elevation of serum ALT levels and massive necrosis in the liver, which were markedly decreased in mice co-treated with prednisolone (supporting Figs. 3A-B). Prednisolone treatment also markedly prevented ConA-induced elevation of serum levels of various cytokines (supporting Fig. 3C). Similarly, prednisolone treatment prevented the NKT activator α -GalCer-induced systemic inflammatory responses, liver inflammation, and injury (supporting Fig. 4).

Co-treatment with prednisolone delays liver repair in CCl₄-induced acute hepatitis

CCl₄ is a hepatotoxic agent that has been widely used to induce liver injury in a hepatotoxin-mediated hepatitis model that allows for the evaluation of both necrosis and subsequent inflammation.²⁷ To determine the role of prednisolone in toxin-induced acute liver injury, mice were injected with CCl₄ and co-treated with prednisolone or vehicle (supporting Fig. 1C). Liver histology analyses in Figs. 1A-B shows that in CCl₄+vehicle group, injection of a single dose of CCl₄ induced massive liver necrosis 24, 48, and 72h post injection, but such necrosis was markedly recovered 96h post injection; surprisingly, in CCl₄+prednisolone group, massive liver necrosis was not improved 96h post injection and still remained until 120h. In agreement with liver histology data, prednisolone co-treatment induced higher levels of serum ALT levels 48h post CCl₄ injection (Fig. 1C). Treatment with prednisolone alone did not induce obvious liver necrosis (Fig. 1B) nor affected serum ALT levels (Fig. 1C). Moreover, the serum levels of several pro-inflammatory cytokines (e.g., TNF- α and IL-6), which are important for liver regeneration,^{33, 34} were elevated after CCl₄ injection with vehicle co-treatment. These effects were completely attenuated in prednisolone co-

treated mice (Fig. 1D). Finally, treatment of mice with prednisolone did not affect hepatic expression of p450CYP2E1, a key enzyme to metabolize CCl₄ (supporting Fig. 5). This suggests that prednisolone exacerbation of CCl₄-induced liver injury is not mediated via the regulation of CCl₄ metabolism.

In addition, we also examined the effects of prednisolone treatment on APAP-induced liver injury (supporting Fig. 6). Liver histology analyses shows a single injection of APAP induced massive liver necrosis 48h post injection, but such necrosis was still remained until 72h in APAP+prednisolone group (supporting Fig. 6C). In addition, prednisolone co-treatment markedly attenuated BrdU incorporation in hepatocytes 48h after APAP injection (supporting Figs. 6C-D).

Co-treatment with prednisolone exacerbates ethanol or ethanol plus CCl₄-induced chronic liver injury and fibrosis

To investigate the effect of prednisolone on alcohol-induced liver injury, mice were fed an ethanol diet for 10 days, followed by a single gavage of ethanol plus prednisolone or vehicle treatment (supporting Fig. 7A). Chronic-binge ethanol feeding elevated serum ALT levels, which were markedly increased in mice co-treated with prednisolone (supporting Fig. 7B). In addition, H&E and Oil Red O staining revealed higher degree of steatosis in mice co-treated with prednisolone than in mice co-treated with vehicle (supporting Fig. 7C).

To further investigate the effect of prednisolone in chronic liver injury and fibrosis, mice were treated with ethanol plus CCl₄ and co-injected with prednisolone or vehicle (supporting Fig. 8A). As illustrated in supporting Fig. 8B, prednisolone co-treatment induced higher levels of serum ALT levels, greater levels of Sirius red and α -SMA staining, and higher hepatic expression levels of α -SMA compared with vehicle co-treated groups (supporting Figs. 8E-F).

Prednisolone administration inhibits acute CCl₄-induced macrophage and neutrophil recruitment in the liver

CCl₄-induced liver injury is associated with activation of Kupffer cells and trigger migration of macrophages into hepatic cords. As shown in Fig. 2A, administration of CCl₄ plus vehicle co-treatment induced accumulation of mononuclear cells (MNCs) and Gr-1^{int}CD11b⁺ macrophages in the liver 24h post treatment, but such accumulation was attenuated in prednisolone co-treated mice. This reduction of macrophages infiltration in prednisolone-treated mice was not due to the increased macrophage apoptosis because there were no differences in macrophage apoptosis between prednisolone co-treated and vehicle co-treated groups (supporting Fig. 9).

