18β-Glycyrrhetinic Acid Ameliorates Acute 
Propionibacterium acnes-induced Liver Injury through 
Inhibition of Macrophage Inflammatory Protein-1α*

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18β-Glycyrrhetinic acid (GA), the major bioactive component of licorice root extract, has a protective effect on hepatic injury and exhibits antiinflammatory activity. Here, we investigate the effect of GA in Propionibacterium acnes-induced acute inflammatory liver injury. C57BL/6 mice were primed with P. acnes followed by lipopolysaccharide challenge to induce fulminant hepatitis. GA (75 mg/kg) or vehicle control was administered intraperitoneally daily 1 day after P. acnes priming, and GA significantly improved mouse mortality. Then, to investigate the underlying mechanisms of GA in this acute inflammatory liver injury model, we primed C57BL/6 mice with P. acnes only. We propose that GA ameliorates acute P. acnes-induced liver injury through reduced macrophage inflammatory protein (MIP)-1α expression in Kupffer cells by down-regulating MyD88 expression and inhibiting NF-κB activation. Reduced MIP-1α expression lowered the recruitment of CD11c+B220− dendritic cell precursors into the liver. Consequently, GA treatment inhibits the activation and proliferation of liver-infiltrating CD4+ T cells and reduces the production of serum alanine aminotransferase and proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α. Moreover, anti-MIP-1α treatment in P. acnes-primed mice inhibits the recruitment of dendritic cell precursors into the liver and suppresses mouse mortality as GA does. Taken together, our results suggest that GA exhibits antiinflammatory effects through inhibition of MIP-1α in a mouse model of acute P. acnes-induced inflammatory liver injury.

Fulminant hepatitis, developing secondary to infection, toxin, or immune-mediated attack, is a rare but potentially fatal disease associated with failure of hepatic regeneration. Mortality without supportive management and/or liver transplantation can be high, and the processes leading to such profound hepatic damage are unknown (1).

The molecular pathogenesis of massive hepatic necrosis is currently under extensive investigation using several animal models (2–4). Mice injected with heat-killed Propionibacterium acnes followed by lipopolysaccharide (LPS)² is one of the most commonly used animal models of fulminant hepatitis (5–7), which can be pathophysiologically classified into two phases: the priming phase induced by P. acnes from day 0 to day 7, and the eliciting phase induced by LPS injection on day 7. At the priming phase, liver macrophages, known as Kupffer cells, continually screen and capture P. acnes from blood and are activated upon P. acnes stimulation (5, 6). Kupffer cells then secrete chemokines such as macrophage inflammatory protein (MIP)-1α to recruit a subset of CD11c+B220− dendritic cell (DC) precursors in the liver, which is an initial event and a prerequisite for liver injury in this model (5, 6). DC precursors differentiate into mature DCs and migrate into hepatic lymph nodes to activate P. acnes-specific CD4+ T cells, which are then recruited to the liver. The accumulation of T cells, macrophages, and DCs produces various proinflammatory molecules and forms granulomas (8). At the eliciting phase, LPS injection enlarges liver inflammation and enhances granuloma formation, leading to massive hepatocellular damage due to necrosis and apoptosis around the granulomas. Within a few days, the dramatically altered internal environment finally results in acute liver failure (5–8).

Traditional Chinese herbal medicine has a history spanning >1000 years (9). As a traditional Chinese medicine, licorice (Glycyrrhiza glabra L.) has been used in the treatment of various inflammatory diseases since ancient times (10), although its therapeutic mechanism remains unknown. Glycyrrhizin, a major bioactive triterpene glycoside of lico- root extract, exhibits its pharmacological functions through its biologically active metabolite, 18β-glycyrrhetinic acid (GA) (11). GA is known to exhibit a variety of phar-

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‡ The abbreviations used are: LPS, lipopolysaccharide; MIP-1α, macrophage inflammatory protein-1α; DC, dendritic cell; GA, 18β-glycyrrhetinic acid; MNC, mononuclear cell; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL, interleukin.
macological effects, with the most dramatic being its antiinflammatory effect (12–15). Recently, Yang et al. (15) found that the stronger neo-minophagen C, a glycyrrhizin preparation, could effectively protect liver against LPS/α-GalN-induced fulminant hepatitis by inhibiting the production of proinflammatory molecules. However, the molecular mechanisms of GA in suppressing acute inflammatory liver injury still need to be elucidated further.

In this study, we found that GA can significantly improve mouse mortality in the model of fulminant hepatitis. In addition, we propose that within 7 days after P. acnes priming, GA ameliorates the clinical symptoms and disease progression of liver injury through inhibition of MIP-1α.