Neutrophil infiltration was determined by immunohistochemical analyses of MPO (a marker for neutrophils). As illustrated in Fig. 2B and supporting Fig. 10, CCl₄ injection resulted in neutrophil infiltration in the liver. The number of MPO⁺ cells was significantly increased 24 and 48h post CCl₄ injection and declined thereafter in the CCl₄+vehicle group. Prednisolone co-treatment delayed hepatic neutrophil recruitment, with a lower number of MPO⁺ cells at 24 and 48h but a higher number at 72h post CCl₄ administration compared with the vehicle co-treated group. Furthermore, FACS analyses also confirmed that prednisolone co-treatment attenuated hepatic neutrophil infiltration post CCl₄ injection. As illustrated in Fig. 2C, in the CCl₄+vehicle group, the total number of Gr-1^{hi}CD11b⁺ neutrophils was elevated, with a peak effect occurring 24h and then declining 72h post injection. Prednisolone co-treatment resulted in a lower number of neutrophils 24h but a slightly higher number 72h post CCl₄ injection compared with the vehicle co-treated mice. Such prednisolone-mediated inhibition of hepatic neutrophil number was not due to promotion of neutrophil apoptosis

because FACS analyses showed that prednisolone co-treatment delayed liver neutrophil apoptosis (supporting Fig. 11).

Finally, the hepatic expression of several cytokines and chemokines, which are associated with macrophage and neutrophil recruitment,³⁵ was suppressed in the prednisolone co-treated mice compared with those in the vehicle co-treated groups (Fig. 2D).

Co-administration with prednisolone attenuates macrophage and neutrophil functions

Macrophages and neutrophils are known to be professional phagocytes that play a significant role in the clearance of apoptotic cells and in the resolution of inflammation. To analyze the effect of prednisolone on macrophage and neutrophil phagocytic activity, mice were injected with latex beads post CCl₄ plus prednisolone or vehicle co-treatment. As illustrated in Figs. 3A-B, CCl₄ injection increased the percentage of macrophage or neutrophil engulfing latex beads, which was significantly reduced in the prednisolone co-treated group. Moreover, an *in vitro* phagocytosis assay (Figs. 3C-D) revealed that prednisolone exposure decreased the fluorescence intensity of latex beads in peritoneal macrophages and neutrophils.

Kupffer cells, liver resident macrophages stimulate tissue damage and repair by secreting cytokines. Therefore, we examined whether prednisolone treatment altered cytokine production by Kupffer cells as well as hepatic stellate cells (HSCs) and hepatocytes. As illustrated in supporting Fig. 12, Kupffer cells produced highest levels of TNF- α and IL-6 after CCl₄ treatment, followed by HSCs and hepatocytes. Such expression in Kupffer cells from CCl₄ plus prednisolone co-treated mice was much lower than those from CCl₄ plus vehicle co-treated mice. Expression of TNF- α but not IL-6 was also attenuated in HSCs from the CCl₄ plus prednisolone group compared with the CCl₄ plus vehicle.

Finally, the effects of prednisolone on neutrophil-mediated ROS production were also examined. As illustrated in Fig. 3E, without *in vitro* treatment with PMA, neutrophils from CCl₄-treated mice produced a slightly higher ROS burst compared with those from mice without CCl₄ treatment. This ROS burst production was suppressed in prednisolone co-treated mice compared with vehicle co-treated mice 24h but not 72h post CCl₄ injection. *In vitro* incubation with PMA, which induces cells to undergo a NOX-dependent respiratory burst, markedly increased the ROS levels of neutrophils (Fig. 3E). This PMA-mediated elevation of ROS production was lower in neutrophils from prednisolone co-treated mice compared with those from vehicle co-treated mice 24h post CCl₄ injection (Fig. 3E).

Prednisolone treatment delays liver regeneration by inhibiting hepatic STAT3 and pNF- κ B activation in CCl₄-induced acute hepatitis

The effect of prednisolone on liver regeneration was examined to further understand why prednisolone treatment delayed liver repair post CCl₄ injection as observed in Fig. 1. As illustrated in Fig. 4A, CCl₄ challenge markedly increased BrdU incorporation in hepatocytes, with a peak effect 48h post challenge. However, prednisolone co-treatment delayed this peak to 72h.