**EXPERIMENTAL PROCEDURES**

**Animals and Experimental Protocol**—Female C57BL/6 mice (8–10 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME) and were kept under pathogen-free conditions in the animal center of the Shanghai Jiao Tong University School of Medicine (Shanghai, China). To induce severe liver injury, mice were injected with 1 mg of heat-killed P. acnes via the tail vein. For survival analysis, mice were given an intravenous injection of 1 μg of LPS 7 days after P. acnes priming. At the indicated time intervals, at least five mice were killed at each time point. Approximately 0.8–1 ml of blood was obtained by cardiac puncture under ether anesthesia, and liver specimens were sampled. Hepatocellular damage was determined by serum alanine aminotransferase levels. For the treatment experiments, GA (75 mg/kg) (GA mice) or dimethyl sulfoxide (control mice) was administered intraperitoneally daily 1 day after P. acnes priming. In some experiments, 200 μg/100 μl anti-MIP-1α polyclonal antibody or control rabbit IgG in phosphate-buffered saline was administered 0 and 2 days after P. acnes injection. For in vivo proliferation assays, mice were injected intraperitoneally with BrdUrd (Sigma-Aldrich) 1 day before sacrifice. All animal experiments complied with the animal protocols approved by the Institutional Review Board of the Institute of Health Sciences (Shanghai, China).

**Histology and Immunohistochemistry**—Liver specimens were fixed in 10% neutral buffered formalin and paraffin-embbeded. Deparaffinized sections (5–10 μm) were stained with hematoxylin and eosin and analyzed by light microscopy. For immunostaining, frozen sections (8 μm) from liver specimens were incubated with rat anti-mouse CD4 or CD11c antibody (BD Biosciences, San Jose, CA) and were then labeled with Cy3-conjugated rabbit anti-rat IgG (Jackson Laboratories) and examined by immunofluorescence microscopy (Nikon, Tokyo, Japan).

**Preparation of Mononuclear Cells (MNCs) from Liver and Flow Cytometric Analysis**—Liver samples from mice were minced and pressed through a 70-μm nylon mesh (BD Falcon, Franklin Lakes, NJ). The cell suspension was treated with 33% Percoll and centrifuged at 2000 rpm at 20 °C for 20 min with break off to remove liver parenchymal cells. The pellets were treated with an red blood cell lysis solution and then washed and resuspended.

Suspensions of cells were stained with fluorescein isothiocyanate-labeled anti-CD4, anti-CD80, anti-CD86, phycoerythrin-conjugated anti-major histocompatibility complex II, anti-B220, anti-CD62L, anti-CD11b, allophycocyanin-conjugated anti-CD44, or biotin-conjugated anti-CD11c and anti-CD69. For biotin-conjugated antibodies, incubation with allophycocyanin-conjugated streptavidin (all from BD Pharmingen, San Diego, CA) was then performed. Isotype controls were used for determination of negative cells. The stained cells were analyzed on a FACSAria instrument (BD Biosciences). In some experiments, the absolute number of DC precursors was determined by multiplying the total MNC number by the fraction of CD11c+ cells in complete Dulbecco’s modified Eagle’s medium at 37 °C under an atmosphere of 5% CO2/95% air for 72 h. For the inhibition assay, either GA at the indicated concentrations or vehicle was added to the culture. Cell proliferation was measured using CCK8 reagent (Dojindo, Kumamoto, Japan).

**Kupffer Cell Isolation**—Liver Kupffer cells were prepared as described previously with some modifications (16). In brief, livers were dissected, homogenized in ice-cold phosphate-buffered saline, and centrifuged. The pellet was resuspended and overlaid on 4 ml of 70% Percoll and 4 ml of 30% Percoll. The Percoll gradient was centrifuged at 2000 rpm for 20 min at 20 °C, without braking, and cells were collected from the 30%/70% Percoll interface, washed with phosphate-buffered saline, and resuspended.

**Real Time PCR**—Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions and reverse transcribed. mRNA expression of IL-2, MIP-1α, MyD88, and β-actin was determined by real time PCR using SYBR Green Master Mix (ABI, Foster City, CA). The primers for IL-2 were 5'-CCTGAGCAGATGGAGAATTACA-3' and 5'-TCCAGAACATCGCCGAG-3'. The primers for MIP-1α were 5'-ACCATGACACTTCTGAACCA-3' and 5'-GTGGATCTTCCGGCTGTAG-3'. The primers for MyD88 were 5'-CAGGAGATGATCGGCAACT-3' and 5'-CTGGCA-AATGAGCAGACACA-3'. The primers for β-actin were 5'-ATGGAGGGAATACGCCC-3' and 5'-TCTTCTGCACTTCCGTGTT-3'. Data were collected and quantitatively analyzed on an ABI Prism 7900 sequence detection system. Mouse β-actin gene was used as an endogenous control for sample normalization.

**Western Blot Analysis and Electrophoretic Mobility Shift Assay**—For Western blot analysis, protein extracts were resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The protein of interest was detected by immunostaining with specific primary anti-
bodies followed by horseradish peroxidase-labeled secondary antibodies using chemiluminescence labeling.

The detection of NF-κB activation was performed with a commercial LightShift Chemiluminescent electrophoretic mobility shift assay kit (Viagene), according to the manufacturer’s instruction. The consensus NF-κB oligonucleotides included in the kit was 5′-AGTTGAGGGACTTCACCAGGC-3′.

Statistical Analysis—The significance between two groups was examined using Student’s t test after analyzing the variance. A p value of <0.05 was considered significant.

RESULTS

Suppression of Mortality and Severity of Liver Injury by GA—The chemical form of GA is shown in Fig. 1 (11). The compound had a low LD$_{50}$ of 308 mg/kg when administered by intraperitoneal injection in mice (17). To determine whether GA alleviates fulminant hepatitis, GA (75 mg/kg) (GA mice) or vehicle control (control mice) was administered intraperitoneally daily 1 day after $P. acnes$ priming, then on day 7, LPS was injected to induce fulminant hepatitis, and the effect of GA on the survival rate of mice was investigated. As illustrated in Fig. 2A, 60% of control mice died within 12 h, and all control mice died within 18 h in response to subsequent LPS injection. In contrast, all GA mice survived for 24 h, and no GA mouse died in the subsequent time observed (data not shown), suggesting that GA treatment could dramatically promote the survival rate of $P. acnes$/LPS-induced fulminant hepatitis in mice. To investigate the mechanism of GA on decreased mouse mortality, we analyzed serum alanine aminotransferase levels within 7 days after $P. acnes$ priming (18). Unexpectedly, upon $P. acnes$ stimulation, there was almost no effect on expression of the other two activation markers, CD62L and CD69. GA treatment also significantly decreased after GA treatment, whereas there was no significant difference in proliferation ability between control mice and GA-treated mice (Fig. 4B) as well as their IL-2 mRNA expression (Fig. 4C) as indicated by BrdUrd incorporation and real time PCR assay.

GA Reduces the Recruitment of CD11c$^+$ B220$^-$ DC Precursors through Inhibition of MIP-1α—We next examined the changes in $P. acnes$-specific proliferation of MNCs evoked by GA treatment in vitro. Unexpectedly, upon $P. acnes$ stimulation, there was no significant difference in proliferation ability between MNCs from control and GA-treated mice (Fig. 4D). Meanwhile, GA did not exhibit any inhibitory effect on the proliferation of MNCs from control mice upon $P. acnes$ stimulation in vitro (Fig. 4E). These results indicate that the inhibition of CD4$^+$ T cell activation and proliferation in $P. acnes$-primed mice after GA treatment is independent of a direct effect on CD4$^+$ T cells.
FIGURE 2. **GA treatment suppressed mouse mortality and severity of *P. acnes*-induced acute liver injury.** Mice were injected with *P. acnes*. GA or vehicle control was administered intraperitoneally daily 1 day after *P. acnes* injection. **A**, cumulative survival rates of control and GA mice after LPS injection. **B**, serum alanine aminotransferase (ALT) levels of normal, control, or GA mice on day 7 after *P. acnes* priming. The data represent the means ± S.D. (error bar) from three to five mice. *, *p* < 0.05. **C**, histopathological analysis of liver specimens obtained from normal, control, or GA mice on day 7 after *P. acnes* priming (magnification, ×200). **D**, histopathological analysis of liver specimens obtained from control or GA mice on days 3, 5, and 7 after *P. acnes* priming (magnification, ×400).
We have reported previously that \textit{P. acnes}-induced recruitment of CD11c/B220\textsuperscript{−} DC precursors from blood is an initial event and is a prerequisite for liver injury in this \textit{P. acnes}-induced liver injury model (6). Here, we investigate whether GA influences the recruitment of CD11c/B220\textsuperscript{−} DC precursors from blood. As we found previously, CD11c/B220\textsuperscript{−} DC precursors increase markedly and peak at day 7 after \textit{P. acnes} priming (Fig. 5, \textit{A}, \textit{B}, and \textit{D}). In contrast, GA treatment dramatically inhibits the recruitment of DC precursors from peripheral blood, and there was no significant increase in the number during the 7 days after \textit{P. acnes} priming (Fig. 5, \textit{C} and \textit{D}), suggesting that impaired activation and proliferation of liver-infiltrating CD4\textsuperscript{+} T cells in GA mice result from the reduced recruitment of CD11c/B220\textsuperscript{−} DC precursors. We then analyzed the expression status of chemokine MIP-1\(\alpha\), which is known to influence the recruitment of CD11c/B220\textsuperscript{−} DC precursors. As expected, liver MIP-1\(\alpha\) mRNA expression was significantly inhibited, and the inhibitory effect was in parallel with the tendency toward decreased recruitment of DC precursors during the 7 days after \textit{P. acnes} priming (Fig. 5, \textit{D} and \textit{E}).