We next investigated the mechanisms underlying the prednisolone-mediated interruption of liver regeneration in CCl₄-induced acute hepatitis by examining the hepatic expression of pSTAT3, pNF- κ B, and proliferative genes. As shown in Fig. 4B, hepatic STAT3 was activated, with a peak effect occurring at 3 to 12h after CCl₄ injection in the CCl₄+vehicle group. The hepatic expression levels of pSTAT3 were lower at 6 and 12h but higher at 24 to 72h after CCl₄ injection in the CCl₄+prednisolone group, which suggests that prednisolone treatment caused a delay in hepatic STAT3 activation. The hepatic expression of pNF- κ B

was also decreased at 3 and 6h in CCl₄+prednisolone group compared with the CCl₄+vehicle group (Fig. 4C). In addition, prednisolone treatment slightly reduced NF- κ B acetylation at 6h post CCl₄ injection, but it did not affect STAT3 acetylation (supporting Fig. 13). The induction of PCNA and cyclin D1 expression was delayed in the prednisolone co-treated mice compared with the vehicle co-treated mice (Fig. 4B).

Neutrophil and/or macrophage depletion aggravates CCl₄-induced acute liver injury and impedes liver regeneration

The above findings suggest that prednisolone inhibits neutrophil and macrophage recruitment and functions; however, the roles of these inflammatory cells in CCl₄-induced liver injury and regeneration remain largely unclear. To understand the functions of macrophages and neutrophils, we used anti-Gr1Ab antibody to deplete these cells prior to CCl₄ challenge. As illustrated in supporting Fig. 14A, pretreatment with anti-Gr1Ab markedly reduced both macrophages (Gr1^{low}CD11b⁺) and neutrophils (Gr1^{hi}CD11b⁺) in the liver. The depletion of both macrophages and neutrophils with anti-Gr-1 Ab induced greater elevation of serum ALT and liver injury (supporting Fig. 14B), increased the area of necrosis (supporting Fig. 14C), and delayed liver regeneration as determined by BrdU incorporation (supporting Fig. 14D).

To further dissect the roles of macrophages and neutrophils, we used anti-Ly6G and clodronate to deplete specifically neutrophils and macrophages, respectively. As illustrated in Fig. 5A, treatment with anti-Ly6G depleted neutrophils (Gr1^{hi}CD11b⁺) without affecting macrophages (Gr1^{low}CD11b⁺) in the liver. Such treatment increased CCl₄-induced elevation of serum ALT and liver necrosis, and delayed liver regeneration (Figs. 5B-D). Additionally, treatment with clodronate depleted F4/80 positive macrophages in the liver (Fig. 6A) and also exacerbated CCl₄-induced elevation of serum ALT and liver necrosis but attenuated liver regeneration (Figs. 6B-D).

Treatment with prednisolone does not affect CCl₄-induced liver injury and regeneration in mGR KO mice

To further explore whether the detrimental effects of prednisolone on CCl₄-induced hepatitis are mediated by targeting macrophages and/or neutrophils, we used myeloid-specific GR knockout (mGR KO) mice, in which the GR gene was deleted in macrophages and neutrophils.³² Western blot analyses confirmed a loss of GR protein expression in both macrophages and neutrophils from mGR KO mice (Fig. 7A). Moreover, the effects of prednisolone on CCl₄-induced acute hepatitis were compared in WT and mGR KO mice. As illustrated in Fig. 7B, CCl₄ treatment resulted in an elevation of serum ALT levels in WT mice, and such elevation was higher in prednisolone co-treated WT mice 48h post CCl₄ injection. However, prednisolone co-treatment did not affect CCl₄-induced elevation of serum ALT levels in mGR KO mice. Liver histology analyses in Fig. 7C show that prednisolone co-treatment increased CCl₄-induced necrotic area in WT mice but did not affect this in mGR KO mice. Immunohistochemical analyses in Fig. 7D show that prednisolone co-treatment delayed neutrophil infiltration in CCl₄-treated WT mice (the peak of neutrophil infiltration occurred 48h post CCl₄ injection alone but occurred 72h post CCl₄ plus prednisolone co-treatment). In contrast, prednisolone co-treatment did not have an effect on neutrophil infiltration in CCl₄-treated mGR KO mice. Similarly, prednisolone co-treatment delayed liver regeneration in CCl₄-treated WT mice but not in mGR KO mice (Fig. 7E).

Discussion

Prednisolone has been used in the treatment of various types of liver diseases, especially autoimmune/inflammatory conditions in which the immune system is overactive.²⁴ However, these treatments have varying degrees of responsiveness among individuals and in different types of liver diseases. This is partly because the effects of prednisolone treatment on liver disease pathogenesis remain unknown. In the current study, several novel findings were demonstrated. First, prednisolone treatment is very effective in preventing T/NKT cell hepatitis but exacerbates hepatotoxin-induced hepatitis and fibrosis. Second, prednisolone treatment inhibits macrophage- and neutrophil-mediated phagocytosis. Third, depletion of macrophages or neutrophils exacerbates liver injury and attenuates liver regeneration in CCl₄-induced liver injury. Fourth, prednisolone treatment delays liver regeneration in CCl₄-induced liver injury. We have integrated all of these findings into a model in Fig. 8.