We further examined whether the inhibited recruitment of CD11c/B220\textsuperscript{−} DC precursors into GA-treated mouse liver is due to decreased production of MIP-1\(\alpha\). As shown in Fig. 5\textit{F}, anti-MIP-1\(\alpha\) treatment significantly inhibited the recruitment of DC precursors in \textit{P. acnes}-primed mouse livers compared with that in control antibody-treated mouse livers, which has a trend similar to GA mice. Moreover, anti-MIP-1\(\alpha\) treatment also suppressed the mouse mortality as GA did, whereas all control antibody-treated mice died within 12 h after LPS injection (Fig. 5G). These results indicate that the reduced recruitment of CD11c/B220\textsuperscript{−} DC precursors in GA-treated mouse liver is mediated by inhibition of MIP-1\(\alpha\) in the liver.

Inhibition of MyD88 Expression and NF-\(\kappa\)B Activation after GA Treatment—Phagocytic liver macrophages, Kupffer cells, play critical roles in initiation of hepatitis (18). We reported
previously that in this P. acnes-induced liver injury model, Kupffer cells continually screen and capture P. acnes from blood and that Kupffer cells may be the source of MIP-1α to recruit DC precursors (6). As a result, we investigated the effects of GA on Kupffer cell activation and MIP-1α expression. As illustrated in Fig. 6A, GA significantly reduced the expression of major histocompatibility complex II and costimulatory molecules (CD80 and CD86) in Kupffer cells. Additionally, mRNA expression of MIP-1α was also inhibited significantly (Fig. 6C).

MyD88 has been reported to play important roles in P. acnes-induced liver injury, and MyD88-deficient mice showed impaired liver inflammation and granuloma formation after P. acnes induction (19, 20). We examined the mRNA expression of MyD88 in Kupffer cells on day 7 after P. acnes priming and found that GA significantly reduced MyD88 mRNA expression in Kupffer cells (Fig. 6C). In addition, GA significantly inhibited the activation of NF-κB, a downstream signal of MyD88, as indicated by inhibition of IκBα degradation and NF-κB activity (Fig. 6, D and E).

DISCUSSION

P. acnes-induced liver injury is reported to be a Th1 cell-mediated inflammatory response (5–7). During this process,
numerous MNCs accumulate in the liver and secrete proinflammatory cytokines, which promote the activation and proliferation of CD4⁺ T cells and liver inflammation. When followed by LPS injection on day 7, massive liver damage ensues and will cause mouse death in a short time (4–7). In this study, we found that GA treatment suppresses P. acnes/LPS-induced mouse mortality and ameliorated acute liver damage. In addition, we discovered that GA treatment significantly inhibits CD4⁺ T cell infiltration in the liver after P. acnes priming. Moreover, the activation status and prolifer-
ation ability of liver-infiltrating CD4$^{+}$ T cells were also impaired as indicated by the down-regulation of an activation marker CD44 expression and BrdUrd incorporation. However, there were no significant differences found in the P. acnes-specific proliferation rate of MNCs between GA mice and control mice in ex vivo studies. Furthermore, GA failed to inhibit directly the proliferation of MNCs from control mice upon P. acnes restimulation in vitro. These results suggest that the amelioration of liver injury by GA treatment is not the result of direct action on the proliferation and activation of liver-infiltrating CD4$^{+}$ T cells and that GA functions in an upstream event in this P. acnes-induced liver injury model.