ConA- and α -GalCer-induced hepatitis are mouse models of T/NKT cell-mediated liver injury that resemble autoimmune hepatitis in humans.^{36, 37} Both models are characterized by the rapid activation of NKT and T cells and subsequent production of various pro-inflammatory cells and infiltration of neutrophils and macrophages, followed by hepatocyte necrosis and apoptosis.^{36, 37} ConA activates a wide array of T cells regardless of their antigen specificity, and α -GalCer is not an endogenous lipid antigen for NKT cells; thus, neither ConA- nor α -GalCer-induced hepatitis represent autoimmune liver disease models in a strict sense. However, many features of autoimmune hepatitis are observed in these models. Thus, both of these models are often considered to represent autoimmune hepatitis models and are used to study the mechanisms underlying T/NKT cell-mediated liver injury and test the effectiveness of immunosuppressive drugs for the treatment of autoimmune hepatitis.^{36, 37} Here, we demonstrated that co-treating mice with prednisolone markedly prevented ConA- and α -GalCer-induced liver inflammation and injury (supporting Figs. 1-2). This finding is consistent with the experimental and clinical data that steroid therapy is effective in a mouse autoimmune hepatitis model of adenoviral infection³⁸ and in patients with autoimmune hepatitis.^{39, 40} The protective effects of prednisolone treatment on T/NKT cell hepatitis are likely mediated by its immunosuppressive function on T/NKT cell activation and cytokine production and the subsequent inhibition of T/NKT cell hepatitis.

In contrast to ConA- or α -GalCer-induced hepatitis, which is initiated by activated immune cells, hepatotoxin ethanol and/or CCl₄-induced liver injury is instigated by the direct induction of hepatocyte damage, which subsequently activates and recruits macrophages/Kupffer cells and neutrophils.²⁷ Activated macrophages/Kupffer cells produce free radicals and proinflammatory cytokines that further trigger hepatocellular damage and induce neutrophil accumulation and activation.⁴¹ The resulting neutrophil influx may promote additional tissue damage via the release of oxygen-reactive species and proteases.^{10, 41} However, macrophages and neutrophils also play an important role in removing necrotic cell debris and activating regenerative pathways, ultimately facilitating tissue repair and the resolution of the inflammatory response.^{13, 41-43} In the current study, we demonstrated that the depletion of macrophages and/or neutrophils markedly increased serum levels of ALT and hepatic necrotic areas but delayed liver regeneration in CCl₄-induced acute hepatitis (Figs. 5-6, supporting Fig. 14). These findings suggest that macrophage and neutrophil infiltration contribute to the removal of necrotic hepatocytes and the subsequent promotion of liver repair in this hepatotoxin-induced liver injury model.

Although prednisolone treatment markedly prevented T/NKT cell hepatitis, the same treatment delayed liver repair and exacerbated liver injury in response to CCl₄, APAP, ethanol, or ethanol plus CCl₄. Given our above-mentioned findings that macrophages and neutrophils play important roles in promoting liver repair (Figs. 5-6, supporting Fig. 14), the

inhibition of macrophage and neutrophil infiltration and their functions is the major mechanism contributing to the detrimental effect of prednisolone in hepatotoxin-induced hepatitis. In addition, prednisolone treatment markedly reduced serum levels of TNF- α and IL-6 (Fig. 1) and inhibited the hepatic activation of the major TNF- α and IL-6 downstream signaling pathway STAT3 and NF- κ B, respectively (Fig. 4), which likely contributed to the inhibitory effect of prednisolone on liver regeneration because these factors promote liver regeneration.^{33, 34}

Although GR is expressed ubiquitously in many cell types, we provided evidence suggesting that the detrimental effect of prednisolone on CCl₄-induced hepatitis is mediated, at least in part, by targeting GR in macrophages and neutrophils. First, prednisolone co-treatment exacerbated CCl₄-induced liver injury in WT mice but not in mGR KO mice, in which the GR gene is deleted in macrophages and neutrophils (Fig. 7A). Second, the inhibitory effects of prednisolone on neutrophil infiltration and liver regeneration in CCl₄-treated WT mice were not observed in mGR KO mice (Figs. 7B-E). Third, *in vitro* treatment with prednisolone directly inhibited macrophage and neutrophil phagocytic functions (Fig. 3). Collectively, these findings suggest that prednisolone targets GR in macrophages and neutrophils, and subsequently inhibits their phagocytic and regenerative functions, thereby exacerbating hepatotoxin-induced liver injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the intramural program of the NIAAA, NIH. No conflicts of interest exist for any of the authors.

We wish to thank Dr. Philippe Mathurin (Université Lille 2, CHRU Lille, Lille, France) and Dr. Michael Lucey (University of Wisconsin, Madison) for their great suggestions during this study, and thank Dr. Louis J. Muglia (University of Cincinnati) for providing myeloid-specific GR KO mice.

References

1. Wang HJ, Gao B, Zakhari S, et al. Inflammation in alcoholic liver disease. *Annu Rev Nutr.* 2012; 32:343–68. [PubMed: 22524187]
2. Lucey MR, Mathurin P, Morgan TR. Alcoholic hepatitis. *N Engl J Med.* 2009; 360:2758–69. [PubMed: 19553649]
3. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology.* 2010; 52:1836–46. [PubMed: 21038418]
4. Heydtmann M, Adams DH. Chemokines in the immunopathogenesis of hepatitis C infection. *Hepatology.* 2009; 49:676–88. [PubMed: 19177577]
5. Czaja AJ, Manns MP. Advances in the diagnosis, pathogenesis, and management of autoimmune hepatitis. *Gastroenterology.* 2010; 139:58–72. e4. [PubMed: 20451521]
6. Szabo G, Csak T. Inflammasomes in liver diseases. *J Hepatol.* 2012; 57:642–54. [PubMed: 22634126]
7. Kubes P, Mehal WZ. Sterile inflammation in the liver. *Gastroenterology.* 2012; 143:1158–72. [PubMed: 22982943]
8. Brenner C, Galluzzi L, Kepp O, et al. Decoding cell death signals in liver inflammation. *J Hepatol.* 2013
9. Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology.* 2011; 141:1572–85. [PubMed: 21920463]

10. Jaeschke H. Neutrophil-mediated tissue injury in alcoholic hepatitis. *Alcohol*. 2002; 27:23–7. [PubMed: 12062633]
11. Ramaiah SK, Jaeschke H. Role of neutrophils in the pathogenesis of acute inflammatory liver injury. *Toxicol Pathol*. 2007; 35:757–66. [PubMed: 17943649]
12. French SW. Alcoholic hepatitis: inflammatory cell-mediated hepatocellular injury. *Alcohol*. 2002; 27:43–6. [PubMed: 12062636]
13. Amulic B, Cazalet C, Hayes GL, et al. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol*. 2012; 30:459–89. [PubMed: 22224774]
14. Karkhanis J, Verna EC, Chang MS, et al. Steroid use in acute liver failure. *Hepatology*. 2013
15. Mathurin P, O'Grady J, Carithers RL, et al. Corticosteroids improve short-term survival in patients with severe alcoholic hepatitis: meta-analysis of individual patient data. *Gut*. 2011; 60:255–60. [PubMed: 20940288]
16. Nguyen-Khac E, Thevenot T, Piquet MA, et al. Glucocorticoids plus N-acetylcysteine in severe alcoholic hepatitis. *N Engl J Med*. 2011; 365:1781–9. [PubMed: 22070475]
17. Christensen E. Glucocorticosteroids in acute alcoholic hepatitis: the evidence of a beneficial effect is getting even weaker. *J Hepatol*. 2010; 53:390–1. [PubMed: 20466448]
18. di Mambro AJ, Parker R, McCune A, et al. In vitro steroid resistance correlates with outcome in severe alcoholic hepatitis. *Hepatology*. 2011; 53:1316–22. [PubMed: 21400552]
19. Louvet A, Wartel F, Castel H, et al. Infection in patients with severe alcoholic hepatitis treated with steroids: early response to therapy is the key factor. *Gastroenterology*. 2009; 137:541–8. [PubMed: 19445945]
20. Singal AK, Shah VH. Alcoholic hepatitis: prognostic models and treatment. *Gastroenterol Clin North Am*. 2011; 40:611–39. [PubMed: 21893277]
21. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N Engl J Med*. 2005; 353:1711–23. [PubMed: 16236742]
22. Fede G, Spadaro L, Tomaselli T, et al. Adrenocortical dysfunction in liver disease: a systematic review. *Hepatology*. 2012; 55:1282–91. [PubMed: 22234976]
23. Mukherjee S, Mukherjee U. A comprehensive review of immunosuppression used for liver transplantation. *J Transplant*. 2009; 2009:701464. [PubMed: 20130772]
24. Tanner AR, Powell LW. Corticosteroids in liver disease: possible mechanisms of action, pharmacology, and rational use. *Gut*. 1979; 20:1109–24. [PubMed: 393573]
25. Tiegs G, Hentschel J, Wendel A. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J Clin Invest*. 1992; 90:196–203. [PubMed: 1634608]
26. Biburger M, Tiegs G. Alpha-galactosylceramide-induced liver injury in mice is mediated by TNF- α but independent of Kupffer cells. *J Immunol*. 2005; 175:1540–50. [PubMed: 16034092]
27. Basu S. Carbon tetrachloride-induced lipid peroxidation: eicosanoid formation and their regulation by antioxidant nutrients. *Toxicology*. 2003; 189:113–27. [PubMed: 12821287]
28. Larson AM. Acetaminophen hepatotoxicity. *Clin Liver Dis*. 2007; 11:525–48. vi. [PubMed: 17723918]
29. Bertola A, Mathews S, Ki SH, et al. Mouse model of chronic and binge ethanol feeding (the NIAAA model). *Nat Protoc*. 2013; 8:627–37. [PubMed: 23449255]
30. Bertola A, Park O, Gao B. Chronic plus binge ethanol feeding synergistically induces neutrophil infiltration and liver injury: A critical role for E-selectin. *Hepatology*. 2013
31. Jeong WI, Park O, Gao B. Abrogation of the antifibrotic effects of natural killer cells/interferon- γ contributes to alcohol acceleration of liver fibrosis. *Gastroenterology*. 2008; 134:248–58. [PubMed: 18166357]
32. Bhattacharyya S, Brown DE, Brewer JA, et al. Macrophage glucocorticoid receptors regulate Toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38 MAP kinase. *Blood*. 2007; 109:4313–9. [PubMed: 17255352]
33. Michalopoulos GK. Liver regeneration. *J Cell Physiol*. 2007; 213:286–300. [PubMed: 17559071]
34. Michalopoulos GK. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am J Pathol*. 2010; 176:2–13. [PubMed: 20019184]