The inflammatory microenvironment provides optimal conditions for emigration of lymphocytes from blood (21, 22). Accumulating evidence indicates that inflammatory or microbial stimuli could induce the production of various chemokines, which play essential roles in regulating the extravasation and tissue accumulation of lymphocytes during disease progression (21, 23–26). In this model of P. acnes-induced liver injury, we demonstrated previously that chemokine MIP-1$\alpha$ plays an important role in the recruitment of CD11c$^{+}$B200$^{-}$ DC precursors, which then induce a Th1 response and inflammation in the liver (6, 8). Our results showed that GA treatment inhibits the recruitment of CD11c$^{+}$B200$^{-}$ DC precursors, which provides a reason for the impaired activation and proliferation of liver-infiltrating CD4$^{+}$ T cells in GA mice. In addition, the inhibitory tendency toward recruitment of DC precursors is in parallel with the reduced MIP-1$\alpha$ expression in the liver after GA treatment, implying that the impaired recruitment of DC precursors is due to inhibition of MIP-1$\alpha$ secretion in the liver. Moreover, anti-MIP-1$\alpha$ treatment also inhibits the recruitment of CD11c$^{+}$B200$^{-}$ DC precursors and suppresses mouse mortality as GA did. These results collectively suggest that GA-mediated amelioration of mouse liver inflammation is caused by inhibition of MIP-1$\alpha$.

Liver resident macrophages, Kupffer cells, play important roles in initiation of many forms of hepatitis (18). We previously reported that in (the) mouse model of P. acnes-induced liver injury, Kupffer cells could capture heat-killed P. acnes from blood and secrete MIP-1$\alpha$, which is responsible for the recruitment of circulating DC precursors (6, 27). In this study, we found that the activation status and MIP-1$\alpha$ mRNA expression of Kupffer cells from GA mice were significantly inhibited com-
pared with those of control mice, suggesting that GA exhibits the ability to inhibit Kupffer cell activation after \( P. acnes \) priming and, as a result, induces a series of subsequent events that ameliorate liver injury.

GA, the major active component of the medicinal plant licorice, has been reported to exhibit a variety of pharmacological effects, including antitumor, antihepatotoxic, and immunomodulatory activities (14, 28–30). However, little is known about the mechanisms by which GA accomplishes its immunomodulatory effect. MyD88, the common adaptor of Toll-like receptors, has been demonstrated to play a critical role in the induction of liver injury and the formation of granulomas after \( P. acnes \) priming. In this mouse model of \( P. acnes \)-induced inflammatory liver injury, MyD88 expression up-regulated markedly (19, 20). Besides, we found that GA can inhibit \( P. acnes \)-induced up-regulation of MyD88 expression. As a result, GA treatment inhibited liver granuloma formation and production of inflammatory cytokines, such as IFN-\( \gamma \) and TNF-\( \alpha \), which was in accordance with the results observed previously in MyD88-deficient mice (19, 20, 31). In addition, it was reported that GA could inhibit TNF-\( \alpha \)-induced chemokine expression through inhibition of NF-\( \kappa B \) activation in vitro (32). In this study, we found that GA could also effectively inhibit \( P. acnes \)-induced NF-\( \kappa B \) activation and chemokine MIP-1\( \alpha \) expression in vivo, which might be mediated by the down-regulation of MyD88 expression.

The MyD88 protein is an adaptor molecule that participates in \( P. acnes \)-induced acute inflammatory liver injury (19, 20). It contains two protein-protein interaction domains, an N-terminal death domain and a C-terminal Toll/IL-1R homology domain separated by a short linker region. MyD88 is recruited to the receptor complexes as a dimer, which is stabilized by homophilic interactions occurring between the death domain and Toll/IL-1R homology domains. Once recruited, it leads to the activation of a series of kinases, and finally targets to degradation the IkB, thereby allowing the NF-\( \kappa B \) enter the nucleus and activate transcription (33). We found in this study that GA could significantly alleviate \( P. acnes \)-induced liver injury through inhibition of MyD88 expression and NF-\( \kappa B \) activation, which suggests that MyD88 might be a potential target of GA for its antiinflammatory activity. Recent evidence revealed that a novel synthesized compound inhibited MyD88 dimerization and IL-1\( \beta \)-mediated activation of NF-\( \kappa B \) transcriptional activity, suggestive of potential antiinflammatory activity (34, 35). Therefore, GA might interact with the MyD88 protein domain and disrupt its dimerization, which led to decreased activation of NF-\( \kappa B \) and reduced inflammatory transcription, and finally alleviated acute liver injury.

In conclusion, we find that GA exhibits antiinflammatory effects through reduced MIP-1\( \alpha \) expression that mediated by inhibiting MyD88 expression and NF-\( \kappa B \) activation in immunopathogenesis in a mouse model of \( P. acnes \)-induced acute inflammatory liver injury. This study provides new insights into the mechanisms of antiinflammatory effects of GA and a novel potential therapeutic drug from natural compounds for the treatment of acute inflammatory liver damage and other inflammatory diseases.

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