35. Williams MR, Azcutia V, Newton G, et al. Emerging mechanisms of neutrophil recruitment across endothelium. *Trends Immunol.* 2011; 32:461–9. [PubMed: 21839681]
36. Tiegs G. Cellular and cytokine-mediated mechanisms of inflammation and its modulation in immune-mediated liver injury. *Z Gastroenterol.* 2007; 45:63–70. [PubMed: 17236122]
37. Dennert G, Aswad F. The role of NKT cells in animal models of autoimmune hepatitis. *Crit Rev Immunol.* 2006; 26:453–73. [PubMed: 17341188]
38. Hardtke-Wolenski M, Fischer K, Noyan F, et al. Genetic predisposition and environmental danger signals initiate chronic autoimmune hepatitis driven by CD4(+) T cells. *Hepatology.* 2013; 58:718–28. [PubMed: 23475565]
39. Czaja AJ. Drug choices in autoimmune hepatitis: part A--Steroids. *Expert Rev Gastroenterol Hepatol.* 2012; 6:603–15. [PubMed: 23061711]
40. Yoshizawa K, Matsumoto A, Ichijo T, et al. Long-term outcome of Japanese patients with type 1 autoimmune hepatitis. *Hepatology.* 2012; 56:668–76. [PubMed: 22334246]
41. Jaeschke H, Hasegawa T. Role of neutrophils in acute inflammatory liver injury. *Liver Int.* 2006; 26:912–9. [PubMed: 16953830]
42. Possamai LA, Antoniadis CG, Anstee QM, et al. Role of monocytes and macrophages in experimental and human acute liver failure. *World J Gastroenterol.* 2010; 16:1811–9. [PubMed: 20397256]
43. Antoniadis CG, Quaglia A, Taams LS, et al. Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology.* 2012; 56:735–46. [PubMed: 22334567]
44. Albano E. Role of adaptive immunity in alcoholic liver disease. *Int J Hepatol.* 2012; 2012:893026. [PubMed: 22229098]
45. Lin F, Taylor NJ, Su H, et al. Alcohol dehydrogenase-specific T-cell responses are associated with alcohol consumption in patients with alcohol-related cirrhosis. *Hepatology.* 2013; 58:314–24. [PubMed: 23424168]

Abbreviations

α-GalCer	α-galactosylceramide
ALT	alanine transaminase
APAP	acetaminophen
ConA	concanavalin A
CCl₄	carbon tetrachloride
FACS	fluorescence-activated cell sorting
CCR2	CC chemokine receptor 2
CXCR2	CXC chemokine receptor 2
GCs	glucocorticoids
GR	GC receptor
HSC	hepatic stellate cell
ICAM1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IL-6	interleukin-6
MCP-1	monocyte chemoattractant protein 1
MIP-2	macrophage inflammatory protein-2
MNCs	mononuclear cells

MPO	myeloperoxidase
ROS	reactive oxygen species
STAT3	signal transducer and activator of transcription 3
TNF-α	tumor necrosis factor- α

Clinical significance

To our knowledge, this is the first study to extensively examine the effects of the glucocorticoid drug prednisolone on liver injury and inflammation in several mouse models. Our findings may help us to not only understand the effects of steroid therapy on liver disease pathogenesis and progression but also select steroid therapy for liver disease treatments. For example, prednisolone treatment effectively prevented ConA- and α -GalCer-induced hepatitis, which are two models that resemble human autoimmune hepatitis.^{36, 37} These findings are in agreement with other clinical results that demonstrate that steroids are effective in many patients with this disease when they are included in the standard treatment regimens of autoimmune hepatitis.³⁹ In contrast, reports regarding steroid therapy for alcoholic hepatitis have been controversial,¹⁵⁻²⁰ Alcoholic hepatitis is a syndrome characterized by inflammatory cell infiltration in the liver (mainly neutrophils) and hepatocellular injury; however, the mechanisms underlying the development of alcoholic hepatitis remain obscure, and there are no animal models of the severe form of this disease.^{1, 2, 9} Alcohol is a hepatotoxin that is primarily metabolized by hepatocytes. This metabolism produces oxidative stress and inflammation, followed by hepatocellular damage and neutrophil infiltration, mechanisms that are very similar to those involved in hepatotoxin CCl₄-induced liver damage. Therefore, inflammation is likely not the primary source of hepatocellular damage in alcoholic hepatitis, and it may occur as a result of hepatocyte necrosis and contribute to tissue repair and the healing process. If this is the case, steroid treatment may exacerbate liver injury and delay liver regeneration in alcoholic hepatitis. However, steroid treatment may retain some beneficial effects in attenuating the systemic inflammatory responses and improving short-term survival in patients with severe alcoholic hepatitis.^{15, 16} Interestingly, patients with alcoholic hepatitis often have increased levels of circulating antibodies against lipid peroxidation adducts and an increased number of T cells in the liver, suggesting that autoimmune responses may contribute to the pathogenesis of alcoholic hepatitis.^{44, 45} Steroid therapy may be effective if autoimmune responses are the primary source of liver injury in alcoholic hepatitis. Future studies are urgently needed to identify the major mechanisms that contribute to hepatocellular damage in alcoholic hepatitis, which may help us to create a more effective strategy using steroids to treat these patients.

Fig. 1. Treatment with prednisolone delays liver repair in CCl₄-induced acute hepatitis
Mice were injected with CCl₄ and co-treated with prednisolone or vehicle, as described in supporting Fig. 1. (A) H&E staining of liver tissues. Arrows indicate necrotic area. (B) The necrotic area was calculated in 20 randomly selected fields (×100 magnification) per section. (C) Serum ALT levels. (D) Serum levels of cytokines. **P* < 0.05 in comparison with the corresponding CCl₄+Predn group.

Fig. 2. Treatment with prednisolone suppresses acute CCl₄-induced macrophage and neutrophil recruitment

Mice were injected with CCl₄ and co-treated with prednisolone or vehicle, as described in supporting Fig. 1. (A) Liver MNCs were isolated after CCl₄ injection with prednisolone or vehicle co-treatment. The cells were stained with anti-CD11b and Gr-1 antibodies and analyzed by flow cytometry. (B) Liver tissues were stained with an anti-MPO Ab, and MPO⁺ cells were counted in 10 microscopy fields ($\times 200$). (C) Liver leukocytes were isolated and stained with CD11b and Gr-1 antibodies and analyzed by flow cytometry. (D) Real-time PCR analyses of hepatic cytokines and chemokines. * $P < 0.05$ in comparison with the corresponding CCl₄+Predn group.

Fig. 3. Treatment with prednisolone suppresses macrophage and neutrophil functions in CCl₄-induced hepatitis

(A, B) *In vivo* analysis of phagocytosis. Mice were injected with latex beads 22h after CCl₄ injection with prednisolone or vehicle co-treatment. Liver leukocytes were isolated 2h after injection of the latex beads and stained with anti-CD11b and Gr-1 antibodies. (C, D) *In vitro* analysis of phagocytosis. Peritoneal macrophages and neutrophils were cultured with latex beads in media with prednisolone or vehicle and flow cytometric analyses of macrophages and neutrophils were performed. The mean fluorescence intensity was determined. (E) Neutrophils were isolated from mice treated with CCl₄ and prednisolone as described in Panel D, and incubated with or without PMA *in vitro*. The production of ROS was then measured. **P* < 0.05.

Fig. 4. Treatment with prednisolone delays liver regeneration by attenuating hepatic pSTAT3 and NF-κB activation in CCl₄-induced acute hepatitis

Mice were injected with CCl₄ and co-treated with prednisolone or vehicle. (A) Liver regeneration was determined by measuring BrdU incorporation. Representative photographs of BrdU immunostaining of liver tissues are shown in the left panel. The percentage of BrdU⁺ hepatocytes was calculated and is shown in the right panel. (B) Western blot analyses of pSTAT3 and proliferative proteins. Densitometric analyses are shown in the right panel. (C) Western blot analyses of pNF-κB proteins. Densitometric analyses are shown in the right panel. **P* < 0.05 in comparison with the corresponding CCl₄+Predn group.

Fig. 5. Neutrophil depletion enhances CCl₄-induced liver injury and inhibits liver regeneration Anti-Ly6G Ab or control Ab was injected i.v. 24h prior to CCl₄ treatment. (A) Liver leukocytes were isolated 24h post CCl₄ treatment and subjected to flow cytometric analyses of macrophages (Gr-1^{int}CD11b⁺) and neutrophils (Gr-1^{hi}CD11b⁺). (B) Serum ALT levels after CCl₄ treatment. (C) H&E staining of liver tissues after CCl₄ treatment. The necrotic area was calculated in 20 randomly selected fields (x100 magnification) per section. Arrows indicate the necrotic area. (D) Representative photographs of the BrdU immunostaining of liver tissues. The percentage of BrdU⁺ hepatocytes was calculated and is shown in the right panel. **P* < 0.05.

Fig. 6. Depletion of macrophage enhances CCl₄-induced liver injury and inhibits liver regeneration

Clodronate was injected i.v. 24h prior to CCl₄ treatment. (A) Representative immunohistochemistry staining of F4/80. (B) Serum ALT levels after CCl₄ treatment. (C) H&E staining of liver tissues. Necrotic area was calculated in 20 randomly selected fields (X100 magnification) per section. Arrows indicate necrotic area. (D) Representative BrdU immunostaining analyses. The percentage of BrdU-positive hepatocytes was counted in 10 microscopy fields (×200) per section. **P* < 0.05.

Fig. 7. Treatment with prednisolone does not influence CCl₄-induced liver injury in mGR KO mice

(A) Western blot analyses of GR protein expression in peritoneal macrophages and neutrophils from thioglycollate-treated WT and mGR KO mice. (B) Serum ALT levels. (C) Representative photograph of H&E staining of liver tissues. The necrotic area was calculated in 20 randomly selected fields ($\times 100$) and is shown in the right panel. (D) Representative immunohistochemical staining of liver tissues with an anti-MPO Ab. The number of MPO⁺ cells was counted in 10 microscopy fields ($\times 200$) and is shown in the right panel. (E) Liver regeneration was determined by measuring BrdU incorporation. The percentage of BrdU⁺ hepatocytes was calculated ($\times 200$) and is shown in the right panel.

* $P < 0.05$

Fig. 8. Prednisolone delays liver repair in hepatotoxin-induced hepatitis by targeting GR in neutrophils and macrophages

Injection of hepatotoxin CCl₄ leads to hepatocyte injury followed by the activation of macrophages/Kupffer cells and ROS production, which may further amplify liver injury. Activated macrophages/Kupffer cells also produce a variety of cytokines and chemokines, followed by the recruitment of neutrophils into the hepatic vasculature. Macrophages and neutrophils may not only contribute to hepatocyte damage but also play an important role in repairing the injured liver by removing necrotic hepatocytes and promoting hepatocyte proliferation. Prednisolone treatment exacerbates the hepatotoxin-induced liver injury by blocking many pathways, as indicated by X.